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ABOUT COVER

Editorial Board Member of *World Journal of Gastroenterology*, Professor Udo Rolle is a Distinguished Professor at the University Hospital of the Goethe-University in Frankfurt, Germany. Having received his doctoral degree from the University of Leipzig in 1994, Prof. Rolle undertook his postgraduate training, first at the University of Leipzig (Germany) and then at the University Hospital Dublin (Ireland), receiving his habilitation in 2003. He rose to Full Professor and Chief Surgeon in the Department of Paediatric Surgery and Paediatric Urology at the University Hospital Frankfurt in 2008. His ongoing research interests involve neonatal GI surgery, pediatric GI motility disorders with long-term follow-up in various congenital anomalies. Currently, he serves as President of the German Association of Paediatric Surgery, General Secretary of the World Federation of Associations of Paediatric Surgery, and General Secretary of the UEMS section of Paediatric Surgery. (L-Editor: Filipodia)

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GUIDELINES

Chinese guidelines on the management of liver cirrhosis (abbreviated version)

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Abstract

Based on reviews of the literature and experts' consensus, the Chinese Society of Hepatology developed guidelines for the diagnosis and treatment of liver cirrhosis, in order to improve clinical practice. In addition to what has been covered in previously published guidelines on the management of cirrhosis complications, these guidelines add new sections and provide updates. The guidelines emphasize the early diagnosis of the cause and assessment of complications. Comprehensive treatments including etiological treatment and

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complication management should be initiated immediately. In addition, regular monitoring, especially surveillance of hepatocellular carcinoma, is crucial for managing patients.

Key Words: Liver cirrhosis; Diagnosis; Therapy; Guidelines; Hypertension portal; Recompensated stage

Core Tip: Based on reviews of the literature and experts' consensus, the Chinese Society of Hepatology developed guidelines for the diagnosis and treatment of liver cirrhosis, in order to improve clinical practice. In addition to what has been covered in previously published guidelines on the management of cirrhosis complications, the guidelines adds new sections and provides updates. The guidelines emphasizes the early diagnosis of the cause and assessment of complications. Comprehensive treatment including etiological treatment and complication management should be initiated immediately. In addition, regular monitoring, especially surveillance of hepatocellular carcinoma, is crucial to manage patients.

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INTRODUCTION

Cirrhosis is currently the 11th most common cause of death globally and accounts for approximately 2 million deaths per year worldwide[1]. The American Association for the Study of Liver Disease, World Gastroenterology Organization, European Association for the Study of the Liver, and International Club of Ascites have developed and updated multiple guidelines and consensuses for the diagnosis and treatment of cirrhosis and its complications[2-4].

To promote diagnosis and treatment of cirrhosis, the Chinese Society of Hepatology (CSH) and the Chinese Society of Gastroenterology (CSG) of Chinese Medical Association (CMA) developed the Chinese Guidelines for the Diagnosis and Treatment of Esophageal and Gastric Variceal Bleeding in Cirrhotic Portal Hypertension[5], Guidelines on the Management of Ascites and Its Related Complications in Cirrhosis[6], and Guidelines on Management of Hepatic Encephalopathy in Cirrhosis[7] recently. The present guideline adds new sections and provides updates.

These guidelines were developed according to evidence-based medicine and Appraisal of Guidelines Research and Evaluation Instrument (AGREE II). A guidance group, secretary group (writing group), and expert group (including corresponding experts) were established and included experts in the fields of liver disease, gastroenterology, infectious disease, surgery, intervention therapy, oncology, traditional Chinese medicine, pharmacology, nursing and clinical study methodology.

The evidence and recommendations mentioned in these guidelines are graded according to the grading of recommendations assessment, development and evaluation (GRADE) system[8].

ETIOLOGY

Common causes of cirrhosis are: Hepatitis B and C, alcoholic consumption, non-alcoholic fatty liver disease, autoimmune liver diseases, Wilson's disease, hemochromatosis, and chronic drug-induced liver injury. Other causes include hepatic amyloidosis, a1-antitrypsin deficiency, hepatic porphyrian, parasitic infections mainly including schistosomiasis and clonorchiosis, circulatory disturbance such as Budd-

Chiari syndrome, and right heart failure. Some patients with cirrhosis have no unknown cause.

Most cases of cirrhosis have a single cause, but sometimes multiple causes co-exist. Superinfection of hepatitis B and C and alcohol consumption in patients with hepatitis B or C are common examples. In addition, based on the primary cause, some synergetic factors can contribute to the exacerbation of cirrhosis such as obesity, insulin resistance, and some drugs[9-13].

EVALUATION OF LIVER FUNCTION AND PORTAL HYPERTENSION

Evaluation of liver function and compensatory capacity

The indicators reflecting hepatic synthetic function include serum albumin (ALB), pre-ALB, coagulation factors, cholesterol, and cholinesterase[14]. With the short half-life, coagulation factors is an early indicator. Prothrombin activity (PTA) and prothrombin international normalized ratio (PT-INR) are the most commonly used.

Scoring systems for assessing the prognosis in cirrhosis patients

Scoring systems for assessing prognosis in cirrhosis patients include Child-Pugh score[15] (Table 1), model for end-stage liver disease (MELD), and MELD-Na score[16] (Table 2).

Common methods of imaging assessments in cirrhosis

Common imaging assessments methods in cirrhosis include sonography, computed tomography (CT) scan, and magnetic resonance imaging (MRI) scan, which can be used to screen the liver tumor and evaluate portal hypertension[17,18]. Recently, liver stiffness measurement (LSM) and transient elastography (TE) have become the most convenient non-invasive diagnostic methods for hepatic fibrosis and early cirrhosis. Fibroscan® (FS) and Fibrotouch® (FT) are the most commonly used LSM tools in the clinic. Detailed cutoff values and the diagnostic value of LSM in hepatic fibrosis and cirrhosis can be found in the Consensus on clinical application of transient elastography detecting liver fibrosis: A 2018 update[19]. Magnetic resonance elastography (MRE), a more recently developed non-invasive staging and diagnosis method for hepatic fibrosis, can be used in patients with ascites and obesity or metabolic syndrome, and can test the whole liver. However, MRE is costly, and its value in staging and diagnosing early cirrhosis and hepatic fibrosis should be further studied. At present, it is not suitable for conventional monitoring of hepatic fibrosis in Chinese patients with chronic liver diseases.

Histological evaluation

Histological evaluation is a "gold standard" for diagnosing and evaluating cirrhosis. Adoption of the Laennec cirrhosis scoring system is suggested (Table 3)[20,21].

Evaluation of portal hypertension

In addition to imaging technology including sonography, LSM, CT, MRI, and MRE, endoscopy including gastroscopy and enteroscopy[5] and hepatic venous pressure gradient (HVPG) measurement[22] are reliable for evaluating the severity of portal hypertension.

Nutritional risk screening and malnutrition evaluation

Nutritional risk screening and malnutrition evaluation in patients with cirrhosis is detailed in Chinese clinical guidelines on nutrition in end-stage liver disease (2019)[23].

DIAGNOSIS

The diagnosis of cirrhosis should comprehensively take into account etiologies, medical history, clinical manifestations, complications, treatment process, laboratory tests, imaging, and histological examinations. Traditionally, cirrhosis can be classified into compensated stage and decompensated stage. However, recent data have shown that some patients with decompensated cirrhosis became recompensated, which is defined as no more decompensation for years with the development in the treatment of cirrhosis. In addition, some patients with cirrhosis present with reversion of

Table 1 Child-Pugh score[15]

Clinical biochemical indicators	1 point	2 points	3 points
Hepatic encephalopathy, grade	None	1-2	3-4
Ascites	None	Mild	Moderate to severe
Total bilirubin, μmol/L	< 34	34-51	> 51
Albumin, g/L	> 35	28-35	< 28
Prothrombin time prolonged, sec	1-3	4-6	> 6

Child Pugh grading criteria for cirrhosis: Grade A: Child-Pugh score 5-6; Grade B: Child-Pugh score 7-9; Grade C: Child-Pugh score 10-15.

Table 2 Model for end-stage liver disease score[16]

Score, points	Significance
< 12	
12-18	Included into the waiting list of liver transplantation
18-25	Require liver transplantation
25-30	Require emergent liver transplantation
> 30	Require emergent liver transplantation for rescue

Model for end-stage liver disease (MELD) score = 3.8 × loge [bilirubin (mg/dL)] + 11.2 × loge (INR) + 9.6 × loge [creatinine (mg/dL) + 6.4 × (causes of disease: Biliary or alcoholic 0, other 1). Bilirubin (mg/dL) = bilirubin (μmol/L) ÷ 17.1; Creatinine (mg/dL) = creatinine (μmol/L) ÷ 88.4.

Table 3 Laennec F1-F4 staging system, Laennec fibrosis staging scoring system in hepatic puncture tissue[20,21]

Stage	Name	Septum, thickness and amount	Criteria	Score
0	No clear fibrosis			0
1	Very mild fibrosis	+/-	No septum or very few thin septum, portal area enlarged or mild peri-sinusoid fibrosis	1
2	Mild fibrosis	+	Occasionally thin septum, portal area enlarged or mild peri-sinusoid fibrosis	2
3	Moderate fibrosis	++	A medium amount of thin septum, even incomplete cirrhosis	3
4A	Cirrhosis, mild, definite or possible	+++	Obvious septum, with circle outline or obvious nodules, thin septum mostly (one wide septum allowable)	4
4B	Moderate cirrhosis	++++	At least two wide septum, but no very wide septum; less than 1/2 of puncture tissues in length are composed of nodules	5
4C	Severe cirrhosis	+++++	At least one very wide septum, or more than 1/2 of puncture tissues in length are composed of nodules (micronodular cirrhosis)	6

cirrhosis[24]. Thus, we suggest that stages of cirrhosis include compensated stage, decompensated stage, recompensated stage, and/or cirrhosis reversion.

Diagnosis of compensated cirrhosis is based on one of the following criteria: (1) Histologically cirrhosis; (2) Gastroesophageal varices or digestive tract ectopic varices on the basis of excluding noncirrhotic portal hypertension; (3) Imaging of cirrhosis or portal hypertension, *e.g.*, splenomegaly, portal vein ≥ 1.3 cm; (4) LSM result complying with diagnostic cutoff of cirrhosis of different causes; and (5) Meeting two or more of the following criteria: (a) Platelet (PLT) < 100 × 10^9/L, without any other reasons; (b) Serum ALB < 35 g/L, excluding malnutrition or kidney diseases; (c) INR > 1.3 or PT prolonged (discontinuing thrombolysis or anticoagulant drugs for over 7 d); and (d) Aspartate aminotransferase (AST)/PLT ratio index (APRI): Adult APRI score > 2.

Diagnosis of decompensated cirrhosis is based on the existence of cirrhosis and any one of the complications including ascites, gastroesophageal varices hemorrhage, sepsis, hepatic encephalopathy, and hepatorenal syndrome.

Cirrhotic recompensation and/or reversion: Accumulating evidence has demonstrated that effective antiviral treatment in decompensated HBV and HCV cirrhosis patients can lead to the recompensation of the liver function and reduce liver transplantation. Even though the definition of recompensation of decompensated cirrhosis has not been established, Chinese experts agree with the following preliminary criteria: When decompensation events (ascites, digestive tract hemorrhage, hepatic encephalopathy) have not recurred for a long time period (at least 1 year) after effective treatment in patients with decompensated cirrhosis, these patients will be classified as recompensated population.

Similarly, clinical data have provided evidence of cirrhosis reversion[25-29] defined as: Ishak fibrosis staging decreases by ≥ 1 stage, or P-I-R classification after treatment decreases.

Recommendation 1: Cirrhosis can be classified into compensated stage, decompensated stage, recompensation stage and/or cirrhosis reversion (B, 1).

Recommendation 2: Diagnosis of compensated cirrhosis: (1) Histologically cirrhosis (A, 1); (2) Gastroesophageal varices or digestive tract ectopic varices on the basis of excluding non-cirrhotic portal hypertension (B, 1); (3) Imaging reveals cirrhosis or portal hypertension (B, 1); and (4) Meeting two or more of the four criteria: (a) PLT < 100 × 10^9/L without any other reasons; (b) ALB < 35 g/L, excluding malnutrition or kidney diseases; (c) INR > 1.3 or PT prolonged; (d) APRI > 2 (B, 1).

Recommendation 3: Diagnosis of decompensated cirrhosis: (1) Cirrhosis; and (2) Any one of the complications of portal hypertension including ascites, gastroesophageal varices hemorrhage, sepsis, hepatic encephalopathy, and hepatorenal syndrome (B, 1).

CIRRHOSIS-RELATED COMPLICATIONS

Serous effusion

Serous effusion of patients with cirrhosis includes ascites, pleural effusion, and pericardial effusion. Refer to the Chinese guidelines on the management of ascites and its related complications in cirrhosis for the diagnosis of cirrhotic ascites[6]. Chylous ascites (CA), hemorrhagic ascites and pleural effusion are discussed here.

The diagnosis of CA is based on the distinct characteristic of the ascitic fluid which includes a milky appearance and a triglyceride level > 200 mg/dL. Though CA can occur in all stages of cirrhosis, it is necessary to exclude other underlying etiologies such as trauma, congenital diseases, infections (especially pulmonary tuberculosis and filariasis), neoplasms, operations, or heart diseases.

Hemorrhagic ascites is diagnosed with a red blood cell count level > 50000/mm^3 in ascites. It is necessary to exclude other underlying etiologies such as tumor, severe infection (including tuberculous peritonitis), coagulopathy, and peritoneal varicose vein rupture.

Pleural effusion in patients with cirrhosis is more common in the right side, but can be bilateral when severe. It can be caused by etiologies other than cirrhosis, such as tuberculosis, which should be excluded. Diagnosis of pleural effusion can be based on chest ultrasonography or X-ray[30].

Gastrointestinal tract hemorrhage

Esophageal and/or gastric variceal rupture is the most common reason for gastrointestinal tract hemorrhage in patients with cirrhosis. See the guidelines for the diagnosis and treatment of esophageal and gastric variceal bleeding in cirrhotic portal hypertension 2016[5] for details. Other reasons include portal hypertensive gastropathy (PHG), portal hypertensive enteropathy(PHE), and internal hemorrhoid[31-34].

Spontaneous bacterial peritonitis and other infections

Refer to the relevant Chinese guidelines for the diagnosis[6]. In addition to spontaneous bacterial peritonitis, common infections in patients with cirrhosis include urinary, biliary, gastrointestinal, respiratory, skin soft tissue infections, and sepsis.

Hepatic encephalopathy

Refer to the relevant Chinese guidelines for the diagnosis[7].

Renal impairment

Renal impairment in cirrhosis patients includes acute kidney injury (AKI), hepatorenal syndrome-acute kidney injury (HRS-AKI), HRS-non-AKI (HRS-NAKI), and chronic kidney disease (CKD)[35,36].

The diagnosis of AKI is based on either of the two criteria[37]: Serum creatinine (Scr) within 48 h after admission increases ≥ 26.5 μmol/L (0.3 mg/dL) from baseline, or Scr within 7 d increases ≥ 50% from baseline (last available Scr within 3 mo can be taken as the baseline value).

The diagnostic criteria of HRS-AKI are as follows: (1) Patients with cirrhosis and ascites; (2) Patients meeting the criteria for AKI; (3) No response after discontinuing diuretics and plasma volume expansion by intravenous infusion of ALB at a dose of 1 g/kg for 48 h; (4) No shock; (5) Patients not using nephrotoxic drugs currently or recently; and (6) no signs of renal structural injury: (a) No proteinuria (< 500 mg/d); (b) No minor hematuria (< 50 red blood cells per high power field); and (c) Normal sonography of the kidneys.

HRS-NAKI[38] (including HRS-AKD and HRS-CKD) is diagnosed if: (1) Patients have cirrhosis with or without ascites; (2) HRS-AKI is excluded; and (3) Estimated glomerular filtration rate (eGFR) < 60 mL/min/1.73 m^2 in the absence of structural injury or Scr increases by < 50% from baseline (last available Scr within 3 mo can be taken as the baseline value).

CKD is defined as an eGFR level of < 60 mL/min/1.73 m^2 for 3 mo regardless of the structural injury of the kidneys.

Cirrhotic cardiomyopathy

Cirrhotic cardiomyopathy (CCM) is cardiac dysfunction characterized by suboptimal contractile response to stress and impaired diastolic function in the absence of previous cardiac diseases[39,40]. Given that most patients are asymptomatic in the initial stages of CCM, clinical, laboratory, electrocardiographic and imaging evaluations are necessary for early diagnosis. Diagnostic criteria of CCM are as follows: (1) Systolic dysfunction characterized by no increase of cardiac output induced by physical or pharmacological stress; (2) Diastolic dysfunction[41]: E/A ratio < 1.0, deceleration time > 200 ms, and isovolumic relaxation time > 80 ms; and (3) Supporting criteria: Electrophysiology abnormality, myocardial chronotropism, QT interval prolongation, non-synchronous electromechanical systole, left atrial enlargement, myocardial hypertrophy, brain natriuretic peptide and its precursor increased, and troponin increased.

Hepatopulmonary syndrome

Diagnostic criteria of hepatopulmonary syndrome (HPS) are detailed in the International liver transplant society practice guidelines: Diagnosis and management of HPS and portopulmonary hypertension 2016[42].

Other complications of cirrhosis

Other complications of cirrhosis include portal vein thrombosis (PVT) which can be classified into acute and chronic PVT[43-45], primary liver cancer[46], hepatic osteopathy[47] and cirrhotic amyotrophy[48,49].

Recommendation 4: AKI diagnosis: Scr within 48 h after admission increases by ≥ 26.5 μmol/L (0.3 mg/dL) from baseline, or Scr within 7 d increases by ≥ 50% than last available Scr within 3 mo or from the baseline value (B, 1). CKD diagnosis: regardless of structural injury of the kidneys, eGFR < 60 mL/min/1.73 m^2 lasts longer than 3 mo, and patients have refractory ascites (B, 1).

Recommendation 5: HRS-AKI diagnosis: (1) cirrhosis and ascites; (2) AKI; (3) No response after discontinuing diuretics and supplementing ALB (20-40 g/d) and expanding the blood volume for 48 h; (4) No shock; (5) No use of nephrotoxic drugs currently or recently; and (6) No evidence of structural injury of the kidneys (A, 1).

Recommendation 6: Diagnosis of HRS-NAKI: (1) cirrhosis with or without ascites; (2) HRS-AKI is excluded; and (3) eGFR < 60 mL/min/1.73 m^2 in the absence of structural injury or Scr increases by < 50% from baseline (last available Scr within 3 mo can be taken as the baseline value) (C, 1).

Recommendation 7: The incidence of abnormal electrophysiology in patients with cirrhosis is high, and screening and monitoring of cirrhotic cardiomyopathy should be emphasized (C, 1).

Recommendation 8: PVT includes acute and chronic PVT (B, 1).

Recommendation 9: Once cirrhosis is diagnosed, it is necessary to closely screen liver cancer (B, 1) by sonography and AFP test every 3-6 mo (C, 1).

Recommendation 10: Cirrhotic osteoporosis is positively correlated with the severity of liver disease. Bone density should be monitored in patients who are newly diagnosed with PBC or cirrhosis and in those after liver transplantation. Moreover, bone density should also be monitored in patients with a history of fragility fracture, postmenopausal women and those who use glucocorticoids in the long term (> 3 mo) (B, 2).

TREATMENT OF CIRRHOSIS

When patients are diagnosed with cirrhosis, comprehensive treatments should be commenced as soon as possible. If feasible, etiological treatment should be started immediately. Anti-inflammation and anti-hepatic fibrosis therapy are options for those who present persistent inflammation and/or fibrosis but are not suitable for or do not respond to etiological treatment. Prevention and treatment of complications play an important role in extending lifespan and improving life quality of patients with cirrhosis.

Etiological treatment

Etiological treatment is key for the treatment of cirrhosis whenever feasible. Detailed recommendations can be found in relevant Chinese guidelines[50-53] and Consensus[54-56]. In terms of immunoglobulin G4-associated cholangitis, immunosuppressant, interventional therapies or surgical intervention may be indicated[57].

D-penicillamine and trientine is indicated in cirrhosis patients due to Wilson's disease. In addition, these patients should avoid the intake of copper-rich foods. Oral zinc formulations (*e.g.*, zinc acetate, zinc gluconate) are recommended to reduce copper absorption[58,59].

For cirrhosis patients due to hemochromatosis, it is necessary to restrict the intake of iron-rich food and prohibit the transfusion of red blood cells. Other options include therapeutic venesection and iron chelators (*e.g.*, deferoxamine or deferasirox)[60].

Treatment of drug-induced cirrhosis is detailed in diagnosis and treatment guidelines on drug-induced liver injury[61].

Anti-inflammation and anti-hepatic fibrosis therapy

Anti-inflammation and anti-hepatic fibrosis therapy are indicated when patients present persistent inflammation and/or fibrosis but are not suitable for or do not respond to etiological treatment. The most commonly used anti-inflammation drugs include glycyrrhizic acid preparation, bicyclol, polyene phosphatidyl choline, silymarin, ademetionine and reduced glutathione[62]. Anti-fibrosis medicines include Anluohuaxian capsule, Fuzheng huayu capsule, and compound Biejiaruangan tablet[63-66].

Prevention and treatment of complications

Ascites: Refer to the Chinese guidelines on the management of ascites and its related complications in cirrhosis[6]. Combination of diuretics, ALB, and vasoconstrictor is recommended to treat refractory ascites.

For patients with chylous ascites, nutritional support with a low-salt, low-fat, medium chain triglyceride and high-protein diet and management of the underlying etiology are the cornerstones of therapy. When these measures fail, other interventions such as octreotide/somatostatin analogues, terlipressin, surgical ligation, embolization and transjugular, intrahepatic, portosystemic shunt (TIPS) can be considered[67-69].

For patients with hemorrhagic ascites, the key therapy is to control the basic causes[70,71]. When these measures fail, terlipressin and somatostatin can be used.

The treatment for cirrhotic patients with pleural effusion is similar to that for cirrhotic ascites. Treatment for chylous pleural effusion is similar to that for chylous ascites.

Gastrointestinal bleeding: Refer to Guidelines for the Diagnosis and Treatment of Esophageal and Gastric Variceal Bleeding in Cirrhotic Portal Hypertension 2016[5]. (1) Esophagogastric variceal bleeding, terlipressin, somatostatin and its analogs, and

pituitrin are used to treat patients with esophagogastric variceal bleeding. When drug therapy fails, other interventions can be considered including a Sengstaken-Blakemore tube, endoscopic variceal ligation or tissue glue injection, TIPS, and surgical therapy. High-risk patients with acute hemorrhage should receive TIPS therapy as early as possible (within 72 h). Balloon-occluded retrograde transvenous obliteration is preferred in patients with gastric variceal bleeding[72]; and (2) PHG and PHE bleeding: non-selective beta blocker (NSBB) is preferred to treat patients with bleeding due to PHG and PHE, and iron supplement is recommended[73,74]. Terlipressin, somatostatin, and its analogs can be considered[75-77].

Infections: Refer to the Chinese guidelines on the management of ascites and its related complications in cirrhosis[6] and Expert Consensus on Diagnosis and Treatment of End-stage Liver Diseases Complicated With Infections[78].

Norepinephrine is a first-line vasoactive drug for the treatment of infective shock. Bothe the dose of catecholamines and the risk of cardiac arrhythmia can be reduced when vasopressin (maximum dose 0.03 U/min) is added to norepinephrine[79]. With a longer half-life and similar effect, terlipressin is more effective in raising blood pressure and is more long-acting.

For patients with sepsis and severe infection, high-dose ALB can be combined with antibacterial agents.

Hepatic encephalopathy: Refer to the Guidelines on the management of hepatic encephalopathy in cirrhosis 2018[7].

Renal impairment: Refer to the Chinese guidelines on the management of ascites and its related complications in cirrhosis[6] and the Guidelines for Diagnosis and Treatment of Liver Failure (2018)[80].

Terlipressin combined with ALB is superior to placebo, ALB alone, octreotide or triple therapy with midodrine, octreotide and ALB in reversing HRS-AKI and HRS-NAKI and improving renal function[81-87]. Terlipressin at a dose of 1 mg per 4-6 h can be administered in combination with ALB (20-40 g per d) for 3 d. With a < 25% decrease of Scr level, the dose of terlipressin can gradually increase to 2 mg per 4 h. If effective (Scr decreases to < 133 μmol/L, along with the increase of arterial pressure, urine output and serum sodium level), the treatment duration is 7-14 d. Otherwise, terlipressin is discontinued. Norepinephrine (0.5-3.0 mg/h) in combination with ALB (10-20 g/L) can also be used.

TIPS can improve the renal function of patients with HRS-AKI and HRS-NAKI[88]. However, there are usually contraindications for TIPS in patients with HRS-AKI. Blood purification therapy can improve the renal function of some HRS-AKI patients. Liver transplantation is the preferred treatment for HRS-AKI and HRS-NAKI.

Cirrhotic cardiomyopathy: Current pharmacological treatment is not specific. Liver transplantation is the only proven treatment with specific effect on CCM[40,89].

HPS: Other than long-term supplemental oxygen, there are no effective therapies for HPS currently. The only definitive therapy is liver transplantation. Thus patients with HPS are recommended for liver transplant evaluation[90,91].

Portal vein thrombosis: Anticoagulant therapy or thrombolytic therapy can be adopted for patients with cirrhotic acute PVT. Low-molecular-weight heparin is preferred. Warfarin can be considered. Treatment duration ranges from 3 to 6 mo. Other interventions include TIPS, thrombolysis, and surgery. Individualized treatment is required for the treatment of chronic PVT[92-94].

Hepatic osteopathy: Diphosphonate can be used in patients with osteoporosis on the basis of calcium preparation and vitamin D. Oral alendronate sodium may lead to variceal bleeding. Zoledronic acid, a new intravenous bisphosphonate is effective in reducing fracture risk and does not have risk of variceal bleeding[95-98].

Nutritional support: Refer to the Clinical guidelines on nutrition in end-stage liver disease 2019, *etc*[7,23].

Nursing of digestive tract hemorrhage.

Recommendation 11: Etiological treatment is the key. If etiological treatment is not available or hepatic fibrosis persists or deteriorates after etiological treatment, anti-fibrosis therapy, such as Anluohuaxian capsule, Fuzhenghuayu capsule, and compound Biejiaruangan tablet can be used (B, 1).

Recommendation 12: In terms of refractory ascites, triple therapy including diuretics, ALB, and vasoconstrictors is recommended (B, 1).

Recommendation 13: In terms of cirrhotic chylous ascites or chylous pleural effusion, a low-salt, low-fat, medium chain triglyceride high-protein diet is recommended (B, 1). Terlipressin and somatostatin may be used (B, 2). A portal-systemic shunt procedure can be performed. If indicated, surgical intervention can be performed (C, 1).

Recommendation 14: For cirrhosis patients with hemorrhagic ascites, the primary treatment is to control the underlying causes. Terlipressin and somatostatin can be used (B, 2).

Recommendation 15: In the case of cirrhotic upper gastrointestinal hemorrhage, terlipressin, somatostatin analogs, proton pump inhibitor or H2 receptor blocker can be used (A, 1).

Recommendation 16: If drugs fail in treating cirrhotic esophageal and gastric variceal bleeding, the Sengstaken-Blakemore tube, endoscopic variceal ligation or tissue glue injection (B, 1), interventional therapies (C, 1) and surgery (C, 2) can be performed.

Recommendation 17: At 5 to 7 d after stopping cirrhotic gastrointestinal bleeding, secondary prevention should be performed with NSBB (A, 1) or carvedilol (B, 1). In patients with gastrointestinal bleeding and ascites, carvedilol is not recommended and the dose of NSBB should be reduced (B, 2).

Recommendation 18: In patients with PHG bleeding, NSBB and iron preparations are recommended (B; 1). In the case of acute hemorrhage, terlipressin or somatostatin analogs can be used (B; 2).

Recommendation 19: In cirrhosis patients with infections, empirical anti-infective therapy should be started as soon as possible. Based on the etiological results, switch to target therapy as soon as possible (B, 1).

Recommendation 20: In the case of sepsis, severe infection or shock, it is recommended to adopt triple therapy including antibacterial agents, ALB, and vasoactive drugs (B, 1).

Recommendation 21: HRS can be treated with terlipressin (1 mg/4-6 h) in combination with ALB (20-40 g/d) for 7 - 14 d, and the therapy can be repeated if HRS recurs (B, 1).

Recommendation 22: For HRS-NAKI patients with a large amount of ascites who do not respond to vasoconstrictors, TIPS can be performed (B, 1). TIPS is not recommended for HRS-AKI patients (C, 1).

Recommendation 23: For HRS-AKI patients who do not respond to vasoconstrictors, renal replacement therapy or artificial liver support can be selected. It is not recommended to perform renal replacement therapy in HRS-NAKI patients. HRS-AKI and HRS-NAKI patients should be preferentially included into the liver transplantation plan (B, 1).

Recommendation 24: There is no effective drug for cirrhotic cardiomyopathy. Patients should be included into the liver transplantation plan (B, 1).

Recommendation 25: There is no effective drug for HPS. Long-term supplemental oxygen is recommended (C, 1). Patients should be included into the liver transplantation plan (B, 1).

Recommendation 26: Anticoagulant therapy or thrombolytic therapy can be adopted for patients with acute PVT and progressive PVT(C, 1). Low-molecular-weight heparin alone or in combination with warfarin may be used (A, 1).

Recommendation 27: In terms of hepatic osteopathy or osteoporosis, bisphosphonates can be used on the basis of calcium preparation and vitamin D (C, 2). Nutritional support is important (B, 1).

PROBLEMS TO BE SOLVED

Test technology

(1) Smart reader of hepatic pathology; (2) Non-invasive monitoring of HVPG; (3) New tools of liver stiffness measurement regardless of ascites, jaundice or inflammation; and (4) Specific and sensitive test of MHE.

Diagnostic method and criteria

(1) Clarification of diagnostic criteria for recompensation and cirrhosis reversion; and (2) Early identification and diagnosis of HRS.

Therapeutic and preventive measures

(1) Evaluation of traditional Chinese medicine in anti-hepatic fibrosis and anti-cirrhosis; (2) Evaluation of diuretics, ALB, and vasoactive drugs in refractory ascites; (3) Evaluation of antibacterial agents, ALB, and vasoactive drugs in sepsis and severe infections; and (4) Primary and secondary prevention of cirrhotic upper gastrointestinal hemorrhage.

CONCLUSION

Liver cirrhosis is a substantial health burden worldwide. To promote diagnosis and treatment of cirrhosis, the CSH and the CSG of CMA developed the Chinese Guidelines for the diagnosis and treatment of cirrhosis complications including esophageal and gastric variceal bleeding, ascites, and hepatic encephalopathy recently[5-7]. However, there is a great need for updates with the development in these areas. In addition, some important topics, especially those on rare complications, have not been covered in those guidelines. Therefore, the present guidelines adds new sections and provides updates, focusing on the early identification of the causes, evaluation of disease severity, comprehensive assessment of complications, etiological treatment, and complication management. Lastly, problems to be solved are addressed.

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MINIREVIEWS

Role of pancreatography in the endoscopic management of encapsulated pancreatic collections – review and new proposed classification

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Abstract

Pancreatic fluids collections are local complications related to acute or chronic pancreatitis and may require intervention when symptomatic and/or complicated. Within the last decade, endoscopic management of these collections *via* endoscopic ultrasound-guided transmural drainage has become the gold standard treatment for encapsulated pancreatic collections with high clinical success and lower morbidity compared to traditional surgery and percutaneous drainage. Proper understanding of anatomic landmarks, including assessment of the main pancreatic duct and any associated lesions – such as disruptions and strictures – are key to achieving clinical success, reducing the need for reintervention or recurrence, especially in cases with suspected disconnected pancreatic duct syndrome. Additionally, proper review of imaging and anatomic landmarks, including collection location, are pivotal to determine type and size of pancreatic stenting as well as approach using long-term transmural indwelling plastic stents. Pancreatography to adequately assess the main pancreatic duct may be performed by two methods: Either non-invasively using magnetic resonance cholangiopancreatography or endoscopically *via* retrograde cholangiopancreatography. Despite the critical need to understand anatomy *via* pancrea-

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tography and assess the main pancreatic duct, a standardized approach or uniform assessment strategy has not been described in the literature. Therefore, the aim of this review was to clarify the role of pancreatography in the endoscopic management of encapsulated pancreatic collections and to propose a new classification system to aid in proper assessment and endoscopic treatment.

Key Words: Endoscopic retrograde cholangiopancreatography; Endoscopy; Endoscopic ultrasound; Pseudocyst; Endosonography; Pancreatic ducts

Core Tip: This review investigates the role of pancreatography in the endoscopic management of encapsulated pancreatic collections and proposes a new simplified classification system for endoscopic pancreatography findings as well as endoscopic management.

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INTRODUCTION

Pancreatic collections

Pancreatic and peripancreatic fluid collections (PFCs) are local complications due to acute or chronic pancreatitis and should be classified by the revised Atlanta Classification considering the time of presentation (more or less than 4 wk) and content (fluid alone or solid component). Before 4 wk, these collections are classified as acute, while after 4 wk collections are designated as late or chronic pancreatic collections. Once a fluid collection has become organized and developed a well-defined wall, these are then termed Encapsulated Pancreatic Collections (EPCs). EPCs are further subdivided into Pseudocyst – fluid containing only - and Walled-off Necrosis (WON) – containing the presence of fluid and a solid or necrotic content[1]. While a majority of these collections will resolve spontaneously, especially during the early phase (< 4 wk), persistent symptoms, complications, or infection may occur prompting treatment[2].

At present, there are 3 therapeutic approaches – surgery, percutaneous drainage and endoscopic drainage – for the treatment of EPCs, each of which may be used independently or in combination with another therapy. For many decades, surgery was considered the standard treatment modality and evolved from an open surgical technique to minimally invasive surgery, combining percutaneous drainage in a step-up manner[3]. More recently, the development of endoscopic drainage using endoscopic ultrasound (EUS) to achieve successful transmural drainage has overcome many complications related to surgery and percutaneous drainage and has demonstrated improved efficacy safety compared to more invasive approaches. At this time, endoscopic treatment of EPCs has become the first-line therapy for both pseudocyst and WON, when technically feasible[4,5].

Pancreatography

Since the 1970's pancreatography by Endoscopic Retrograde Cholangio-pancreatography (ERCP) has been reported as a useful tool for the management of pancreatic pseudocysts. In 1979, Sugawa *et al*[6] demonstrated pre-operative endoscopic pancreatography was a preferred strategy among 83 patients prior to surgical treatment of pseudocysts. In 1988, Nordback *et al*[7] again reported endoscopic pancreatography to be a useful tool to guide the best approach to PFCs, one that could predict response to percutaneous drainage or surgery. Since that time, from the 1990s and 2000s, pancreatography has helped clinicians determine if an endoscopic

transpapillary approach could be performed[8-10]. In addition to the potential therapeutic approach by transpapillary drainage, pancreatography has being reported to be an important prognostic factor to determine treatment success and recurrence, especially when Disconnected Pancreatic Duct Syndrome (DPDS) is diagnosed[11,12]. Along with ERCP, magnetic resonance cholangiopancreatography (MRCP) has increasingly become a non-invasive alternative to assess the main pancreatic duct (MPD), especially when secretin-enhanced is available. MRCP has the additional advantage of evaluating the MPD distal to a complete disruption and the pancreatic parenchyma; however, this imaging modality continues to have a lower sensitivity when compared to endoscopic pancreatography[13,14].

Disconnected pancreatic duct syndrome

DPDS was first described in 1989 by Smedh *et al*[15] in a case series of three patients[15]. It can be defined by (A) a complete MPD disruption and (B) a viable pancreatic tissue upstream from the disruption, resulting in a collection or fistula[11,16]. Therefore, in order to properly diagnose DPDS it remains essential to adequately assess the MPD and pancreatic parenchyma, usually performed by MRCP or computerized tomography (CT) combined with ERCP. DPDS has a tremendous impact on potential treatment and prognosis of EPCs and directly affects outcomes such as clinical success, recurrence, need for repeat interventions - including surgery - and duration of hospital stay. Thus, proper recognition and diagnosis of DPDS is fundamental in order to achieve the best outcomes for EPCs[11].

Objectives

The objective of this study was to perform a literature review including current recommendations and best practices regarding pancreatography and classifications in the context of endoscopic treatment of EPCs.

This review will be structured in to three main parts. First we aim to discuss the background information regarding pancreatography for EPCs, followed by our proposed classification, where we describe and propose a new practical and simple classification for pancreatography findings and their therapeutics implications. Lastly, we compare all previous classifications and our new proposed one and detail how this will aid endoscopists in daily practice and further improve standardization within the medical literature.

METHODS

All studies describing findings of pancreatography and the resulting endoscopic management of EPCs were included in this review. A protocolized search of MEDLINE (*via* PubMed) and Embase databases was performed through August 20, 2020.

The search strategy for MEDLINE was: "(Pancreatic duct OR Minor duodenal papilla OR Wirsung duct OR Wirsung's duct OR Cholangiopancreatography, endoscopic retrograde OR Cholangiopancreatographies, endoscopic retrograde OR ERCP) AND (Pancreatic pseudocyst OR Pancreatic pseudocysts OR Walled off necrosis)". All types of study were included.

After the initial search, duplicate studies were removed and selected studies were examined for information including: Indication and moment of pancreatography, study modality (*i.e.*, ERCP or MRCP), pancreatography findings and descriptors, pancreatography classification, and findings that directly influenced the plan to pursue an endoscopic approach. All relevant information was extracted using Excel spreadsheets for future analysis.

BACKGROUND

Indication

Since most PFCs will resolve spontaneously, there was no indication measure to routinely evaluate the MPD. Although pancreatography is not always considered for the evaluation of EPCs, the general consensus at this time is that pancreatography should be performed for symptomatic patients with EPC that will undergo endoscopic intervention[11,17]. Yet, despite its importance, consensus and guideline recommen-dations remain highly variable. Currently, the European Society Gastroin-testinal

Endoscopy (ESGE) recommends pancreatography for WON that undergo endoscopic treatment[18]; however, there are no recommendations regarding pancreatic pseudocysts. At present, the American Society for Gastrointestinal Endoscopy does not comment on the topic or importance of pancreatography in its most recent guideline[2]. The Asian EUS group experts guideline implicitly recommends pancreatography suggesting pancreatic duct stent for partial disruption and acknowledging higher recurrence rates among patients with MPD disruption[19]. The rational to evaluate the MPD *via* pancreatography – either by ERCP or MRCP - for all cases of EPCs treated endoscopically is to appropriately assess for DPDS, and to assist endoscopists as to which lesions should or may benefit from treatment[20]. Thus, pancreatography may impact therapeutic, diagnostic, and prognostic outcomes for the management of EPCs and should always be performed in this context[21].

Time

The decision as to when to perform pancreatography remains a highly controversial topic. Many individuals may prefer pancreatography prior to endoscopic drainage[22], peri-procedurally at the same time as drainage[17], or post-drainage[23]. Authors advocating for pancreatography prior to drainage typically perform MRCP to evaluate both the collection and the MPD – allowing for information gathering, planning of the therapeutic approach, and potentially avoiding an unnecessary ERCP and complications related to it[20,24,25]. The rational for performing pancreatography at the same procedure as endoscopic drainage is to optimize the approach in a single procedure, which may result in a shorter hospital stay and lower overall cost[26]. It should be noted that this approach may not be feasible in cases of gastric outlet obstruction due to inflammation which may precludes passage of duodenoscope. In regards to pancreatography post-drainage, this strategy may provide the added advantage of increased accuracy given compression by the pancreatic collection and local inflammation may limit interpretation of the MPD prior to drainage[14,23]. Although concerns have been raised regarding ERCP in the setting of a PFC, studies have shown it to be a safe procedure with no negative impact[17]. Presently, the ESGE recommends pancreatography, either by MRCP (preferably) or ERCP, prior to transmural stent removal after endoscopic drainage[18]. At this time, there is no prospective study comparing the ideal strategy or time to perform pancreatography, with the decision largely driven by expert consensus, provider familiarity, anecdotal evidence, or institution protocol.

Study modality

As discussed previously, pancreatography should be performed *via* either ERCP (Supplementary Video 1) or MRCP[27]. CT has been reported as an option to evaluation of the MPD; however, its accuracy is less than ideal and not adequate to rule out MPD lesions[28]. Therefore, these authors do not currently recommend the use of CT to evaluate the pancreatic duct. At present, ERCP remains the gold standard to perform pancreatography due to higher sensitivity to detect ductal leaks when compared to MRCP and may be cost-effective and more convenient since it can be performed at this same time as other endoscopic procedures or drainage[14,23]. Yet despite these advantages of ERCP, it is not without certain limitations including the invasiveness of approach and risk for complications, including pancreatitis, bleeding, and perforation – and may not be able to accurately evaluate the MPD distal to a total disruption.

MRCP has the advantage of being a non-invasive exam, without significative associated adverse events and enables investigation of the MPD distal to a complete disruption and the pancreatic parenchyma – fundamental for the diagnosis of DPDS. Furthermore secretin-enhanced MRCP has been shown to increase the sensitivity for MPD disruptions[29]; however, this may not be widely available at most institutions. Currently, MRCP is recommended as the preferable method to evaluate the MPD after endoscopic drainage by the ESGE[18].

More recently, EUS has also been reported to be an effective alternative method to closely provide a detailed assessment of the MPD in the context of PFCs, although the sensitivity and specificity remains poorly evaluated to date[11,30]. Thus, these authors believe it is reasonable to perform a secretin-enhanced MRCP as the first line strategy to evaluate the pancreatic duct, if available[20]. Otherwise endoscopic pancreatography *via* an ERCP approach should be performed as the procedure of choice with patients fully aware of the potential for adverse events, though these remain acceptably low[17,31-34].

Descriptors

Despite the importance of pancreatography, description of findings is largely heterogeneous and not uniform in the current literature. Although some terms are often used by various authors and clinicians, terminology and descriptor language has not been standardized[35]. The most commonly utilized terms to describe abnormalities in literature are: Disruption (some authors dived into partial/incomplete and total/complete disruptions), disconnection, DPDS, transected, leak, fistula, rupture, stricture, stenosis, cut-off, obstruction and communication/non-communication with collection[9,13,14,18,19,22,36-40]. This heterogeneity may lead to confusion when reporting and interpreting data[8]. Although some terms are presumed to have the same meaning - such as partial disruption and partial leak, complete disruption and disconnection, cut-off and obstruction, stricture and stenosis – others seem to be uncertain - such as disruption, rupture, transection. It is also critically important to underscore that DPDS is an incorrect term to describe endoscopic pancreatography findings. The complete disruption of the MPD is one of two necessary conditions to diagnose DPDS. An image study showing a functional pancreatic tissue upstream to the complete disruption is necessary to define DPDS[11,40]. Therefore, ERCP alone cannot appropriately describe this phenomena; however, when pancreatography is performed by MRCP it is possible to diagnose DPDS since it allows study the MPD upstream the disruptions and the pancreatic tissue[13].

Classifications

Five classifications on pancreatography findings have been described. The main characteristics of these classifications are summarized on Table 1. One was published in India[23], one in Italy[35], two in the United States by the same group[37,41] and one in Finland[7].

The first study to classify findings on pancreatography was a Finnish retrospective study published in 1988[7]. This group analyzed 15 patients with pancreatic pseudocysts who had undergone endoscopic pancreatography and were treated either by surgery or percutaneous external drainage. These authors then identified five patterns noted on pancreatography and classified these findings into three types, two of them with two subtypes (Figure 1). Based on the results observed, Nordback and colleagues suggested the best approach for each pancreatography type. Type I would benefit from percutaneous drainage, Type II from conservative management for 12 wk, and Type III from internal drainage (usually by surgery) or caudal pancreatic resection.

In the United States, Nealon *et al*[37,41] published two retrospective studies in 2002 and again in 2009, showing the impact pancreatography in the context of pancreatic pseudocyst to determine the best approach and estimate prognosis[37,41]. The second study[37], that can be interpreted as an updated of the first one[41], analyzed 563 patients with pseudocysts that underwent ERCP, MRCP, or contrast injection within an external drain placed percutaneous or surgically and described four pancreatography types (Figure 2). Type I findings would benefit most from endoscopic or percutaneous drainage; Type II recommending endoscopic management; and types III and IV planned for surgical intervention.

More recently, in 2017, Mutignani *et al*[35] published a review on pancreatic fistulae and proposed a complete classification considering etiology and pancreatography, recommending an endoscopic approach for each type. These authors first divided pancreatic fistulas into three possible etiologies. Type I and type III were not related to pancreatitis and are beyond of the scope of this review. However, Type II were classified as injury to the MPD, usually related to PFCs and were dived into "open proximal stump" (IIO) and "closed proximal stump" (IIC) (Figure 3). For Type IIO, Mutignani and colleagues suggested bridging stent (first choice), transpapillary stent, or nasopancreatic drainage. For Type IIC, these authors recommended transmural EUS-drainage of the caudal collection with plastic stents, EUS-guided pancreaticogastrostomy, or a conversion to an IIO type and then treat accordingly.

In an prospective series of 88 patients with symptomatic WON, Dhir *et al*[23] demonstrated EUS-drainage with metal stents and pancreatography was performed *via* ERCP and MRCP. This group proposed four types on pancreatography using findings of ERCP and MRCP (Figure 4) and showed higher recurrence when there was MPD disconnection, regardless of whether WON was proximal (Type I) or distal (Type II) to the disconnection.

Approach to pancreatography findings

Endoscopic approaches based upon pancreatography findings continue to be

Table 1 Classifications of pancreatography findings

Ref.	Study object	Study objective	Descriptors	Classification	Practical implication
Dhir *et al*[23], 2018	EUS-drained WON	Pancreatography patters in WON and collection recurrence	-Duct disconnection; -Leaks	-Type I: Disconnection in the neck/body region, with a ductal leak at the proximal end; -Type II: Disconnected duct with a WON distal to the disconnection. It is not possible to ascertain the ductal communication of WON; -Type III: ductal leak without disconnection; -Type IV: Shows a noncommunicating WON, with no disconnection	Recurrence is higher in patters w/ disconnection (types I and II): -Type I: 5/35 patients (14.3%)–62.5% of recurrences; -Type II: 2/18 patients (11.1%) - 25% of recurrences; -Type III: 0/26 patients (0%) - 0% of recurrences; -Type IV: 1/8 patients (12.5%)–12.5% of recurrences
Mutignani *et al*[35], 2017	All pancreatic fistulas	Guide endoscopic approach	-Leakages; -Disruption (partial); -Disconnection (total)	-Type I: Leakages from small side brunches. IH: head | IB: body | IT: tail; -Type II: Leak in the MPD Open (IIO) or Close (IIC); -Type III: leaks after pancreatectomy; IIIP: Proximal pancreas (after distal pancreatectomy); IIID: Distal pancreas (after pancreaticoduodenectomy)	-IH and IB: Bridging OR NPD; -IT: Bridging OR cianoacrilate/fibrin/glue/polymer injection at pancreatic tail; -IIO: Bridging OR NPD OR transpapillary stent; -IIC: EUS transmural drain of collection from excluded gland OR EUS pancreaticogastrostomy OR Conversion to IIO and treat as IIO; -IIIP: Transpapillary stent; -IIID: Few endoscopic options. EUS transmural drainage OR nasojejunal drain at the level of dehiscence in continuous aspiration
Nealon *et al*[37], 2009	Pseudocyst due to pancreatitis[1]	Guide the best approach: endoscopic, interventional radiology or surgical intervention	-Normal[2]; -Stricture; -Chronic pancreatitis; -Occlusion; -Communication / no communication with collection	-Type I for normal ducts, IA: No communication, IB: With communication; -type II for duct strictures; IIA: no communication; IIB: with communication; -Type III for duct occlusion or disconnected duct syndrome; IIIA: no communication; IIIB: with communication; - Type IV for changes of chronic pancreatitis; IVA: no communication, IVB: with communication	-Type I: Endoscopic or percutaneous management; unlikely to require operation; -Type II: Endoscopic management depending on the magnitude and length of the stricture - transpapillary stents for selected ducts; -Type III and type IV: Surgical intervention exclusively
Nealon *et al*[41], 2002	Pseudocyst[1] that underwent pancreatography by ERCP	Guide the best approach between percutaneous drainage or surgical intervention	-Normal[2]; -Strictures; -Complete cutoff; -Chronic pancreatitis;-MPD-pseudocyst communication or not	-Type I: normal duct/no communication with cyst; -Type II: normal duct with duct–cyst communication; -Type III: otherwise normal duct with stricture and no duct–cyst communication; -Type IV: otherwise normal duct with stricture and duct–cyst communication; -Type V: otherwise normal duct with complete cut-off; -Type VI: chronic pancreatitis, no duct–cyst communication; -Type VII: chronic pancreatitis with duct–cyst communication	-Type I: consider percutaneous drainage (PD); -Type II: avoid PD; -Type III: consider PD treatment; -Type IV: surgery (avoid PD); -Type V: surgery (avoid PD); -Type VI: surgery (avoid PD); -Type VII: surgery (avoid PD)
Nordback *et al*[7], 1988	Pseudocyst[1] that underwent pancreatography by ERCP	Guide the best approach	-Stenosis; -Pseudocyst opens to the duct; -Pseudocyst is filled	-Type I: MPD is imaged up to the end without much stenosis, Pseudocyst may (Type IA) or may not (IB) be filled, but is further away from the main pancreatic duct; -Type II: no main duct stenosis and pseudocyst opens to the duct; -Type III: stenosis of the main pancreatic duct, + filling of the pseudocyst behind the stenosis (IIIA), or not (IIIB)	Type I: PD is a good option; Type II: expectant management for 12 wk, if persistent: Internal drainage (PD, endoscopically, surgery); Type III: Internal drainage (external drainage contraindicated); caudal resection

[1]Pseudocyst before Revised Atlanta Classification could involve heterogeneous types of collections.

[2]"Normal duct" means duct without chronic pancreatitis changes. EUS: Endoscopic ultrasound; WON: Walled-off Necrosis; MPD: Main pancreatic duct; PD: Percutaneous drainage; NPD: Nasopancreatic drain.

controversial. Some individuals advocate transpapillary drainage *via* pancreatic stenting for all MPD leaks and disruptions and combined with transmural drainage[20,21,23]. However, it should be noted that transpapillary drainage alone may be considered in specific cases where transmural drainage is not technically possible and there are favorable anatomical features – such as small collection, location in the head or uncinate process of the pancreas, and in cases with evidence of communication with the MPD[12,26]. A meta-analysis including 9 studies, with a total of 604 procedures,

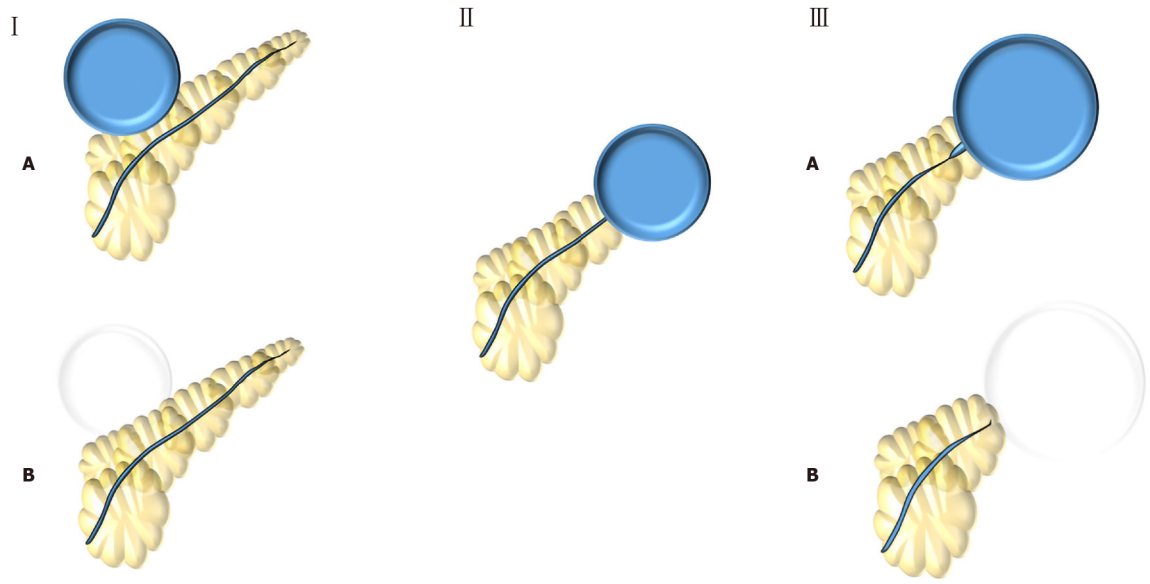

Figure 1 Nordback *et al*[7] (1988) classification. Type I: Normal main pancreatic duct (MPD) contrasting (type IA) or not (type IB) the collection; Type II: MPD opens to the collection; Type III: MPD with stenosis contrasting (type IIIA) or not (type IIIB) the collection.

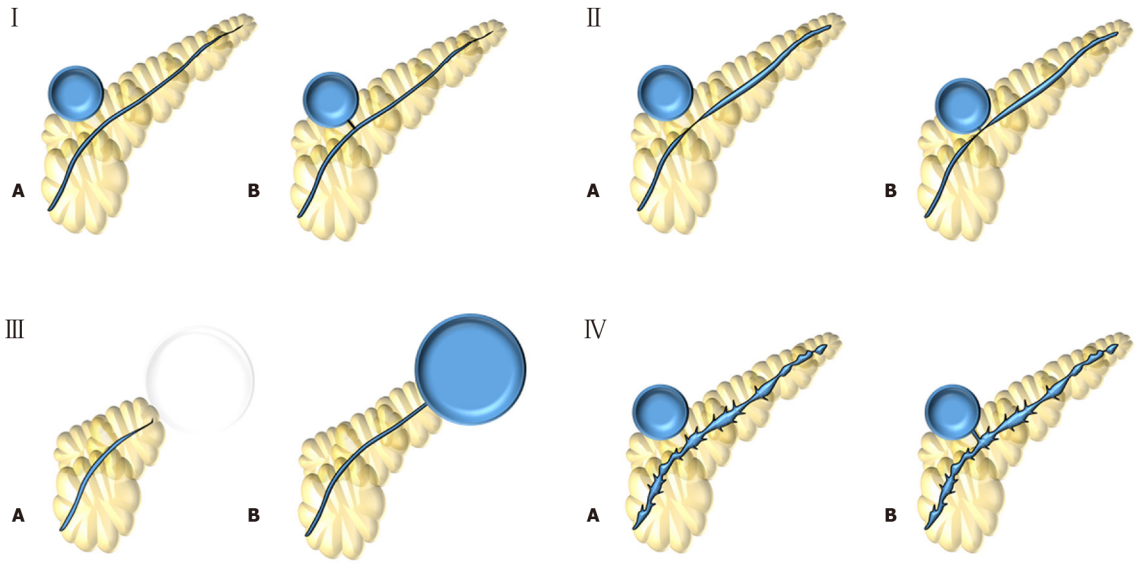

Figure 2 Nealon *et al*[37] (2009) classification. Type I: Normal main pancreatic duct (MPD); Type II: MPD stricture; Type III: MPD occlusion; Type IV: Chronic pancreatitis. All types are subdivided according if they have communication (subtype A) or not (subtype B) with the collection.

concluded that combined drainage with transmural and transpapillary approach does not have any benefits regarding technical success, clinical success, nor recurrence when compared to transmural drainage alone[42]. These findings are important but highly contestable since the majority (7 out of 9) of included studies were retrospective and they did not analyze the results by different pancreatography patterns. Other studies have shown better outcomes when a partial disruption have been treated by pancreatic stent bridging of the MPD[21,43,44] with this strategy currently recommended by the Asian guidelines consensus and considered an option by ESGE[18,19,45].

The optimal management of DPDS also remains controversial. Surgery is still the gold standard treatment, though it is associated with a considerable morbidity and cost[46,47]. Most authors agree that pancreatic stenting is not effective for DPDS and many advocate for long-term transmural indwelling plastic stents – also recommended by ESGE[13,18,48]. Although complications related to long-term transmural indwelling plastic stents have been reported, including migration, gastrointestinal obstruction, perforation, infection, and bleeding, these occurrences are usually mild. Thus, it is

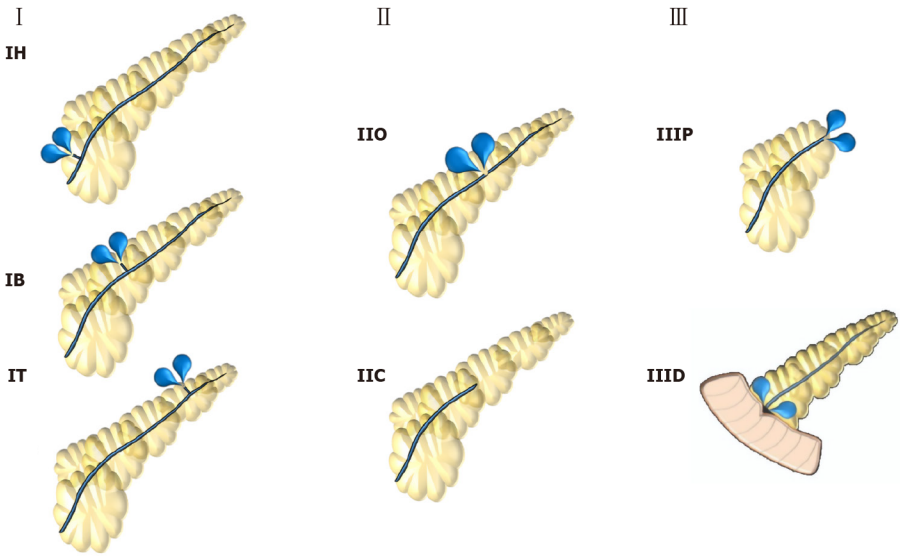

Figure 3 Mutignani *et al*[35] (2017) classification. Type I: Leakages from small side brunches in the pancreatic head (IH), body (IB) or tail (IT); Type II: Leak in the main pancreatic duct that may have an open (IIO) or close (IIC) proximal stump; Type III: Leaks after pancreatectomy that may be after proximal pancreas (IIIP) or distal pancreas (IIID) resection.

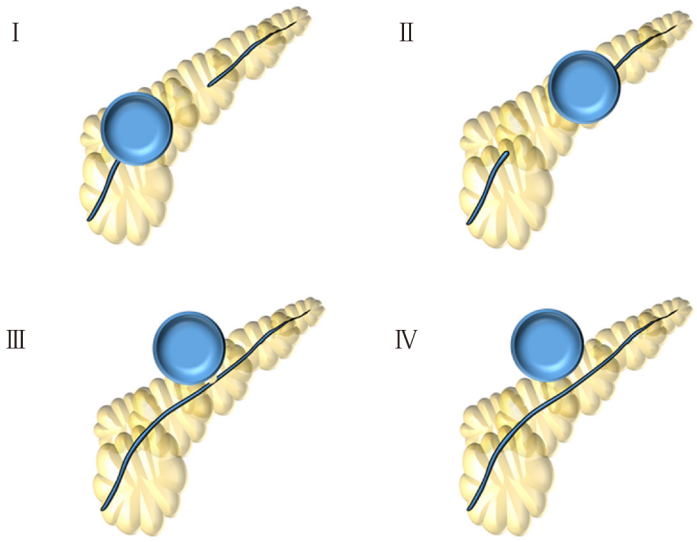

Figure 4 Dhir *et al*[23] (2018) classification. Type I: Disconnection in the neck/body region, with a ductal leak at the proximal end; Type II: Disconnected duct with a Walled-off Necrosis distal to the disconnection – not possible to ascertain ductal communication with collection; Type III: Ductal leak without disconnection; Type IV: Shows a noncommunicating Walled-off Necrosis, with no disconnection.

considered a safe and effective method to prevent recurrence in patients with DPDS[36,38,49]. EUS-guided transluminal-MPD drainage has been reported for external pancreatic fistulas and may be an option for selected patients with DPDS that possess a dilated MPD[50,51]. Recently, Basha *et al*[52] questioned the real importance of transluminal indwelling stenting for DPDS in a study with 274 patients with WON that underwent endoscopic drainage[52]. These authors reported a recurrent rate of 13.2%, in which 97% had DPDS, but only 6.6% (17 patients) required reintervention. This study also suggested that patients with DPDS should be followed and treated if a symptomatic recurrent collection occurs instead of performing any treatment to prevent those recurrences.

Additionally, strictures of the MPD may be treated using pancreatic stenting[8,20,28,40,53]. Although this remains a reasonable approach, there is no comparative study demonstrating the impact of stricture treatment for EPCs management. Currently, the lack of prospective controlled studies comparing the role of pancreatography findings makes most the current recommendations weak with an

overall low-quality of evidence. Therefore, it is necessary to standardize pancreatography findings for better communication and to enable high-quality prospective controlled studies considering those different findings in order to clarify the best endoscopic management towards MPD injuries in the context of EPCs.

NEW CLASSIFICATION PROPOSITION

The classifications found in the literature, despite having value, are burdensome, overly complex, and difficult to apply during routine examinations. Therefore, the translation of these schemes to real-world clinical practice, or even standard for research reporting purposes has remained limited. As such, designing a simple, practical, and applicable classification system to standardize endoscopic pancreatography findings in the context of endoscopic treatment of EPCs is needed. Here we propose a new easy to apply classification for endoscopic pancreatography findings (Figure 5) with translation of these findings to impact endoscopic management (Table 2).

Type I involves a normal MPD, without stricture or disruption (Figure 6A). Therefore, no additional therapy is required. Type II demonstrates a stricture within the MPD (Figure 6B). We recommend treatment involving a pancreatic stent through the area of stenosis. Type III involves a partial disruption of the MPD – the MPD contrasts beyond disruption point (Figure 6C). In these cases, pancreatic stent bridging the rupture should be performed. Type IV shows a complete disruption of the MPD – the MPD does not contrast beyond disruption point. It may be presented with contrast extravasation (Type IV-A) (Figure 6D) or without contrast extravasation and abrupt cut-off (Type IV-B) (Figure 6E). Type IV should alert for the possibility of DPSP and an image study - such as CT or MRI - must be performed to confirm or rule out DPDS. If DPSD is confirmed, long-term transmural indwelling plastic stents should be considered. It is also critically important to recognize that more than one type may be presented simultaneously, such as a pancreatography demonstrating a stricture and a complete disruption with contrast extravasation (Figure 6F) - classified as a type II + IV-A.

DISCUSSION

Classifications are important tools used frequently in all fields of medicine, helping to categorize finding, standardize treatment-specific approaches, and facilitate ease of communication between providers. Furthermore, the better the attempt at classification (*i.e.*, the ability for conditions to fit within pre-determined criteria), the more applicable and clinically relevant these can by to everyday clinical practice. Reviewing literature, there is not any current classification system allow for this to occur – further highlighting why no descriptions and increased confusion regarding the role of pancreatography is present in the literature.

It is well established that EUS-guided transmural drainage is the gold standard approach for both pseudocyst and WON[4,5]. Thus, pancreatography classifications that attempt to guide the best approach – surgery, percutaneous drainage, or endoscopic drainage - no longer have clinical relevance. At present, there is not sufficient evidence or data in the proposed classifications by Nordback[7] and Nealon[37,41], to guide clinicians and endoscopists regarding the best approach decision.

Since EUS-drainage is the gold standard treatment for EPCs, pancreatography classification should ultimately be used to determine the best endoscopic approach. Mutignani´s classification[35] is the only one among the previous classification systems that attempts to guide endoscopic approach accordingly to the findings. Yet despite this, limitations remain.

The endoscopic approaches towards MPD remain controversial in literature since there is no prospective randomized trial comparing the decision to treat MPD lesions. While some retrospective studies and case series suggest better outcomes when a partial disruption of the MPD is treated with a bridging pancreatic stent[12,21,43,44], this data has not yet been studied in prospective studies. Additionally, another important point is to distinguish between a partial and a complete disruption of the MPD. Only the system devised by Dhir *et al*[23] dedicated a specific category (type III) for partial disruption of the MPD.

DPDS has been reported as an important condition that is underdiagnosed – related to an increased need for reintervention, surgery, longer hospital stay, and higher

Table 2 Lera-Proença new proposed classification for endoscopic pancreatography findings

Types	Finding	Endoscopic approaches
Type I	Normal MPD	No additional therapy
Type II	Stricture	Consider pancreatic stent
Type III	Partial disruption (MPD contrasts beyond disruption point)	Pancreatic stent bridging the rupture
Type IV	Complete disruption (MPD does not contrast beyond disruption point), A: With contrast extravasation; B: Without contrast extravasation and abrupt cut-off	CT or MRI to confirm or rule out DPDS; Consider long-term transmural indwelling plastic stents

MPD: Main pancreatic duct, CT: Computerized tomography; MRI: Magnetic resonance imaging; DPDS: Disconnected pancreatic duct syndrome.

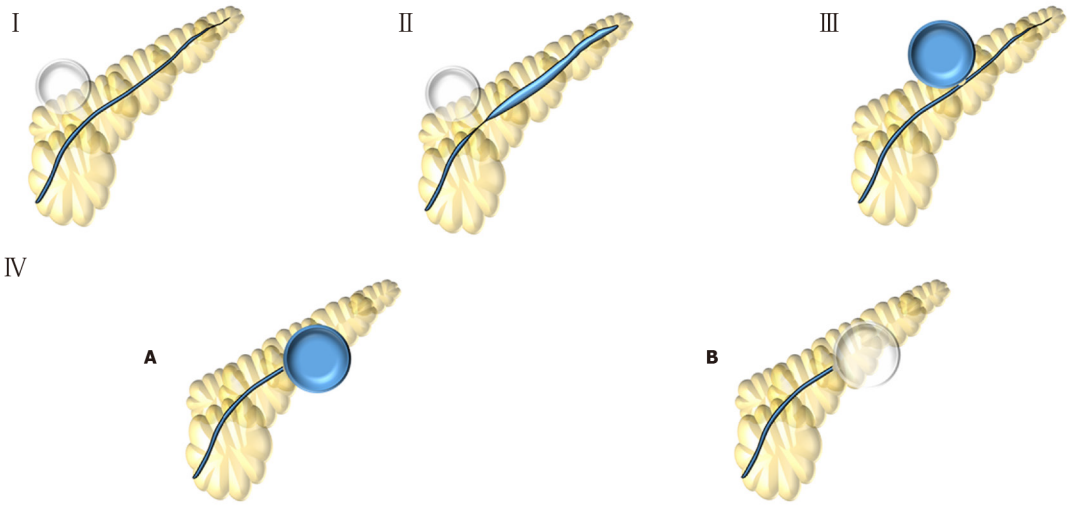

Figure 5 Lera-Proença (2020) new proposed classification. Type I: Normal main pancreatic duct; Type II: Stricture; Type III: Partial disruption – main pancreatic duct contrasts beyond disruption; Type IV: Complete disruption - main pancreatic duct does not contrast beyond disruption. IV-A: with contrast extravasation or IV-B: without contrast extravasation and cut-off.

recurrence[11,48]. Therefore, it remains essential that any pancreatography classification define and categorize lesions with increased ability to differentiate and diagnose DPDS. Among previous classifications, Dhir´s[23] was the only one to correlated properly pancreatography findings and DPDS.

Our new proposed classification aims to determine the best endoscopic treatment based upon pancreatography findings, clearly distinguish between partial and total disruption and suggests cases which should warrant investigation for DPDS. Additionally, this classification system as designed by these atuhors is based upon on endoscopic pancreatography findings, making it easier and more applicable than Dhir´s classification that requires additionally imaging with MRCP. A comparative table between all classifications and the crucial points is presented in Table 3.

CONCLUSION

Evaluation of the MPD *via* pancreatography in the context of endoscopic treatment of EPCs may provide diagnostic, therapeutic, and prognostic implications and should therefore be performed for all cases. This may be performed by ERCP or MRCP, preferably with contrast-enhanced secretin when available. While optimal timing (pre-drainage, peri-drainage, or post-drainage) has not been determined, assessment of the duct, regardless of when, remains key. Although some pancreatography classification have been proposed, none is widely used in literature, likely due to non-standardized approaches or outdated practices not relevant to the modern endoscopist for the management of EPCs. Additionally, it is critically important to understand the significance of DPDS, make a clear distinction between partial and complete MPD disruption, and determine the best endoscopic approach based upon pancreatography

Table 3 Comparation between pancreatography classifications

Ref.	Study modality	Guide endoscopic approach?	Category for partial MPD disruption?	Diagnosis or suspicion of DPDS?
Proença, 2020	ERCP	Yes	Yes	Yes
Dhir *et al*[23], 2018	ERCP + MRCP	No	Yes	Yes
Mutignani *et al*[35], 2017	Not specified	Yes	No	No
Nealon *et al*[37], 2009	ERCP	No	No	No
Nordback *et al*[7], 1988	ERCP	No	No	No

ERCP: Endoscopic retrograde cholangiopancreatography; MRCP: Magnetic resonance cholangiopancreatography; MPD: Main pancreatic duct; DPDS: Disconnected Pancreatic Duct Syndrome.

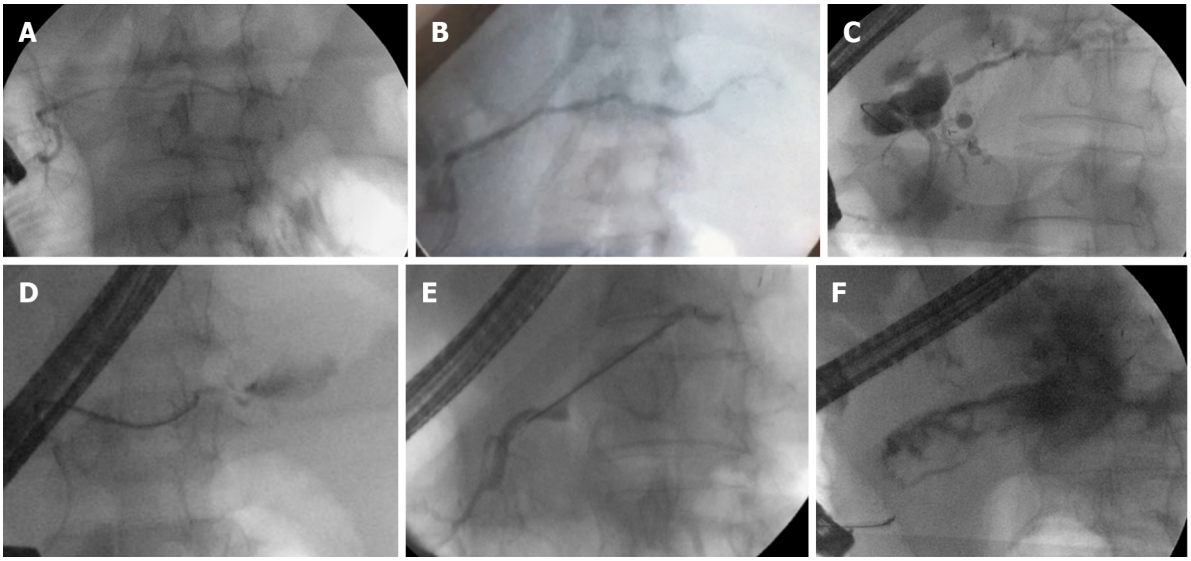

Figure 6 Endoscopic pancreatography classified by Lera-Proença classification. Endoscopic pancreatography findings, A: Normal pancreatography (type I); B: Stricture (type II); C: Partial disruption (type III); D: Complete disruption with contrast extravasation (type IV-A); E: Complete disruption without contrast extravasation and cut-off (Type IV-B); and F: Stricture and complete disruption with contrast extravasation (Type II + IV-A).

findings. Therefore, we propose a simplified and practical classification system to report the findings of pancreatography, improve uniformity for future research, inform guidelines and clinical management, and ultimately guide endoscopic treatment of EPCs.

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ORIGINAL ARTICLE

Basic Study

Discovery of unique African *Helicobacter pylori* CagA-multimerization motif in the Dominican Republic

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Abstract

BACKGROUND

Helicobacter pylori (*H. pylori*) colonizes the human stomach and is a major cause of peptic ulcer disease and gastric cancer. However, although the prevalence of *H. pylori* is high in Africa, the incidence of gastric cancer is low, and this phenomenon is called to be African enigma. The CagA protein produced by *H. pylori* is the most studied virulence factor. The carcinogenic potential of CagA is

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associated with the Glu-Pro-Ile-Tyr-Ala (EPIYA) patterns and CagA-multimerization (CM) motifs.

AIM

To better understand the EPIYA patterns and CM motifs of the *cagA* gene.

METHODS

Gastric mucosal biopsy specimens were obtained from 258 patients with dyspepsia living in the Dominican Republic, from which 120 *H. pylori* strains were cultured. After the bacterial DNA extraction, the EPIYA pattern and CM motif genotypes were determined using a polymerase chain reaction-based sequencing. The population structure of the Dominican Republic strains was analyzed using multilocus sequence typing (MLST). Peptic ulcer disease and gastric cancer were identified *via* endoscopy, and gastric cancer was confirmed by histopathology. Histological scores of the gastric mucosa were evaluated using the updated Sydney system.

RESULTS

All *CagA*-positive strains carried the Western-type CagA according to the identified EPIYA patterns. Twenty-seven kinds of CM motifs were observed. Although the typical Western CM motif (FPLKRHDKVDDLSKVG) was observed most frequently, the typical East Asian CM motif (FPLRRSAAVNDLSKVG) was not observed. However, "FPLRRSAKVEDLSKVG", similar to the typical East Asian CM motif, was found in 21 strains. Since this type was significantly more frequent in strains classified as hpAfrica1 using MLST analysis (P = 0.034), we termed it Africa1-CM (Af1-CM). A few hpEurope strains carried the Af1-CM motif, but they had a significantly higher ancestral Africa1 component than that of those without the Af1-CM motif (P = 0.030). In 30 *cagA*-positive strains, the "GKDKGPE" motif was observed immediately upstream of the EPIYA motif in the EPIYA-A segment, and there was a significant association between strains with the hpAfrica1 population and those containing the "GKDKGPE" motif (P = 0.018). In contrast, there was no significant association between the CM motif patterns and histological scores and clinical outcomes.

CONCLUSION

We found the unique African CM motif in Western-type CagA and termed it Africa1-CM. The less toxicity of this motif could be one reason to explain the African enigma.

Key Words: *Helicobacter pylori*; CagA; *CagA* 3' region; CagA-multimerization motif; Glu-Pro-Ile-Tyr-Ala motif; Dominican Republic

Core Tip: We found the unique African (hpAfrica1-type) CagA-multimerization (CM) motif in Western-type CagA in the Dominican Republic and termed it Africa1-CM (Af1-CM). In addition, a few hpEurope strains carrying the Af1-CM motif had a significantly higher ancestral Africa1 component than those without the Af1-CM motif. This result suggests that the ancestors of these hpEurope strains having the Af1-CM motif underwent genetic recombination with the hpAfrica1 strains in the past. In contrast, there was no significant association between the CM motif patterns and histological scores and clinical outcomes.

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INTRODUCTION

Helicobacter pylori (*H. pylori*) plays an essential role in the development of various gastroduodenal diseases including gastric cancer[1]. However, only a small proportion of people infected with *H. pylori* develop these diseases. Interestingly, the incidence of gastric cancer in Africa is much lower than that in other countries, despite the high prevalence of *H. pylori* infection in this area[1]. This contradictory phenomenon is known as the "African enigma". Geographic differences in the incidence of gastric cancer can be explained, at least in part, by the presence of different types of *H. pylori* virulence factor, especially CagA, VacA and OipA[1].

The *H. pylori* CagA protein is one of the well-known pathogenic virulence factors involved in the pathogenesis of gastric cancer[1]. The *cagA* gene encodes a 120-145 kDa CagA and is located at one end of the *cag* pathogenicity island (PAI) that encodes the type IV secretion system (TFSS)[2]. *CagA* can directly translocate into host gastric epithelial cells *via* the TFSS and undergoes tyrosine phosphorylation by Src family kinases and then binds to various molecules including the Src homology 2-containing protein tyrosine phosphatase-2 (SHP-2)[3-5]. Gastric mucosal epithelial cells form a monolayer of polarized cells through tight junctions. When CagA binds to the kinase domain of partitioning-defective 1b (PAR1b)/microtubule affinity-regulating kinase 2 (MARK2), it destroys the tight junctions in a tyrosine phosphorylation-independent manner and causes loss of epithelial cell polarity[6]. CagA multimerizes in cells independently of tyrosine phosphorylation, and a CagA-multimerization (CM) sequence consisting of 16 amino acids has been identified as its responsible region[7]. There is also a report that the CM sequence is called conserved repeat responsible for phosphorylation-independent activity[8]. This CM sequence is essential for binding between CagA and PAR1. PAR1 forms a homomultimer in the cell. Thus, CagA forms a multimer indirectly through binding to the PAR1 multimer and binds to SHP-2 after being tyrosine-phosphorylated[7].

The tyrosine phosphorylation site of CagA is characterized by the presence of a unique Glu-Pro-Ile-Tyr-Ala (EPIYA) motif, which is present in multiple numbers in the C-terminal region of CagA[1]. The EPIYA-repeat region is composed of various combinations of four distinct EPIYA segments, EPIYA-A, -B, -C and -D, based on the sequences surrounding each of the EPIYA motifs[9]. *H. pylori cagA*-positive strains isolated in Western countries possess the Western CagA, which contains EPIYA-A, EPIYA-B, and EPIYA-C segments, whereas that in East Asian countries possess the East Asian CagA, which contains EPIYA-A, EPIYA-B and EPIYA-D segments. We previously showed that the East Asian CagA was more virulent than the Western CagA[10]. The EPIYA-D motif of the East Asian CagA exhibits a stronger SHP-2 binding affinity than the EPIYA-C motif of the Western CagA[9]. In addition, the number of EPIYA-C sites is directly correlated to the level of tyrosine phosphorylation and SHP-2 binding activity among the Western CagA species.

Eleven of the 16 amino acids in the CM motif are well conserved between the Western and East Asian *cagA* species[7]. The peptide sequence of the typical Western CM motif (W-CM), which is observed in the Western *cagA*, is "FPLKRHDKVD DLSKVG" and that of the typical East Asian CM motif (E-CM), which is observed in the East Asian *cagA*, is "FPLRRSAAVNDLSKVG"[11,12]. In the Western *cagA* species, each EPIYA-C segment contains a single CM motif, and there is also another copy of the CM motif immediately distal to the last repeat of the EPIYA-C segment. In the East Asian *cagA* species, there is a CM motif that is located immediately distal to each repeat of the EPIYA-D segment. Thus, the Western *cagA* carries at least two W-CM sequences, whose number increases in parallel with the number of EPIYA-C segments. The ability of the Western CagA to bind to PAR1 is proportional to the number of W-CM sequences[11]. Meanwhile, a single E-CM sequence has almost twice the PAR1 binding ability of the single W-CM sequence.

We have previously reported that all 64 *H. pylori* strains isolated in 2011 in the Dominican Republic carried the Western *cagA* specific sequences[13]. However, this report did not mention the CM motif. In the subsequent study in the Dominican Republic, we have already performed a multilocus sequence typing (MLST) analysis of seven housekeeping genes for a total of 119 *H. pylori* strains, which were collected in 2011 and 2016, followed by population structure analysis using STRUCTURE software[14]. Therefore, *H. pylori* strains of the Dominican Republic were divided into two populations: 68 strains with hpAfrica1 [46, 5, and 17 with hspWAfrica, hspSAfrica, and a hybrid (hspWAfrica/hpEurope), respectively], and 51 strains with hpEurope (47 and 4 with hspEuropeS and hspEuropeN, respectively). The ethnics in the Dominican Republic consists of 16% of European, 11% of African, and 73% of mixed race[15]. Therefore, it is important to investigate the genetic diversity of the *cagA* gene in the

Dominican Republic, where human demography consists of various ancestries. In this study, we evaluated the correspondence between the CM motif and phylogeographical classification using MLST population structure in *H. pylori* in the Dominican Republic. We also examined the relationship between these types and gastric mucosal damages.

MATERIALS AND METHODS

Study population and DNA extraction

We recruited outpatients with mild dyspeptic symptoms living in the Dominican Republic. Gastric mucosal biopsy specimens were taken from 258 dyspeptic patients (158 in 2011 and 100 in 2016; 86 males and 172 females; age range, 17-91 years; mean age, 46.2 ± 15.8 years) who underwent endoscopy examination at the Digestive Disease Center (Dr Luis E. Aybar Health and Hygiene City, Santo Domingo, Dominican Republic). Patients with a history of partial gastric resection or previous treatment for *H. pylori* infection were excluded. During each endoscopy session, 4 gastric biopsy specimens were obtained (three from the antrum and one from the corpus). The three specimens from the antrum were used for *H. pylori* culture, rapid urease test and histological examination. The specimen from the corpus was used for histological examination.

Gastric biopsy specimens for histological examination were embedded in paraffin after being fixed in 40 g/L formaldehyde. Hematoxylin and eosin and May-Giemsa were selected as stain techniques. The updated Sydney system was used to analyze histological severity[16]. The degree of the bacterial load was classified into four grades: 0, "normal"; 1, "mild"; 2, "moderate"; and 3, "marked". Peptic ulcer and gastric cancer were identified *via* endoscopy, and gastric cancer was confirmed by histopathology. Gastritis was diagnosed in the absence of peptic ulcer or gastric malignancy. For *H. pylori* culture, antral biopsy specimens were homogenized and inoculated onto antibiotic selection plates, and then subcultured on Mueller Hinton II Agar medium (Becton Dickinson, Sparks; MD, United States) supplemented with 7% horse blood without antibiotics. The plates were incubated up to 10 d at 37 °C under microaerophilic conditions (100 mL/L O_2, 50 mL/L CO_2, and 850 mL/L N_2). *H. pylori* isolates were identified based on colony morphology, Gram staining results, and oxidase, catalase, and urease reactions. Isolated strains were stored at −80 °C in *Brucella* broth (Becton Dickinson, Sparks; MD, United States) containing 10% dimethyl sulfoxide and 10% horse serum. Bacterial DNA was extracted using a commercially available kit (QIAGEN Inc.; Valencia, CA, United States).

Eventually, 64 strains were cultured from 158 patients in 2011, and 56 strains were cultured from 100 patients in 2016, thus a total of 120 *H. pylori* strains were obtained. The ethnicity of the 120 patients, based on self-assessment at the time of medical examination, was 114 multiracial (32 males, 82 females) and 6 Africans (3 males, 3 females).

Analysis of H. pylori population structure

A detailed description of *H. pylori* population structure methods has already been published in our previous study[14].

Analysis of H. pylori cagA

The *cagA* gene was determined using polymerase chain reaction PCR-based sequencing as described previously[10,17]. The absence of *cagA* was confirmed by the presence of *cagA* empty site, as previously described[18]. The *cagA* genotype (East Asian-type and Western-type) was confirmed by sequencing the PCR products as described previously[19]. The EPIYA-A region and CM motif from the strains were compared using the program WebLogo (version 3.7.4) (http://weblogo.threeplusone.com/).

Statistical analysis

Statistical significance was tested using Fisher's exact test and Wilcoxon rank-sum test performed in the R package (version 3.4.0). A P value of < 0.05 was accepted as statistically significant.

RESULTS

H. pylori infection rate and endoscopic findings

We performed endoscopy for 258 dyspeptic patients. The prevalence of *H. pylori* infection determined *via* histological examination was 67.8% (175/258). Clinical diagnosis of them were 219 cases with chronic gastritis, 38 cases with peptic ulcer and 1 case with gastric cancer. From 258 dyspeptic patients, a total of 120 strains were isolated (120/258, 46.5%). Of these, 93 strains were isolated from subjects with chronic gastritis, 26 from peptic ulcer subjects, and 1 from a gastric cancer subject (Supplementary Table 1).

H. pylori population structure

As we have already reported[14], the Dominican Republic strains were assigned to either hpAfrica1 (57.1%, 68/119) or hpEurope (42.9%, 51/119) (Supplementary Table 1). From the result of the STRUCTURE run of linkage model at K = 4, the four major ancestral components reported in the previous studies were observed: Ancestral Africa1, ancestral Europe 1, ancestral Europe 2, and ancestral East Asia.

CagA sequences and EPIYA phosphorylation motif patterns

In total, 84 of 120 (70.0%) strains possessed the *cagA* gene. The *cagA* gene was not detected in one strain; however, this strain contained partial *cag* PAI genes, as confirmed by *cag* PAI empty site. Thus, we considered this strain as "*cagA* undetermined" (Supplementary Table 1). The prevalence of *cagA* was compared between the clinical outcomes of patients (Table 1). No significant difference was found between gastritis and peptic ulcer patient groups. All 84 *cagA*-positive strains were classified as Western CagA: ABC (n = 72), ABCC (n = 7), AC (n = 2), AABC (n = 1), AB (n = 1), and ABBC (n = 1). Interestingly, in 30 *cagA*-positive strains, the "GKDKGPE" motif was observed immediately upstream of the EPIYA motif in the EPIYA-A segment, which was not seen in the typical Western-type and East Asian-type CagA (Figure 1, Supplementary Table 1).

CM motifs and patterns

In total, 172 CM motifs were found in 84 *cagA*-positive strains (82 for 1st CM motif, 83 for 2nd CM motif, and 7 for 3rd CM motif). These CM motifs were classified into 27 types. Twelve types were observed in the 1st CM motif, 19 types in the 2nd CM motif, and 3 types in the 3rd CM motif (Table 2). The CM motif type that had the highest number of appearances was the typical W-CM motif (FPLKRHDKVDDLSKVG) observed in strains circulating in Western countries[20], with 45 found in the 1st CM motif, 31 found in the 2nd CM motif, and 4 found in the 3rd CM motif. Interestingly, no typical E-CM motif (FPLRRSAAVNDLSKVG) was observed in strains circulating in East Asian countries. Furthermore, the Amerindian CM motif (Am-CM: xxLKRxAKVDDLxKxG and YTLKMHAGDDNLRSKVG) of Amerindian CagA was not observed[21,22]. However, "FPLRRSAKVEDLSKVG", which was found in 21 strains similar to the typical E-CM motif, was found in the 2nd and 3rd CM motifs. Amino acid sequence comparison with the GenBank BLAST data (http://www.ncbi.nlm.nih.gov/BLAST/) indicated that the "FPLRRSAKVEDLSKVG" sequence mainly matched with the sequences of strains in the African (Senegal, South Africa, and Gambia) and American (United States, Colombia, Mexico, and Cuba) continents (Supplementary Table 1). In previous studies[12,20,23], this motif was classified as a subtype of the typical E-CM motif. Interestingly, there were no East Asian and Amerind strains in the list of matches from the GenBank BLAST search. As reported in our previous study[14], many of the strains used in this study were African type (hpAfrica1) (Supplementary Table 1). Therefore, we hypothesized that "FPLRRSAKVEDLSKVG" is the typical Africa1 CM (Af1-CM) motif specific to a strain derived from Africa (hpAfrica1 strain). A previous study[11] noted that W-CM and E-CM varied at positions 4, 6, 7, 8 and 10 (FPLxRxxxVxDLSKVG). In the type classification of CM motif in this study, we focused on these five sequences, and when four or more positions of the sequences of a strain matched the typical W-CM, we designated a classification criterion that this strain belongs to W-CM. Similarly, when four or more positions of the sequences of a strain match the typical E-CM or the typical Af1-CM, we designated a classification criterion that this strain belongs to E-CM or Af1-CM, respectively. Other types of sequences not classified by these criteria were assigned as different CM (D-CM).

As shown in Table 2, in the 1st CM motif, 76 strains were W-CM and 6 were D-CM. In the 2nd CM motif, 37 strains were W-CM, 34 were Af1-CM, 1 was E-CM and 11 were D-CM. In the 3rd CM motif, 5 strains were W-CM and 2 were Af1-CM. Table 3 shows

Table 1 Association between the prevalence of *cagA* and clinical outcomes

	Total	Gastritis	Peptic ulcer	Gastric cancer
Number of strains	120	93	26	1
cagA positive	84 (70.0%)	66 (71.0%)	18 (69.2%)	0
cagA negative	35 (29.2%)	26 (28.0%)	8 (30.8%)	1 (100%)
cagA undetermined	1 (0.008%)	1 (1.1%)	0	0

Table 2 Peptide sequences and types of CagA-multimerization motif in *Helicobacter pylori* strains

First motif			Second motif			Third motif		
Peptide sequences	**Type**	**No.**	**Peptide sequences**	**Type**	**No.**	**Peptide sequences**	**Type**	**No.**
FPLKRHDKVDDLSKVG	W-CM	45	FPLKRHDKVDDLSKVG	W-CM	31	FPLKRHDKVDDLSKVG	W-CM	4
FPLKRHDKVEDLSKVG	W-CM	17	FPLRRSAKVEDLSKVG	Af1-CM	21	FPLRRSAKVEDLSKVG	Af1-CM	2
FPLKRHAKVDDLSKVG	W-CM	4	FPLKKHAKVEDLSKVG	D-CM	7	FLLKRHDKVDDLSKVG	W-CM	1
FPLKKHAKVEDLSKVG	D-CM	3	FPLRRSAKVEDLSKAG	Af1-CM	4			
FPLKKHDKVDDLSKVG	W-CM	3	FPLKKHDKVEDLSKVG	W-CM	2			
FPLKKHDKVEDLSKVG	W-CM	3	FPLKRSAKVEDLSKVG	Af1-CM	2			
FPLKKHAKVDDLSKVG	W-CM	2	FPLKRSAKVDDLSKVG	D-CM	2			
FLLKRHDKVDNLSKVG	W-CM	1	FPLRRSAKVDDLSKVG	Af1-CM	2			
FPLRRSDKVDDLSKVG	D-CM	1	FPLKRYDKVDNLSKVG	W-CM	2			
FPLKKHAKVEDLSEVG	D-CM	1	FPLKRHDKVEDLSKVG	W-CM	1			
FPLKRHDKIDDLSKVG	W-CM	1	FPLKKHDKVDDLSKVG	W-CM	1			
FPLKKHAKVEDLSKAG	D-CM	1	FPLRRSTKVEDLSKAG	Af1-CM	1			
Nothing (Deletion)		2	FPLRRSAAVDDLSKVG	E-CM	1			
			FPLRRGAKVEDLSKVG	Af1-CM	1			
			FPLRKSAKVEDLSKVG	Af1-CM	1			
			FPLRRSDKVDNLSKVG	D-CM	1			
			FPSKKHAKVEDLSKVG	D-CM	1			
			FPFRRSDKVEDLSKVG	Af1-CM	1			
			FPLRRSDKVEDLSKVG	Af1-CM	1			
Total		84	Total		83	Total		7

The CagA-multimerization (CM) motif types were determined based on comparison with the typical Western CM motif (FPLKRHDKVDDLSKVG), the typical East Asian CM motif (FPLRRSAAVNDLSKVG), and the typical Africa1 CM motif (FPLRRSAKVEDLSKVG). W-CM: Western CM motif; E-CM: East-Asian CM motif; Af1-CM: Africa1-CM motif; D-CM: Different CM motif.

the combinations of the 1st, 2nd and 3rd CM motifs. The three frequent types were W-CM:W-CM (n = 30), followed by W-CM:Af1-CM (n = 27), and W-CM:D-CM (n = 10).

Number of strains containing Af1-CM, D-CM and E-CM as observed in the GenBank BLAST data

To count the appearance frequencies of 27 kinds of CM motifs observed in the Dominican Republic in each country of the world, a GenBank BLAST search was performed. The results are shown in Supplementary Table 2. The three W-CM peptide sequences of FPLKRHDKVDDLSKVG, FPLKRHDKVEDLSKVG and FPLKKHDKVDDLSKVG had more than 100 matches in the database, therefore, it was difficult to create a detailed list. However, these three peptide sequences were contained in strains around the world, with no regional bias.

Table 3 *CagA*-multimerization motif patterns in *Helicobacter pylori* strains

CM motif pattern	Occurrence	Frequency (%)
W-W	30	35.7
W-Af1	27	32.1
W-D	10	11.9
D-Af1	5	6.0
W-W-W	5	6.0
W-Af1-Af1	2	2.4
-W	2	2.4
D-D	1	1.2
W-E	1	1.2
W-	1	1.2
Total	84	

CagA-multimerization (CM) motif patterns were determined based on the identification of CM sequences that were located before and after the (Glu-Pro-Ile-Tyr-Ala)-C motif. W-W: Strains with two Western CM motifs in their CagA; W-Af1: Strains with one Western and one Africa1 CM motif in their CagA; W-D: Strains with one Western and one Different CM motif in their CagA; D-Af1: Strains with one Different and one Africa1 CM motif in their CagA; W-W-W: Strains with three Western CM motifs in their CagA; W-Af1-Af1: Strains with one Western and two Africa1 CM motifs in their CagA; -W: Indicates that only one Western CM motif was observed following the Glu-Pro-Ile-Tyr-Ala (EPIYA)-C motif, in the absence of a CM motif before the EPIYA-C motif; D-D: Strains with two Different CM motifs in their CagA; W-E: Strains with one Western and one East Asian CM motif in their CagA; W-: Indicates that only one Western CM motif was observed before the EPIYA-C motif, in the absence of a CM motif following the EPIYA-C motif.

Relationship between cagA genotypes and phylogeographical classification using MLST population structure

Of the 68 hpAfrica1 strains, 61 were *cagA* positive (92.8%), 6 were *cagA* negative (9.8%), and one could not determine the presence of *cagA* (Supplementary Table 1). In contrast, of the 51 hpEurope strains, only 23 were *cagA* positive (45.1%) and 28 were *cagA* negative (54.9%). The difference in *cagA* prevalence between the hpAfrica1 and the hpEurope strains was significant ($P < 0.001$, Fisher's exact test). Next, to verify whether the Af1-CM motif was related to the hpAfrica1 strain, it was compared with the phylogeographical classification using MLST population structure. Among 34 strains containing Af1-CM in the 2nd CM motif, 29 were hpAfrica1 (85.3%) and 5 were hpEurope (14.7%). In contrast, of the 37 strains containing the W-CM motif in the 2nd CM motif, 23 were hpAfrica1 (62.2%) and 14 were hpEurope (37.8%). The difference in CM motif type between the hpAfrica1 and the hpEurope strains was significant ($P = 0.034$, Fisher's exact test). Next, among 23 *cagA*-positive strains of hpEurope, the ratio of the ancestral Africa1 component, which was calculated using the STRUCTURE run of linkage model, was compared between 5 strains having Af1-CM motif and 18 strains without Af1-CM motif. From the result, the 5 strains having the Af1-CM motif had a significantly higher ancestral Africa1 component than the 18 strains without the Af1-CM motif ($P = 0.030$, Wilcoxon rank-sum test; Figure 2).

"GKDKGPE" motif unique to hpAfrica1 strains

Harrison *et al*[24] hypothesized that the "KDKGPE" motif in the EPIYA-A segment of some Western CagA strains could be a unique African motif. Beltrán-anaya *et al*[25] have also noted the appearance of "GKDKGPE" in some Mexican strains. Amino acid sequence comparison with the GenBank BLAST data (http://www.ncbi.nlm.nih.gov/BLAST//BLAST/) indicated that the "GKDKGPEEPIY AQVNK" sequence observed in many of the strains in this study was mainly matched with the sequences of strains in the African (Senegal, Gambia, and Sudan) and American (United States, Colombia, Mexico, and Cuba) continents. To verify whether the "GKDKGPE" motif was related to the hpAfrica1 strain, it was compared with the phylogeographical classification using MLST population structure. Among 30 strains containing the "GKDKGPE" motif, 28 were hpAfrica1 and 2 were hpEurope. In contrast, after excluding the two AC-type strains, among the 52 strains without the "GKDKGPE" motif, 33 were hpAfrica1 and 19 were hpEurope. The presence of the "GKDKGPE" motif was significant between the hpAfrica1 and the hpEurope strains ($P = 0.003$,

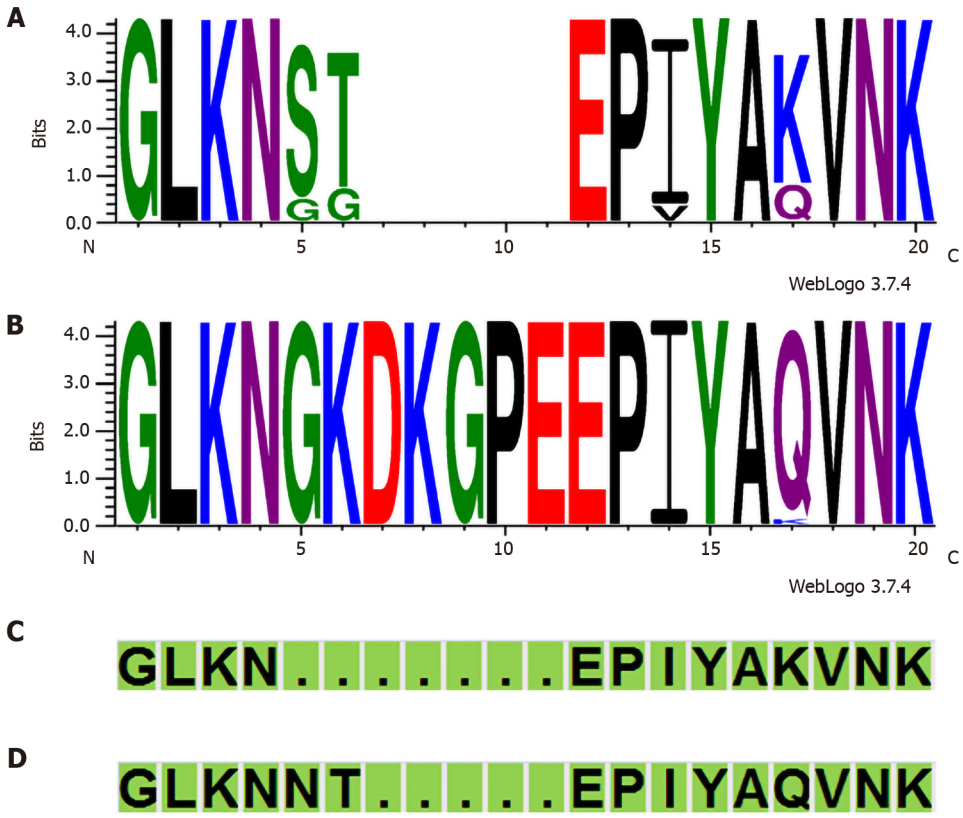

Figure 1 Variation in the *CagA* (Glu-Pro-Ile-Tyr-Ala)-A region from the Dominican Republic strains. A: CagA Glu-Pro-Ile-Tyr-Ala (EPIYA)-A region in 52 strains without containing "GKDKGPE" motif (excluding two AC-type strains); B: CagA EPIYA-A region in 30 strains containing "GKDKGPE" motif. The reference sequences[19] are shown at the bottom of the figure (green); C: Most frequent Western-type CagA EPIYA-A region; D: Most frequent East Asian-type CagA EPIYA-A region.

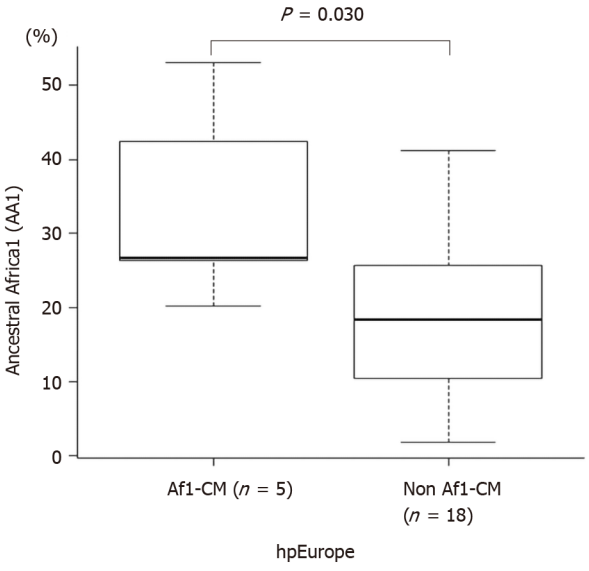

Figure 2 Box plot diagram of the ancestral Africa1 components in the hpEurope population classified by the presence or absence of the Africa1 CagA-multimerization motif. The difference in the ancestral Africa1 ratio between the bacterial populations was investigated using the Wilcoxon rank-sum test. AA1: Ancestral Africa1; Af1-CM: Africa1 CagA-multimerization.

Fisher's exact test). In addition, another characteristic was observed in the four amino acid sequences immediately upstream of the EPIYA motif in the EPIYA-A segment among the 30 strains containing the "GKDKGPE" motif; 29 were QVNK and 1 was KVNK (Supplementary Table 1). In contrast, among 52 strains in which the "GKDKGPE" motif was not conserved, 12 strains were QVNK, and 40 strains were KVNK. The presence of the "GKDKGPE" motif was significant between the QVNK and KVNK motifs ($P < 0.001$, Fisher's exact test).

Relationship between the CM and "GKDKGPE" motifs

Among 37 strains containing the W-CM motif in the 2nd CM motif, the "GKDKGPE" motif was conserved in 3 strains and not in 34 strains (Supplementary Table 1). In contrast, among 34 strains containing the Af1-CM motif in the 2nd CM motif, the "GKDKGPE" motif was conserved in 22 strains and not in 12 strains. The presence of the "GKDKGPE" motif was significant between the two CM motif types in the 2nd CM motif ($P < 0.001$, Fisher's exact test).

CM motif patterns and gastric disorders

Histological scores according to the CM motif patterns are shown in Supplementary Table 3. No significant difference was observed between the two groups in all histological scores. Association of CM motif patterns with clinical outcomes is shown in Supplementary Table 4. No correlation was found between clinical results and CM motif patterns.

Nucleotide sequencing

Sequencing data for the *cagA* genes of *H. pylori* is available under DDBJ accession numbers AB860373-AB860407, AB860409-AB860411, and LC546765-LC546810.

DISCUSSION

All 84 *cagA*-positive strains in this study were classified as Western CagA. The hpAfrica1 strains had a significantly higher ratio of having the *cagA* gene than the hpEurope strains ($P < 0.001$, Fisher's exact test). This ratio was similar to that of the previous study[26]. Twenty-seven kinds of CM motifs were observed in 84 *cagA*-positive strains isolated in the Dominican Republic, which is higher in number than those of reports in many countries around the world[21,27-30]. In contrast, this rich variety of CM motifs in the Dominican Republic was similar to that of reports in Colombia and New York[12,23]. The CM motif type with the highest number of occurrences was the typical W-CM motif, but no typical E-CM motif or Am-CM was observed. Interestingly, a large number of CM motifs (FPLRRSAKVEDLSKVG) having a sequence similar to that of the typical E-CM motif were observed in the 2nd CM motif (also in some 3rd CM motifs), and we termed it Africa1-CM (Af1-CM). The proportion of Af1-CM in the 2nd CM motif in the hpAfrica1 strains was significantly higher than that in the hpEurope strains ($P = 0.034$, Fisher's exact test). This significant difference supported our hypothesis that Af1-CM is a unique CM motif in an African-derived strain (hpAfrica1). This Af1-CM does not appear in the 1st CM motif but seems to appear in the 2nd or 3rd CM motif. Interestingly, no strain with the same sequence as the five kinds of Af1-CM subtypes was found in the African continent using the GenBank BLAST search (Supplementary Table 2). Thus, although similar in sequence to the original Af1-CM motif, these strains may not be of African origin and hence further investigation is required. No typical E-CM (FPLRRSAAVNDLSKVG) was observed in the 84 *cagA*-positive strains isolated in the Dominican Republic, but a subtype of E-CM (FPLRRSAAVDDLSKVG), one amino acid difference from E-CM, was found. However, the fourth Arginine and the sixth Serine were also included in the sequence of the typical Af1-CM, and the tenth was Aspartic acid instead of Asparagine specific to E-CM. Therefore, if different classification criteria are set, this motif may be classified into the Af1-CM subtype. Seven kinds of CM motifs were classified into D-CM. Since these motifs contain amino acids characteristic of W-CM and Af1-CM respectively, their origin may be a recombinant strain, or a completely different ancestral strain that has not yet been surveyed. The same reason may be assigned to why many types of subtypes of W-CM, E-CM, and Af1-CM appeared.

Olbermann *et al*[26] compared the CM motifs of 38 representative *cagA*-positive strains of the world. Among them, strain CC42C (FPLRRSAKVEDLSKVG, hpAfrica1, South Africa) and strain D3a (FPLKRSAKVEDLSKVG, hpAfrica1, Senegal) had the same motif as Af1-CM proposed by our group. The fact that the phylogeographical

classification types of two strains are hpAfrica1 supports our Af1-CM hypothesis. Strain LSU2003-1 (FPLRKSAKVDDLSKVG, hpAfrica1, United States) is classified as D-CM according to the classification criteria defined in our study, but this strain also contains some amino acids characteristic of Af1-CM.

Sicinschi *et al*[12] reported that of the 42 Western CagA (ABC) strains collected in Colombia, 13 had the E-CM motif, of which 12 were collected from the low-risk gastric cancer region (residents of Pacific coast). According to the classification criteria defined in our study, it follows that 13 strains classified as E-CM in their paper are classified as Af1-CM. Thus, these observations in Colombia support our hypothesis. Hatakeyama[20,31] considered in a review paper that the 1st CM and 2nd CM of two strains of Western CagA (ABC) collected in Mexico were both E-CM, which is a rare case. However, this E-CM motif has the same sequence as the typical Af1-CM motif proposed by our group. It makes sense when we consider that these strains are descendants of African strains from the slave trade era. Ogorodnik *et al*[23] reported that 27 out of 42 Western CagA strains collected at a New York Hospital had an E-CM motif. In addition, when compared with the clinical diagnosis, the strain containing E-CM motif was less toxic than that containing W-CM motif. The main E-CM motifs of these strains are FPLRRSAKVEDLSKVG, FPLRRSAKVDDLSKVG, and FPLRKSAKVELSKVG, which are the same motifs as Af1-CM proposed by our group. The majority of *H. pylori* infected people treated at this New York hospital are immigrants from the Caribbean, such as Jamaica, the Dominican Republic, and Haiti. Thus, it also makes sense when we consider that these strains are descendants of African strains from the slave trade era.

Ahire *et al*[32] created both E-CM motif-transfected and W-CM motif-transfected strains and then compared the toxicities of these strains by testing with gastric epithelial cells. The E-CM motif used in that cloning study had the same sequence as the typical Af1-CM motif proposed by our group. This study also reported that strains with the E-CM motif (Af1-CM motif in the classification criteria in our paper) induced less hummingbird elongation than strains with the W-CM motif. Nesić *et al*[33] showed that the third Leucine, fifth Arginine, ninth Valine, and twelfth Leucine of the 14 amino acid CM motifs were four critical amino acid residues involved in the MARK2 kinase binding domain of CagA, *via* crystal structure analysis using X-ray diffraction. In the Af1-CM motif that we found in our study, these four amino acids were always conserved, similar to the E-CM and W-CM motifs, and thus it does not seem to affect this interaction.

Harrison *et al*[24] hypothesized that the "KDKGPE" motif in the EPIYA-A segment of some Western CagA strains could be a unique African motif. Amino acid sequence comparison with the GenBank BLAST data indicated that the "GKDKGPEEPIY AQVNK" sequence observed in many strains in this study mainly matched with sequences of strains in the African and American continents. Furthermore, there was a significant association between strains with the hpAfrica1 population and those containing the “GKDKGPE” motif (P = 0.003, Fisher’s exact test). These results supported the hypothesis by Harrison *et al* that "GKDKGPE" is a unique motif in an African-derived strain (hpAfrica1). In addition, many of the four amino acid sequences immediately downstream of the “GKDKGPEEPIYA” motif were “QVNK” (P < 0.001, Fisher’s exact test). According to Xia *et al*[19], “KVNK” predominates in the Western CagA (ABC), while “QVNK“ predominates in the East Asian CagA (ABD). Interestingly, all CagA types in our study are Western CagA. Therefore, “QVNK” dominates the four amino acid sequences immediately downstream of the “GKDKGPEEPIYA” motif of the Western CagA strain derived from Africa (hpAfrica1) having the “GKDKGPE” motif.

In our study, a few hpEurope strains had the Af1-CM and “GKDKGPE” motifs. *H. pylori* frequently undergoes recombination among unrelated strains[34,35]. Among the hpEurope strains, having the Af1-CM motif had a significantly higher ancestral Africa1 component than not having it (P = 0.030, Wilcoxon rank-sum test). This result suggests that the ancestors of the hpEurope strains that had the Af1-CM motif underwent genetic recombination with the hpAfrica1 strains in the past.

In this study, no association was found between the CM motif patterns and gastric disorders. According to previous studies[12,23], *H. pylori* strains with Af1-CM are less toxic compared to strains with the W-CM motif. Thus, the less toxicity of Af1-CM motif could be one reason to explain the African enigma. Twenty-seven kinds of CM motifs were observed in 84 strains of the Western-type CagA in the Dominican Republic. In the future, it is necessary to evaluate the relationship between these various CM motifs and their toxicity by conducting tests such as hummingbird phenotype *in vitro*.

To our knowledge, this report is the first study to elucidate the diverse

characteristics of the CM motif types of the Western-type CagA strains in the Dominican Republic. However, there are several limitations in this study. Firstly, the number of strains involved was small. For a better understanding, further studies should use a large number of samples, balanced for each diagnosis. Secondly, although our samples were taken at a national reference hospital in Santo Domingo city, the capital of the Dominican Republic, our results cannot be generalized across the entire region of the Dominican Republic since the physical and cultural landscape varies by the geographic region in the Dominican Republic.

CONCLUSION

In conclusion, we found the unique African (hpAfrica1-type) CM motif in the Western-type CagA in the Dominican Republic and termed it Africa1-CM. The less toxicity of this motif could be one reason to explain the African enigma. In addition, a few hpEurope strains carrying the Af1-CM motif had a significantly higher ancestral Africa1 component than those without the Af1-CM motif. This result suggests that the ancestors of these hpEurope strains, having the Af1-CM motif, underwent genetic recombination with the hpAfrica1 strains in the past.

ARTICLE HIGHLIGHTS

Research background

Helicobacter pylori (*H. pylori*) plays an essential role in the development of peptic ulcer disease and gastric cancer. The CagA protein produced by *H. pylori* is the most studied virulence factor.

Research motivation

Gastric cancer is one of the most common cancers.

Research objectives

The present study aimed to evaluate the correspondence between the CagA-multimerization (CM) motif and phylogeographical classification using multilocus sequence typing (MLST) population structure in *H. pylori* in the Dominican Republic, which could be helpful to elucidate the African enigma.

Research methods

The Glu-Pro-Ile-Tyr-Ala (EPIYA) pattern and CM motif genotypes were determined using a polymerase chain reaction-based sequencing. The population structure was analyzed using MLST. Peptic ulcer disease and gastric cancer were identified *via* endoscopy, and gastric cancer was confirmed by histopathology.

Research results

Many CM motifs, which are the amino acid sequences of "FPLRRSAKVEDLSKVG", were found. This type was significantly more frequent in strains classified as hpAfrica1 using MLST analysis ($P = 0.034$).

Research conclusions

We found the unique African CM motif in the Western-type CagA in the Dominican Republic and termed it Africa1-CM.

Research perspectives

In the future, it is necessary to evaluate the relationship between these various CM motifs and their toxicity by conducting tests such as hummingbird phenotype *in vitro*.

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ORIGINAL ARTICLE

Basic Study

Nimbolide inhibits tumor growth by restoring hepatic tight junction protein expression and reduced inflammation in an experimental hepatocarcinogenesis

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Abstract

BACKGROUND

Altered tight junction (TJ) proteins are correlated with carcinogenesis and tumor development. Nimbolide is a tetranotriterpenoid that has been shown to have antioxidant and anti-proliferative properties; however, its anticancer effects and molecular mechanism in hepatocellular carcinoma (HCC) remains obscure.

AIM

To investigate the effect of nimbolide on TJ proteins, cell cycle progression, and hepatic inflammation in a mouse model of HCC.

METHODS

HCC was induced in male Swiss albino mice (CD-1 strain) by a single intraperitoneal injection of 100 mg/kg diethylnitrosamine (DEN) followed by 80 ppm N-nitrosomorpholine (NMOR) in drinking water for 28 wk. After 28 wk, nimbolide (6 mg/kg) was given orally for four consecutive weeks in DEN/NMOR induced HCC mice. At the end of the 32nd week, all the mice were sacrificed and blood and liver samples were collected for various analyses. Macroscopic examinations of hepatic nodules were assessed. Liver histology and HCC tumor markers such as alpha-fetoprotein (AFP) and glypican-3 were measured. Expression of TJ proteins, cell proliferation, and cell cycle markers, inflammatory markers, and oxidative stress markers were analyzed. In silico analysis was performed to confirm the binding and modulatory effect of nimbolide on zonula occludens 1 (ZO-1), nuclear factor of kappa light polypeptide gene enhancer in B-

institutional and national guidelines for the care and use of laboratory animals were followed.

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cells (NF-κB), and tumor necrosis factor alpha (TNF-α).

RESULTS

We found nimbolide treatment at a concentration of 6 mg/kg to HCC mice reduced hepatic tumor size by 52.08% and tumor volume ($P < 0.01$), and delayed tumor growth in HCC mice with a concomitant reduction in tumor markers such as AFP levels ($P < 0.01$) and glypican-3 expression ($P < 0.05$). Furthermore, nimbolide treatment increased tight junction proteins such as ZO-1 and occludin expression ($P < 0.05$, respectively) and reduced ZO-1 associated nucleic acid binding protein expression ($P < 0.001$) in HCC mice liver. Nimbolide treatment to HCC mice also inhibited cell proliferation and suppressed cell cycle progression by attenuating proliferating cell nuclear antigen ($P < 0.01$), cyclin dependent kinase ($P < 0.05$), and CyclinD1 ($P < 0.05$) expression. In addition, nimbolide treatment to HCC mice ameliorated hepatic inflammation by reducing NF-κB, interleukin 1 beta and TNF-α expression ($P < 0.05$, respectively) and abrogated oxidative stress by attenuating 4-hydroxynonenal expression ($P < 0.01$). Molecular docking studies further confirmed that nimbolide interacts with ZO-1, NF-κB, and TNF-α.

CONCLUSION

Our current study showed for the first time that nimbolide exhibits anticancer effect by reducing tumor size, tumor burden and by suppressing cell cycle progression in HCC mice. Furthermore, nimbolide treatment to HCC mice ameliorated inflammation and oxidative stress, and improved TJ proteins expression. Consequently, nimbolide could be potentially used as a natural therapeutic agent for HCC treatment, however further human studies are warranted.

Key Words: Hepatocellular carcinoma; Nimbolide; Tight junction; Inflammation; Oxidative stress; Zonula occludens 1 associated nucleic acid binding protein

Core Tip: The effects of nimbolide on tight junction (TJ) proteins, cell cycle progression, and hepatic inflammation in diethylnitrosamine (DEN) and N-nitrosomorpholine (NMOR) induced hepatocellular carcinoma (HCC) mouse model is unknown. This study revealed for the first time that nimbolide suppresses tumor growth by restoring hepatic TJ proteins, inhibiting cell proliferation and cell cycle progression, and attenuating inflammation and oxidative stress in HCC mice. Moreover, nimbolide showed a modulatory effect on zonula occludens 1, nuclear factor of kappa light polypeptide gene enhancer in B-cells and tumor necrosis factor alpha proteins confirmed by in silico analysis. All the above results clarified that nimbolide abolished DEN and NMOR induced hepatocarcinogenesis and suggested that nimbolide could be a future potential drug candidate for the treatment of human HCC.

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INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most lethal malignancies and ranks third in the annual cancer mortality rate with overall 6-20 months of median survival after diagnosis[1]. The etiological factors such as hepatitis viral infection and alcohol abuse instigate chronic inflammation in the liver and lead to cirrhosis and ultimately HCC at the terminal stage[2]. The treatment modalities for HCC are limited with

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surgical resection and liver transplantation. The frequency of recurrence of HCC is high[1]. Since most of the HCC patients are diagnosed in an advanced stage, surgical options are unsuitable and only systemic chemotherapy (doxorubicin or sorafenib) and supportive care are the best available treatment options, however, only minimally increase the lifespan. Moreover, chemotherapy has low efficiency and side effects, and do not improve the 5-year survival rates[3]. Besides, due to the development of drug resistance and the existence of genetic heterogeneity within the individuals, the clinical outcome of targeted therapies is less effective[4]. Therefore, the discovery and development of successful new molecular chemotherapeutic agents targeting multiple pathways simultaneously are an immediate need to prevent the disease progression and recurrence in HCC patients.

Tight junction (TJ) proteins are intercellular adhesion molecules located at the apical and basolateral membrane and play a fundamental role in maintaining tissue barrier and cellular integrity[5]. It regulates paracellular movement in endothelial cells, whereas in epithelial cells it tightly seals the gap between adjacent cells in an adhesive manner and prevents cell dissociation[6]. Of note, HCC is a type of epithelial cell carcinoma[7]. In the liver, TJ proteins are present in the hepatocytes and biliary epithelial cells, which form a blood-biliary barrier[8]. Zonula occludens (ZO)-1, a membrane-associated scaffolding protein that belongs to membrane associated guanylate kinase family, contains three PDZ domains, a single SH3 domain and a GK domain[9]. ZO-1 binds to occludin (transmembrane protein) *via* its GK domain and interacts with homologous family members ZO-2 and ZO-3 *via* the second PDZ domain[10,11]. Apart from barrier function, TJs proteins have been implicated in cellular signal transduction to modulate cell proliferation and differentiation[12]. It has been found that SH3 domain of ZO-1 shares homology with SH3 domain of lethal disc large-1 tumor suppressor gene of drosophila, and its deletion or mutation leads to cell overgrowth and neoplastic changes, suggesting ZO-1 acts as a tumor suppressor[13]. ZO-1 binds to Y-box transcription factor ZO-1 associated nucleic acid binding protein (ZONAB), which interacts with the cell cycle kinase cyclin dependent kinase (CDK4), and regulates transcription of cell cycle related genes such as proliferating cell nuclear antigen (PCNA) and CyclinD1, providing a molecular link between ZO-1 and ZONAB, thereby regulating cellular proliferation[14,15]. Alteration in TJ proteins leads to a decrease in cell-cell adhesion, which is a crucial step to the cellular phenotypic transformation for tumor initiation and progression. Indeed, loss of TJ proteins such as ZO-1, occludin, and claudin have been shown to correlate with increased invasiveness, metastasis, and poor prognosis in breast cancer and colorectal cancer[16-18]. Similarly, although occludin$^{-/-}$ mice exhibited no gross anatomical defects, the gastric epithelial cells became hyperplastic after 40 wk[19]. Of note, our human study in HCC patients indicated a significant correlation between hepatic ZO-1 and high-sensitive C-reactive protein, however, the causal relationship remains unclear[20].

Nimbolide is a tetranotriterpenoid, and an active compound isolated from leaves and flowers of the Neem tree (*Azadirachta indica*), which is a traditional medicinal plant of Meliaceae family which is widely distributed in Asia, Africa, and other tropical parts of the world[21]. Several published studies have shown evidence that nimbolide induces apoptosis, inhibits cell proliferation, and metastasis in a variety of cancer cells including prostate, breast cancer, and choriocarcinoma cell lines[22-24]. Furthermore, the antitumor activities of nimbolide have been shown to have beneficial effects in pre-clinical models of various cancers such as oral squamous cell carcinoma[25], prostate cancer, and pancreatic cancer[26,27]. Nimbolide also induces cell cycle arrest and apoptosis in renal cell carcinoma[28] and its antioxidant and anti-inflammatory activities were widely studied using *in vivo* and *in vitro* models[29,30]. In the current study, we aimed to investigate the effect of nimbolide on TJ protein expression, cell cycle progression, and inflammation in diethylnitrosamine (DEN) and N-nitrosomorpholine (NMOR) induced experimental HCC in mice.

MATERIALS AND METHODS

Materials

DEN (N0756) and NMOR (N7382) were purchased from Sigma-Aldrich, St. Louis, Missouri, United States. Nimbolide of 97% purity (AHRF/NP/001) was procured from Asthagiri Herbal Research Foundation, Chennai, Tamilnadu, India. Doxorubicin hydrochloride (10 mg vial, Biochem pharmaceutical industries Ltd., Mumbai, India) was obtained from the Department of Medical Oncology, JIPMER.

Animals and treatment procedure

Male Swiss mice (CD-1 strain) weighing 10-15 g were obtained from the central animal facility, JIPMER, and maintained in a specific pathogen-free environment. All the animals were housed in polypropylene cages and maintained at 21-25 °C with 12 h light/dark cycle and free (*ad libitum*) access to water and standard rodent pellets. All experimental study was approved by JIPMER Institute Animal Ethics Committee and was carried out in accordance with the Committee for the Purpose of Control and Supervision of Experiments in Animals (CPCSEA) guidelines.

Induction of HCC: Hepatocellular carcinoma was induced in mice by administering a single intraperitoneal (i.p.) injection of DEN [100 mg/kg body weight in phosphate buffer saline (PBS)] and subsequent administration of NMOR in the drinking water (80 ppm) for 28 wk. After tumor formation at 28th week, the mice were randomized into five groups. Group I Naive mice continued to receive standard pellet diet and water *ad libitum* for four weeks. Group II naïve + treatment mice continued to receive standard pellet diet and water *ad libitum*, and were administered with nimbolide (6 mg/kg body weight in PBS) daily for four weeks through oral gavage. Group III HCC mice continued to receive standard pellet diet and water *ad libitum*, and were administered PBS (oral) for four weeks. Group IV HCC mice were administered with nimbolide (6 mg/kg body weight in PBS) daily for four weeks through oral gavage. Group V HCC mice were treated with the standard anticancer drug doxorubicin hydrochloride (1 mg/kg body weight in saline; i.p.) weekly thrice for the next four weeks to compare the chemotherapeutic efficacy of nimbolide. The final study groups were: (1) Naïve (n = 8); (2) Naïve + nimbolide (n = 8); (3) HCC (n = 10); (4) HCC + nimbolide (n = 10); and (5) HCC + doxorubicin (n = 10). The experimental protocol and treatment schedule is depicted in Figure 1A.

At termination (end of 32nd week) mice in different study groups were sacrificed by exsanguination under anaesthesia (ketamine hydrochloride 100 mg/kg). Blood was collected from the cardiac puncture in a heparinized tube, centrifuged at 3500 rpm at 4 °C for 10 min; plasma separated was stored at -80 °C for further analysis. After weighing liver tissues, parts of hepatic tumor nodules were excised, washed with PBS, and stored in 10% neutral buffered formalin for histopathological analysis, and the remaining liver tissue/nodules were snap-frozen in liquid nitrogen and stored in -80 °C for molecular studies.

Morphological and histological assessment of liver tumor

The entire visible and macroscopic nodules on the surface of the liver were counted for each mouse in all experimental groups. The length (L) and width (W) of superficial tumor nodules were measured by using a digital vernier caliper. The largest length (L) of the nodule was considered as the maximum tumor diameter. The volume of the tumor was calculated using the formula; volume (V) = L × (W)^2/2. Tumor burden was calculated by multiplying mean tumor volume with the no. of nodules for each mouse in the experimental groups. Percentage tumor growth inhibition (% TGI) was defined as the difference between mean tumor volume (MTV) of DEN + NMOR treated (MTVD) and chemotherapeutic group (MTVT) at 32nd week, using the formula: % TGI = [(MTVD - MTVT)/MTVD] × 100.

For histopathological analysis, 10% formalin-fixed liver tissues were dehydrated with a series of graded alcohol, cleared in xylene, and embedded in paraffin wax. Thereafter, 5 μm tissue sections were cut and stained with hematoxylin and eosin (HE) with standard protocol. HE stained sections were assessed for histological features of HCC by an independent pathologist in a blind fashion. Numbers of mitotic cells in 10 high-power fields (HPF, 400 ×) were counted on HE stained slides and the highest number of mitotic cells among them was defined as the mitotic index.

Analysis of liver function parameters and alpha-fetoprotein

Plasma liver function parameters such as aspartate aminotransferase (AST), alanine aminotransferase (ALT) and alkaline phosphatase (ALP) were measured by autoanalyzer using Beckman coulter kits (United States), according to manufacturer instruction. HCC tumor marker alpha-fetoprotein (AFP) was measured in plasma by ELISA kit (CKbio-15638; Shanghai, China).

Western blotting

Liver tissues were snap-frozen in liquid nitrogen and stored at -80 °C for western blot analysis. About 150 mg of tissue was weighed and homogenized in 500 μL ice-cold RIPA buffer (G-Biosciences, St. Louis, MO, United States) with protease inhibitor

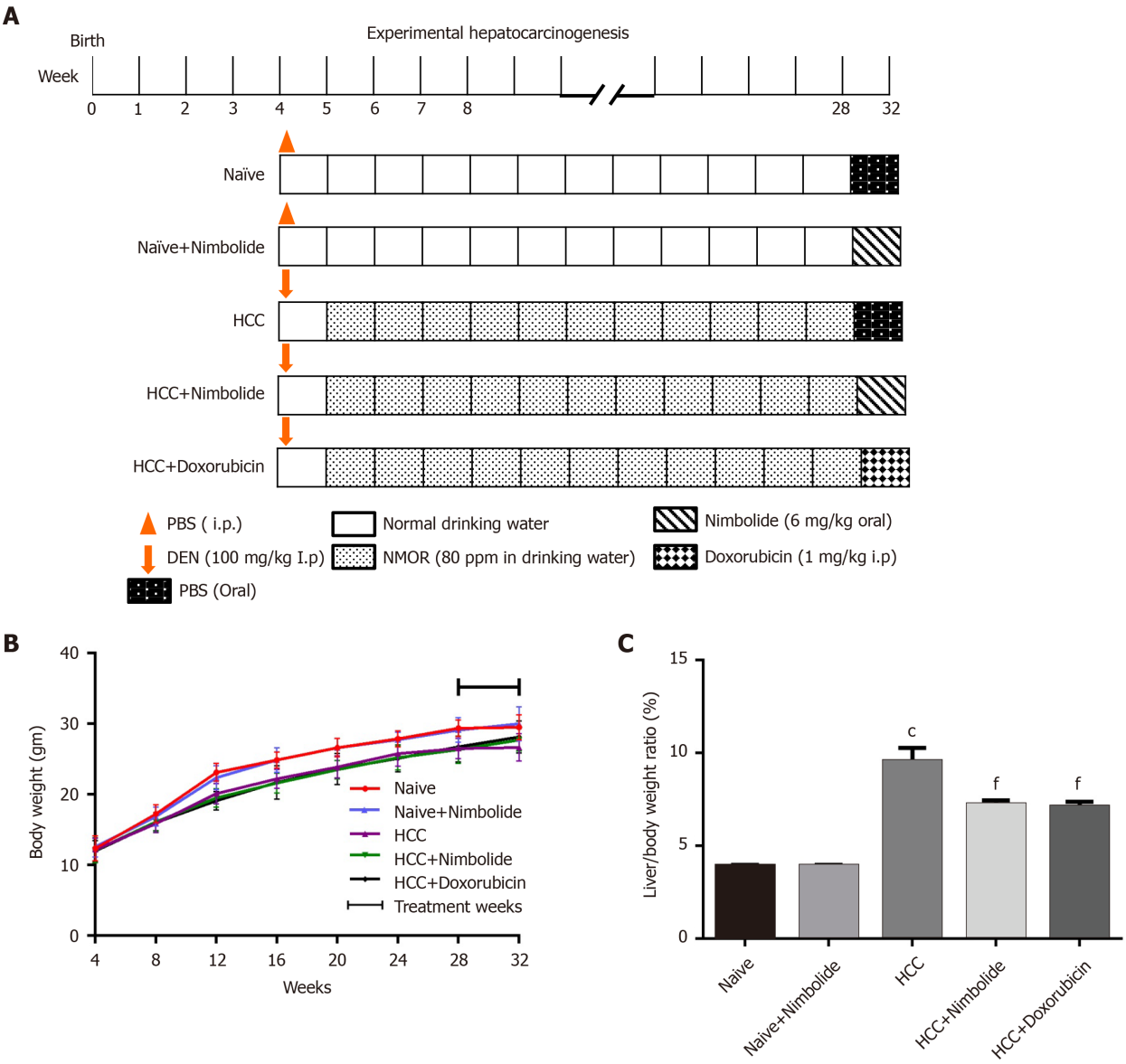


Figure 1 Effect of nimbolide on body weight and liver coefficient in diethylnitrosamine and N-nitrosomorpholine induced hepatocellular carcinoma mice. A: Flowchart for the experimental hepatocarcinogenesis model; B: Average weekly body weight of naïve and experimental mice; C: Liver coefficient of naïve and experimental mice. All the data are expressed as mean ± SEM (n = 6-10). The comparison between the groups was analyzed by one-way ANOVA followed by Tukey's multiple comparison test post-hoc test or Kruskal-Wallis followed by Dunn's multiple comparison post-hoc test. °P < 0.0001 compared to naïve group; ^{f}P < 0.001 compared to hepatocellular carcinoma group. DEN: Diethylnitrosamine; HCC: Hepatocellular carcinoma; NMOR: N-nitrosomorpholine; PBS: Phosphate buffer saline.

cocktail (P8340, Sigma-Aldrich, St. Louis, MO, United States) and 10% protein methyl sulfonyl fluoride (in ethanol). Tissue homogenate was centrifuged at 14000 g for 10 min at 4 °C and supernatant was collected. The protein content of the individual sample was estimated by Bradford assay using the Pierce coomassie protein assay kit (23200, Thermo Fisher Scientific, Rockford, IL, United States). About 30 μg of total protein of individual sample was diluted in SDS sample buffer and were resolved using SDS-polyacrylamide gel electrophoresis and blotted onto a nitrocellulose membrane (S045A330R, Advantec, Tokyo, Japan). Then the membranes were blocked for non-specific binding with 5% non-fat dry milk (GRM1254; Himedia, Mumbai, India) in Tris-buffered saline containing 0.01% Tween 20 (TBST) for 1 h at room temperature, followed by overnight incubation with primary antibody at 4 °C. The primary antibodies used were rabbit polyclonal anti-ZO1 (1:1000, 61-7300; Thermo Fisher, Rockford, IL, United States), rabbit polyclonal anti-ZONAB (1:500, 40-2800; Thermo Fisher, Rockford, IL, United States), mouse monoclonal anti-occludin (1:1000, sc-133256; Santa Cruz, Dallas, TX, United States), rabbit polyclonal anti-CDK4 (1:1000, A0366; Abclonal, Woburn, Massachusetts, United States), rabbit polyclonal anti-CyclinD1 (1:1000, A2708; Abclonal, Woburn, MA, United States), rabbit monoclonal

anti-nuclear factor of kappa light polypeptide gene enhancer in B-cells (NF-κB)p65 (1:1000, 8242S; CST, Danvers, MA, United States), rabbit polyclonal anti-tumor necrosis factor alpha (TNF-α) (1:1000, 2081R; BIOSS, Woburn, Massachusetts, United States), rabbit polyclonal anti-Interleukin 1 beta (IL-1β) (1:1000, A11370; Abclonal, Woburn, MA, United States) and mouse monoclonal anti-β-actin (1:1000, AC004; Abclonal, Woburn, MA, United States). After overnight incubation, membranes were washed with TBST three times and incubated with peroxidase-conjugated goat anti-rabbit (1:10000, 406401; BioLegend, San Diego, CA, United States) or goat anti-mouse (1:10000, 405306; BioLegend, San Diego, CA, United States) for 1 h at room temperature and the bands were visualized using an enhanced chemiluminescent substrate (34087; West pico detection kit, Thermo Scientific, Rockford, IL, United States) in Chemidoc system (Bio-rad, United States). Protein bands were quantified using Image Lab software (Bio-rad, United States).

Immunohistochemistry

3-5 μm thick sections were cut using an automated microtome (Leica Biosystems) and mounted on a silane coated slide. After deparaffinization, slides were immersed in xylene and rehydrated in different graded ethanol followed by 3% H_2O_2 in methanol for 15 min to quench the endogenous peroxidase activity. Then the sections were rinsed with PBS, and antigen retrieval was performed using citrate buffer (PH = 6.0) in the decloaking system at 110°C for 10 min. After blocking, the sections were incubated with primary antibody overnight at 4°C. Primary antibodies used were rabbit polyclonal anti-glypican-3 (1:100, A13988; Abclonal, Woburn, MA, United States), rabbit polyclonal anti-PCNA (1:100, A0264; Abclonal, Woburn, MA, United States), and rabbit polyclonal anti-4-Hydroxynonenal (4HNE) (1:100, bs-6313R; Bioss, Woburn, Massachusetts, United States). After washing with PBS, sections were incubated with universal anti-mouse/rabbit Ig (MP-7500; Vector lab, Burlingame, CA, United States) for 30 min at room temperature and immunostaining was performed with ImmPACT DAB kit (SK4105; Vector lab, Burlingame, CA, United States) and counterstained with hematoxylin. After mounting with Histamount solution, sectioned were examined under EVOS FLc imaging system (Life Techonologies, United States).

Molecular docking analysis

Molecular docking using Schodinger AutoDock 4.0 software (United States) was performed to predict the binding affinity of nimbolide with ZO-1, NF-κB, and TNF-α. The X-ray crystal structure of ZO-1 [Protein Data Bank archive (PDB) ID: 2RRM], NF-κB (PDB ID: 1VKX), and TNF-α (PDB ID: 2TNF) were retrieved from RCSB protein data bank (http://www.rcsb.org). The proteins were prepared before molecular docking using PyMol software (Schrodinger LLC, Cambridge, United States). Nimbolide (CID: 100017) 2D structure was retrieved from the Pubchem database (https://pubchem.ncbi.nlm.nih.gov/compound) in SDF file format and then converted to .mol2 format using Open Babel software version 2.4.1. After the simulation, different docked conformations were obtained and conformation with the strongest binding affinity towards ligand binding cavity of ZO-1, NF-κB, and TNF-α were selected as possible binding conformations and taken for further analysis. The final assessment of protein-ligand interaction was done and interpreted as Glide score (kcal/moL).

Statistical analysis

All the data are expressed as mean ± standard error of mean (SEM). Results were analyzed using GraphPad Prism 6.0 software. The comparison between different groups was analyzed by one-way ANOVA followed by Tukey's multiple comparison post-hoc test or by Kruskal-Wallis followed by Dunn's multiple comparison post-hoc test. A $P < 0.05$ was considered as statistical significance.

RESULTS

Effect of nimbolide on body weight, liver weight and liver coefficient in DEN/NMOR induced HCC mice

Experimental mice were treated with nimbolide or doxorubicin (Figure 1A) and the average weekly bodyweight of the naive and experimental mice was represented in Figure 1B. HCC induction in mice showed a decreased bodyweight throughout the experimental period compared to naive mice. Nimbolide or doxorubicin treatment to

HCC mice showed a trend in increased body weight from 28th to 32nd week ($P = 0.6781$) (Figure 1B). Furthermore, the liver/body weight ratio measured was higher ($P < 0.0001$) in HCC mice compared to naïve mice and this may be due to the induction of tumors by DEN and NMOR. On the contrary, treatment with nimbolide to HCC mice showed reduced liver/body weight ratio ($P < 0.001$) when compared to DEN/NMOR induced HCC mice, suggesting nimbolide possibly alleviated tumor burden (Figure 1C). Similar effects were seen in mice treated with doxorubicin in DEN/NMOR induced HCC mice (Figure 1C). Naïve mice treated with nimbolide shows similar body weight and liver/body weight ratio when compared to the naïve mice alone group.

Nimbolide inhibits DEN/NMOR induced hepatocarcinogenesis

Macroscopically visible hepatic nodules were evident on the liver surface of HCC mice, whereas, treatment with nimbolide or doxorubicin showed much lesser hepatic nodules (Figure 2A). Moreover, the maximum tumor diameter measured was higher in HCC mice and was reduced following treatment with nimbolide ($P < 0.05$) or doxorubicin ($P < 0.05$) to HCC mice (Figure 2B). The calculated tumor burden was also higher in HCC mice. Treatment with nimbolide ($P < 0.05$) or doxorubicin ($P < 0.05$) to HCC mice showed reduced tumor burden compared to HCC mice alone group (Figure 2C). Also, nimbolide or doxorubicin treatment to HCC mice inhibited tumor growth by 52.08% and 54.45%, respectively, with the reduction in mean tumor volume ($P < 0.01$) compared to HCC mice alone group (Table 1).

To identify the effective dose of nimbolide, we performed a dose-response study with three different doses of nimbolide (1.5 mg/kg, 3 mg/kg, and 6 mg/kg body weight) in DEN/NMOR induced HCC mice. We found treatment with 6 mg/kg nimbolide reduced maximum tumor diameter ($P < 0.05$) (Supplementary Figure 1) and tumor volume ($P < 0.05$) (Supplementary Figure 2) in HCC mice, while 1.5 mg/kg and 3 mg/kg nimbolide treatment were not significant compared to HCC mice alone group. Liver function parameters and AFP levels were also significantly decreased to HCC mice following 6 mg/kg b.w. nimbolide treatment (Supplementary Table 1). Based on the above preliminary data, the effective dose of 6 mg/kg b.w. nimbolide was chosen for subsequent experiments.

To confirm the malignancy potential of DEN/NMOR induced hepatocarcinogenesis, representative hematoxylin and eosin stained sections of the liver was studied and the results were shown in Figure 2D. Naive (Figure 2D, panel a) and naïve + nimbolide (Figure 2D, panel b) mice liver showed normal hepatic lobular architecture whereas, HCC mice liver showed neoplastic changes (Figure 2D, panel c). The neoplastic changes in the liver showed classical features of HCC characterized by hepatocyte arranged in cords (3 cell layer), cellular atypia, eosinophilic to clear cytoplasm, enlarged nucleus, and hyperchromasia. Treatment with nimbolide or doxorubicin showed minimal cellular atypia and decreased nuclear: Cytoplasmic ratio (Figure 2D, panel d and e). Furthermore, the mitotic index is a simple method for the evaluation of cellular proliferation rate and is a hallmark feature of cancer. Indeed, the mitotic index is a predictor of prognosis in patients with HCC[31]. Hematoxylin and eosin stained liver section of HCC mice showed a higher mitotic index (3-4/10 HPF) whereas, a lower mitotic index (1-2/10 HPF) was observed in nimbolide or doxorubicin treated HCC mice liver (Table 2).

Effect of nimbolide on liver function parameters and tumor and cell proliferation markers in DEN/NMOR induced HCC mice

Elevated levels of hepatic dysfunction marker enzymes such as AST, ALT, and ALP are widely used to assess hepatic dysfunction, and are associated with HCC progression[32]. DEN/NMOR induced HCC mice showed elevated levels of AST, ALT, and ALP ($P < 0.0001$) when compared to naive mice. Treatment with nimbolide or doxorubicin to HCC mice lowered the plasma levels of AST, ALT, and ALP ($P < 0.01$) compared to HCC mice alone group (Figure 3A-C). Plasma alpha-fetoprotein (AFP) levels and glypican-3 are clinically used as tumor markers for the diagnosis and prognosis of HCC[33,34]. Reduced AFP levels was reported to have prognostic significance after hepatic tumor resection and transarterial chemoembolization in HCC patients[35,36]. We found a higher plasma AFP levels in HCC mice ($P < 0.0001$) compared to naive mice. Nimbolide or doxorubicin treatment to HCC mice showed decreased plasma AFP levels ($P < 0.01$) compared to HCC mice alone group (Figure 3D). Increased hepatic glypican-3 expression was associated with a significantly lower 5-year survival rate and poor prognosis in HCC patients[34]. Figure 3E shows the immunostaining of glypican-3 on the liver sections of naive and experimental mice.

Table 1 Mean tumor volume and tumor growth inhibition (%) in the experimental mice

Experimental groups	Mean tumor volume (mm^3)	Tumor growth inhibition (%)
HCC	78.45 ± 11.87	-
HCC + nimbolide	37.60 ± 5.26[e]	52.08
HCC + doxorubicin	35.73 ± 8.09[e]	54.45

[e]$P < 0.01$. HCC: Hepatocellular carcinoma.

Table 2 Mitotic index in the naïve and experimental mice

Experimental groups	Mitotic index/10 HPF
Naïve	0/10 HPF
Naïve + nimbolide	0/10 HPF
HCC	3-4/10 HPF
HCC + nimbolide	1-2/10 HPF
HCC + doxorubicin	1-2/10 HPF

HCC: Hepatocellular carcinoma; 10/HPF: 10 high power field (400 ×).

Naive mice (Figure 3E, panel a) and naïve + nimbolide mice (Figure 3E, panel b) liver showed negative reactivity to glypican-3 whereas, strong glypican-3 cytoplasmic positivity was seen in HCC mice liver (Figure 3E, panel c), while nimbolide or doxorubicin treatment to HCC mice showed reduced glypican-3 cytoplasmic positivity (Figure 3E, panel d and e). Quantification of glypican-3 positive cells in the experimental group was represented in Figure 3F. PCNA is a putative marker for cell proliferation and hepatocyte damage and its high expression was associated with poor survival in HCC patients[37]. Figure 3G shows the immunostaining of PCNA on the liver sections of naive and experimental mice. Negative staining was observed in naïve (Figure 3G, panel a) and naïve + nimbolide (Figure 3G, panel b) mice liver whereas, HCC mice liver (Figure 3G, panel c) displayed much higher PCNA-positive nuclei, while treatment with nimbolide (Figure 3G, panel d) or doxorubicin (Figure 3G, panel e) to HCC mice showed reduced PCNA-positive nuclei. Quantification of PCNA positive nuclei in the experimental group was represented in Figure 3H.

Nimbolide ameliorates hepatic inflammation and oxidative stress in DEN/NMOR induced HCC mice

Figure 4A-C shows the hepatic NF-κB, TNF-α, and IL-1β protein expressions by western blot in the naive and experimental mice. The hepatic protein expression of NF-κB ($P < 0.01$), IL-1β ($P < 0.001$), and TNF-α ($P < 0.01$) were increased in HCC mice compared to naive mice. Nimbolide or doxorubicin treatment to HCC mice showed decreased NF-κB ($P < 0.05$), IL-1β ($P < 0.05$), and TNF-α ($P < 0.05$) protein expressions compared to HCC mice alone. Figure 4D shows immunostaining of 4HNE on the liver sections of naive and experimental mice. Naive (Figure 4D, panel a) and naïve + nimbolide (Figure 4D, panel b) mice liver showed negative reactivity to 4HNE while 4HNE expression has increased in HCC mice liver (Figure 4D, panel c). Nimbolide (Figure 4D, panel d) or doxorubicin (Figure 4D, panel e) treatment to HCC mice showed reduced hepatic 4HNE nuclear positivity compared to HCC mice alone. Moreover, 4HNE positive nuclei quantification was done and represented in Figure 4E.

Nimbolide treatment attenuates hepatic ZONAB and cell cycle markers proteins expression in DEN/NMOR induced HCC mice

Figure 5A shows the hepatic ZONAB protein expression by western blot in naive and experimental mice. The hepatic ZONAB protein expression was higher ($P < 0.0001$) in HCC mice compared to naive mice while treatment with nimbolide ($P < 0.001$) or doxorubicin ($P < 0.01$) to HCC mice showed reduced ZONAB protein expression

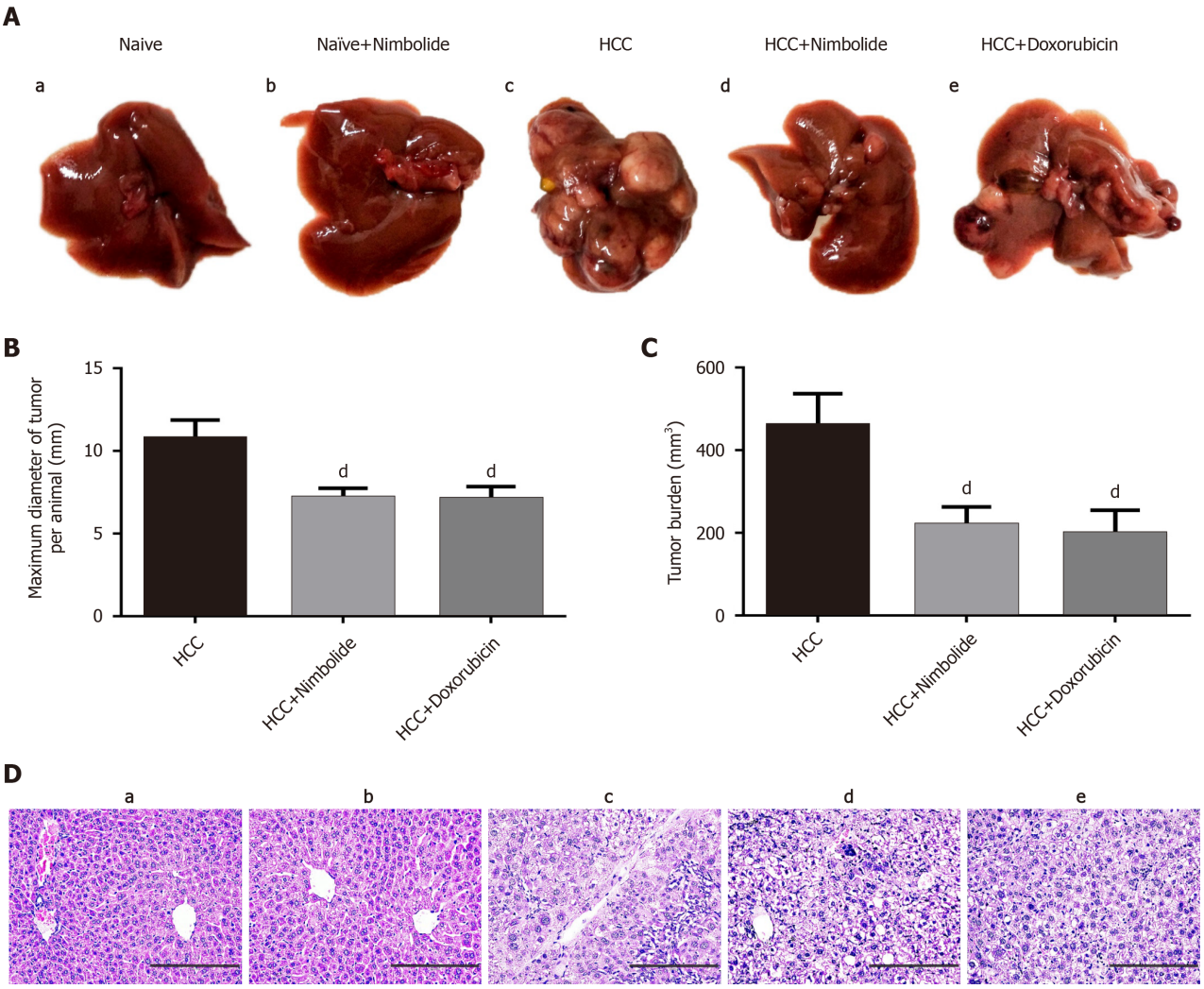


Figure 2 Nimbolide inhibits diethylnitrosamine and N-nitrosomorpholine induced hepatocarcinogenesis. A: Macroscopic images of mice liver from naïve and experimental groups; B: Maximum tumor diameter in naïve and experimental groups; C: Tumor burden in naïve and experimental groups; D: Representative images of hematoxylin and eosin stained liver sections from naive and experimental groups (200 × magnification). Scale bar: 200 μm; all the data are expressed as mean ± SEM (n = 6-7). The comparison between the groups was analyzed by one-way ANOVA followed by Tukey's multiple comparison post-hoc test or Kruskal-Wallis followed by Dunn's multiple comparison post-hoc test. $^{d}P < 0.05$ compared to hepatocellular carcinoma group. HCC: Hepatocellular carcinoma.

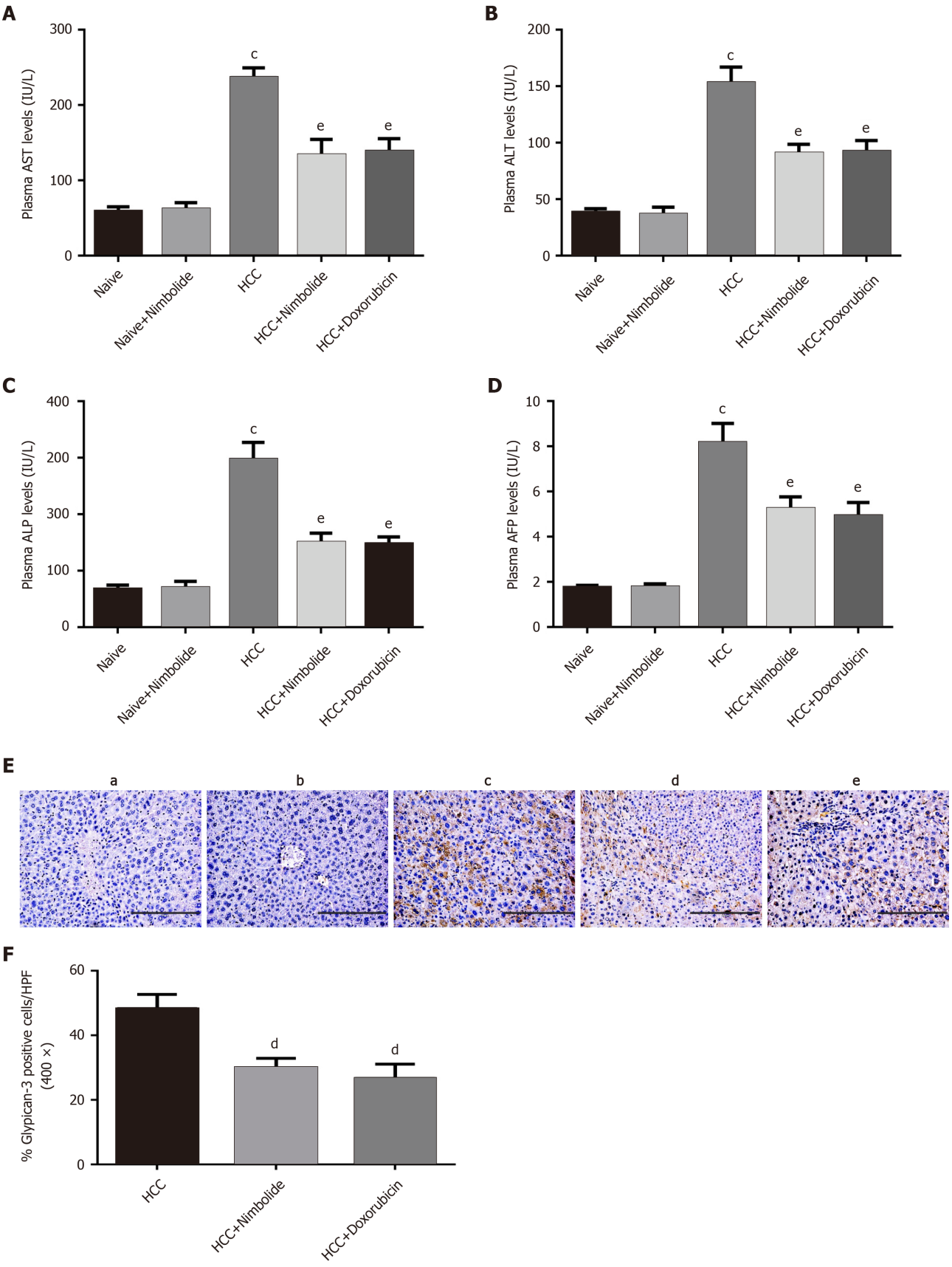
compared to HCC mice alone. Figure 5B and C shows the cell cycle markers CDK4 and CyclinD1 protein expressions by western blot in naive and experimental mice. The hepatic CDK4 ($P < 0.01$) and CyclinD1 ($P < 0.001$) protein expressions were increased in HCC mice compared to naive mice. Treatment with nimbolide ($P < 0.05$) or doxorubicin ($P < 0.05$) to HCC mice showed decreased CDK4 and cyclinD1 protein expression compared to HCC mice alone.

Nimbolide treatment restores hepatic tight junction protein expressions in DEN/NMOR induced HCC mice

Figure 6A shows the hepatic occludin protein expression by western blot in naive and experimental mice. The hepatic occludin protein expression was down-regulated ($P < 0.01$) in HCC mice compared to naive mice while treatment with nimbolide ($P < 0.05$) or doxorubicin ($P < 0.05$) to HCC mice, occludin protein expression was up-regulated compared to HCC mice alone. Figure 6B shows the hepatic ZO-1 protein expression by western blot in naive and experimental mice. Moreover, hepatic ZO-1 protein expression was reduced ($P < 0.01$) in HCC mice compared to naive mice. Nimbolide ($P < 0.05$) or doxorubicin ($P < 0.05$) treatment to HCC mice showed significantly increased ZO-1 protein expression when compared to lone HCC.

Nimbolide has a higher binding affinity towards ZO-1, NF-κB, and TNF-α proteins

The 2D structure of nimbolide is represented in Supplementary Figure 3. The 3D structure and ligand binding cavity of ZO-1, NF-κB, and TNF-α was shown in


A
Plasma AST levels (IU/L)
300
200
100
0
c
e
e
Naive
Naive+Nimbolide
HCC
HCC+Nimbolide
HCC+Doxorubicin
B
Plasma ALT levels (IU/L)
200
150
100
50
0
c
e
e
Naive
Naive+Nimbolide
HCC
HCC+Nimbolide
HCC+Doxorubicin
C
Plasma ALP levels (IU/L)
400
300
200
100
0
c
e
e
Naive
Naive+Nimbolide
HCC
HCC+Nimbolide
HCC+Doxorubicin
D
Plasma AFP levels (IU/L)
10
8
6
4
2
0
c
e
e
Naive
Naive+Nimbolide
HCC
HCC+Nimbolide
HCC+Doxorubicin
E
a
b
c
d
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% Glypican-3 positive cells/HPF (400 ×)
60
40
20
0
d
d
HCC
HCC+Nimbolide
HCC+Doxorubicin

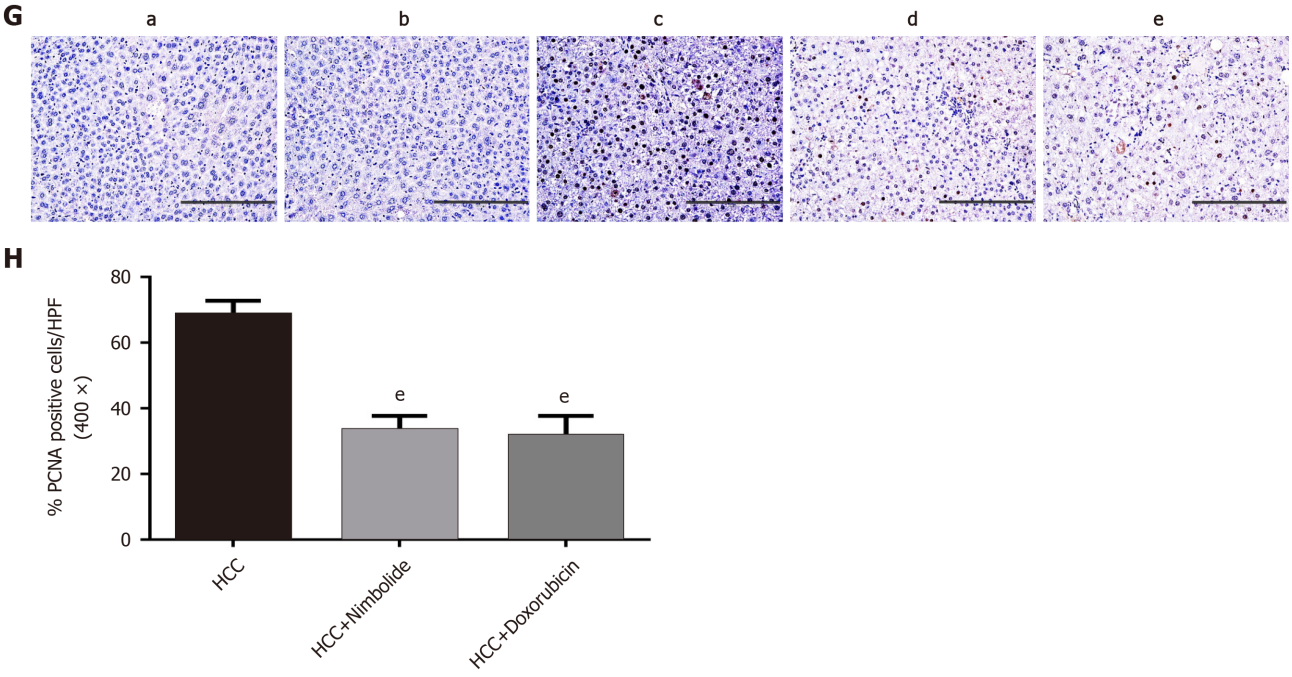

Figure 3 Effect of nimbolide on liver function parameters and tumor and cell proliferation markers in diethylnitrosamine and N-nitrosomorpholine induced hepatocellular carcinoma mice. A: Plasma aspartate aminotransferase level in naïve and experimental groups; B: Plasma alanine aminotransferase level in naïve and experimental groups; C: Plasma alkaline phosphatase level in naïve and experimental groups; D: Plasma alpha-fetoprotein level in naïve and experimental groups; E: Representative images of glypican-3 immunostaining of mice liver from naive and experimental groups (200 × magnification); F: Quantification of glypican-3 positive cells in experimental groups by counting five 400 × fields of each liver section; G: Representative images of proliferating hepatocytes by proliferating cell nuclear antigen (PCNA) immunostaining of mice liver from naive and experimental groups (200 × magnification); H: Quantification of PCNA positive nuclei in experimental groups by counting five 400 × fields of each liver section. Scale bar: 200 μm; all the data are expressed as mean ± SEM (n = 3-6). The comparison between the groups was analyzed by one-way ANOVA followed by Tukey's multiple comparison post-hoc test or Kruskal-Wallis followed by Dunn's multiple comparison post-hoc test. $^{c}P < 0.0001$ compared to naïve group; $^{d}P < 0.05$, $^{e}P < 0.01$ compared to hepatocellular carcinoma group. AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; ALP: Alkaline phosphatase; AFP: Alpha-fetoprotein; PCNA: Proliferating cell nuclear antigen; HPF: High-power fields; HCC: Hepatocellular carcinoma.

Supplementary Figure 4A-F. Docked complexes of nimbolide and ZO-1 protein showed nimbolide formed hydrogen bonds with amino acid residues Arg 757, Lys 760, Asn 764, Asn 765 and hydrophobic interaction with amino acid residues Ala 623, Pro 710, Lys 760, Leu 761 towards predicted ligand-binding cavity of ZO-1 with a glide score of -9.28 kcal/moL which indicates nimbolide has a strong binding affinity and modulatory effect on ZO-1 protein (Figure 7A-C and Table 3). Molecular docking also revealed nimbolide formed hydrogen bonds with amino acid residues Lys 28, Arg 33, Ser 45 and hydrophobic interaction with amino acid residues Arg 33, Arg 35, Pro 47 towards predicted ligand-binding cavity of NF-κB protein with a glide score of -7.62 kcal/moL suggesting nimbolide has a modulatory effect on NF-κB protein (Figure 7D-F and Table 3). Besides, nimbolide also formed hydrogen bonds with amino acid residues Lys 98, Tyr 119 and hydrophobic interaction with amino acid residues Ala 96, Pro 117, Ile 118, Tyr 119 towards predicted ligand-binding cavity of TNF-α protein with a glide score of -8.84 kcal/moL indicating nimbolide has a strong binding and modulatory effect on TNF-α protein (Figure 7G-I and Table 3).

DISCUSSION

Our study showed for the first time that nimbolide treatment to DEN and NMOR induced HCC mice exhibits anticancer effect as evidenced by decreased plasma AFP levels and hepatic glypican-3 expression, inhibition of cell cycle progression (ZONAB, CDK4, and cyclinD1), and amelioration of inflammation and oxidative stress. Nimbolide treatment also reduced tumor size, tumor burden, and pathological grade in HCC mice. The other major finding of the current study is increased TJ proteins such as ZO-1 and occludin following treatment with nimbolide to HCC mice. Further, the molecular docking study revealed that nimbolide formed several hydrogen bonds and hydrophobic interactions with amino acid residues of ligand binding cavity of

Table 3 Binding affinity and interaction of nimbolide with different amino-acid residues of zonula occludens 1, nuclear factor of kappa light polypeptide gene enhancer in B-cells and tumor necrosis factor alpha proteins. Binding affinity is represented as Glide score (kcal/moL)

S. No.	Proteins	Nimbolide (CID: 100017)		
		Glide score (kcal/moL)	Hydrogen bonds	Hydrophobic interaction
1	ZO-1 (PDB ID: 2RRM)	-9.28	Arg 757, Lys 760, Asn 764, Asn 765	Ala 623, Pro 710, Lys 760, Leu 761
2	NF-κB (PDB ID: 1VKX)	-7.62	Lys 28, Arg 33, Ser 45	Arg 33, Arg 35, Pro 47
3	TNF-α (PDB ID: 2TNF)	-8.84	Lys 98, Tyr 119	Ala 96, Pro 117, Ile 118, Tyr 119

ZO-1: Zonula occludens 1; PDB: Protein data bank archive; NF-κB: Nuclear factor of kappa light polypeptide gene enhancer in B-cells; TNF-α: Tumor necrosis factor alpha.

ZO-1, NF-κB, and TNF-α, showing higher binding affinity as observed by high glide score between the docked complexes.

Doxorubicin is a standard anticancer drug that was also administered to HCC mice to compare the effectiveness of nimbolide. Our results revealed that almost 52.08% of tumor growth was inhibited by nimbolide, whereas doxorubicin inhibited 54.45% tumor growth in HCC mice. Additionally, reduction in liver coefficient, tumor diameter, tumor burden, and tumor volume in HCC mice by nimbolide treatment was also comparable with the standard anticancer drug doxorubicin.

A key finding of this study was that nimbolide treatment improved hepatic TJ protein (ZO-1 and occludin) expression in HCC mice. Our previous human study showed evidence that decreased hepatic ZO-1 expression was positively correlated with disease severity in HCC patients[20]. Moreover, occludin was also found to be decreased in HCC patients[38]. Of note, our previous study also showed evidence that cirrhotic mice brain exhibited significantly decreased ZO-1, occludin, and claudin-5 protein expression, which were associated with increased inflammation observed in cirrhotic mice[39,40]. The TJ protein ZO-1 has been reported to be involved in cell proliferation[12]. ZO-1 knock-down in mice showing embryonically lethal and was associated with defective angiogenesis of the yolk sac and impaired apoptosis of the embryonic cells[41]. Besides, the deletion of ZO-1 showed increased cell proliferation and neoplastic changes which suggests ZO-1 may have a tumor suppressor role[13]. In this context, lycopene (carotenoid) administration inhibited human cutaneous squamous cell carcinoma (cSCC) cell proliferation by up-regulating ZO-1 expression[42]. Moreover, earlier studies have suggested that ZO-1 regulates cell proliferation by binding to ZONAB, a Y-box transcription factor that has DNA binding element inverted CCAAT box, whose nuclear accumulation leads to transcription of cell cycle related genes such as CDK4, Cyclin D1, and PCNA[14,15]. This was supported by the fact that, in a sparse culture where the proliferation rate is high, the cellular ZONAB expression is high and the ZO-1 expression is low. By contrast, the ZONAB expression is down-regulated and ZO-1 expression is up-regulated in the confluent quiescent monolayer cells[43]. Knock-down of ZO-1 or overexpression of ZONAB resulted in increased proliferation of retinal pigment epithelial cells and induced epithelial to mesenchymal transition phenotype in mice[44]. In the present study, we found increased hepatic protein expression of ZONAB and its transcriptionally regulated cell cycle mediators such as CDK4, cyclinD1, and PCNA expressions in HCC mice, which were decreased following nimbolide treatment. Furthermore, we noted a reciprocal relationship between the protein expression pattern of ZO-1 and ZONAB in HCC mice which was reversed by nimbolide treatment. Thus, our study demonstrated for the first time that ZO-1/ZONAB pathway plays a crucial role in the pathogenesis of HCC by regulating cell cycle progression. Moreover, we also identified that nimbolide interacts with the ligand-binding cavity of ZO-1, forming hydrogen bonds and hydrophobic interaction with amino acids which may have a modulatory effect.

Another finding in our study is that nimbolide could regulate cell cycle progression in DEN and NMOR induced HCC in mice. Cell cycle is a major event for cell proliferation and an uncontrolled tumor cell proliferation is responsible for HCC progression[45,46]. CDK4 and cyclinD1 are important mediators of cell cycle progression which are found to be overexpressed and associated with poor prognosis in HCC patients[45,46]. In the presence of extracellular stimuli such as cytokines (TNF-α), the active complex is formed by CDK4/CyclinD1 which phosphorylates retinoblastoma

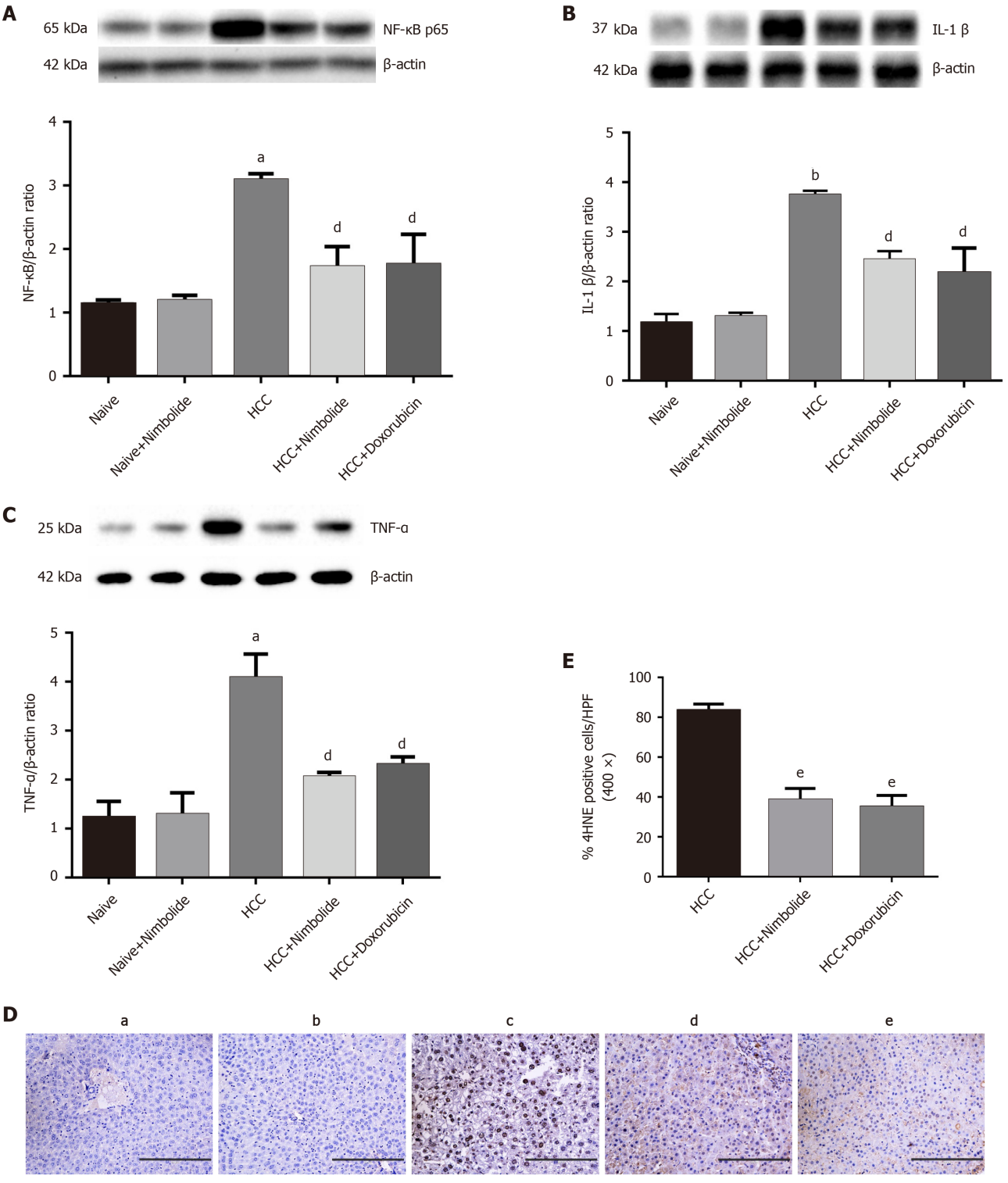

Figure 4 Nimbolide ameliorates hepatic inflammation and oxidative stress in diethylnitrosamine and N-nitrosomorpholine hepatocellular carcinoma mice. A-C: Hepatic protein expression levels of nuclear factor of kappa light polypeptide gene enhancer in B-cells, interleukin 1 beta and tumor necrosis factor alpha by western blot in naive and experimental groups; D: Representative images of 4-Hydroxynonenal (4HNE) immunostaining of mice liver from naive and experimental groups (200 × magnification); E: Quantification of 4HNE positive nuclei in experimental groups by counting five 400 × fields of each liver section. Scale bar: 200 μm; all the data are expressed as mean ± SEM (n = 3). The comparison between the groups was analyzed by one-way ANOVA followed by Tukey's multiple comparison post-hoc test or Kruskal-Wallis followed by Dunn's multiple comparison post-hoc test. $^{a}P < 0.01$, $^{b}P < 0.001$ compared to naïve group; $^{d}P < 0.05$, $^{e}P < 0.01$ compared to hepatocellular carcinoma group. NF-κB: Nuclear factor of kappa light polypeptide gene enhancer in B-cells; IL-1β: Interleukin 1 beta; TNF-α: Tumor necrosis factor alpha; 4HNE: 4-Hydroxynonenal; HCC: Hepatocellular carcinoma.

protein (pRb), and promotes the transition of the cell cycle from G1 to S phase through the restriction (R) checkpoint. The pRb induces gene expression necessary for DNA replication such as PCNA during S phase[47]. A previous study has shown that nimbolide regulates cell cycle progression in both cell lines and animal models[48]. In glioblastoma, nimbolide inhibited CDK4/CDK6 kinase activity and arrested cell cycle at the G1 phase[49]. Priyadarsini *et al*[50] reported that in HeLa cells nimbolide treatment inhibited major cell cycle regulatory proteins such as cyclin D1, cyclin B, and PCNA and restricted cell cycle at G0/G1 phase[50]. Concurrent with the above studies, we also observed that nimbolide treatment reduces CDK4 and Cyclin D1 protein expression in HCC mice. Therefore, we speculate that nimbolide could inhibit cell proliferation by preventing cell cycle progression from G1 to S phase and prevents HCC pathogenesis.

In the current study, we found nimbolide might dampen the inflammation through NF-κB pathway in HCC mice. Inflammation has a wide impact on the pathogenesis of cancer development starting from initiation to progression and metastasis. 70% of HCC develops in cirrhotic patients on the background of inflammation[1]. Activation of the NF-κB pathway plays a critical role in inflammation-induced cancer by affecting cell proliferation, apoptosis, angiogenesis, and metastasis[2]. Stimulation of hepatic kupffer cells during chronic inflammation releases proinflammatory cytokines such as TNF-α and IL-1β *via* the NF-κB pathway which is known to regulate cell proliferation, survival, and inhibition of apoptosis[2]. In this study, we found increased hepatic protein expression of NF-κB and its downstream mediator proinflammatory cytokines TNF-α and IL-1β in HCC mice, indicating the hyperinflammatory state of hepatic tissue. A previous study indicated that nimbolide administration inhibits NF-κB signaling and proinflammatory cytokines in both cell lines and animal models[48]. In line with this evidence, Kavitha *et al*[51] reported that nimbolide exerts anticancer effects in HepG2 cells by inhibiting NF-κB signaling and its downstream mediator Wnt/β-catenin pathway[51]. Similarly, Gupta *et al*[52] reported that nimbolide treatment inhibited xenograft progression in colorectal cancer by targeting the NF-κB pathway and by suppressing pro-inflammatory microenvironment[52]. Concomitant with the above results we also proved that nimbolide treatment attenuated the protein expression of NF-κB, TNF-α, and IL-1β in HCC mice. Moreover, molecular docking study revealed that nimbolide formed hydrogen bonds and hydrophobic interaction with amino acid residues of the ligand binding cavity of NF-κB and TNF-α. These results suggest that nimbolide ameliorated inflammation and suppressed NF-κB signaling in DEN and NMOR induced HCC mice. Therefore it is postulated that nimbolide might regulate cell proliferation by targeting the NF-κB signaling pathway which is considered as a central pathway linking inflammation and cancer[2]. The proposed mechanism of action by which nimbolide exerts its therapeutic effects in HCC mice is depicted in Figure 8.

DEN is known to induce HCC by generating reactive oxygen species (ROS) which causes oxidative stress in the cell leading to oxidative DNA damage, membrane lipid peroxidation, and mitochondrial damage favoring cell survival and proliferation[53]. 4HNE, a potent and stable by-product of lipid peroxidation, is considered as a marker of oxidative stress, and can independently form DNA adducts, and causes mutation and genetic instability, thus favoring hepatocarcinogenesis[54]. In the current study, we found increased nuclear 4HNE protein expression in HCC mice liver while nimbolide treatment to HCC mice significantly reduced hepatic 4HNE protein expression. This suggests that nimbolide could protect ROS induced oxidative damage and lipid peroxidation in HCC. In this context, Alshammari *et al*[29] reported that nimbolide treatment abrogates oxidative stress and promotes antioxidants capacity in the primary hepatocytes[29]. Similarly, another study in DMBA induced oral cancer showed that nimbolide inhibited oxidative stress-induced DNA damage by improving antioxidant enzymes[30]. Thus, collectively our results indicate that nimbolide has a potent antioxidant activity and abrogates oxidative stress by quenching free radicals and might restore cellular redox balance thereby attenuating the progression of carcinogenesis. Our study has some limitations that we did not study the nimbolide effects in ZO-1 or ZONAB conditional knock-out mice to prove the exact mechanism of HCC regulation. Indeed, our future experimental approach would delineate this either *in vivo* or in vitro. Moreover, with data obtained in this work, it is not possible to establish the mechanisms involved in the anticancer effect of nimbolide. The study show different mechanisms that occur at the same time than the anticancer effect but a direct relationship between them has not been showed. The efficacy of doxorubicin is very limited in the treatment of human HCC so a limited effect of nimbolide could be also expected.

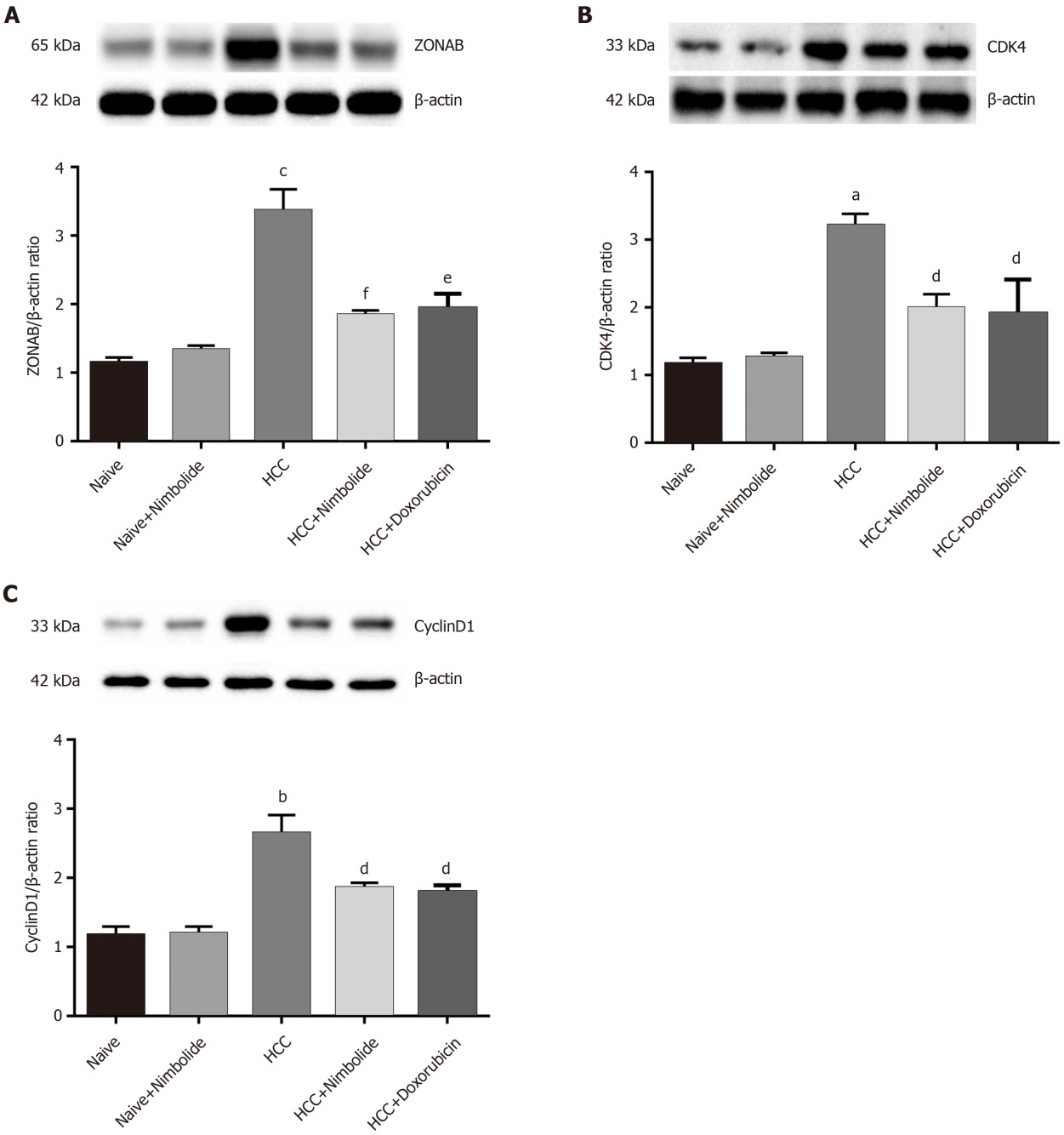

Figure 5 Nimbolide treatment attenuates hepatic zonula occludens 1 associated nucleic acid binding protein and cell cycle markers proteins expression in diethylnitrosamine and N-nitrosomorpholine induced hepatocellular carcinoma mice. A: Hepatic protein expression levels of zonula occludens 1 associated nucleic acid binding protein by western blot in naive and experimental groups; B: Hepatic protein expression levels of cyclin dependent kinase 4 by western blot in naive and experimental groups; C: Hepatic protein expression levels of CyclinD1 by western blot in naive and experimental groups. All the data are expressed as mean ± SEM (n = 3). The comparison between the groups was analyzed by one-way ANOVA followed by Tukey's multiple comparison post-hoc test or Kruskal-Wallis followed by Dunn's multiple comparison post-hoc test. [a]P < 0.01, [b]P < 0.001, [c]P < 0.0001 compared to naïve group; [d]P < 0.05, [e]P < 0.01, [f]P < 0.001 compared to HCC group. ZONAB: Zonula occludens 1 associated nucleic acid binding protein; CDK4: Cyclin dependent kinase; HCC: Hepatocellular carcinoma.

CONCLUSION

In conclusion, the results of our study showed evidence in HCC mice that nimbolide treatment suppresses tumor growth and cell cycle progression, and ameliorates inflammation and oxidative stress. Importantly, nimbolide treatment restored hepatic TJ proteins expression and regulates ZO-1/ZONAB pathway in HCC. Our study also demonstrated that the anticancer efficiency of nimbolide was comparable with standard anticancer drug doxorubicin in a preclinical model of HCC. Treatment with natural bioactive compounds, therefore, represents promising chemoprevention agents with minimal side effects, and thus nimbolide could be used as a novel

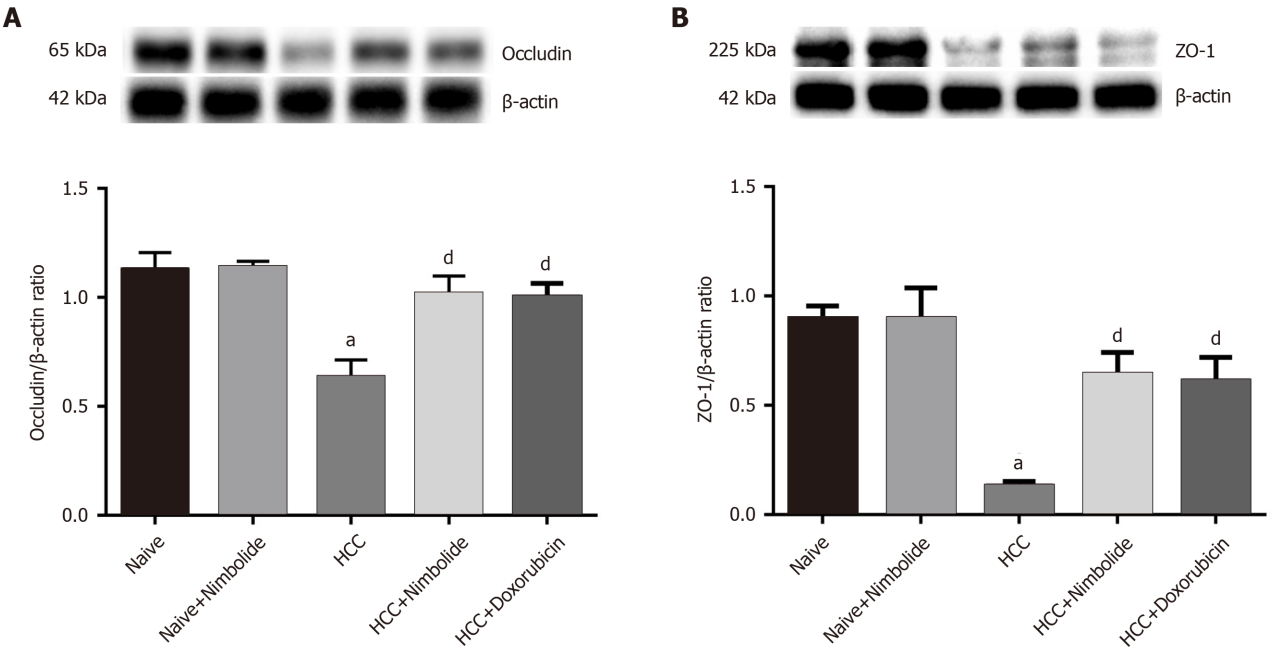

Figure 6 Nimbolide treatment restores hepatic tight junction protein expression in diethylnitrosamine and N-nitrosomorpholine induced hepatocellular carcinoma mice. A and B: Hepatic protein expression levels of occludin and zonula occludens 1 by western blot in naive and experimental groups. All the data are expressed as mean ± SEM (n = 3). The comparison between the groups was analyzed by one-way ANOVA followed by Tukey's multiple comparison post-hoc test or Kruskal-Wallis followed by Dunn's multiple comparison post-hoc test. $^{a}P < 0.01$ compared to naïve group; $^{d}P<0.05$ compared to hepatocellular carcinoma group. ZO-1: Zonula occludens 1; HCC: Hepatocellular carcinoma.

potential candidate drug against HCC progression. Indeed, further molecular and population-based studies are warranted.

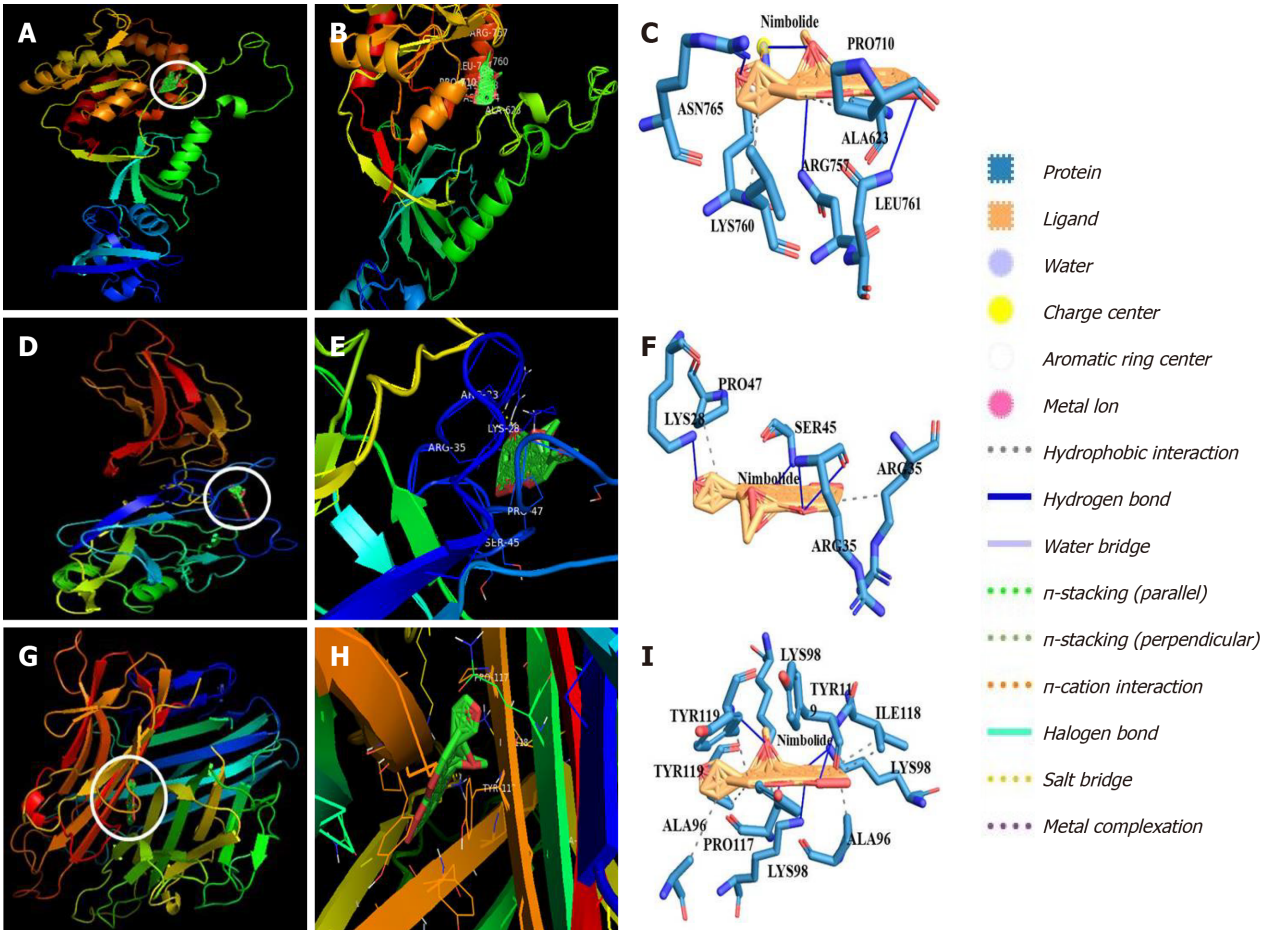

Figure 7 Molecular docking analysis of nimbolide with zonula occludens 1, nuclear factor of kappa light polypeptide gene enhancer in B-cells and tumor necrosis factor alpha. A and B: Representation of nimbolide and zonula occludens 1 (ZO-1) docking complex; C: Interaction of nimbolide with amino acid residues of ZO-1; D and E: Representation of nimbolide and nuclear factor of kappa light polypeptide gene enhancer in B-cells (NF-κB) docking complex; F: Interaction of nimbolide with amino acid residues of NF-κB; G and H: Representation of nimbolide and tumor necrosis factor alpha (TNF-α) docking complex; I: Interaction of nimbolide with amino acid residues of TNF-α.

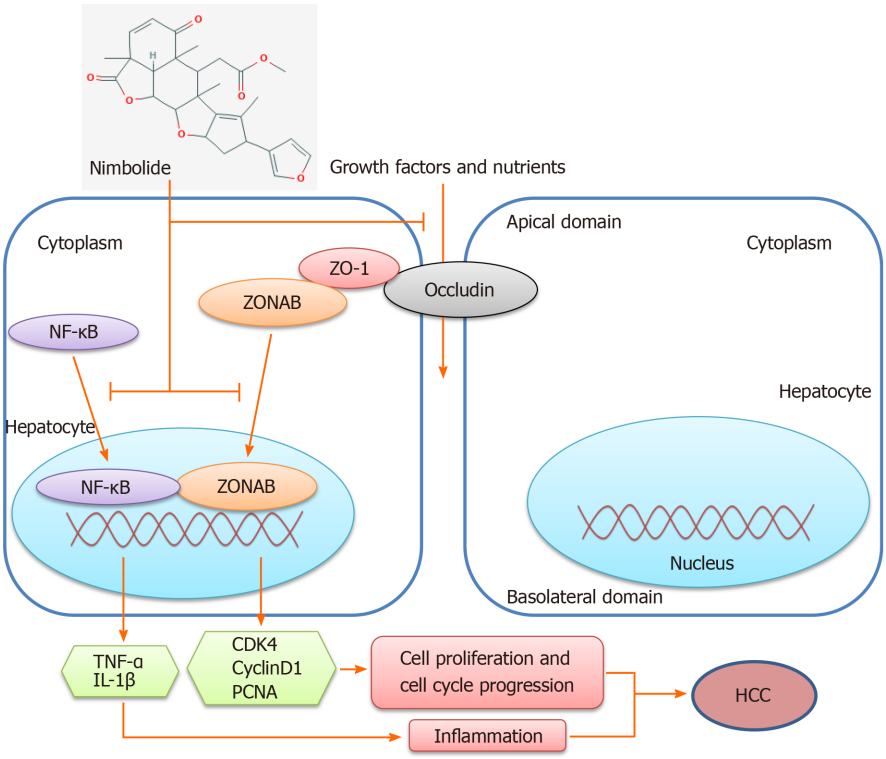

Figure 8 Schematic representation of the mechanism of action of nimbolide in diethylnitrosamine and N-nitrosomorpholine induced hepatocellular carcinoma mice. Nimbolide treatment inhibits tumor growth in diethylnitrosamine and N-nitrosomorpholine induced Hepatocellular carcinoma (HCC) mice by targeting tight junctions (TJs) proteins [Zonula occludens-1 (ZO-1) and Occludin]. Nimbolide mediated up-regulation of ZO-1 represses the transcriptional activity of ZO-1 associated nucleic acid binding protein at a junctional site preventing its nuclear accumulation and thus preventing the induction of cell cycle related genes cyclin dependent kinase 4, cyclinD1 and proliferating cell nuclear antigen. Nimbolide treatment also restores barrier integrity by up-regulation TJs proteins which prevent translocation of growth factors and nutrients necessary for cell growth from apical to the basolateral domain. Further, nimbolide also targets the nuclear factor of kappa light polypeptide gene enhancer in B-cells pathway ameliorating inflammation in HCC mice. ZO-1: Zonula occludens 1; ZONAB: ZO-1 associated nucleic acid binding protein; CDK4: Cyclin dependent kinase; PCNA: Proliferating cell nuclear antigen; NF-κB: Nuclear factor of kappa light polypeptide gene enhancer in B-cells; IL-1β: Interleukin 1 beta; TNF-α: Tumor necrosis factor alpha; HCC: Hepatocellular carcinoma.

ARTICLE HIGHLIGHTS

Research background

Worldwide, hepatocellular carcinoma (HCC) is a prevalent lethal disease exhibiting the highest cancer mortality rate with limited chemotherapeutic options. The discovery of a new chemotherapeutic agent is an urgent demand for the treatment of HCC patients.

Research motivation

Tight Junction (TJ) proteins have been implicated to regulate various signal transductions in carcinogenesis. Recent evidence has shown that nimbolide possesses anticancer activity in various cancers; however, its specific mechanism in HCC remains elusive.

Research objectives

We aimed to study the effect of nimbolide on TJ proteins, cell cycle progression, and hepatic inflammation in an experimental hepatocarcinogenesis mouse model.

Research methods

Diethylnitrosamine (DEN) and N-nitrosomorpholine (NMOR) induced mouse model of HCC was performed in the present study. Nimbolide was given orally for four consecutive weeks to DEN/NMOR induced HCC mice from 28th to 32nd week. At the end of the study period (32nd week), all the mice were sacrificed, blood and liver samples were collected for various analysis. HCC tumor markers such as alpha-fetoprotein (AFP) levels and glypican-3 protein expression were analysed. Hepatic TJ proteins, cell cycle, and inflammatory marker protein expressions were evaluated by western blot analysis. Cell proliferation and oxidative stress markers were analyzed by

immunohistochemistry. Molecular docking study was performed to confirm the interaction of nimbolide with zonula occludens 1 (ZO-1), nuclear factor of kappa light polypeptide gene enhancer in B-cells (NF-κB) and tumor necrosis factor alpha (TNF-α).

Research results

Hepatic tumor size and HCC tumor markers AFP and glypican-3 were reduced following nimbolide treatment. TJ protein (ZO-1 and occludin) expression levels were restored after nimbolide treatment, while ZO-1 associated nucleic acid binding protein expression was attenuated. Additionally, nimbolide treatment also reduced cell proliferation and cell cycle markers. Moreover, nimbolide treatment ameliorated hepatic inflammation and oxidative stress in HCC mice. The binding affinity and modulatory effects of nimbolide with ZO-1, NF-κB, and TNF-α were confirmed by molecular docking analysis.

Research conclusions

Nimbolide treatment showed anticancer effects by improving TJ proteins expression, suppressing cell cycle and cell proliferation, and ameliorating inflammation and oxidative stress in DEN and NMOR induced HCC mice.

Research perspectives

Nimbolide may be used as a potential chemotherapeutic agent for HCC treatment. Further molecular research and human studies may delineate nimbolide as a candidate drug for HCC treatment.

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ORIGINAL ARTICLE

Case Control Study

Altered metabolism of bile acids correlates with clinical parameters and the gut microbiota in patients with diarrhea-predominant irritable bowel syndrome

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Author contributions: Wei W designed and performed the study, analyzed the data, and drafted the manuscript; Wei W, Wang HF, Zhang YL, and Niu BY collected the clinical data and fecal samples from subjects; Zhang Y gave guidance on experiment operation and data interpretation, and contributed to manuscript revision; Yao SK designed the study, supervised the study performance, revised the manuscript, and obtained the funding.

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Abstract

BACKGROUND

Bile acids (BAs) have attracted attention in the research of irritable bowel syndrome with predominant diarrhea (IBS-D) due to their ability to modulate bowel function and their tight connection with the gut microbiota. The composition of the fecal BA pool in IBS-D patients is reportedly different from that in healthy populations. We hypothesized that BAs may participate in the pathogenesis of IBS-D and the altered BA profile may be correlated with the gut microbiome.

AIM

To investigate the role of BAs in the pathogenesis of IBS-D and the correlation between fecal BAs and gut microbiota.

METHODS

Fifty-five IBS-D patients diagnosed according to the Rome IV criteria and twenty-eight age-, sex-, and body mass index-matched healthy controls (HCs) were enrolled in this study at the gastroenterology department of China-Japan Friendship Hospital. First, clinical manifestations were assessed with standardized questionnaires, and visceral sensitivity was evaluated *via* the rectal distension test using a high-resolution manometry system. Fecal primary BAs including cholic acid (CA) and chenodeoxycholic acid (CDCA), secondary BAs including deoxycholic acid (DCA), lithocholic acid (LCA), and ursodeoxycholic acid (UDCA) as well as the corresponding tauro- and glyco-BAs were examined

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by ultraperformance liquid chromatography coupled to tandem mass spectrometry. The gut microbiota was analyzed using 16S rRNA gene sequencing. Correlations between fecal BAs with clinical features and gut microbiota were explored.

RESULTS

Fecal CA (IBS-D: 3037.66 [282.82, 6917.47] nmol/g, HC: 20.19 [5.03, 1304.28] nmol/g; $P < 0.001$) and CDCA (IBS-D: 1721.86 [352.80, 2613.83] nmol/g, HC: 57.16 [13.76, 1639.92] nmol/g; $P < 0.001$) were significantly increased, while LCA (IBS-D: 1621.65 [58.99, 2396.49] nmol/g, HC: 2339.24 [1737.09, 2782.40]; $P = 0.002$] and UDCA (IBS-D: 8.92 [2.33, 23.93] nmol/g, HC: 17.21 [8.76, 33.48] nmol/g; $P = 0.025$) were significantly decreased in IBS-D patients compared to HCs. Defecation frequency was positively associated with CA ($r = 0.294$, $P = 0.030$) and CDCA ($r = 0.290$, $P = 0.032$) and negatively associated with DCA ($r = -0.332$, $P = 0.013$) and LCA ($r = -0.326$, $P = 0.015$) in IBS-D patients. In total, 23 of 55 IBS-D patients and 15 of 28 HCs participated in the visceral sensitivity test. The first sensation threshold was negatively correlated with CDCA ($r = -0.459$, $P = 0.028$) in IBS-D patients. Furthermore, the relative abundance of the family *Ruminococcaceae* was significantly decreased in IBS-D patients ($P < 0.001$), and 12 genera were significantly lower in IBS-D patients than in HCs ($P < 0.05$), with 6 belonging to *Ruminococcaceae*. Eleven of these genera were negatively correlated with primary BAs and positively correlated with secondary BAs in all subjects.

CONCLUSION

The altered metabolism of BAs in the gut of IBS-D patients was associated with diarrhea and visceral hypersensitivity and might be ascribed to dysbiosis, especially the reduction of genera in *Ruminococcaceae*.

Key Words: Bile acids; Irritable bowel syndrome; Diarrhea; Visceral hypersensitivity; Microbiota; Dysbiosis

Core Tip: This study comprehensively investigated the fecal bile acid profile of irritable bowel syndrome with predominant diarrhea (IBS-D) patients and healthy controls, and the correlations between bile acids (BAs) and clinical characteristics as well as the gut microbiota of IBS-D patients. We found that the composition of fecal BAs in IBS-D patients is featured by increased primary BAs and decreased secondary BAs, which was associated with diarrhea and visceral hypersensitivity. The abnormality of BAs might be induced by dysbiosis in IBS-D patients, especially the reduction of genera in the *Ruminococcaceae* family, which contains the majority of bacteria that are capable of converting primary BAs into secondary BAs.

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INTRODUCTION

Irritable bowel syndrome (IBS) is a chronic and sometimes intractable functional bowel disorder, characterized by recurrent abdominal pain related to defecation or accompanied by changes in stool frequency or form. It affects 1.1%–35.5% of the population across different countries[1], with a prevalence of 6.5% in the Chinese population[2]. IBS is classified into four subtypes based on the predominant stool form of the patients: IBS with predominant diarrhea (IBS-D), IBS with predominant constipation (IBS-C), IBS with mixed bowel habits, and IBS unclassified[3]. As the most

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common subtype in China, IBS-D accounts for 47.1%–66.3% of all IBS cases[4,5]. The exact pathogenesis of IBS is incompletely understood. Motility disturbances and visceral hypersensitivity are generally thought to be involved, and gut dysbiosis, immune dysregulation, intestinal barrier dysfunction, and brain-gut interaction disorder may also be implicated in the pathophysiology of IBS[6].

Recently, bile acids (BAs) metabolism has attracted attention in IBS. Apart from facilitating the absorption of lipids, BAs can also affect gastrointestinal motility, mucosal permeability, and the secretion of water, electrolytes, and mucus in the gut[7]. Changes in BAs metabolism between IBS patients and healthy populations have been reported[8-10], but the data delineating BAs metabolism in Chinese IBS-D patients are still sparse. Moreover, BA sequestrants, which can bind intraluminal BAs, can attenuate diarrhea and decrease the score of the IBS Symptom Severity Scale (IBS-SSS) in IBS-D patients[11-13]. Therefore, the involvement of BAs in the pathogenesis of IBS-D is worthy of particular note.

Primary BAs, including cholic acid (CA) and chenodeoxycholic acid (CDCA), are formed from cholesterol in hepatocytes, excreted through the biliary tree into the gallbladder after conjugation with glycine or taurine, and further released into the duodenum in response to meal ingestion. Conjugated CA and CDCA are then chemically modified by the intestinal microbiota through deconjugation and 7α-dehydroxylation, converting them into secondary BAs, *i.e.* deoxycholic acid (DCA) and lithocholic acid (LCA), respectively. Apart from LCA, a small part of CDCA is transformed into another kind of secondary BA, ursodeoxycholic acid (UDCA), by microbiota through 7α/β-epimerization[14,15]. DCA, LCA, and UDCA can also be conjugated with glycine or taurine. Over 90% of conjugated BAs are reabsorbed in the terminal ileum and return to the liver through the portal circulation, while unconjugated BAs can be reabsorbed by passive diffusion across the epithelium of the small intestine and colon[7,16]. Because of the critical position of the gut microbiome in BAs synthetic regulation, gut dysbiosis may lead to dysmetabolism of BAs[14]. Ample evidence has suggested that the gut microbiota of IBS-D patients differs from that of healthy controls (HCs)[17-21], and gut dysbiosis has been considered to be involved in the putative pathophysiology of IBS[22-24]. However, information about correlations between fecal BAs and the gut flora in IBS-D patients is limited.

Due to the evidence of altered fecal BA profile and gut microbiota in IBS-D patients, we hypothesized that BAs may participate in the pathogenesis of IBS-D, and the gut dysbiosis may contribute to the abnormity of BAs metabolism. To investigate this hypothesis, BAs-related metabolomic analyses and 16S rRNA gene sequencing of feces were performed in an IBS-D cohort and matched HCs to explore the association between the composition of fecal BA pool and the gut microbiota.

MATERIALS AND METHODS

Subject recruitment and sample collection

A total of 55 IBS-D patients aged between 18 and 60 years along with 28 age-, sex-, and body mass index (BMI)-matched HCs were recruited in this study. The sample size was calculated with PASS 08.0.3 (NCSS LLC., Kaysville, UT, United States), based on the proportions of primary BAs, secondary BAs, and conjugated BAs in IBS-D patients and HCs in a previous study[25]. A sample size of 22 patients and 11 HCs will have 90% power to detect the difference in fecal BAs composition at a significance level of 0.05.

All patients visited the gastroenterology department of China-Japan Friendship Hospital from May 2019 to October 2019 and were diagnosed with IBS-D according to the Rome IV criteria. The score of IBS-SSS of the patients was required to be above 75 to ensure that they were symptomatic at the study entry. HCs were recruited through public advertisements. Subjects with a history of organic diseases (including gastrointestinal diseases and other major organ injuries), major abdominal surgery, endoscopic retrograde cholangiopancreatography, psychiatric disorders, and abuse of alcohol were excluded. Pregnant or lactating female subjects and those with dysmenorrhea were also excluded. Subjects who took probiotics, antibiotics, prokinetics, antispasmodics, analgesics, non-steroidal anti-inflammatory drugs, and antidepressants within 4 wk, or corticosteroids, immunosuppressants, BA sequestrants, and lipid-lowering agents within 6 mo before the study were excluded. Informed consent was obtained from each subject before his/her entry to the study. This study was approved by the Ethics Committee of the China-Japan Friendship Hospital (No. 2019-64-K44).

Fresh stool samples were collected with sterile plastic tubes from all subjects in

China-Japan Friendship Hospital and were immediately transferred to the laboratory and stored at −80°C until analysis. Each sample was homogenized and divided into at least 2 parts for BAs and microbiota analyses. Considering the circadian rhythm in BAs synthesis and metabolism[26], all of the samples were collected between 07:00 am and 10:00 am. All subjects were asked to maintain their usual dietary habits at least 1 wk before the collection of the stool samples and until all of the assessments were finished. The pharmacological agents aforementioned were not allowed throughout the study period.

Clinical and psychological assessments

Disease severity was evaluated with the IBS-SSS, a validated questionnaire including five items (severity and frequency of abdominal pain, abdominal bloating, bowel habit dissatisfaction, and overall interference with quality of life)[27]. The highest score was 100 for each item and the total score ranges from 0 to 500. All subjects were asked to report their stool consistency according to the Bristol stool form scale (BSFS) and defecation frequency per day during the preceding 2 wk.

Psychological characteristics were assessed with the hospital anxiety and depression scale (HADS) consisting of 7 items for anxiety and 7 items for depression. The maximum score is 21 for both of the dimensions, with a score of less than 8 for non-cases, a score of 8-10 for doubtful cases, and a score of more than 10 for definite cases of anxiety or depression[28]. In addition, the visceral sensitivity index (VSI) scale was used to measure gastrointestinal symptoms-specific anxiety (GSA)[29], with 15 items ranging from score 0 (no GSA) to 5 (severe GSA) and a maximum score of 75.

Visceral sensitivity was assessed with a high-resolution manometry system that has been used previously in our laboratory[30]. Briefly, after completely emptying the rectum with a glycerin enema, an anorectal catheter with a latex balloon at the tip was inserted into the rectum of subjects. After subjects adapted to the catheter in the rectum, the balloon was manually inflated with air through a 100-mL syringe at a speed of 10 mL/5 s, and subjects were asked to report their feelings of initial perception, defecation sensation, and discomfort/pain during the process, with the corresponding balloon volumes recorded as the first sensation threshold, defecating sensation threshold, and maximum tolerable threshold, respectively.

BAs analysis

Chemicals: All 15 standards were obtained from Steraloids Inc. (Newport, RI, United States) and TRC Chemicals (Toronto, ON, Canada) including CA, CDCA, DCA, LCA, UDCA, glyco-cholic acid, glyco-chenodeoxycholic acid, glyco-deoxycholic acid, glyco-lithocholic acid, glyco-ursodeoxycholic acid (GUDCA), tauro-cholic acid (TCA), tauro-chenodeoxycholic acid, tauro-deoxycholic acid, tauro-lithocholic acid, and tauro-ursodeoxycholic acid (TUDCA). Six deuterated internal standards were obtained from Steraloids Inc. (Newport, RI, United States) and C/D/N Isotopes Inc. (Quebec, Canada).

Sample preparation: To diminish sample degradation, feces samples were thawed in an ice-bath at first. Next, approximately 10 mg of each sample was weighed and transferred to a tube together with 25 mg zirconium oxide beads and 200 μL acetonitrile/methanol (8/2) containing 10 μL internal standards. The sample was centrifuged at 13500 *g* for 20 min at 4 °C after homogenization. Then, 10 μL supernatant was diluted with 90 μL of the mixture containing the equal amount of acetonitrile/methanol (8/2) and ultrapure water. After centrifugation, the supernatant was used to quantitate the BAs with the ultraperformance liquid chromatography coupled to tandem mass spectrometry (UPLC-MS/MS) system (ACQUITY UPLC-Xevo TQ-S, Waters Corp., MA, United States).

UPLC-MS/MS analysis: Each sample was injected splitless into an ACQUITY UPLC Cortecs C18 1.6 μM VanGuard precolumn (2.1 mm × 5 mm) and an ACQUITY UPLC Cortecs C18 1.6 μM analytical column (2.1 mm × 100 mm). The mobile phases consisted of 10 mmol/L ammonium acetate with 0.25% acetate acid (mobile phase A) and acetonitrile/methanol/isopropanol (8/1/1) (mobile phase B). The flow rate was 0.40 mL/min with the following mobile phase gradient: 0-0.3 min (5% B), 0.3-0.5 min (5%-10% B), 0.5-2 min (10%-15% B), 2-3 min (15%-30% B), 3-6 min (30% B), 6-8 min (30%-35% B), 8-9 min (35%-40% B), 9-10 min (40% B), 10-15 min (40%-75% B), 15-15.5 min (75%-100% B), 15.5-16.2 min (100% B), 16.2-16.3 min (100%-5% B), 16.3-17 min (5% B). The column temperature was 30°C and the injection volume of each sample was 5 μL. The capillary voltage was 2.0 kV in negative ion mode. The source temperature was maintained at 150°C and the desolvation gas temperature was maintained at

550°C.

Data processing: The raw data files generated by UPLC-MS/MS were processed using MassLynx software (v4.1; Waters, MA, United States) to perform peak integration, calibration, and quantitation for BAs in the samples. The BAs analysis was conducted by the Metabo-profile Biotechnology (Shanghai, China).

16S rRNA gene sequencing analysis

Microbial DNA was extracted from the fecal samples of the subjects using a QIAamp Fast DNA Stool Mini Kit (Qiagen, Valencia, United States). DNA quality was determined by 1% agarose gel electrophoresis and Thermo NanoDrop 2000 (Thermo Fisher Scientific, MA, United States). The amplification of the hypervariable V3-V4 region was conducted using primers 341F (5′-CCTACGGGRSGCAGCAG-3′) and 806R (5′-GGACTACVVGGGTATCTAATC-3′). The KAPA HiFi Hotstart ReadyMix PCR Kit (Kapa Biosystems, Massachusetts, United States) was used for PCR, containing 15 μL of 2 × Kapa Library Amplification ReadyMix, 1 μL forward primer, 1 μL reverse primer, and 50 ng template DNA. The amplicons were then extracted with 2% agarose gels and further purified using an AxyPrep DNA Gel Extraction Kit (Axygen, CA, United States), and quantified using a Qubit dsDNA HS Assay Kit (Invitrogen, MA, United States). After the library was constructed, sequencing was performed using the Illumina NovaSeq PE250 platform (Illumina, CA, United States). Fecal 16S rRNA analysis was conducted by the Realbio Genomics Institute (Shanghai, China).

Sequences with similarity ≥ 97% were defined as operational taxonomic units. Alpha-diversity was analyzed with QIIME (V1.9.1), including the Shannon and Simpson indexes to depict microbial diversity as well as the Chao1 index to depict microbial richness. Beta diversity was assessed by principal coordinate analysis (PCoA) based on weighted and unweighted UniFrac distance metrics analysis. The Mann-Whitney *U*-test was employed to compare the relative abundance of bacterial taxa between the IBS-D group and the control group after logarithmic transformation. The distinguishing features of the fecal microbiota were analyzed using linear discriminant analysis (LDA) effect size (LEfSe), which can identify floras both significantly different and biologically meaningful[31]. The LEfSe *P* value of 0.05 and the LDA score threshold of 2.0 were used in this study.

Statistical analysis

Data are presented as the mean ± SD or the median (Q1, Q3). Comparisons between groups were performed using the independent samples t-test or the nonparametric Mann-Whitney U-test, and qualitative data were analyzed using the Chi-squared test, with a two-sided $P < 0.05$ considered statistically significant. False discovery rate (FDR) correction following the Benjamin-Hochberg method was applied when comparing the concentrations of BAs and the relative abundances of gut microbiota in the two groups. The relationships of BAs with the other clinical and microbial parameters were analyzed by Spearman's correlation. Statistical analyses were performed using SPSS version 23.0 (SPSS Inc., Chicago, IL, United States). Statistical charts were generated using Graph Prism version 6.0 (GraphPad Software Inc., La Jolla, CA, United States).

Apart from the absolute concentrations of the different BAs, several parameters were also analyzed. The total BAs is the sum of the 15 BAs. The unconjugated BAs is the sum of CA, CDCA, DCA, LCA, and UDCA, and the conjugated BAs is the sum of their corresponding glyco- and tauro-BAs. The primary BAs is the sum of CA and CDCA and their glyco- and tauro- derivatives, and similarly, the secondary BAs is the sum of DCA, LCA, UDCA, and their glyco- and tauro-derivatives.

RESULTS

Demographics and clinical characteristics

Fifty-five IBS-D patients (41 men, 14 women) and 28 age-, sex-, and BMI-matched HCs (20 men, 8 women) participated in the study. Their demographic and clinical characteristics are presented in Table 1. The median duration of disease was 3.0 years (range, 0.5–30 years) and the median IBS-SSS score was 180.0 (range, 100–410) in the IBS-D group. The scores of abdominal pain severity and frequency on the IBS-SSS were 40.0 (20.0, 50.0) and 30.0 (20.0, 40.0) in the IBS-D group, respectively. The defecation frequency and BSFS scores were both significantly higher in IBS-D patients

Table 1 Demographics and clinical characteristics of patients with diarrhea-predominant irritable bowel syndrome and healthy controls

Features	IBS-D patients	Controls	*P* value
n	55	28	NA
Age in yr	35.1 ± 9.8	34.4 ± 10.6	0.754
Gender, male: Female	41: 14	5: 2	0.761
Body mass index in kg/m^2	23.3 ± 3.4	22.6 ± 3.1	0.335
Duration of disease in yr	3.0 (1.5, 7.0)	NA	NA
Defecation frequency	3.5 (2.5, 4.0)	1.0 (0.7, 1.0)	< 0.001
BSFS score	5.5 (5.0, 6.0)	4.0 (4.0, 4.0)	< 0.001
IBS-SSS	180.0 (150.0, 240.0)	NA	NA
Abdominal pain severity	40.0 (20.0, 50.0)	NA	NA
Abdominal pain frequency	30.0 (20.0, 40.0)	NA	NA
Abdominal bloating	10.0 (0.0, 20.0)	NA	NA
Bowel habit dissatisfaction	60.0 (60.0, 70.0)	NA	NA
Overall interference with QOL	30.0 (30.0, 50.0)	NA	NA
HADS anxiety score	5.0 (3.0, 10.0)	2.0 (0.0, 3.8)	< 0.001
HADS depression score	4.0 (1.0, 7.0)	1.5 (0.0, 3.8)	0.009
VSI score	30.0 (19.0, 42.0)	4.0 (0.0, 13.3)	< 0.001

The data are presented as the mean ± SD or the median (Q1, Q3). BSFS: Bristol stool form scale; HADS: Hospital anxiety and depression scale; IBS-D: Irritable bowel syndrome with predominant diarrhea; IBS-SSS: Irritable bowel syndrome symptom severity scale; NA: Not applicable; QOL: Quality of life; VSI: Visceral sensitivity index.

than in HCs ($P < 0.001$). Meanwhile, scores of HADS-anxiety, HADS-depression, and VSI were significantly increased in patients compared to controls ($P < 0.01$), indicating that IBS-D patients were prone to suffering from comorbid anxiety and depression.

In total, 23 of the 55 IBS-D patients and 15 of the 28 HCs participated in the visceral sensitivity test, while the other subjects refused this examination due to the concern of the discomfort caused by the catheter in the rectum. The maximum tolerable threshold was significantly lower in the patients compared to that in HCs ($P < 0.001$). The defecating sensation threshold tended to decrease in the patients, but the difference was not statistically significant ($P = 0.073$). The difference between the first sensation thresholds of the two groups was not significant (Table 2).

Fecal BA pool composition

The absolute concentrations of the 15 fecal BAs measured were available for all of the subjects and are summarized in Table 3. Compared to HCs, primary BAs, including CA, CDCA, and corresponding conjugated BAs, were significantly elevated in IBS-D patients ($P < 0.01$). Moreover, IBS-D patients displayed a significant decrease of LCA ($P < 0.01$) and a decreased trend of DCA although not significantly ($P = 0.084$). In addition, TUDCA and GUDCA were also significantly increased ($P < 0.01$), while UDCA was significantly decreased in IBS-D patients compared to HCs ($P < 0.05$). The level of total fecal BAs was significantly elevated in the IBS-D group ($P < 0.05$).

In terms of unconjugated BAs and conjugated BAs, the fecal BA pool of the IBS-D group and the HC group were both predominantly composed of unconjugated BAs. The ratio of conjugated BAs to unconjugated BAs (CBA/UBA) was significantly increased in the IBS-D group ($P < 0.01$), with the levels of unconjugated BAs and conjugated BAs both significantly higher in IBS-D patients compared to HCs ($P < 0.05$). Meanwhile, the level of fecal secondary BAs was much higher than primary BAs in the control group; however, this trend was inversed in some IBS-D patients, leading to a significantly increased ratio of primary BAs to secondary BAs (PBA/SBA) in the IBS-D group ($P < 0.001$).

To further explore the proportion of IBS-D patients with a remarkably imbalanced fecal BAs composition, the 90th percentiles of the CBA/UBA ratio (0.0299) and the PBA/SBA ratio (1.40) in the control group were determined as cutoff values, and we

Table 2 Visceral sensation thresholds in patients with diarrhea-predominant irritable bowel syndrome and healthy controls

Visceral sensation threshold in mL	IBS-D patients, *n* = 23	Controls, *n* = 15	*P* value
First sensation threshold	40 (20, 50)	40 (35, 60)	0.235
Defecating sensation threshold	60 (50, 85)	70 (65, 100)	0.073
Maximum sensation threshold	105 (90, 120)	160 (145, 190)	< 0.001

The data are expressed as median (Q1, Q3). IBS-D: Irritable bowel syndrome with predominant diarrhea.

Table 3 Levels of fecal bile acids in patients with diarrhea-predominant irritable bowel syndrome and controls

Bile acids in nmol/g	IBS-D patients, *n* = 55	Controls, *n* = 28	*P* value
CA	3037.66 (282.82, 6917.47)	20.19 (5.03, 1304.28)	< 0.001
CDCA	1721.86 (352.80, 2613.83)	57.16 (13.76, 1639.92)	< 0.001
DCA	2012.66 (232.57, 2659.34)	2159.78 (1676.03, 3094.08)	0.084
LCA	1621.65 (58.99, 2396.49)	2339.24 (1737.09, 2782.40)	0.002
UDCA	8.92 (2.33, 23.93)	17.21 (8.76, 33.48)	0.025
TCA	5.36 (0.62, 26.39)	0.72 (0.46, 2.11)	0.004
TCDCA	6.85 (1.54, 22.89)	1.41 (0.37, 3.58)	0.001
TDCA	1.53 (0.93, 8.08)	1.75 (0.86, 6.63)	1.000
TLCA	0.88 (0.57, 1.85)	1.03 (0.36, 2.80)	0.908
TUDCA	1.43 (0.68, 2.61)	0.37 (0.07, 1.23)	0.002
GCA	4.36 (2.31, 17.52)	2.23 (1.39, 3.55)	< 0.001
GCDCA	17.47 (5.61, 51.56)	5.17 (2.56, 10.51)	< 0.001
GDCA	3.32 (0.63, 10.63)	2.67 (1.44, 6.83)	0.867
GLCA	0.64 (0.39, 1.61)	0.91 (0.41, 1.28)	0.282
GUDCA	1.27 (0.56, 4.76)	0.65 (0.38, 0.87)	0.002
Total BAs	8227.35 (5218.49, 12464.03)	5220.28 (3971.35, 8272.29)	0.011
Unconjugated BAs	8197.73 (5135.49, 11622.72)	5183.71 (3945.65, 8253.23)	0.017
Conjugated BAs	55.28 (27.09, 198.08)	28.51 (9.68, 36.32)	0.002
Tauro-BAs	23.00 (5.03, 58.65)	6.34 (2.52, 18.05)	0.006
Glyco-BAs	28.03 (13.20, 69.07)	12.77 (6.18, 23.15)	< 0.001
CBA/UBA ratio	0.0080 (0.0036, 0.0172)	0.0035 (0.0019, 0.0058)	0.004
Primary BAs	5534.22 (672.24, 10546.54)	85.88 (27.08, 3197.10)	< 0.001
Secondary BAs	3759.10 (336.36, 4694.96)	4675.59 (3543.60, 5623.73)	0.009
PBA/SBA ratio	1.66 (0.22, 11.14)	0.02 (0.01, 0.57)	< 0.001

The bile acids data are presented as the median (Q1, Q3). The *P* value after FDR correction was presented in the table. BAs: Bile acids; CA: Cholic acid; CBA/UBA ratio: The ratio of conjugated BAs to unconjugated BAs; CDCA: Chenodeoxycholic acid; DCA: Deoxycholic acid; GCA: Glyco-cholic acid; GCDCA: Glyco-chenodeoxycholic acid; GDCA: Glyco-deoxycholic acid; GLCA: Glyco-lithocholic acid; GUDCA: Glyco-ursodeoxycholic acid; IBS-D: Irritable bowel syndrome with predominant diarrhea; LCA: Lithocholic acid; PBA/SBA ratio: The ratio of primary BAs to secondary Bas; TCA: Tauro-cholic acid; TCDCA: Tauro-chenodeoxycholic acid; TDCA: Tauro-deoxycholic acid; TLCA: Tauro-lithocholic acid; TUDCA: Tauro-ursodeoxycholic acid; UDCA: Ursodeoxycholic acid.

found that 10 (18.2%) and 30 (54.5%) patients had a high CBA/UBA ratio (≥ 0.0299) and a high PBA/SBA ratio (≥ 1.40) among the 55 IBS-D patients, respectively. There were no significant differences in demographic indices, BMI, or duration of disease between the high CBA/UBA and low CBA/UBA groups in patients, as well as between the high PBA/SBA and low PBA/SBA groups in patients.

Correlations between fecal BAs and clinical parameters

We analyzed the correlations between the fecal BAs and clinical parameters in the IBS-D group. Defecation frequency was positively associated with the levels of CA and CDCA and inversely associated with the levels of DCA and LCA ($P < 0.05$) (Figure 1A-D). Moreover, we observed an inverse correlation between the first sensation threshold and the concentration of CDCA ($P < 0.05$) (Figure 1E). The defecating sensation threshold also tended to be negatively correlated with CDCA ($P = 0.060$) (Figure 1F). However, the duration of disease, IBS-SSS, abdominal pain severity, abdominal pain frequency, the BSFS score, and the maximum tolerable threshold showed no significant correlations with fecal BAs. Additionally, there were no significant correlations between the HADS-anxiety score, HADS-depression score, or VSI and fecal BAs, in line with the earlier studies[9,32].

In the IBS-D group, patients with a high PBA/SBA ratio had a significantly higher defecation frequency than those with a low PBA/SBA ratio (3.5 [2.5, 4.5] *vs* 3.0 [1.5, 3.5]; $P = 0.038$), along with an increasing trend of bowel habit dissatisfaction (70.0 [60.0, 72.5] *vs* 60.0 [45.0, 70.0]; $P = 0.059$] and abdominal pain severity (40.0 [30.0, 60.0] *vs* 30.0 [20.0, 50.0]; $P = 0.077$). However, all of the clinical parameters aforementioned showed no significant difference between patients with high and low CBA/UBA ratios.

Characteristics of the gut microbiome of IBS-D patients

We performed 16S rRNA gene sequencing of the fecal samples from all subjects. Chao1 analysis exhibited a decrease of microbiota richness in IBS-D patients compared with HCs ($P < 0.05$) (Figure 2A), whereas the results of the Shannon and Simpson indices showed no evident differences in the diversity and evenness of the colonic microbiota. Unweighted and weighted UniFrac PCoA showed that the global microbiota structure of the samples of IBS-D patients differed significantly from that of HCs ($P < 0.05$) (Figure 2B and C). The configuration of the gut microbiota in the two groups is shown in Figure 3.

Furthermore, distinguishing phylotypes at the phylum, class, order, family, and genus levels were analyzed *via* LEfSe (Figure 4). As dominant phyla in human feces, the relative abundance of *Proteobacteria* was significantly higher in IBS-D group ($P < 0.001$), and *Bacteroidetes* and *Actinobacteria* tended to increase while *Firmicutes* tended to decrease in the IBS-D group. The gut microbiota of IBS-D patients were characterized by decreased relative abundance of the class *clostridia* (LDA score [log10] > 4.8, $P = 0.011$), the order *Clostridiales* (LDA score [log10] > 4.8, $P = 0.011$), and the family *Ruminococcaceae* (LDA score [log10] > 4.8, $P < 0.001$), and increased relative abundances of the class *Gammaproteobacteria* (LDA score [log10] > 3.6, $P < 0.001$), the order *Enterobacteriales* (LDA score [log10] > 3.6, $P < 0.001$), and the family *Enterobacteriaceae* (LDA score [log10] > 3.6, $P < 0.001$). At the genus level, 12 genera were significantly less abundant ($P < 0.05$) in the fecal microbiota of IBS-D patients, including 6 genera in *Ruminococcaceae* (*Anaerofilum*, *Anaerotruncus*, *Clostridium IV*, *Faecalibacterium*, *Gemmiger*, and *Oscillibacter*), 2 genera in the family *Lachnospiraceae* (*Clostridium XlVb* and *Coprococcus*), 2 genera in the family *Porphyromonadaceae* (*Barnesiella* and *Odoribacter*), 1 genus in the family *Rikenellaceae* (*Alistipes*) and 1 genus in the family *Synergistaceae* (*Cloacibacillus*), yet the differences of *Clostridium IV*, *Clostridium XlVb* and *Barnesiella* lost significance after FDR correction. In parallel, 8 genera were significantly more abundant in the IBS-D group ($P < 0.05$), including *Escherichia/shigella*, *Enterococcus*, *Streptococcus, Rothia*, *Klebsiella*, *Saccharibacteria genera incertae sedis*, *Fusobacterium*, and *Veillonella. Ruminococcus* belonging to *Ruminococcaceae* was also increased in the IBS-D group ($P = 0.049$), but it lost significance after FDR correction.

Correlations between fecal BAs and microbiota composition

To explore whether the changes in fecal BA profile correlated with the configuration of gut flora, and given that conjugated BAs only account for a minor part of fecal BA pool, we analyzed the associations between the five unconjugated BAs (CA, CDCA, DCA, LCA, and UDCA) and the distinguishing genera in all subjects. Markedly, the genera reduced in the IBS-D group except for *Cloacibacillus* exhibited a negative correlation with primary BAs and a positive correlation with secondary BAs, and *Ruminococcus* presented a similar trend (Figure 5).

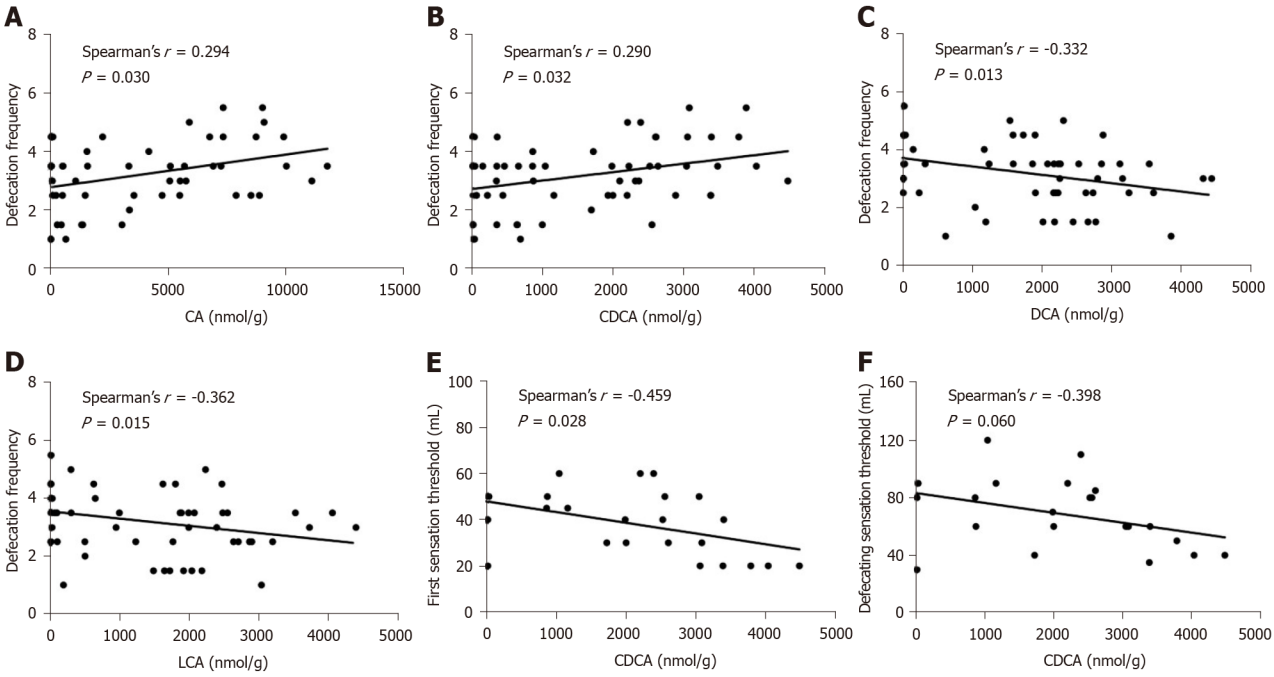


Figure 1 Correlations between fecal bile acids and clinical parameters in patients with diarrhea-predominant irritable bowel syndrome. A-D: Defecation frequency was positively correlated with the level of cholic acid and chenodeoxycholic acid (CDCA), and negatively correlated with the level of deoxycholic acid and lithocholic acid; E: The first sensation threshold was negatively correlated with CDCA; F: The defecating sensation threshold tended to be negatively correlated with CDCA. CA: Cholic acid; CDCA: Chenodeoxycholic acid; DCA: Deoxycholic acid; LCA: Lithocholic acid.

DISCUSSION

In this study, we comprehensively assessed the composition of the fecal BA pool of IBS-D patients, the correlations between clinical features and the imbalance of fecal BAs, and the correlations between fecal BAs and the gut microbiome. The fecal BA pool of IBS-D patients was characterized by increased primary BAs and decreased secondary BAs, along with increased total BAs. Correlations between defecation frequency and thresholds of rectal distention testing with fecal BAs were observed. Furthermore, we found that several genera with discrepant relative abundances between IBS-D patients and HCs were significantly related to fecal BAs, especially the genera within the family *Ruminococcaceae*.

Evidence is accumulating that fecal primary BAs are increased in IBS-D[9,10,25,33]. A study with 14 IBS-D patients and 18 HCs by Duboc *et al*[33] reported that fecal DCA was reduced in IBS-D patients, and the levels of LCA and UDCA in IBS-D patients were similar to those in HCs, whereas our results showed that LCA and UDCA were decreased significantly in IBS-D patients and DCA tended to decrease. Over half of the subjects in the IBS-D group had a higher PBA/SBA ratio than the 90th percentiles of the PBA/SBA ratio in HCs in the current study, suggesting that the imbalance between primary BAs and secondary BAs in IBS-D deserves more attention. The level of total fecal BAs in IBS-D is inconsistent in different studies, with several reporting an increase[34,35] while others showing no significant difference compared with HCs[8,10,25,33]. A meta-analysis reported that BA malabsorption (BAM) appeared in 28.1% of IBS-D patients[36]. However, Peleman *et al*[37] demonstrated that fecal CA and CDCA concentrations in IBS-D patients without BAM were still significantly higher than those in HCs, suggesting that abnormal BA metabolism might exist in IBS-D patients whether they have co-occurring BAM or not.

Both fecal conjugated and unconjugated BAs were increased significantly in the IBS-D group in the present study. One likely explanation is that the BA biosynthesis increases in IBS-D patients, as evidenced by elevated serum 7α-hydroxy-4-cholesten-3-one (C4, the precursor of primary BAs in the liver and thus a marker of hepatic BA synthesis[38]) in several studies[8,11,35], thereby the conjugated BAs entering the intestine may also increase, leading to more unconjugated BAs. However, only a minority of the IBS-D patients had a CBA/UBA ratio above the 90th percentiles of the CBA/UBA ratio in HC, and we found no significant difference in clinical manifestations between patients with high and low CBA/UBA ratios, suggesting that an abnormality of conversion from conjugated BAs to unconjugated BAs may not widely exist in IBS-D

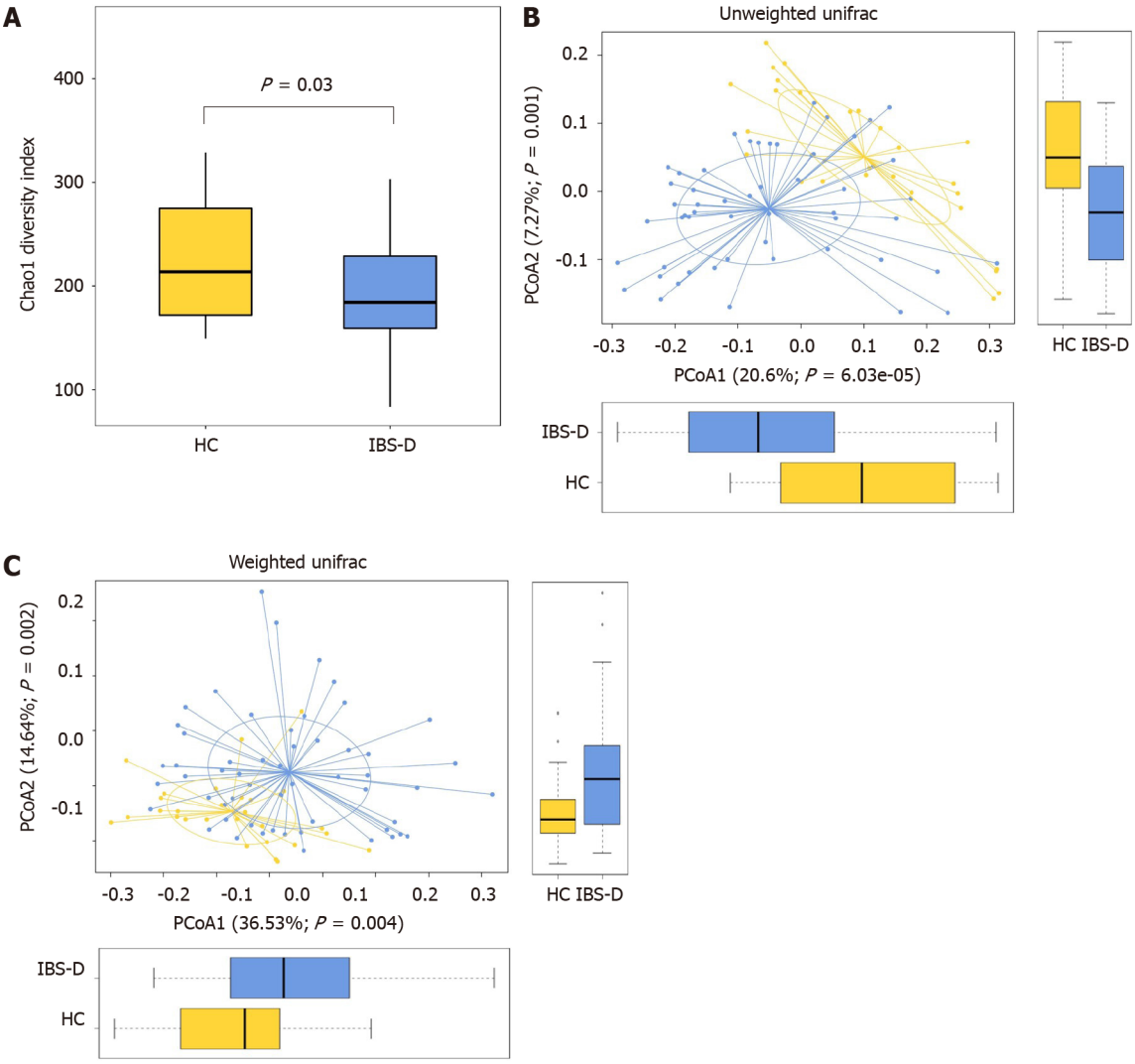


Figure 2 Fecal bacterial structures of patients with diarrhea-predominant irritable bowel syndrome and healthy controls. A: Chao1 index in the irritable bowel syndrome with predominant diarrhea (IBS-D) group and the healthy control (HC) group, Chao1 index was decreased significantly in the IBS-D group; B and C: Weighted and unweighted principal coordinate analysis of fecal bacterial in the IBS-D and HC groups, both differed significantly between the two groups. Boxes indicate the interquartile range; lines inside the boxes indicate the medians; the two whiskers indicate the maximum and minimum of the data; the points outside the box indicate outliers. PCoA: Principal coordinate analysis.

patients.

BAs have been previously shown as an intraluminal factor influencing the secretion function of the colon. Rectal perfusion of CDCA, DCA, and their conjugated BAs in healthy volunteers can induce water and chloride secretion[39]. Experiments *in vitro* revealed that CDCA, DCA, and their conjugated patterns can inhibit Cl^-/OH^- exchange and activate the cystic fibrosis transmembrane conductance regulator of colonic epithelial cells, attenuating the absorption and stimulating the secretion of chloride, respectively[40-42]. Besides, several studies found that instillation of TCA into the sigmoid colon and oral administration of CA and CDCA could accelerate colon motility[43-45], and the colonic transit rate of IBS-D patients showed positive correlation with the proportion of primary BAs in feces[37], indicating that primary BAs can modulate colonic motor function. The positive association between defecation frequency and primary BAs found in the current study, consistent with previous reports[9,33], might result from the promotion of secretion and motility of the colon by CA and CDCA. It is noteworthy that the relationship between colonic motility and luminal BAs is not unidirectional. Aside from BAs, abnormal motility may be induced by other factors such as neuromuscular dysfunction and chronic stress in IBS-D patients[46,47]. The variance in colonic transit may influence the passive absorption of BAs across the colon epithelium and result in changes of BAs excreted in feces[37], which is corroborated by the decrease or increase of total BAs in the feces of healthy volunteers when intervened with loperamide or senna, respectively[48]. Therefore, correlations cannot be equated with causal associations in this observational study,

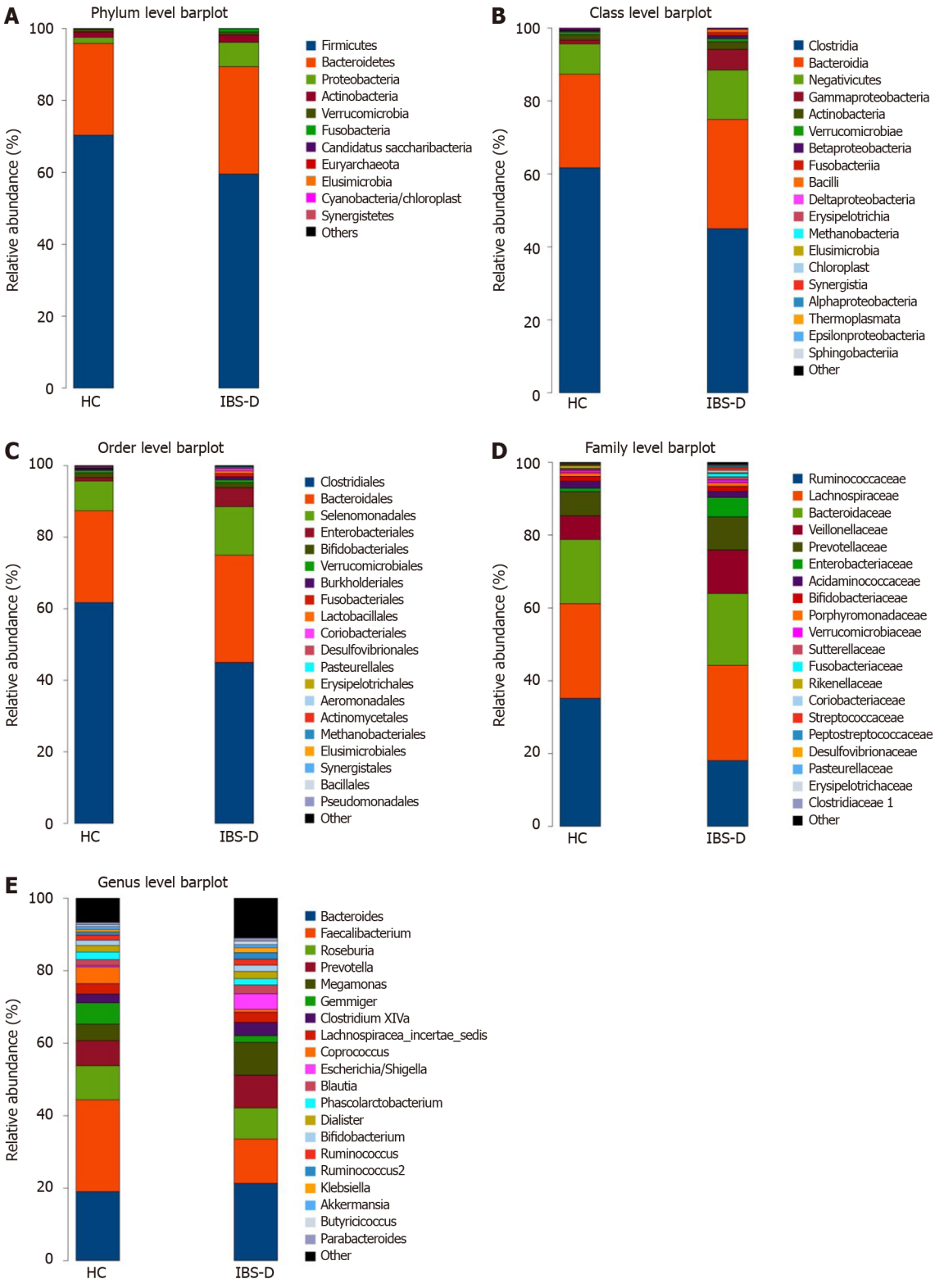
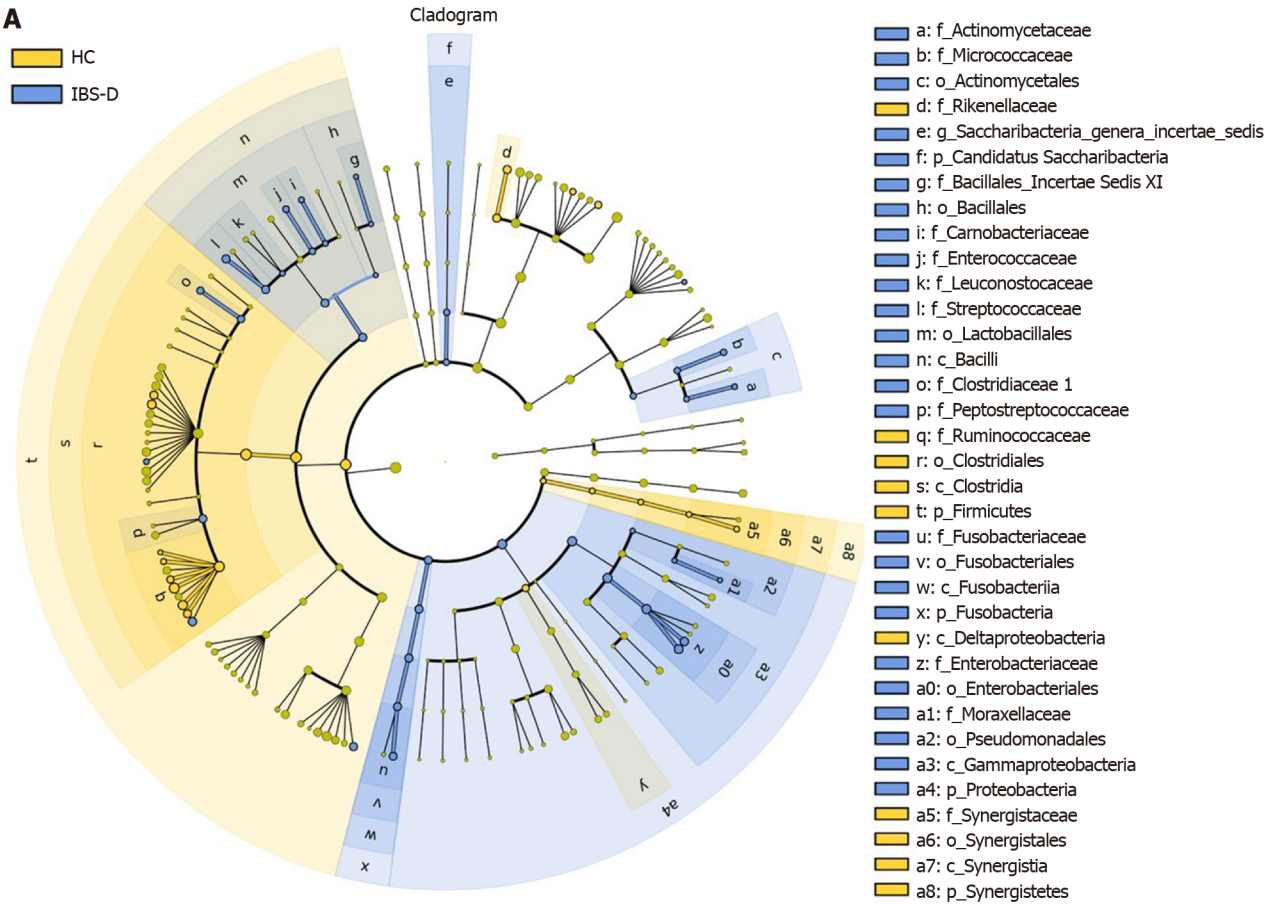


Figure 3 Configuration of fecal bacterial of patients with diarrhea-predominant irritable bowel syndrome and healthy controls. A-E: Relative bacterial abundance of fecal bacterial at phylum (A), class (B), order (C), family (D), and genus (E) levels in the irritable bowel syndrome with predominant diarrhea group and the healthy control group. IBS-D: Irritable bowel syndrome with predominant diarrhea; HC: Healthy control.

and a longitudinal study is required to verify these results.

Previous studies mainly focused on the relationship between gastrointestinal motility rather than visceral sensitivity and BAs in IBS-D[9,10,35]. Experiments in animals and healthy volunteers demonstrated that instillation of CDCA and DCA could increase the sensitivity to rectal distension[49-51]. Consistently, we observed that the first sensation threshold and the defecating sensation threshold inversely associated with


A
HC
IBS-D
Cladogram
a: f_Actinomycetaceae
b: f_Micrococcaceae
c: o_Actinomycetales
d: f_Rikenellaceae
e: g_Saccharibacteria_genera_incertae_sedis
f: p_Candidatus Saccharibacteria
g: f_Bacillales_Incertae Sedis XI
h: o_Bacillales
i: f_Carnobacteriaceae
j: f_Enterococcaceae
k: f_Leuconostocaceae
l: f_Streptococcaceae
m: o_Lactobacillales
n: c_Bacilli
o: f_Clostridiaceae 1
p: f_Peptostreptococcaceae
q: f_Ruminococcaceae
r: o_Clostridiales
s: c_Clostridia
t: p_Firmicutes
u: f_Fusobacteriaceae
v: o_Fusobacteriales
w: c_Fusobacteriia
x: p_Fusobacteria
y: c_Deltaproteobacteria
z: f_Enterobacteriaceae
a0: o_Enterobacteriales
a1: f_Moraxellaceae
a2: o_Pseudomonadales
a3: c_Gammaproteobacteria
a4: p_Proteobacteria
a5: f_Synergistaceae
a6: o_Synergistales
a7: c_Synergistia
a8: p_Synergistetes

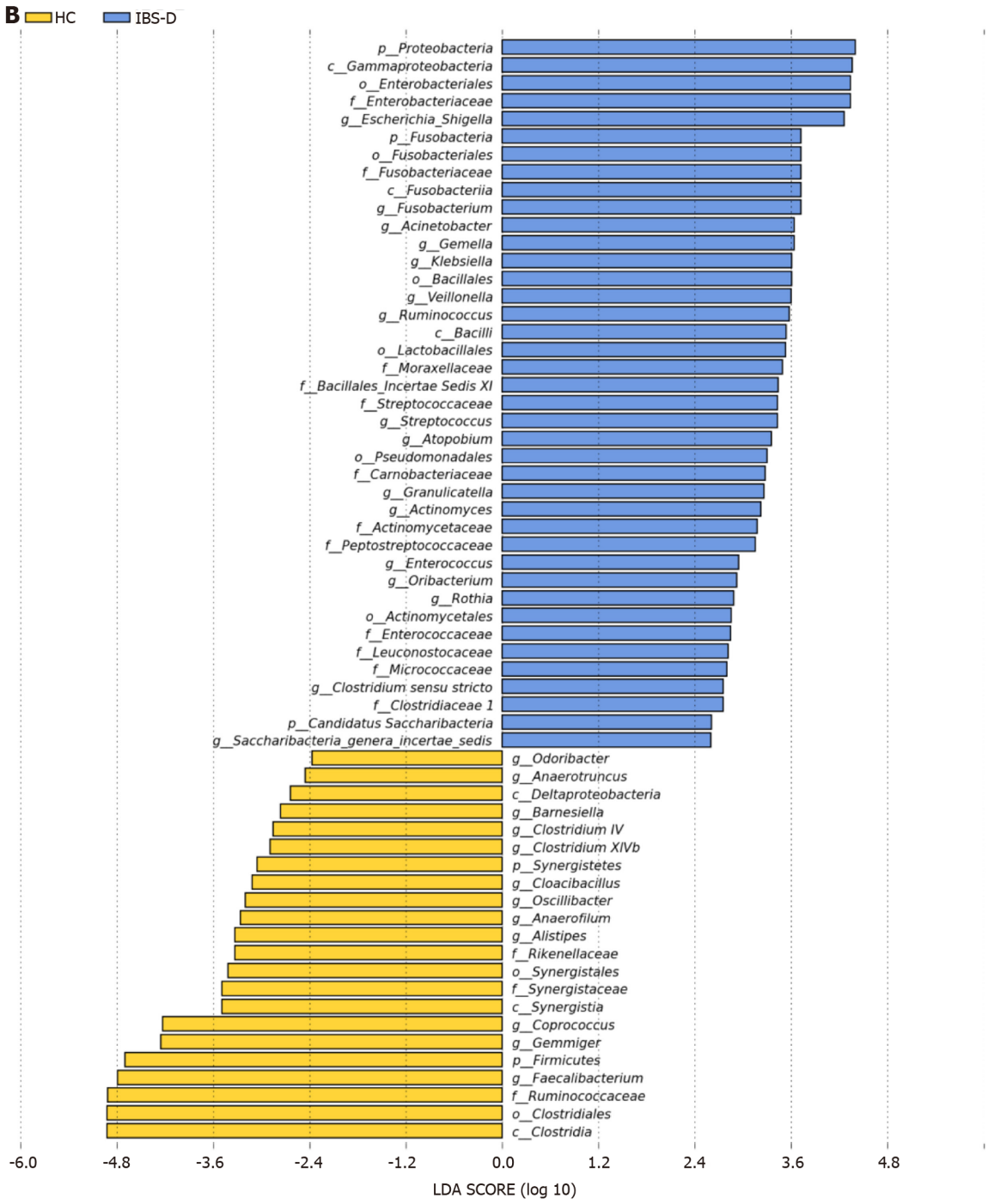


Figure 4 Linear discriminant analysis effect size analysis of fecal bacterial of patients with diarrhea-predominant irritable bowel syndrome and healthy controls. A: The cladogram showed enriched taxa in the irritable bowel syndrome with predominant diarrhea (IBS-D) group (blue) and the healthy control (HC) group (yellow); the taxa represented by the English letters in the cladogram are shown in the legend on the right; B: Taxa enriched in the IBS-D group are indicated with a positive linear discriminant analysis (LDA) score (blue) and taxa enriched in the HC group are indicated with a negative score (yellow); only taxa with LDA effect size (LEfSe) *P* values < 0.05 and LDA scores ≥ 2.0 are presented.

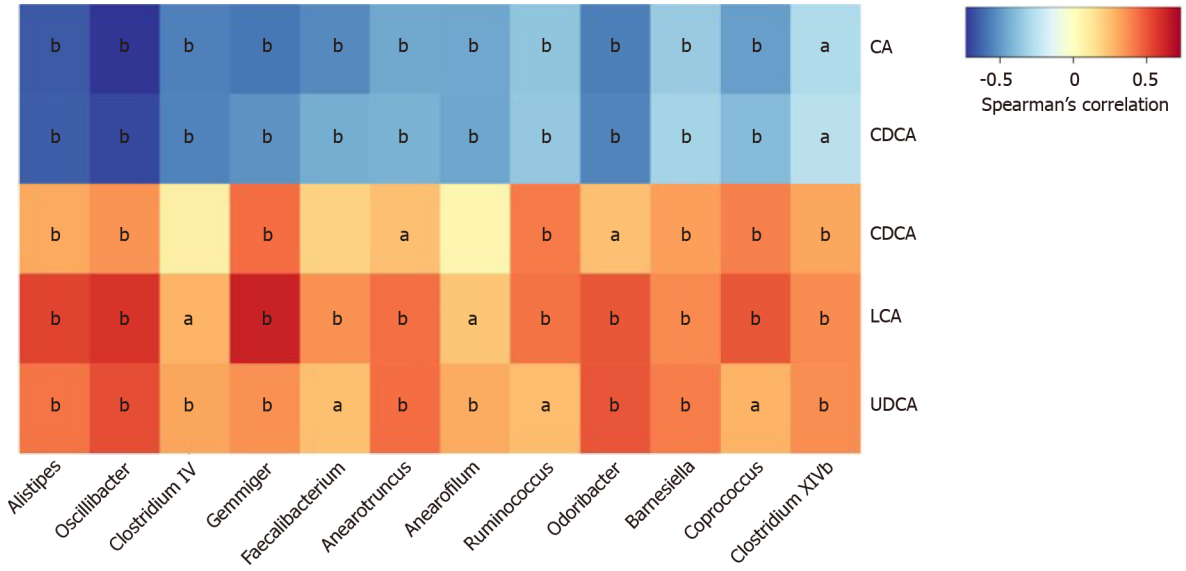


Figure 5 Correlations between fecal bile acids and microbiota composition in all subjects. The heatmap presents the Spearman correlation coefficients between unconjugated fecal bile acids and the distinguishing genera in all subject. Seven genera belong to the family *Ruminococcaceae* (*Oscillibacter*, *Clostridium IV*, *Gemmiger*, *Anaerofilum*, *Anaerotruncus*, *Faecalibacterium*, and *Ruminococcus*); one genus belongs to the family *Rikenellaceae* (*Alistipes*); two genera belong to the family *Porphyromonadaceae* (*Odoribacter* and *Barnesiella*); two genera belong to the family *Lachnospiraceae* (*Coprococcus* and *Clostridium XIVb*). All of the genera were decreased significantly in the IBS-D group except for *Ruminococcus*. $^{a}P < 0.05$, $^{b}P < 0.01$. CA: Cholic acid; CDCA: Chenodeoxycholic acid; DCA: Deoxycholic acid; LCA: Lithocholic acid; UDCA: Ursodeoxycholic acid.

fecal CDCA in IBS-D patients, suggesting that increased CDCA in the colon might induce a decline in the colonic sensory thresholds, which could be one of the potential mechanisms of visceral hypersensitivity of IBS-D patients. Still, these observational results should be viewed with caution, and further studies on the link between hypersensitivity and BAs in IBS-D are warranted.

We next compared the composition of the gut microbiome in IBS-D patients and HCs. As expected, gut dysbiosis was observed in IBS-D patients. In agreement with a previous study[52], we found that the richness of the gut flora was reduced in IBS-D while the diversity and evenness showed no significant difference between IBS-D patients and HCs, and we also found that *Firmicutes* tended to decrease while *Bacteroidetes* tended to increase in IBS-D. The proportions of the *Clostridiales* order and the *Ruminococcaceae* family within it have been reported to decrease in IBS[52,53], which is supported by our findings. In particular, we observed six genera in *Ruminococcaceae* were significantly decreased in IBS-D, including *Anaerofilum*, *Anaerotruncus*, *Clostridium IV*, *Faecalibacterium*, *Gemmiger*, and *Oscillibacter*.

The conversion of BAs in the intestine depends on the microbial community. Tauro- and glyco- BAs are first deconjugated into taurine/glycine and unconjugated BAs under the action of bile salt hydrolase (BSH) enzymes in various bacteria, as a prerequisite for further BAs metabolism by the microbiome[14]. Subsequently, the formation of DCA and LCA from CA and CDCA through 7α-dehydroxylation was carried out by bacteria with BA-inducible (*bai*) genes[14]. BSH distributes widely at the phylum level including *Firmicutes*, *Bacteroidetes*, and *Actinobacteria* in humans[54], while the capability to convert primary BAs to secondary BAs is limited to only a small number of species, mainly in *Clostridiales*, with an overwhelming majority of the strains belonging to *Ruminococcaceae*, and a marginal part belonging to the *Lachnospiraceae* and *Peptostreptococcaceae* families[55-57].

Given the imbalance of primary and secondary BAs in IBS-D and the essential position of microbiota in the metabolism of BAs, we further investigated the association between fecal BAs and the gut microbiota. The multiple genera of *Ruminococcaceae* and *Lachnospiraceae* decreased in IBS-D patients were positively associated with secondary BAs while negatively associated with primary BAs, indicating that the altered composition of the fecal BA pool might be ascribed to the reduction of bacteria with *bai*, which could lower the efficiency of conversion from primary BAs to secondary BAs. *Ruminococcaceae* has been reported to be positively associated with DCA in the feces of cirrhotic patients[58]. Kwan *et al*[59] also provided evidence that DCA and LCA in serum were positively correlated with *Ruminococcaceae* and *Lachnospiraceae* in feces although fecal BAs were not reported, supporting the

importance of these two families in the metabolism of BAs. However, aside from microbial factors, faster colonic transit can result in shorter time for biotransformation of BAs and may be another cause for the imbalance between primary and secondary BAs[37], and the inverse association between DCA and defecation frequency in IBS-D patients in our study may be explained by the shortened CA exposure time caused by accelerated colonic transit, despite that DCA is considered as a secretory BA.

It must be noted that there are reciprocal regulations between the microorganism and BAs[60,61]. The antibacterial activity of BAs[62,63] may affect the gut community structure, thus the abnormal BAs concentration in the gut may, in turn, influence the gut microbiota in IBS-D patients. Increased CA in the gut could induce increased *Gammaproteobacteria*[64], which could be a potential reason for increased *Gammaproteobacteria* in IBS-D patients. Interestingly, animal studies found that CA intake induced expansion of *Firmicutes*, especially the groups capable of 7α-dehydroxylation, and reduction of *Bacteroidetes*[64,65], contrary to the features of the microflora we observed in IBS-D patients, which support to some extent that the reduction of the bacteria with *bai* genes might happen prior to the increase of CA in the gut of IBS-D patients. However, further animal experiments and longitudinal research in humans are required, and BA sequestrants may contribute to the exploration of causal associations between BAs dysmetabolism and gut dysbiosis in IBS-D patients.

There were several limitations in this preliminary study. First, in consideration that women only accounted for approximately 25% of our patients, the findings in this study need to be verified in the future with more female patients recruited. The sex disparity in the distribution of IBS subtypes may be the major reason for the sex imbalance in this study, with IBS-D more prevalent in men and IBS-C more prevalent in women[66,67]. Besides, women with dysmenorrhea were excluded in this study, resulting in a further reduction of available female patients. Second, although BAs can theoretically affect the secretory function of the colon, we failed to observe a correlation between stool form and BAs, which might be a result of the similarity in the BSFS score in most IBS-D patients. Hence, feces moisture, a more precise parameter than the BSFS score, can be a better choice in a future study. Third, we did not supply a standardized diet for subjects during the study period, though subjects were required not to change their daily dietary habits throughout the study period, considering that the short-term modification of a diet can rapidly disturb the gut microbiota[68]. A standardized diet can largely control the influence of disparate eating habits on the microbiota and BA profile. However, the difference between the standardized diet and the usual dietary habits of patients may cause perturbation to the gut microbiota, masking the gut microbiota under usual dietary habits. Therefore, the measures of gut microbiota and BAs before and after a standardized diet combined with a detailed assessment of the usual dietary habits of patients are necessary for a future study.

CONCLUSION

In conclusion, our study depicted the composition of fecal BA pool in IBS-D patients, which was characterized by increased primary BAs and decreased secondary BAs and associated with diarrhea as well as visceral hypersensitivity. The imbalance of primary and secondary BAs might be induced by dysbiosis in IBS-D, especially the reduction of *Ruminococcaceae*. These preliminary findings may offer insight into the complicated pathophysiology of IBS-D, and provide evidence for BAs modulation in the treatment of IBS-D.

ARTICLE HIGHLIGHTS

Research background

Bile acids (BAs) have attracted attention in irritable bowel syndrome with predominant diarrhea (IBS-D) because of the effects on gastrointestinal motility and secretion function of the intestine. Experiments in animals and healthy volunteers also indicated that hypersensitivity can be caused by BAs, which is a major pathophysiological abnormality in IBS-D. The metabolism of BAs in the intestine depends on the gut microbiota. Therefore, it could be hypothesized that BAs may be involved in the pathogenesis of IBS-D and the altered BA profile in the intestine may be associated with microbiota.

Research motivation

Although a few studies have portrayed the composition of fecal BAs in IBS-D patients, the data in Chinese IBS-D patients are still sparse. Besides, few studies have explored the correlations between the gut flora and BAs in IBS-D patients. The main topics of this study included identifying the correlations of BAs with clinical features containing rectoanal sensory parameters of IBS-D patients, and exploring the correlations between the composition of fecal BAs and the gut microbiome. The findings may add insight to the pathogenesis of IBS-D, and provide evidence for regulating intestinal BAs to treat IBS-D.

Research objectives

The present study aimed to evaluate the correlations of BAs with clinical features of IBS-D patients and to explore whether the composition of fecal BAs was associated with the gut microbiome in IBS-D patients.

Research methods

Subjects underwent clinical and psychological assessments, including IBS symptom severity system, the grade of Bristol stool form scale and defecation frequency per day in the preceding 2 wk, hospital anxiety and depression scale, and visceral sensitivity index, along with visceral sensitivity testing with a high-resolution manometry system. Fecal BAs were measured by ultraperformance liquid chromatography coupled to tandem mass spectrometry. The gut microbiota was analyzed using 16S rRNA gene sequencing. Relationships between fecal BAs and clinical characteristics as well as gut microbiota were explored.

Research results

Cholic acid, chenodeoxycholic acid (CDCA), and their conjugated BAs were significantly increased, while lithocholic acid (LCA) and ursodeoxycholic acid were significantly decreased and deoxycholic acid (DCA) tended to decrease in IBS-D patients. Defecation frequency was positively associated with primary BAs and inversely associated with DCA and LCA in IBS-D patients. The first sensation threshold was negatively correlated, and the defecating sensation threshold tended to be negatively correlated with CDCA in IBS-D patients. Furthermore, several genera were significantly reduced in IBS-D patients compared with HCs and exhibited a negative correlation with primary BAs and a positive correlation with secondary BAs, especially the genera in the *Ruminococcaceae* family.

Research conclusions

This study presented evidence that the composition of the fecal BA pool was characterized by increased primary BAs and decreased secondary BAs in IBS-D, which was associated with diarrhea and visceral hypersensitivity in IBS-D. The dysmetabolism of BAs in IBS-D might be ascribed to gut dysbiosis especially the reduction of *Ruminococcaceae*.

Research perspectives

In the future, careful evaluation of the usual dietary habit of subjects is required, and diet needs to be standardized during the study period. Large multicenter studies are also necessary to verify the conclusions drawn in this study. Notably, BA sequestrant may contribute to the studies on the involvement of BAs in IBS-D pathophysiology as well as the association between BAs and microbiota.

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ORIGINAL ARTICLE

Case Control Study

Alteration of fecal tryptophan metabolism correlates with shifted microbiota and may be involved in pathogenesis of colorectal cancer

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Author contributions: Sun XZ designed and performed the study, analyzed the data, and drafted the manuscript; Sun XZ, Zhou YC, and Qin G collected the clinical data and fecal samples from the subjects; Zhao DY gave guidance on experiment operation and data interpretation, and contributed to manuscript revision; Yao SK designed the study, supervised the study performance, revised the manuscript, and obtained the funding.

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Abstract

BACKGROUND

Gut tryptophan (Trp) metabolites are produced by microbiota and/or host metabolism. Some of them have been proven to promote or inhibit colorectal cancer (CRC) *in vitro* and animal models. We hypothesized that there is an alteration of gut Trp metabolism mediated by microbiota and that it might be involved in the pathogenesis of cancer in patients with CRC.

AIM

To investigate the features of Trp metabolism in CRC and the correlation between fecal Trp metabolites and gut microbiota.

METHODS

Seventy-nine patients with colorectal neoplastic lesions (33 with colon adenoma and 46 with sporadic CRC) and 38 healthy controls (HCs) meeting the inclusion and exclusion criteria were included in the study. Their demographic and clinical features were collected. Fecal Trp, kynurenine (KYN), and indoles (metabolites of Trp metabolized by gut microbiota) were examined by ultraperformance liquid chromatography coupled to tandem mass spectrometry. Gut barrier marker and indoleamine 2,3-dioxygenase 1 (*IDO1*) mRNA were analyzed by quantitative real-time polymerase chain reaction. Zonula occludens-1 (ZO-1) protein expression was analyzed by immunohistochemistry. The gut microbiota was detected by 16S ribosomal RNA gene sequencing. Correlations between fecal metabolites and other parameters were examined in all patients.

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RESULTS

The absolute concentration of KYN [1.51 (0.70, 3.46) nmol/g *vs* 0.81 (0.64, 1.57) nmol/g, $P = 0.036$] and the ratio of KYN to Trp [7.39 (4.12, 11.72) × 10^{-3} *vs* 5.23 (1.86, 7.99) × 10^{-3}, $P = 0.032$] were increased in the feces of patients with CRC compared to HCs, while the indoles to Trp ratio was decreased [1.34 (0.70, 2.63) *vs* 2.46 (1.25, 4.10), $P = 0.029$]. The relative *ZO-1* mRNA levels in patients with CRC (0.27 ± 0.24) were significantly lower than those in HCs (1.00 ± 0.31) ($P < 0.001$), and the relative *IDO1* mRNA levels in patients with CRC [1.65 (0.47-2.46)] were increased ($P = 0.035$). *IDO1* mRNA levels were positively associated with the KYN/Trp ratio ($r = 0.327$, $P = 0.003$). ZO-1 mRNA and protein levels were positively correlated with the indoles/Trp ratio ($P = 0.035$ and $P = 0.009$, respectively). In addition, the genera *Asaccharobacter* (Actinobacteria) and *Parabacteroides* (Bacteroidetes), and members of the phylum Firmicutes (*Clostridium XlVb, Fusicatenibacter, Anaerofilum,* and *Anaerostipes*) decreased in CRC and exhibited a positive correlation with indoles in all subjects.

CONCLUSION

Alteration of fecal Trp metabolism mediated by microbiota is associated with intestinal barrier function and tissue Trp metabolism, and may be involved in the pathogenesis of CRC.

Key Words: Tryptophan metabolism; Colorectal cancer; Kynurenine; Indoles; Microbiota; Colorectal adenoma

Core Tip: This study comprehensively assessed the profiles of fecal tryptophan (Trp) metabolism in patients with colorectal cancer (CRC) and to explore the potential correlations between the gut microbiome, intestinal barrier function, tissue kynurenine pathway (KP), and alterations in fecal Trp metabolism. We found that CRC gut Trp metabolism was characterized by a decreased Trp indole pathway, which was positively correlated with bowel gut barrier function, and an increased KP in colon tissue. In addition, the decreased indoles-producing bacteria may lead to downregulation of the Trp indole metabolic pathway, allowing more Trp to be metabolized along the KP.

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INTRODUCTION

Population aging has resulted in a substantial increase in the number of new colorectal cancer (CRC) cases globally. It is estimated that in 2030, approximately 2.2 million new cases will be diagnosed with CRC and that 1.1 million will die from the disease worldwide[1]. Despite a slight decrease in the death rate[2], CRC is still ranking third in morbidity and second among all cancer-related death cases[3,4]. The development of CRC results from the progressive accumulation of genetic and environmental alterations, which drive the malignant evolution of the colon from normal mucosa through early adenoma/polyp to cancer[5]. The complicated process is considered to be associated with the following risk factors: Diet (high intake of fat/protein and low consumption of dietary fibers), sociodemographic and medical factors, lifestyle (exercise, smoking, and drinking history), and so on[6].

A growing number of prior studies have indicated that diet, gut microbiota, and metabolite interactions are involved in the pathogenesis of CRC *via* damage to the epithelial barrier and mucus barrier, gut inflammation, immune escape, genetic/epigenetic alteration[7-9], *etc*. Most of the topics were focused on short-chain

fatty acids[10], amino acids[11], and bile acids (BAs)[12]. Although tryptophan (Trp) metabolites have been proven to promote or inhibit CRC *in vitro* and animal models[13,14], few studies on gut Trp metabolism have been found, especially its interaction with gut microbiota. Therefore, it is meaningful to study the characteristics of gut Trp metabolism in patients with CRC.

In addition to being excreted in the stool and used for protein synthesis, gut Trp is catabolized mainly along the following three pathways[15]: (1) Trp indole pathway: Trp in feces can be metabolized into indole and indole derivatives by gut microorganisms, such as *Clostridium sporogenes* and *E. coli*[16], *Achromobacter liquefaciens, Bacteroides spp*[17], and *Bifidobacterium spp*[18]. Indoles, including indole, indole-3-acid-acetic (IAA), skatole (3-methylindole), indole-3-propionic acid (IPA), indole-3-aldehyde (IALD), indole-3-acetaldehyde (IAALD), and so on[11], play an important role in modulating the expression of inflammation-related genes[19], strengthening the epithelial cell barrier[20], and inhibiting the growth of CRC cells[21] in an aryl hydrocarbon receptor (AHR)-dependent way; (2) Kynurenine (KYN) pathway (KP): Most Trp in tissue is metabolized to KYN along the KP, which is regulated by the main rate-limited enzymes, including indoleamine 2,3-dioxygenase 1 (IDO1) in almost all tissue and Trp 2,3-dioxygenase in the liver[22]. Intestinal bacteria can indirectly participate in the KP by affecting the activity of IDO or the concentration of other metabolites[23]; and (3) Serotonin pathway (SP): Trp can be converted to 5-hydroxytryptophan (5-HTP) by Trp hydroxylase, followed by the decarboxylation of 5-HTP through the activation of aromatic L-amino acid decarboxylase[15]. Spore-forming bacteria promote serotonin synthesis from enterochromaffin cells (ECs) in the colon[24]. Since the first two signaling pathways have been shown to be associated with an impaired gut barrier, overactivation of WNT/β-catenin, and immune escape in CRC mice[13,25,26], we hypothesized that they may also be involved in the development of CRC in humans.

The primary aim of this study was to investigate the alteration of gut Trp metabolism and microbiota in patients with CRC. The secondary purpose was to explore the potential correlations between the gut microbiome, intestinal barrier function, tissue KP, and alterations in fecal Trp metabolism, respectively.

MATERIALS AND METHODS

Subjects and sample collection

In this study, we used the biological samples of 79 patients with colorectal neoplastic lesions [33 with colon adenoma (ADA) and 46 with sporadic CRC] and 38 age-, sex-, and body mass index (BMI)-matched healthy controls (HCs). The patients were recruited between March 2019 and December 2019 at The China-Japan Friendship Hospital. The HCs were recruited through public posters. We estimated the sample size with PASS 08.0.3 (NCSS LLC, Kaysville, UT, United Satets) based on the KYN content in the pretest.

All the patients included in our study were newly diagnosed and did not receive chemotherapy or any other therapy. All patients were hospitalized in the gastroenterology department and underwent neoplastic lesion resection.

The inclusion criteria were as follows: (1) Patients whose tumors were removed and pathologically diagnosed with adenomatous polyp or colorectal adenocarcinoma; (2) HCs were recruited from asymptomatic individuals who had undergone complete colonoscopy and had negative results; and (3) Age of 18-80 years old, regardless of sex. Subjects with the following conditions were excluded: (1) Subjects who had a history of psychiatric disorders or were pregnant or lactating; (2) Subjects with other primary digestive disorders or patients in whom neoplastic lesions developed in the context of hereditary disease; (3) Patients with current evidence of inflammatory or infective diseases; and (4) Those unable to come off any of the following medications: Antibiotics, nonsteroidal anti-inflammatory drugs, immune modulators, probiotics, corticosteroids, prokinetics, or antispasmodics within 4 wk. All patients and HCs volunteered from similar geographic areas and had similar eating habits.

The demographic and clinical data, such as age, sex, BMI, history of smoking, drinking, and detailed family history, were collected. Pathological data were also included for analysis. The tumors were staged according to the American Joint Commission on Cancer (AJCC) TNM staging system[27].

After signing an informed consent form, fecal samples and colon tissues of each subjects were collected. Fecal samples were collected from asymptomatic volunteers before colonoscopy or patients before resection. Each qualified fecal sample was collected in 7:00-9:00 in the morning and divided into two parts with sterile stool

collection tubes. Then, samples were frozen in liquid nitrogen immediately and stored at -80 °C for further testing. Two pieces of colon tissues were taken from the adenoma or cancer of patients and the rectosigmoid junction of HCs. One specimen was immediately fixed in formalin for 3 d, embedded in paraffin, and sectioned (4 μm) for immunohistochemistry. Other tissues were then immediately immersed in RNAstorage reagent (RNAwait; Solarbio, Beijing, China) and stored at -80 °C for real-time polymerase chain reaction (RT-PCR).

Our study was approved by the Ethics Committee of China-Japan Friendship Hospital (No. 2018-116-K85), and written informed consent was obtained from all subjects.

Targeted metabolomics profiling

Chemicals and reagents: We used targeted metabolomics methods to quantitate the Trp and Trp metabolites in this study. The metabolites detected are as follows: L-Trp, L-KYN, indole, skatole, indole-3-carboxylic acid (I3CA), IALD, IAA, IPA, indoxyl-3-sulfate (I3S), and IAALD. All standards used were obtained from Sigma-Aldrich (St. Louis, MO, United States) and Steraloids Inc. (Newport, RI, United States). Formic acid (Optima LC-MS) was obtained from Sigma-Aldrich (St. Louis, MO, United States). Methanol, isopropanol, and acetonitrile (Optima LC-MS) were purchased from Thermo-Fisher Scientific (Fair Lawn, NJ, United States).

Sample preparation: Standards were precisely weighed and dissolved in 50% methanol, mixed into a concentration of 5.0 mg/mL, and then diluted into a series of standard samples to obtain standard curves of concentration. Fecal samples were thawed in an ice bath to diminish degradation. For indoles, 10-mg fecal samples were accurately weighed and transferred into a grinding tube, 15 μL of water was added and homogenized for 3 min, and then 150 μL of methanol containing internal standards was added. After 3 min of homogenizing, the supernatant was transferred to a 96-well plate by high-speed centrifugation (18000 *g*, 20 min). Next, 50 μL of ultrapure water was added, and the samples were oscillated at a speed of 650 rpm for 10 min at 10 °C. For Trp and KYN, 5 mg of samples were weighed and transferred to a new tube. After homogenization and centrifugation, 20 μL of ultrapure water was added, and then the plate was sealed. The derivatized samples were oscillated at a speed of 650 rpm for 10 min at 10 °C. After centrifugation, 135 μL of supernatant was transferred to a new plate for LC-MS analysis.

Ultra-performance liquid chromatography coupled to tandem mass spectrometry data acquisition and processing: In addition to indole and skatole, other metabolites were detected with an ultra-performance liquid chromatography coupled to tandem mass spectrometry (UPLC-MS/MS) system (ACQUITY UPLC-Xevo TQ-S, Waters Corp., Milford, MA, United States). The concentrations of indole and skatole were detected by UPLC-Photo-Diode Array at a wavelength of 218 nm.

The optimized instrument settings of indoles are briefly described below: A 10-uL sample at a temperature of 10 °C was injected in the splitless mode into an ACQUITY UPLC Cortecs C18 1.7 um VanGuard precolumn (2.1 mm × 5 mm) and an ACQUITY UPLC Cortecs C18 1.7 um analytical column (2.1 mm × 100 mm). The mobile phase consisted of 10 mmol/L ammonium acetate with water with 0.1% formic acid (mobile phase A) and acetonitrile/IPA (70:30) or acetonitrile with 0.1% formic acid (mobile phase B) run at a flow rate of 0.3 mL/min. The elution gradients were 0-3 min (10%-30% B), 3-4.5 min (30%-45% B), 4.5-6.5 min (45%-100% B), 6.5-7 min (100% B), 7-8 min (100%-10% B), and 8-9 min (10% B). The column was maintained at 40 °C. The mass spectrometer was operated with source and desolvation temperatures set at 150 °C and 550 °C, respectively. The capillary voltage was 2.0 (ESI-/+) kV mode. The settings for Trp and KYN are similar to those of indoles. However, they have a different mobile phase gradient: 0-1 min (5% B), 1-11 min (5%-78% B), 11-13.5 min (78%-95% B), 13.5-14 min (95%-100% B), 14-16 min (100% B), 16-16.1 min (100%-5% B), and 16.1-18 min (5% B).

The sample control procedure and data control procedure refer to criterion (ISO9001, QAIC/CN/170149). The raw data files generated by UPLC-MS/MS were processed using MassLynx software (v4.1, Waters, Milford, MA, United States) to perform peak integration, calibration, and quantitation for each metabolite.

Zonula occludens-1 and IDO1 mRNA analysis by quantitative RT-PCR

Gut barrier function was assessed by measuring Zonula occludens-1 (ZO-1). The levels of *ZO-1* and *IDO1* mRNA in tissues were analyzed by RT-PCR. Total RNA in colon tissues was isolated with TRIzol Reagent (Servicebio, Wuhan, China). A RevertAid

First Strand cDNA Synthesis Kit was used to perform reverse transcription (Thermo Scientific, Waltham, MA, United States). Then, RT-PCR was performed using the StepOnePlus Real-Time PCR System. The primers (GAPDH: forward, 5′-GGAAGCTTGTCATCAATGGAAATC-3′and reverse, 5′-TGATGACCCT TTTGGCTCCC-3′. IDO1: forward, 5′-GGATTCTTCCTG GTCTCTCTATT GG-3′ and reverse, 5′-GGTCTTCCC AGAACCCTTCATAC-3′. ZO-1: forward, 5′-TTCCAG CCAGCCTGCTAAAC-3′ and reverse, 5′-CAATA GCGTAGCCCGTTCATCT-3′) for PCR were from Biotechnology (Shanghai, China). All samples were amplified with the following thermal cycling conditions: 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s and then 60 °C for 60 s. All results were normalized to the reference gene as a control using the $2^{-\Delta\Delta Ct}$ method[28]. The levels of *ZO-1* and *IDO1* mRNA are expressed as fold changes relative to the mean level of HCs.

ZO-1 protein analysis by immunohistochemistry

Paraffin sections were processed for immunohistochemistry. They were incubated with the primary antibody at a dilution of 1:200 (ZO-1 mouse monoclonal antibody, Proteintech, Wuhan, China). More details were described as previous studies[29,30].

16S ribosomal RNA gene sequencing

DNA extraction and sequencing: Genomic DNA was extracted from 200 mg of fecal samples using a QIAamp Fast DNA Stool Mini Kit (Qiagen, Valencia, United States) following the instructions. The amplification selection region of 16S rDNA was V3-V4, and the general primers used were 341F and 806R. The sequences are 341F (5′-CCTACGGGRSGCAGCAG-3′) and 806R (5′-GGACTACVVGGGTATCTAATC-3′). Individual amplification products were purified, barcoded, and pooled to construct the sequencing library (KAPA HiFi Hotstart ReadyMix PCR kit). Then, Illumina NovaSeq PE250 was used for sequencing (Illumina, CA, United States). The sequenced raw data were then spliced and filtered to obtain clean data. Clustering and species classification were performed. After quality control was performed, reads were first sorted according to their abundance from large to small and then clustered through 97% similarity to obtain operational taxonomic units (OTUs).

Statistical analysis

Chemometric analyses of clinicopathologic and metabolite data were performed using SPSS (IBM-SPSS Statistics, United States) version 22. 0. Quantitative data analyses were carried out by the Kolmogorov-Smirnov to test whether the data were in accord with the normal distribution and are presented as the mean ± SD or the median (Q1, Q3). When comparing between groups, ANOVA were used to analyze data that obeyed the normal distribution. The Mann-Whitney *U* test or Kruskal-Wallis test was used to analyze abnormally distributed data. Qualitative data were analyzed using the chi-squared test or nonparametric test. False discovery rate (FDR) correction was applied when comparing the relative abundances of gut microbiota. The microbiota data were analyzed with R statistical software. Correlations between parameters were explored using Spearman's correlation analysis. A two-tailed $P < 0.05$ was considered statistically significant.

RESULTS

Characteristics of participants

A total of 117 participants were included in the study. There were 46 patients with CRC (32 males and 14 females) and 38 HCs (24 males and 14 females) who participated in the study. In order to gain more insight into the adenoma-carcinoma sequence, 33 patients with ADA (23 males and 10 females) were also included. No significant differences were found between every two groups with regard to age, sex, or BMI ($P > 0.05$). Their demographic and clinical information is presented in Table 1.

Fecal Trp and Trp metabolites

Stool samples with insufficient quantity and unqualified quality were excluded. A total of 91 samples were tested for Trp and Trp metabolites. Metabolites of the 91 subjects were used for correlation analysis with corresponding parameters of colon tissue and shifted microbiota of feces. I3S and IAALD were excluded for analysis due to a detection rate of lower than 85%. The indoles here are the sum of indole, skatole, IAA, IPA, I3CA, and IALD. The absolute concentrations of metabolites are shown in

Table 1 Demographic and clinical information of participants included in the study

Feature	HCs (*n* = 38)	ADA patients (*n* = 33)	CRC patients (*n* = 46)	*P* value
Age (yr)	56.85 ± 10.99	61.18 ± 8.53	63.63 ± 11.39	0.095
Gender (male:female)	24:14	23:10	32:14	0.799
BMI (kg/m^2)	23.86 ± 3.84	24.817 ± 3.31	23.66 ± 2.88	0.676
Smoking history (%)	10 (26.3)	15 (45.5)	17 (37.0)	0.241
Drinking history (%)	14 (36.8)	9 (27.3)	12 (26.1)	0.522
CRC of FDRs (%)	6 (21.4)	8 (24.2)	8 (17.4)	0.753

The data are presented as the mean ± SD. HC: Healthy control; ADA: Colorectal adenoma; CRC: Colorectal cancer; BMI: Body mass index; FDR: False discovery rate.

Table 2. The concentration of fecal KYN was higher in patients with ADA and CRC than in HCs (P = 0.036 and P = 0.627, respectively) but only reached a statistically significant difference in CRC. In detail, the concentrations of IALD and I3CA were significantly elevated in patients with CRC (P = 0.044 and P = 0.084, respectively), while IPA was decreased in both patients with ADA and CRC (P < 0.005). There was no significant difference in the levels of total indoles between patients with ADA and HCs or patients with CRC and HCs (P > 0.05).

Through further analysis, we found that the ratio of KYN to Trp (KYN/Trp ratio) was significantly higher in patients with CRC than in HCs (P < 0.005). In addition, the ratio of indoles to Trp (indoles/Trp ratio) in feces decreased in both patients with ADA and CRC when compared with HCs (P = 0.003 and P = 0.029, respectively). Regarding the concentrations of Trp, indole, IAA, and skatole, no statistically significant difference was found between HCs and patients (P > 0.005). The details are shown in Table 2.

IDO1 mRNA and tight junction protein *ZO-1* mRNA

IDO1 is the main rate-limiting enzyme for Trp metabolism in colon tissues. ZO-1 is a well-accepted marker of gut barrier function. *IDO1* mRNA and *ZO-1* mRNA were detected in colon tissues. The relative *ZO-1* mRNA levels in patients with ADA (0.36 ± 0.21) and CRC (0.27 ± 0.24) were significantly lower than those in HCs (1.00 ± 0.31) (P < 0.001) (Figure 1A). In comparison to HCs [0.93 (0.46-1.28)], the relative *IDO1* mRNA levels in patients with ADA [1.68 (1.28-2.49)] and CRC [1.65 (0.47-2.46)] were increased (P = 0.004 and P = 0.035, respectively) (Figure 1B).

Correlations between fecal Trp metabolism and *ZO-1* and *IDO1* mRNA

We analyzed the correlations between fecal Trp metabolism and other parameters (*IDO1* and *ZO-1* mRNA levels) in all subjects. *IDO1* mRNA levels were positively associated with the KYN/Trp ratio (r = 0.327, P = 0.003), and *ZO-1* mRNA levels were positively correlated with the indoles/Trp ratio (r = 0.279, P = 0.009) (Figure 1C and D).

ZO-1 protein and correlation between indole/Trp ratio and *ZO-1* protein

Representative photos of the immunoreactivity of ZO-1 are showed in Figure 2A-C. Mean optical density of ZO-1 tended to be decreased in ADA [0.34E-2 (0.02E-2 to 0.47E-2)] and CRC [0.08E-2 (0.06E-2 to 0.13E-2)] compared to controls [1.84E-2 (1.22E-2 to 2.56E-2)] (P < 0.001). There was a positive correlation between fecal indoles/Trp ratio and ZO-1 protein in all subjects (r = 0.217, P = 0.045) (Figure 2D).

Features of gut microbiome of patients with colorectal neoplastic lesions

We performed 16S ribosomal RNA gene sequencing on the fecal samples of the three groups of subjects. Microbiome signatures along the adenoma-carcinoma sequence were detected. A total of 883 OTUs were generated from 117 samples. The relative abundances of the microbiota in the three groups at the phylum and genus levels are shown in Figure 3A and B. Firmicutes and Bacteroidetes were the most predominant phyla. The Firmicutes/Bacteroidetes ratio in patients with CRC (49.48%:36.20%) was lower than that in HCs (64.25%:28.06%). Although the richness of flora decreased in the disease groups, it did not reach statistical significance (chao1, P > 0.05). Both the

Table 2 Concentrations of tryptophan and tryptophan metabolites in patients and healthy controls

Metabolite (nmol/L)	Controls (*n* = 28)	ADA patients (*n* = 24)	CRC patients (*n* = 39)	Intergroup comparisons (*P* value)		
				HC/ADA	HC/CRC	ADA/CRC
Trp	196.67 (125.10, 353.30)	289.46 (206.2, 393.72)	241.88 (165.98, 429.16)	0.081	0.118	0.611
KYN	0.81 (0.64, 1.57)	0.94 (0.66, 2.25)	1.51 (0.70, 3.46)	0.627	0.036	0.140
Indole	252.00 (170.00, 545.00)	251.00 (66.50, 366.75)	356.00 (128.50, 729.25)	0.213	0.878	0.170
Skatole	210.00 (172.00, 223.00)	137.00 (115.00, 206.00)	231.00 (147.50, 265.50)	0.111	0.495	0.019
IAA	6.56 (1.25, 20.59)	6.63 (3.35, 18.82)	7.61 (1.85, 16.1)	0.401	0.943	0.876
IPA	10.67 (6.36, 15.48)	7.75 (4.55, 10.38)	7.65 (4.19, 13.11)	0.025	0.025	0.348
IALD	8.00 (4.25 15.50)	19.50 (8.25, 31.75)	37.00 (13.00, 63.00)	0.044	0	0.024
I3CA	1.13 (0.81, 1.71)	1.10 (0.74, 2.11)	3.64 (1.79, 8.15)	0.971	0.011	0.028
Indoles	428.24 (276.45, 963.95)	325.50 (179.82, 504.33)	547.87 (247.97, 823.64)	0.100	0.955	0.044
Indoles/Trp ratio	2.46 (1.25, 4.10)	1.48 (0.58, 1.97)	1.34 (0.70, 2.63)	0.003	0.029	0.413
KYN/Trp ratio ($\times 10^{-3}$)	5.23 (1.86, 7.99)	3.73 (1.99, 11.39)	7.39 (4.12, 11.72)	0.845	0.032	0.105

The data are presented as the median (Q1, Q3). HC: Healthy control; ADA: Colorectal adenoma; CRC: Colorectal cancer; BMI: Body mass index; Trp: Tryptophan; KYN: Kynurenine; IAA: Indole-3-acid-acetic; IPA: Indole-3-propionic acid; IALD: Indole-3-aldehyde; I3CA: Indole-3-carboxylic acid.

Adonis and Anosim analyses indicated that the global microbiota structure of the samples of patients with ADA and CRC differed significantly from that of HCs ($P < 0.05$). There was a trend along the HC-adenoma-carcinoma sequence (Figure 3C and D).

The top 20 different genera among the three groups are shown in Figure 4A ($P < 0.05$). The abundance of six genera decreased gradually, and three genera increased gradually in the HC-adenoma-carcinoma sequence. Then, we focused on differences in bacteria between CRC and HCs. At the genus level, a total of 29 genera were found to have different abundances between the two groups using the Mann-Whitney *U* analysis. After FDR correction, only ten genera reached a significant difference ($P_{Adjueted} < 0.05$).

Linear discriminant analysis (LDA) integrated with effect size (LEfSe) was used to identify the specific bacteria associated with CRC (Figure 4B and C). On the one hand, several bacteria, including the Bacteroidetes phylum, class *Bacteroidia*, order *Bacteroidales*, genus *Escherichia/Shigella* (phylum Proteobacteria), and order *Verrucomicrobiales* (phylum Verrucomicrobia), were all significantly overrepresented [all LDA scores (log10) > 3.6] in the feces of patients with CRC. On the other hand, the phylum Firmicutes, order *Clostridiales* (phylum Firmicutes), and family *Lachnospiraceae* (phylum Firmicutes) were the most abundant microbiota in HCs [LDA scores (log10) > 4.8].

Correlations between bacterial genera and fecal metabolites

We analyzed the correlation between differentially abundant bacterial genera (LEfSe analysis) and Trp metabolites in all subjects. Remarkably, the genera *Asaccharobacter* (Actinobacteria) and *Parabacteroides* (Bacteroidetes) and members of the phylum Firmicutes (*Clostridium XlVb, Fusicatenibacter, Anaerofilum*, and *Anaerostipes*) exhibited a positive correlation with indoles, and the genus *Lactobacillus* was positively correlated with fecal Trp. Other details are shown in Figure 5.

DISCUSSION

This is the first study to investigate the profiles of fecal Trp metabolism in patients with CRC by LC-MS and to explore the potential correlations between the gut microbiome, intestinal barrier function, tissue Trp KP, and alterations in fecal Trp

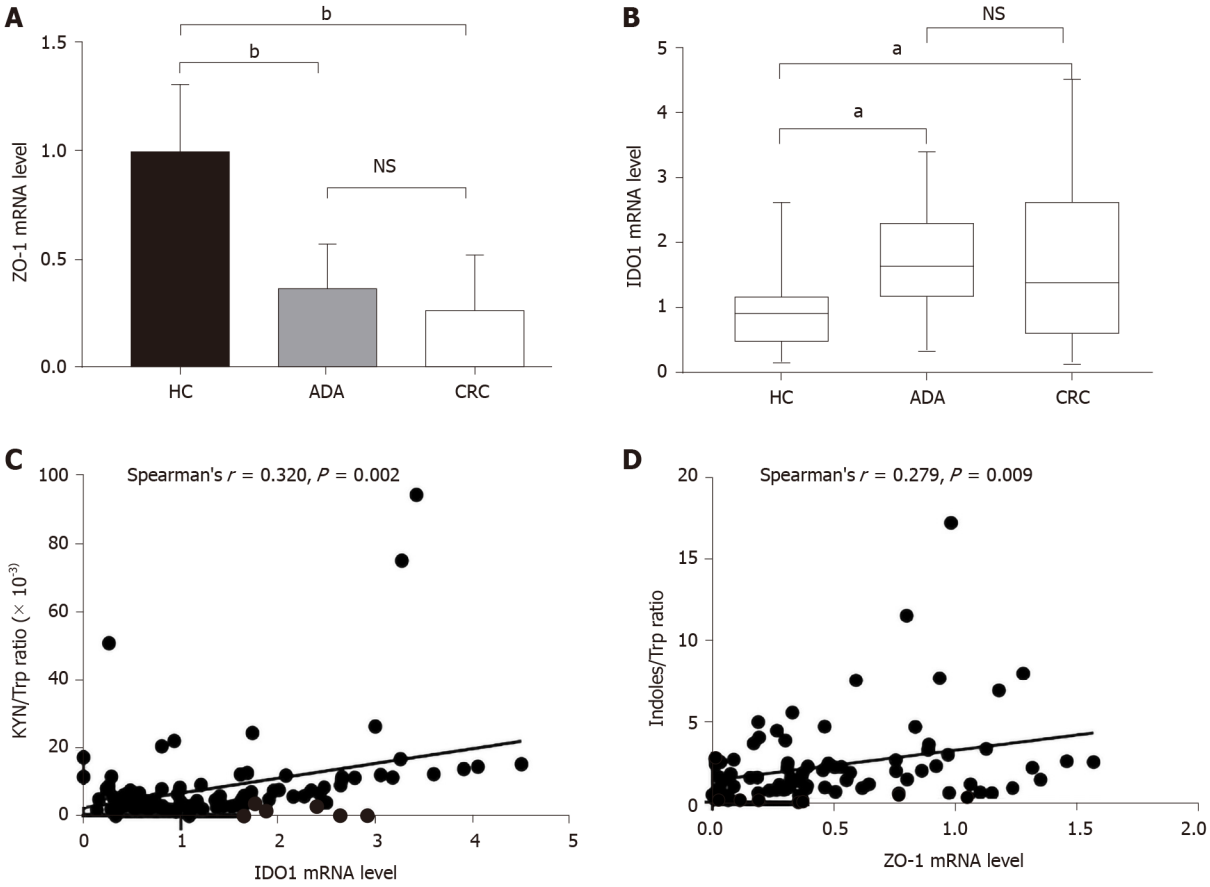


Figure 1 Levels of Zonula occludens-1 and indoleamine 2,3-dioxygenase 1 mRNA in colon tissue and their correlations with fecal tryptophan metabolites. A: The relative Zonula occludens-1 (*ZO-1*) mRNA levels in colorectal adenoma and colorectal cancer (CRC) were significantly lower than those in controls ($P < 0.001$); B: The relative indoleamine 2,3-dioxygenase 1 (*IDO1*) mRNA levels in ARA and CRC were higher than those in controls ($P = 0.04$ and $P = 0.035$, respectively); C: Fecal kynurenine/tryptophan (Trp) ratio was positively correlated with the level of IDO1 *mRNA* in all subjects; D: There was a positive correlation between fecal indoles/Trp ratio and ZO-1 *mRNA* level in all subjects. KYN: Kynurenine; Trp: Tryptophan; HC: Healthy control; ADA: Colorectal adenoma; CRC: Colorectal cancer; ZO-1: Zonula occludens-1; IDO1: Indoleamine 2,3-dioxygenase 1.

metabolism. We found that CRC fecal Trp metabolism was characterized by a decreased Trp indole pathway, which is positively correlated with bowel gut barrier function, and an increased fecal KYN/Trp ratio, which can partially reflect KP activity in colon tissue. In addition, correlations between differentially abundant bacterial genera and imbalanced fecal Trp metabolism were also found in this study. This study provides a preliminary exploration for possible involvement of Trp metabolites in the tumorigenesis of CRC in humans.

In recent years, accumulating evidence suggests that gut indoles, mostly Trp metabolites, are decreased in the feces of patients with metabolic syndrome[31] and play a protective role in DSS-induced colitis or gut pathogen infection[32,33]. There are few clinical studies on indoles in CRC, and one early study detected an increase in indoles in CRC feces and attributed this high concentration to differences in diet[34]. In this study, the fecal indoles/Trp ratio in patients with CRC was much lower than that in healthy subjects, although no differences were found in the absolute indole concentration. This result is supported by a number of *in vitro* and *in vivo* studies. I3C can induce apoptosis and decrease cell viability in several human CRC cell lines[35]. The impaired Trp indole pathway plays a catalytic role in the early stages of CRC[13]. Interruption of Trp indole AHR signaling results in markedly increased *TNF-a, IL-1β*, and *IL-6* mRNA levels in the inflammation-associated colorectal tumorigenesis model. Once activated by indoles, AHR acts directly on intestinal stem cells to strengthen intestinal barrier function and maintain mucin production[25]. Exposure to physiological concentrations of indole results in increased expression of the anti-inflammatory cytokines IL-10[36] and IL-22[37]. Moreover, the positive correlation between the indoles/Trp ratio and ZO-1 in our study is further evidence that indoles are involved in intestinal barrier function. In addition, indole derivatives have anticancer effects *via* the PI3K/Akt/mTOR signaling pathway[38]. Collectively, we concluded that there is an inhibitory effect of fecal indoles on CRC tumorigenesis.

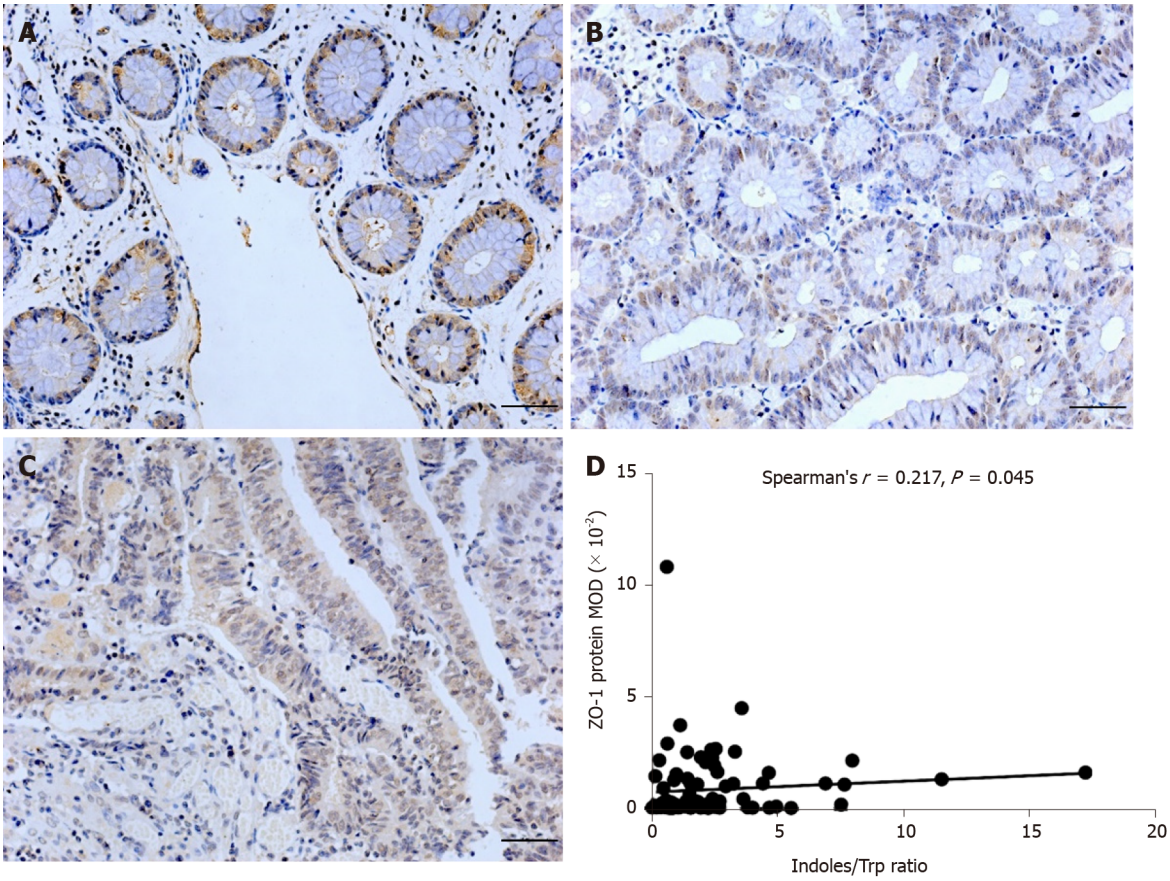


Figure 2 Immunohistochemical staining for Zonula occludens-1 and its correlation with fecal indoles/tryptophan ratio (× 200 magnification). Immunoreactivity of Zonula occludens-1 (ZO-1) tended to be decreased in colorectal adenoma (B) and colorectal cancer (C) compared to controls (A) as shown in the figure (scale bar = 100 μm); D: There was a positive correlation between fecal indoles/tryptophan ratio and ZO-1 protein in all subjects (P = 0.045). MOD: Mean optical density; Trp: Tryptophan; ZO-1: Zonula occludens-1.

KYN is a key metabolite of Trp along the KP[22] and has been shown to be elevated in colon cancer tissue and serum in previous studies[39,40]. KYN metabolized in host epithelial cells or mucus can enter the feces and fecal KYN/Trp ratio can partially reflect KP activity in colon tissue. The KYN/Trp ratio is regarded as a promising surveillance biomarker for cancer[41]. Given its rate-limiting function, the activity of IDO1 can be roughly assessed by the KYN/Trp ratio in serum[42]. The high expression of IDO1 in CRC is consistent with our results and has a positive correlation with poor overall survival outcomes[43]. IDO (mainly IDO1)/KP promotes tumorigenesis through two independent mechanisms. On the one hand, KP metabolites result in the activation of Wnt/β-catenin signaling independent of the effect on adaptive immunity[44]. New discoveries have shown that KP metabolites have the direct function of promoting cancer cell proliferation and inhibiting apoptosis by activating PI3K/AKT signaling in the neoplastic colon epithelium[14]. It has been validated that Wnt/β-catenin and PI3K/AKT are canonical pathways in CRC. On the other hand, activating the KP is thought to facilitate tumor progression by promoting immune suppression or tolerance. IDO1-expressing cells (mainly APCs) help create a tolerogenic microenvironment in the tumor and the tumor-draining lymph nodes that contributes to tumor immune escape and metastasis[26]. We observed not only an increase in KYN and the KYN/Trp ratio in CRC feces but also a positive correlation between the fecal KYN/Trp ratio and tissue IDO1. Therefore, changes in fecal KYN and the KYN/Trp ratio, which can be detected noninvasively and partially reflect tissue KP, may be involved in the development of CRC.

Since Trp in feces is mainly metabolized by bacteria, we analyzed the microflora profiles of healthy people and patients with CRC as well as the relationship between metabolites and differentially abundant bacteria. We observed a decrease in Firmicutes (*Clostridiales* and *Clostridium*) and an increase in Proteobacteria (*Escherichia/Shigella* genus and *Enterobacteriaceae* genus), which was consistent with the results of previous studies[45,46]. The former (mainly *Clostridium*) are known to play an anti-inflammatory role in the gut, while a member of the phylum Proteobacteria *Escherichia coli* [with

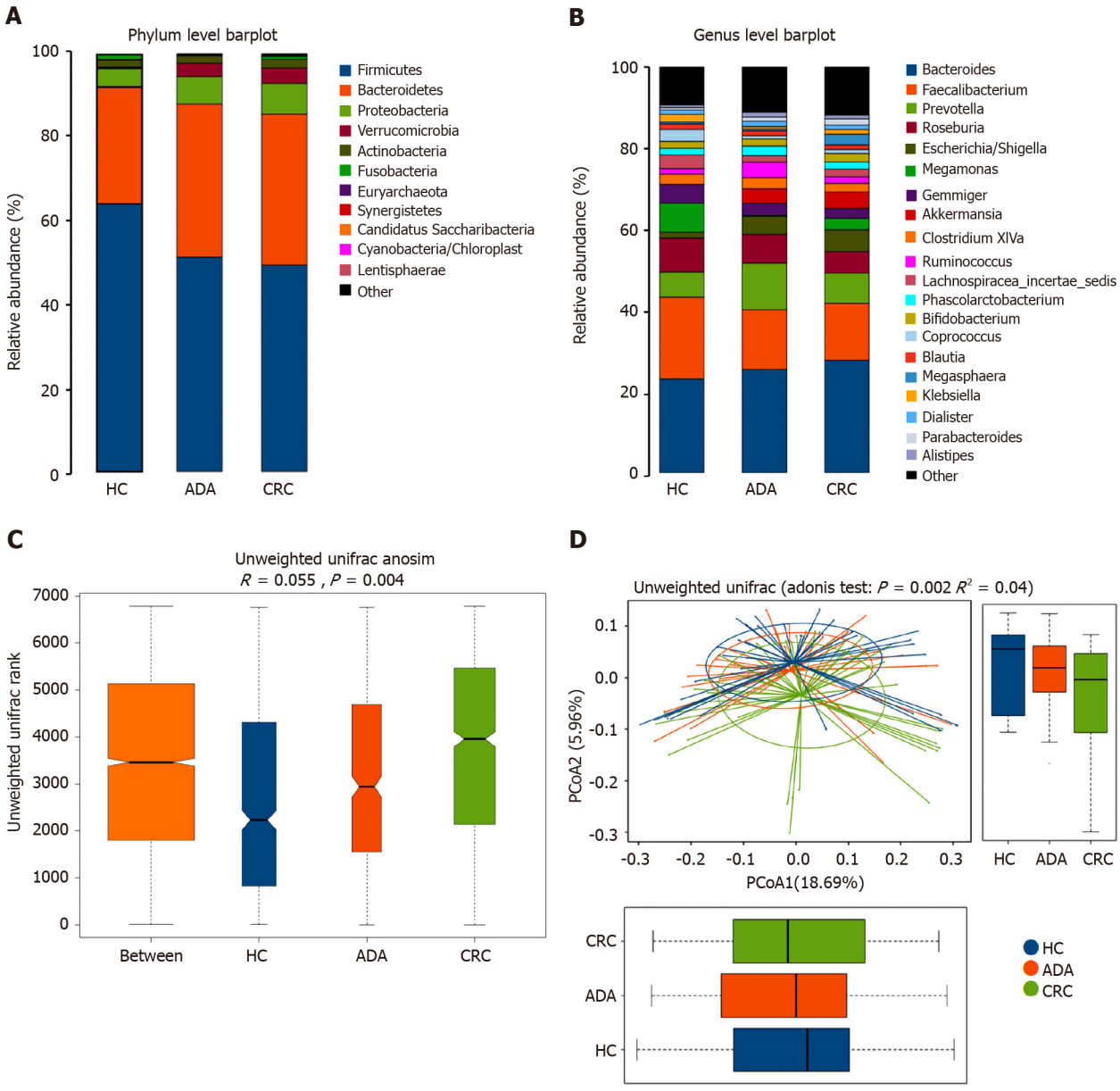
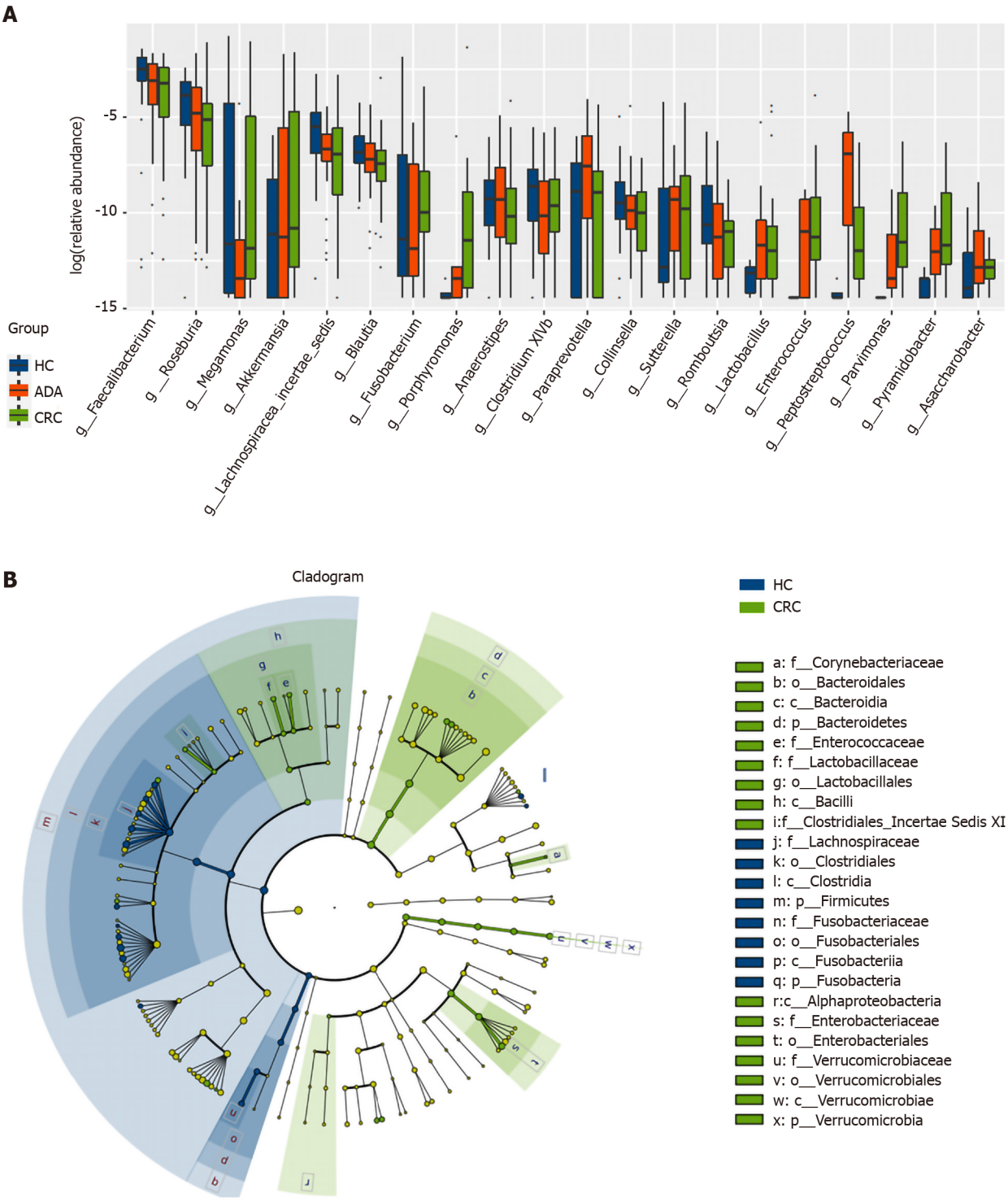


Figure 3 Fecal bacterial structure of patients with colorectal adenoma, colorectal cancer, and healthy controls. A and B: Relative bacterial abundance of fecal bacterial at the phylum and genus levels in the three groups; C and D: Unweighted Unifrac (Anosim and Adonis) analysis of fecal bacterial in the patients and healthy control group ($P < 0.05$). Different colors represent different groups, and the distance between points indicates the degree of difference; boxes indicate the interquartile range; lines inside the boxes indicate the medians; the two whiskers indicate the maximum and minimum of the data; the points outside the box indicate outliers. HC: Healthy control; ADA: Colorectal adenoma; CRC: Colorectal cancer.

expression of the genomic island polyketide synthase (pks+)] can alkylate the DNA of epithelial cells and thus lead to CRC[47,48]. Consistent with a recent study[45], we observed a decline in the Firmicutes/Bacteroidetes ratio in CRC, although an upregulated ratio has been suggested as an indicator of several disorders[49]. In addition, Adonis and Anosim analyses showed a difference between patients with CRC and HCs in bacterial composition. Therefore, the tumor-promoting effects of the gut microbiome may be caused not only by a specific pathogen but also by holistic dysbiosis[50].

In the Trp indole pathway, most gut Trp is converted to indole by tryptophan decarboxylase, to indoleacetamide *via* tryptophan monooxygenase, and to trytamine. In further steps, these metabolites can be metabolized to skatole, IPA, or I3S, serving as the end products of Trp metabolism in the gut[11]. All these enzymes involved in these reactions are produced by the expression of functional genes in gut microbes. We found that indoles were positively related to the genera *Asaccharobacter* (Actinobacteria) and *Parabacteroides* (Bacteroidetes) and members of the phylum Firmicutes (such as *Clostridium XlVb*, *Fusicatenibacter*, *Anaerofilum*, and *Anaerostipes*),


A
log(relative abundance)
-5
-10
-15
Group
HC
ADA
CRC
g__Faecalibacterium
g__Roseburia
g__Megamonas
g__Akkermansia
g__Lachnospiracea_incertae_sedis
g__Blautia
g__Fusobacterium
g__Porphyromonas
g__Anaerostipes
g__Clostridium XlVb
g__Paraprevotella
g__Collinsella
g__Sutterella
g__Romboutsia
g__Lactobacillus
g__Enterococcus
g__Peptostreptococcus
g__Parvimonas
g__Pyramidobacter
g__Asaccharobacter
B
Cladogram
HC
CRC
a: f__Corynebacteriaceae
b: o__Bacteroidales
c: c__Bacteroidia
d: p__Bacteroidetes
e: f__Enterococcaceae
f: f__Lactobacillaceae
g: o__Lactobacillales
h: c__Bacilli
i:f__Clostridiales_Incertae Sedis XI
j: f__Lachnospiraceae
k: o__Clostridiales
l: c__Clostridia
m: p__Firmicutes
n: f__Fusobacteriaceae
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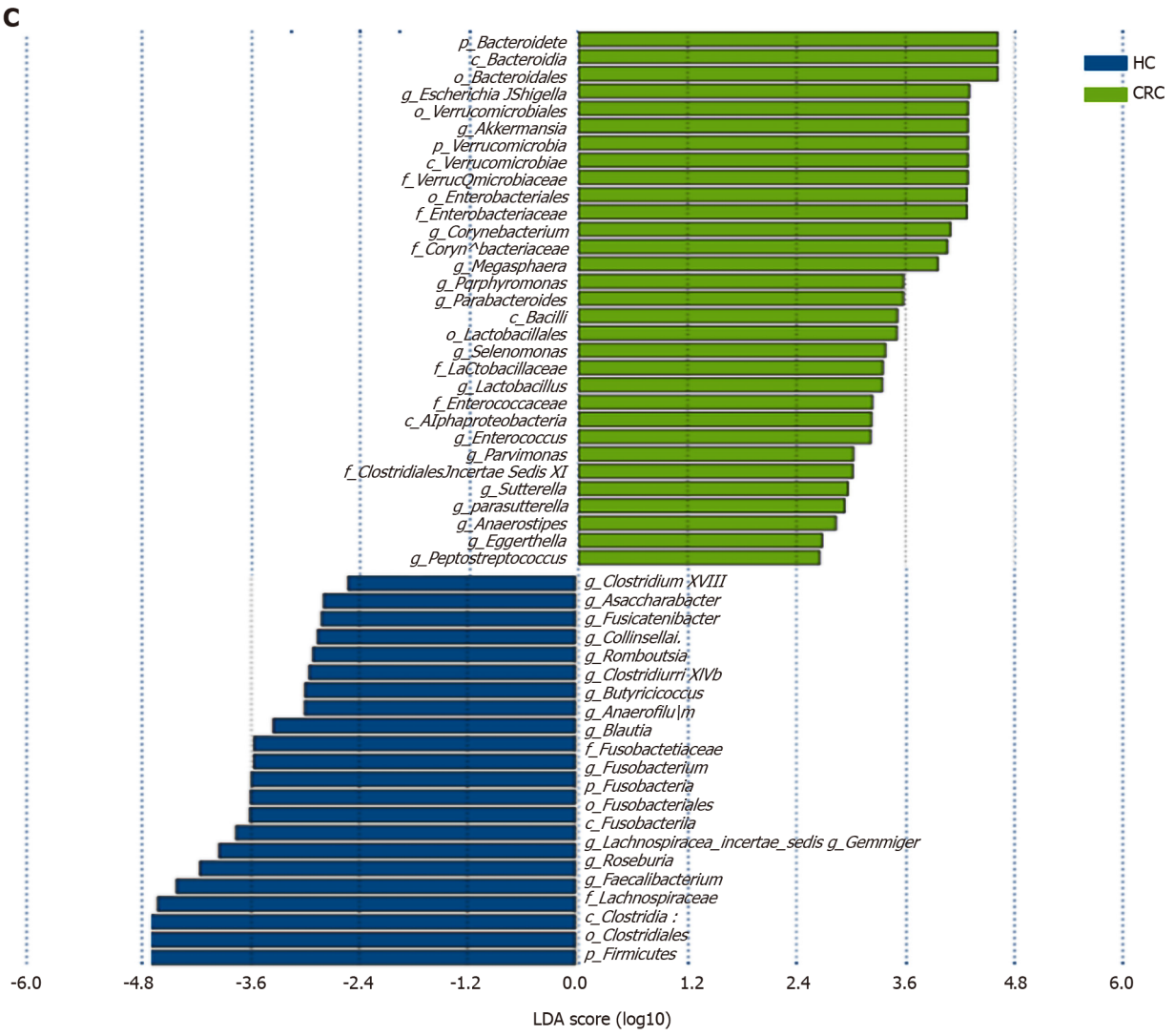

Figure 4 Different genus among the three groups and linear discriminant analysis effect size analysis of fecal bacterial of colorectal cancer patients and healthy controls. A: Top 20 different genus among the three groups with a Kruskal-Wallis's *P* value < 0.05; B: Cladogram showing enriched taxa in the colorectal cancer (CRC) patient and healthy control (HC) groups; the taxa represented by the English letters in the cladogram are shown in the legend on the right; C: Taxa enriched in HCs are indicated with a negative score and the taxa enriched in the CRC group are indicated with a positive linear discriminant analysis (LDA) score; only taxa with an LDA effect size's *P* value < 0.05 and an LDA score ≥ 2.0 are showed. LDA: Linear discriminant analysis; HC: Healthy control; CRC: Colorectal cancer.

whose relative abundances were lower in CRC patients than in HCs in the LEfSe analysis. Actinobacteria[51] and *Clostridium* of Firmicutes[16] have been proven to be involved in the production of indoles. This suggests that decreased indoles-producing or indoles-related bacteria may lead to downregulation of the Trp indole metabolic pathway, allowing more Trp to be metabolized along the KP. In addition, the significant positive correlation between Trp and *Lactobacillus* can be explained by Trp's ability to promote the growth of *Lactobacillus*[33]. The diversity and complexity of intestinal bacteria and metabolites limit our ability to explain all the results and require further study.

To learn more about the adenoma-carcinoma sequence, we also studied the characteristics of gut microbiota and Trp metabolism in patients with colon ADA, which is different from other studies. As expected, some increasing and decreasing changes along the HC-adenoma-carcinoma sequence were found. Changes in the intestinal microenvironment caused by microbiota dysbiosis and shifts in Trp metabolism occurred in the early events of CRC pathogenesis. A previous study suggested that adding indoles to the diet has a certain antitumor effect in $Apc^{Min/+}$ mice[52]. Therefore, supplementation with probiotics or indoles may also have an inhibitory effect on the HC-adenoma-carcinoma sequence in humans.

The limitations of the study are as follows: First, the fecal microbiota and metabolites were not equivalent to the concept of the "tumor microenvironment", and

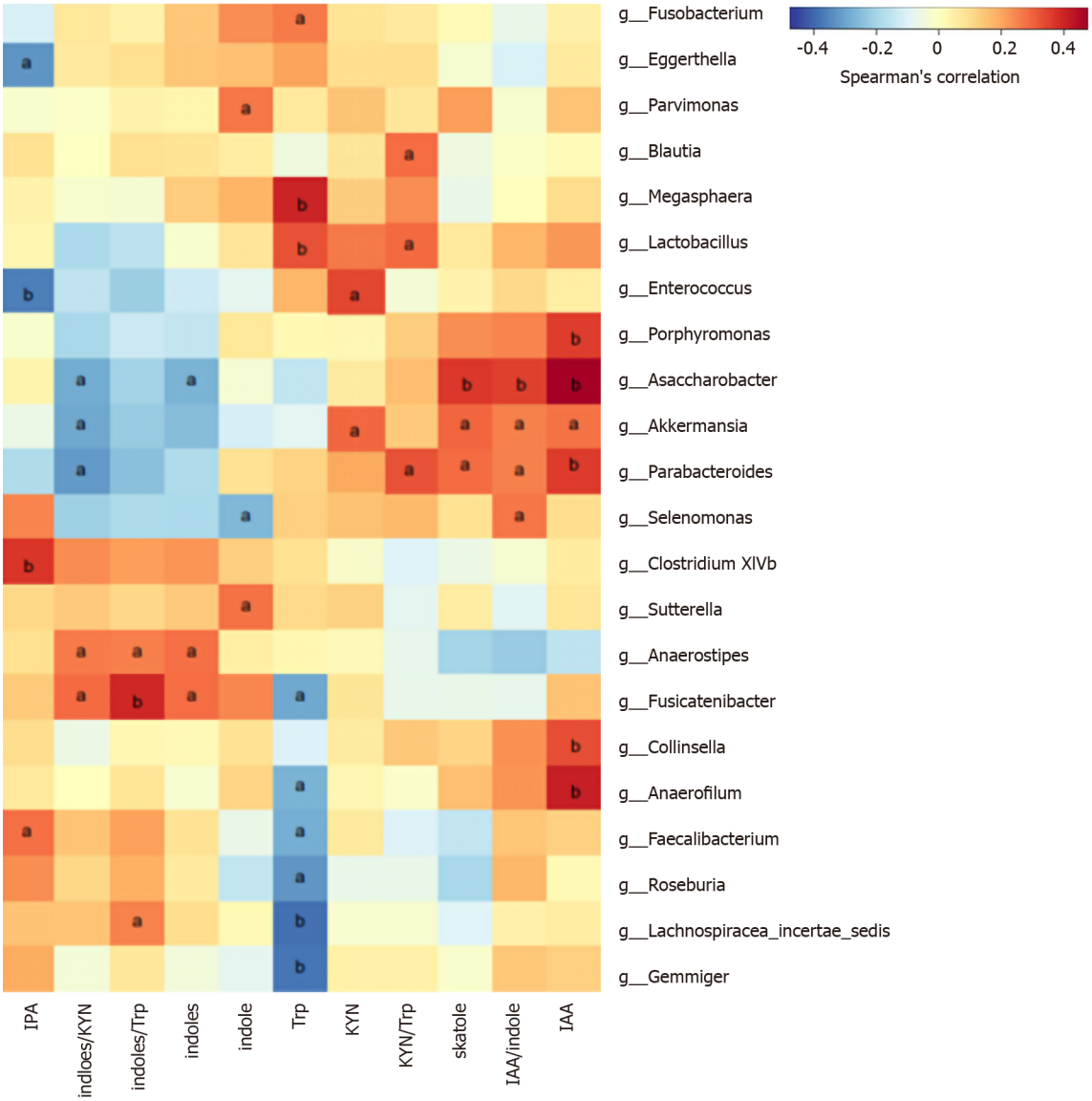

Figure 5 Correlations between differential bacteria at the genus level using linear discriminant analysis effect size analysis and fecal metabolites. The heatmap presents the Spearman correlation coefficients between fecal metabolites and genera with significantly different relative abundance in the colorectal cancer (CRC) patients and healthy controls (HCs). Indoles were positively related to the genera *Asaccharobacter* (Actinobacteria) and *Parabacteroides* (Bacteroidetes), and members of the phylum Firmicutes (such as *Clostridium XlVb*, *Fusicatenibacter*, *Anaerofilum*, and *Anaerostipes*), whose relative abundances were lower in CRC patients than in HCs in the linear discriminant analysis effect size analysis. $^{a}P < 0.05$, $^{b}P < 0.01$. HC: Healthy control; CRC: Colorectal cancer. IPA: Indole-3-propionic acid; IAA: Indole-3-acid-acetic; KYN: Kynurenine; Trp: Tryptophan.

they tend to be influenced by diet and lifestyle[7,53]. Nevertheless, the noninvasiveness makes the results easily verifiable in a larger sample in the future. Second, the conclusions based on this observational study prevent us from determining the causal relationships. Our results showed that fecal Trp metabolism was correlated with shifted microbiota, intestinal barrier function, and tissue Trp KP. There is no evidence that they have participated in the pathogenesis of CRC. It is possible that CRC induced the changes of microbiota and fecal Trp metabolis. Despite some support from preclinical studies[13,52], the results need to be further verified by more evidence. Third, although the levels of mRNA and protein have a similar trend in tissue[54,55], the expression of IDO1 represented by the mRNA level was not accurate. Since this is a preliminary exploratory study, we will detect IDO1 protein expression and other indexes of intestinal barrier function in subsequent validation experiments. Finally, subjects were not given a standardized diet during the study period as in some other studies[45], but were required to maintain their dietary habits before fecal samples were collected. The standardized diet minimizes diet-induced deviations in microbiota and Trp metabolites but does not reflect the long-term status of the patient's gut microenvironment. Although a standardized diet in this study is important, the need

for scheduled surgery for patients with colon cancer makes this difficult to implement.

CONCLUSION

In conclusion, this study provides new evidence that gut Trp metabolism and microbiome compositions are different in subjects with CRC compared to HCs. Reduced fecal Trp indole metabolism and increased tissue KP may be involved in the pathogenesis of CRC by reducing the intestinal barrier and increasing immune escape, respectively. Collectively, these data suggest that restoring the homeostasis of Trp metabolism and the microbiome may have preventive or therapeutic effects on CRC.

ARTICLE HIGHLIGHTS

Research background

Population aging has resulted in a substantial increase in the number of new colorectal cancer (CRC) cases globally. Gut microbiota and metabolite interactions are involved in the pathogenesis of CRC *via* various genetic and epigenetic alterations. Gut tryptophan (Trp) metabolites are produced by microbiota and/or host metabolism. Some of them were thought to play a role in CRC in animal and *in vitro* studies.

Research motivation

Few studies on fecal Trp metabolism have been found, especially its interaction with gut microbiota. Therefore, it is meaningful to study the characteristics of gut Trp metabolism in patients with CRC.

Research objectives

To investigate the features of Trp metabolism in CRC patients and the correlation between fecal Trp metabolites and gut microbiota.

Research methods

Subjects meeting the inclusion and exclusion criteria were included in the study. Their demographic and clinical features were collected. Fecal Trp, kynurenine (KYN), and indoles (metabolites of Trp metabolized by gut microbiota) were examined by ultraperformance liquid chromatography coupled to tandem mass spectrometry. Gut barrier marker and indoleamine 2,3-dioxygenase 1 (*IDO1*) mRNA were analyzed by quantitative real-time polymerase chain reaction. Zonula occludens-1 (ZO-1) protein expression was analyzed by immunohistochemistry. The gut microbiota was detected by 16S ribosomal RNA gene sequencing. Correlations between fecal metabolites and other parameters were examined in all patients.

Research results

The absolute concentration of KYN and the ratio of KYN to Trp were increased in the feces of patients with CRC compared to HCs, while the indoles to Trp ratio was decreased. Colon *IDO1* mRNA levels were positively associated with fecal KYN/Trp ratio and *ZO-1* mRNA levels were positively correlated with the indoles/Trp ratio. The genera *Asaccharobacter* (Actinobacteria) and *Parabacteroides* (Bacteroidetes), and several members of the phylum Firmicutes (*Clostridium XlVb, Fusicatenibacter, Anaerofilum,* and *Anaerostipes*) decreased in CRC and exhibited a positive correlation with indoles in all subjects.

Research conclusions

CRC gut Trp metabolism was characterized by a decreased Trp indole pathway in feces, which is positively correlated with bowel gut barrier function, and an increased kynurenine pathway activity in colon tissue. In addition, correlations between differentially abundant bacterial genera and imbalanced fecal Trp metabolism were also found in this study.

Research perspectives

This preliminary study investigated the alteration of gut Trp metabolism and the possible mechanism of Trp metabolites in CRC pathophysiology. In the future, we will focus on the following aspects. First, we will detect Trp and Trp metabolites in both

feces and colon tissues to further study the "tumor microenvironment". Second, it is necessary to study the dietary habits of CRC patients and explore the relationship between the diet and gut Trp metabolism. Supplementation with indoles in diet may also have an inhibitory effect on the HC-adenoma-carcinoma sequence in humans.

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ORIGINAL ARTICLE

Retrospective Cohort Study

High mortality associated with gram-negative bacterial bloodstream infection in liver transplant recipients undergoing immunosuppression reduction

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Abstract

BACKGROUND

Immunosuppression is an important factor in the incidence of infections in transplant recipient. Few studies are available on the management of immunosuppression (IS) treatment in the liver transplant (LT) recipients complicated with infection. The aim of this study is to describe our experience in the management of IS treatment during bacterial bloodstream infection (BSI) in LT recipients and assess the effect of temporary IS withdrawal on 30 d mortality of recipients presenting with severe infection.

AIM

To assess the effect of temporary IS withdrawal on 30 d mortality of LT recipients presenting with severe infection.

METHODS

A retrospective study was conducted with patients diagnosed with BSI after LT in the Department of Liver Surgery, Renji Hospital from January 1, 2016 through December 31, 2017. All recipients diagnosed with BSI after LT were included. Univariate and multivariate Cox regression analysis of risk factors for 30 d mortality was conducted in the LT recipients with Gram-negative bacterial (GNB) infection.

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RESULTS

Seventy-four episodes of BSI were identified in 70 LT recipients, including 45 episodes of Gram-positive bacterial (GPB) infections in 42 patients and 29 episodes of GNB infections in 28 patients. Overall, IS reduction (at least 50% dose reduction or cessation of one or more immunosuppressive agent) was made in 28 (41.2%) cases, specifically, in 5 (11.9%) cases with GPB infections and 23 (82.1%) cases with GNB infections. The 180 d all-cause mortality rate was 18.5% (13/70). The mortality rate in GNB group (39.3%, 11/28) was significantly higher than that in GPB group (4.8%, 2/42) (P = 0.001). All the deaths in GNB group were attributed to worsening infection secondary to IS withdrawal, but the deaths in GPB group were all due to graft-versus-host disease. GNB group was associated with significantly higher incidence of intra-abdominal infection, IS reduction, and complete IS withdrawal than GPB group (P < 0.05). Cox regression showed that rejection (adjusted hazard ratio 7.021, P = 0.001) and complete IS withdrawal (adjusted hazard ratio 12.65, P = 0.019) were independent risk factors for 30 d mortality in patients with GNB infections after LT.

CONCLUSION

IS reduction is more frequently associated with GNB infection than GPB infection in LT recipients. Complete IS withdrawal should be cautious due to increased risk of mortality in LT recipients complicated with BSI.

Key Words: Immunosuppressive therapy; Liver transplantation; Bloodstream infection; Multidrug-resistant gram-negative bacterium

Core Tip: Bacterial infections are the most common infectious complication after liver transplantation (LT). Immunosuppression (IS) reduction is usually needed in case of bacterial infections in LT recipients. However, we do not know exactly the incidence of IS reduction during bloodstream infection after LT and its effect on patient outcome. This single-center analysis summarized the IS reduction data in 70 LT recipients. We found IS reduction is more frequently associated with Gram-negative bacterial infection than Gram-positive bacterial infection in LT recipients. Complete withdrawal of IS should be done cautiously due to increased risk of mortality.

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INTRODUCTION

Bacterial infections continue to be the most common infectious complication after liver transplantation (LT), usually within 2 mo after LT[1]. Bloodstream infections (BSI) account for 19%-46% of all major infections after LT[2-5] and are associated with a mortality rate of nearly 40%[6].

Several factors are known to be associated with BSI after LT in adults, including intraoperative blood loss, intraoperative transfusion, retransplantation, longer duration of catheterization, and biliary complication. Immunosuppression (IS) is the single most important factor contributing to the incidence of infections in transplant recipients[7]. The commonly used immunosuppressive agents after LT include calcineurin inhibitor, such as tacrolimus (0.1-0.15 mg/kg/d in 2 doses) or ciclosporin (6-8 mg/kg/d in 2 doses), mycophenolate mofetil (500-1000 mg, bid), sirolimus (2 mg/d), and corticosteroids (induction with high dose methylprednisolone 500-1000 mg intravenously, followed by tapering over 5 d to maintenance with prednisone 5-20

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mg/d). The management of IS therapy during infection after LT is highly controversial, although IS reduction (partially discontinue or reduce the dosage of at least one IS agent) or complete withdrawal may be a generally accepted option in life-threatening infections. To date, only few studies have assessed the impact of IS reduction or complete withdrawal of immunosuppressive therapy on infection outcomes in LT recipients[8,9]. In these studies, researchers reported that immunosuppressive agents may be discontinued completely in kidney transplantation recipients since hemodialysis is an effective option in case of rejection. In contrast, complete discontinuation of IS is highly dangerous in liver transplantation because it may lead to graft loss and patient death.

This study aimed to examine the management of immunosuppressive therapy during bacterial BSI in LT recipients in the Department of Liver Surgery, Renji Hospital during a 2-year period and the effect of temporary IS withdrawal on 30 d mortality of recipients presenting with severe infection.

MATERIALS AND METHODS

Study design and population

A retrospective single-center observational cohort study was conducted in the LT recipients diagnosed with BSI in Department of Liver Surgery, Renji Hospital from January 2016 through December 2017. Overall, 1297 LT recipients were identified, including 786 children (650 Living donors and 136 deceased donors) and 511 adults. All the enrolled LT recipients satisfied the inclusion criteria: (1) 18 to 75 years of age; and (2) With diagnosis of bloodstream infection confirmed by blood culture. The patients were excluded if infection was localized or in the brain or patients died on the day of surgery. Seventy patients with 74 episodes of BSI were eligible for inclusion in this analysis. All donor organs registered in the database were donated voluntarily. No donor organs were obtained from executed prisoners.

Patient charts and in-hospital records were carefully reviewed to collect study variables and fill in the pre-determined case reports. The researchers systematically checked the integrity of the data before importing it into the database. The follow-up period was at least 180 d after the onset of index BSI. The study was carried out in accordance with the Declaration of Helsinki and approved by our institutional review board (Approval No. KY2019-160).

Antimicrobial prophylaxis

The perioperative prophylactic antimicrobial therapy included intravenous ampicillin (120 mg/kg/d, q6h) and cefotaxime (120 mg/kg/d, q6h) within 1 h before LT and lasting for 3-5 d. Methicillin-resistant *Staphylococcus aureus* (MRSA) nasal colonization was routinely screened when the patient was included on transplant waiting list and transferred to liver intensive care unit after the operation. Alternative regimen including vancomycin may be considered for the patients with a history of MRSA infection or colonization. The surgeon may modify the prophylactic regimen according to the history of infectious disease based on the experience of our center. Oral acyclovir or valganciclovir after intravenous ganciclovir was administered for prevention of cytomegalovirus. Antiviral prophylaxis and hepatitis B immunoglobulin therapy were given to the patients undergoing LT for managing hepatitis B cirrhosis. Routine antifungal prophylaxis was only applicable to the patients at high risk of invasive aspergillosis or candidiasis, as described elsewhere[10].

Immunosuppression strategy

Standard IS regimens include high-dose prednisone and basiliximab induction, followed by tacrolimus, mycophenolic acid, and prednisone. For the patients with unremarkable post-transplant process, steroids were withdrawn 3-6 mo after LT. A mammalian target of rapamycin inhibitor was added to the treatment regimen after the first month of transplantation if patients were at risk of hepatocellular carcinoma. Liver biopsy was performed in case of elevated transaminases or laboratory results indicative of unexplained cholestasis.

The target serum level of tacrolimus was 8-12 ng/mL during the first month of LT and 6-8 ng/mL during the first 6 mo of LT. The target serum level of cyclosporin was 200-250 mg/mL during the first month and 150-200 mg/mL in the first 6 mo of LT.

Definitions

BSI was defined as the isolation of pathogenic microorganisms from at least one blood culture specimen. Positive blood culture from two separate sites was required for the skin flora associated with contamination. Polymicrobial BSI was defined as two or more microorganisms isolated from the same one blood culture specimen. Intra-abdominal infections include peritonitis, peritoneal abscess, and cholangitis occurring more than 30 d after surgery. BSI was classified as secondary BSI when the pathogens from blood sample originated from the infection in other body site.

BSI source was determined according to the Center for Disease Control and Prevention criteria[11] and considered as primary source when no identifiable source was available.

Multi-drug resistance (MDR) was defined as acquired non-susceptibility to at least one agent in three or more antimicrobial classes. Carbapenem-resistant *Enterobacteriaceae* was defined by current Center for Disease Control and Prevention criteria as *Enterobacteriaceae* strains resistant to at least one carbapenem. For all the Gram-negative isolates, carbapenemase production (*Klebsiella pneumoniae* carbapenemase, New Delhi metallo-b-lactamase, OXA-23, and OXA-51) was confirmed by simplex 'in-house' polymerase chain reaction assays with specific primers, including: bla_{KP}*c*-related sequences (5'-TCTGGACCGCTGGGAGCTGG-3', forward and 5'-TGCCCGTTGACGCCCAATCC-3', reverse); $bla_{OXA\text{-}23}$-related sequences (5'-GATCGGATTGGAGAACCAGA-3', forward and 5'-ATTTCTGACCGCATTTCCAT-3', reverse), and bla_{NDM}-related sequences 5'-GGTTTGGCGATCTGGTTTTC-3', forward and 5'-CGGAATGGCTCATCACGATC-3', reverse). Community-acquired BSI was defined as when positive blood culture was taken within 48 h since hospital admission. Hospital acquired BSI was defined as a positive blood culture obtained from patients who had been hospitalized for 48 h or longer.

For the management of immunosuppressive therapy during BSI episodes, we recorded the changes of index blood culture over a period of 7-10 d. Changes of immunosuppressive therapy were classified as follows: (1) IS was withdrawn completely when all immunosuppressive drugs were discontinued simultaneously; (2) IS therapy was partially discontinued when at least one immunosuppressive drug (steroids, calcineurin inhibitors, or mammalian target of rapamycin inhibitors) was discontinued; (3) IS was reduced when the dosage of at least one immunosuppressive drug was reduced by a minimum of 50%; and (4) IS reduction was defined as at least one of the above situations.

Data collection

All relevant data were collected from the enrolled patients, including demographic data, etiology of liver disease, biopsy-confirmed rejection or medical interventions for elevated liver transaminase, and/or re-transplantation within 90 d after BSI. BSI data included the pathogenic bacterial isolates and their susceptibility patterns, empiric antibiotic treatment, as well as appropriateness and duration of antibiotic treatment. IS data included the dosage, serum level of immunosuppressive agents, and time and duration of discontinuation.

Statistical analysis

Statistical analysis was performed using the SPSS Advanced Statistics Modules, version 20.0 (SPSS, Armonk, NY, United States). Kaplan-Meier analysis was used to determine the effect of MDR infection on patient survival after LT. The normally distributed continuous variables were expressed as mean ± standard deviation and compared by Student's *t*-test. All other non-normally distributed continuous data were presented as median [interquartile range (IQR)] and compared by Mann-Whitney *U*-test.

Univariate analysis was applied to determine the risk factors for 30 d mortality in LT recipients with BSI. Only the variables showing $P < 0.10$ in the univariate analysis were tested in multivariate analysis. Stepwise variable logistic regression model was utilized to identify the independent risk factors for 30 d mortality of Gram-negative bacterial (GNB) infections.

RESULTS

A total of 74 episodes of BSI were identified in 70 LT recipients in the 2-year period. Most of the patients (53, 75.7%) were males with a median (IQR) age of 48 (40-51) years. The etiology of liver disease was mainly hepatitis B virus-related cirrhosis

(33/70, 47.1%) and hepatocellular carcinoma (20/70, 28.6%) (Table 1). The 74 episodes of BSI were classified into Gram-positive bacterial (GPB) infections (45 episodes in 42 patients) and GNB infections (29 episodes in 28 patients) based on the Gram staining of the pathogenic bacteria.

Characteristics of BSI episodes

The median (IQR) time from LT to the onset of BSI was 6 (3-20) d. Majority (67, 90.5%) of the BSI episodes occurred within 180 d after LT and were hospital acquired (94.8%). The BSI source was surgical wound (47.6%), primary (23.8%), respiratory tract (14.3%), biliary tract (11.9%), central venous catheter (4.8%), urinary tract, and intra-abdominal (2.1%) in GPB group. Intra-abdominal infection (32.1%) was the primary site of BSI, followed by biliary tract (25.0%), urinary tract (21.4%), respiratory tract (17.9%), primary (10.7%), and central venous catheter (7.1%) in GNB group. GNB group showed numerically longer withdrawal time than GPB group (12.6 d *vs* 6.3 d) (Table 1).

The median (IQR) time from the day of transplantation (day 0) to onset of BSI was 4 (1-6) d in GPB group (n = 45) and 12 (8-41) d in GNB group (n = 29). The distribution of bacterial species is presented in Table 2. The isolates in GPB group included coagulase-negative *Staphylococcus* (n = 24), *Enterococcus faecalis* (n = 4), *Staphylococcus aureus* (n = 3), *Enterococcus faecium* (n = 4), and *Streptococcus* (n = 2). The pathogenic isolates in GNB group were mostly antibiotic resistant (n = 22, 75.9%). The etiological agents were *Klebsiella pneumoniae* (n = 11, including eight carbapenemase-producing strains and one pandrug resistant strain), *Acinetobacter baumannii* (n = 7, all carbapenemase-producing strains), *Escherichia coli* (n = 5, including two ESBL-producing strains and one extensively drug-resistant strain), and *Pseudomonas aeruginosa* (n = 3, including two multi-drug resistant strains and one carbapenemase-producing strain).

Management of immunosuppressive therapy

IS reduction was found in 28 (41.2%) cases, specifically 5 cases (5/28, 17.9%) in GPB group and 23 cases in GNB group. As for GPB BSIs, dosage reduction was identified in 2 patients (all tacrolimus), and complete IS withdrawal in 3 patients. In the LT recipients with GNB BSIs, dosage reduction (tacrolimus, steroids, ciclosporin, and/or mycophenolate) was made in six patients. At least one immunosuppressive drug was discontinued in one patient. Both dosage reduction and discontinuation of at least one drug were identified in one patient. Complete IS withdrawal was found in 15 patients.

Outcome analysis

Fifty-seven patients completely recovered from infectious complications, including 40 (95.2%) in GPB group and 17 (60.7%) in GNB group. The 180 d all-cause mortality rate was 18.6% (13/70). The 2 deaths in GPB group were due to graft-versus-host disease (GVHD). The 11 deaths in GNB group were attributed to worsening infection secondary to IS withdrawal. Kaplan-Meier analysis showed that the patients with MDR GNB infections had significantly lower 90 d survival rate than the patients without MDR GNB infections (50% *vs* 100%, log-rank test, P = 0.03) after onset of BSI.

Three patients (7.1%) developed suspected rejection episodes in GPB group, while seven patients (25%) developed rejection episodes in GNB group.

In patients with GNB infections, patients who died within 30 d of infection diagnosis showed a higher prevalence of rejection, a higher risk of *Klebsiella pneumoniae* infection, and a more frequent presentation with IS withdrawal; all of these differences reached statistical significance. No differences in the 30 d mortality were found, taking into account patient primary disease or based on the source of infection. In addition, there were no differences between the episodes in which the antimicrobials were used as empiric therapy or target therapy (Table 3).

Univariate analysis showed that rejection within 90 d after BSI, *K. pneumoniae* infection, and complete IS withdrawal were significantly associated with 30 d mortality of GNB infections after LT. Multivariate analysis indicated that rejection within 90 d after BSI (P = 0.01) and complete IS withdrawal (P = 0.019) were independent predictors of 30 d mortality in patients with GNB infections (Table 4).

DISCUSSION

Our data indicate that BSI is a common complication in LT recipients. At least one BSI episode was identified in 14.5% (74/511) of LT recipients in the first year after transplantation. This is consistent with the previous reported incidence of 28%-

Table 1 Characteristics of liver transplant recipients with bloodstream infection in terms of bacterial pathogens, *n* (%)

Variable	Gram-positive BSI, *n* = 42	Gram-negative BSI, *n* = 28	*P* value
Age in yr, mean ± SD	51.0 ± 10.5	47.66 ± 13.01	0.228
Female sex	9 (21.4)	8 (28.6)	0.495
Etiology of liver transplant			
Hepatocellular carcinoma	13 (31.0)	7 (25.0)	0.589
Hepatitis B virus	20 (47.6)	13 (46.4)	0.922
Others	9 (21.4)	8 (28.6)	0.495
Baseline immunosuppressive treatment			
Prednisone	18 (42.9)	12 (42.9)	0.596
Tacrolimus	31 (73.8)	18 (64.3)	0.833
Cyclosporin	7 (16.7)	5 (17.9)	0.897
Mycophenolate mofetil	35 (83.3)	17 (60.7)	0.034
BSI characteristics			
Early BSI[1]	41 (97.6)	26 (92.9)	0.718
Septic shock	0	4 (14.3)	NA
BSI source, episodes	*n* = 45	*n* = 29	
Primary	10 (23.8)	3 (10.7)	0.318
Surgical wound	20 (47.6)	0	NA
Biliary tract	5 (11.9)	7 (25.0)	0.246
Urinary tract	1 (2.4)	6 (21.4)	0.025
Central venous catheter	2 (4.8)	2 (7.1)	0.649
Intra-abdominal	1 (2.4)	9 (32.1)	0.001
Respiratory tract	6 (14.3)	5 (17.9)	0.833
Management of infection			
Empiric therapy	31 (73.8)	14 (50.0)	0.126
Target therapy	11 (26.2)	12 (42.9)	0.201
Source control	2 (4.8)	3 (10.7)	0.608
Length of stay in d, median (IQR)	18 (15-26.8)	24.5 (18-39)	0.055
From the day of transplantation to onset of BSI in d, median (IQR)	4 (1-6)	12 (8-41)	0.048
Patient outcome			
Died	2 (4.8)	11 (39.3)	0.001
Suspected rejection[2]	3 (7.1)	7 (25.0)	0.081
Microbiological clearance	43 (95.6)	18 (62.1)	0.001
IS reduction	5 (11.9)	23 (82.1)	< 0.001
Complete IS withdrawal	3 (7.1)	15 (53.6)	< 0.001
Length of IS withdrawal in d	6.3 (2-10)	12.6 (4-25)	0.171

[1]Within 6 mo after liver transplantation.
[2]Within 90 d after bloodstream infection. BSI: bloodstream infection; IQR: Interquartile range; IS: Immunosuppression; NA: Not applicable; SD: Standard deviation.

46%[5,12]. Previous studies demonstrated that one important high risk factor for bacterial infection in patients after solid organ transplantation was post-transplant IS therapy[13,14], which was supported by a hypothesis that post-transplant IS can reduce inflammatory cascades. This is considered one of the main pathophysiological factors of sepsis. Therefore, it is a common option for clinicians to reduce or discontinue immunosuppressive therapy when transplant recipients experience severe infection.

Nearly half of the LT recipients with BSI in our study were managed with either dosage reduction or discontinuation of IS treatment. Of the 28 patients managed with IS reduction, only 5 were managed with either dosage reduction or discontinuation of immunosuppressive therapy in GPB group. Twenty-three patients were managed with either dosage reduction or discontinuation of immunosuppressive therapy in GNB group. In addition, we found that IS withdrawal was common in the patients with MDR GNB infections and associated with increased risk of mortality. However, discontinuation of immunosuppressive regimens did not increase the risk of death in patients with GPB infection.

Few studies are available to evaluate the effect of IS reduction on the outcome of patients with bacterial infection. Mañez *et al*[8] showed that 31 LT recipients discontinued immunosuppressive drugs temporarily because of severe opportunistic infection, and 41% of these patients died while in the hospital. However, none of them had BSI or sepsis. A recent study[15] described the management of immunosuppressive therapy at the time of diagnosis of BSI in LT recipients. Ninety cases (43%) were managed with "IS reduction", which was associated with worse outcome in LT recipients with BSI. We also found the same negative correlation between IS reduction and 30 d mortality in patients with drug-resistant bacterial infection in GNB group. The patients with severe infections or septic shock in our center were more likely to be managed by lowering the dose of or withdrawing immunosuppressive agents, but such a practice may have led to the worse outcome.

In patients with GPB BSI, the incidence of graft rejection was 7.1%, and mortality was 4.8% (n = 2). Both patients died from GVHD. In the patients with GNB BSI, the risk of graft rejection was earlier and higher (25.0%) and the mortality was 39.3%. All the deaths except one (GVHD) were due to worsening infection secondary to IS withdrawal. These findings suggest that IS less intense in those cases. The deaths were more likely associated with epidemiologic and technical-surgical factors. Another possible explanation is that IS reduction may put the patients at risk of graft rejection, which in turn leads to graft dysfunction, graft loss, or death[16].

We found that all the BSI episodes occurred in the first 180 d after LT. This was consistent with the previous reports, which confirmed early-onset BSI and other complications[3,10,17-19]. Sganga *et al*[20] reported that 28% of transplant recipients developed BSI in the first 60 d after LT. In a Japanese study, 34.3% of LT recipients developed BSI in the first 90 d after LT and had a higher mortality rate than the recipients without BSI[3]. Kim *et al*[2] also reported that recipients with early-onset BSI were at a significantly higher risk of mortality compared to those without infection or infection without bacteremia. Several factors have contributed to the increased risk of early bacterial infection, including complexity of surgical procedures, high level of IS due to rejection, multiple entries for microorganisms (*e.g.*, incisions, catheters, and probes), and poor performance status[21-23].

GPBs were previously considered to be the key BSI pathogens after transplantation[5,24,25]. However, current research identified GNBs as the predominant pathogens[26-28]. We found in this study that GPBs were more frequently isolated than GNBs (60.8% *vs* 39.2%). Meanwhile, we found a high prevalence of infections caused by MDR GNB, including *Acinetobacter* (24.14%), and *Enterobacteriaceae* (37.93%), mainly carbapenem-resistant strains. MDR GNB pathogens in LT recipients have increased worldwide, with a prevalence of over 50%. MDR GNB infections are associated with higher mortality rate than GPB infections[29,30]. Previous studies reported that MDR GNB infections were common in LT recipients[26,31,32]. A cohort study of 475 LT recipients demonstrated that MDR GNB infections were associated with higher mortality (50%)[13].

The common pathogens of infection after LT include *E. coli*, *Klebsiella*, *Enterobacter*, and *S. marcescens*[27,33]. *P. aeruginosa* and *A. baumannii* are also common causes of GNB infection. The prevalence of ESBL-producing GNB, carbapenem-resistant *K. pneumoniae*(CRKP), MDR *Acinetobacter*, and MDR *Pseudomonas* are on the rise and are associated with higher rate of treatment failure[13]. Importantly, we found that infection due to MDR GNB was one of the strongest predictors of post-LT mortality. The 90 d mortality was as high as 50% for the patients with MDR GNB infections. These findings are consistent with two recent studies showing that when LT patients were infected with CRKP, the 1-year survival dropped dramatically from 86 % to 29 % and

Table 2 Distribution of the bacterial pathogens causing bloodstream infections in liver transplant recipients

Gram stain	Bacterial species	*n* (%)	Resistant strain, *n*
Gram-positive, *n* = 45	*Enterococcus faecium*	4 (8.89)	XDR, 1
	Staphylococcus aureus	3 (6.67)	
	Enterococcus faecalis	4 (8.89)	
	Streptococcus	2 (4.44)	
	Coagulase negative *Staphylococcus*	24 (4.44)	
	Others	8 (17.78)	
Gram-negative, *n* = 29	*Klebsiella pneumoniae*	11 (37.93)	CRKP, 8; PDR, 1
	Acinetobacter baumannii	7 (24.14)	CRAB, 7
	Escherichia coli	5(17.24)	ESBL, 2; XDR, 1
	Pseudomonas aeruginosa	3 (10.34)	CRPA, 1; MDR, 2
	Others	3 (10.34)	

CRAB: Carbapenem-resistant *Acinetobacter baumannii*; CRKP: Carbapenem-resistant *Klebsiella pneumoniae*; CRPA: Carbapenem-resistant *Pseudomonas aeruginosa*; ESBL: Extended spectrum beta-lactamase; MDR: Multidrug-resistant; PDR: Pandrug-resistant; XDR: Extensively drug-resistant.

from 93% to 55%, respectively[34,35].

As prior studies reported[2,26,27], the most frequent sources of BSIs in our study were intra-abdominal and biliary tract in GNB group. Intra-abdominal infection largely occurred in the first 3 mo, while cholangitis was the major source of BSI at later time. Reduction of biliary complications was thought to be an important factor for lower incidence of bacteremia, especially in living-donor liver transplantation because biliary tract is one of the most common port of bacterial entry due to the complexity of liver transplantation procedures[2]. Similar to previous reports[36-38], the primary site of infection was not identified in 17.6% of the cases in this study, probably due to early proactive antibiotic therapy and the difficulty of identifying intra-abdominal and biliary sources. George *et al*[38] reported that many episodes of primary bacteremia were associated with biliary leakage, hepatic infarction, or abdominal fluid. Bile leakage or biliary stenosis is a major postoperative complication, with an incidence of 10%-15% in deceased donor LT and 15%-30% in living transplantation recipients[39,40].

There are some limitations in this study. Firstly, this is a retrospective single center and small size study. Secondly, the number of BSI episodes may have been underreported. Finally, variability in immunosuppressive management may exit when comparing our findings with the practice in other medical centers.

CONCLUSION

In conclusion, IS reduction is surprisingly more common in cases of GNB than GPB BSIs in the LT recipients. MDR GNB infection may put LT recipients at higher risk of graft rejection and death than GPB infection. Rejection and complete IS withdrawal are the independent predictors for the 30 d mortality in patients with GNB infection. Complete IS withdrawal should be done cautiously due to increased risk of mortality in the LT recipients complicated with GNB infection. A multidisciplinary approach, timely and appropriate successful antimicrobial therapy, and source control, when necessary, may be safer and more effective than IS reduction therapy in recipients with BSI after LT.

Table 3 Relationship of clinical and therapeutic variables with outcomes in patients with Gram-negative bacterial infections, *n* (%)

Variable	30 d mortality, *n* = 11	30 d survival, *n* = 17	*P* value
Age in yr, mean ± SD	48.09 ± 15.4	47.29 ± 12.2	0.88
Female sex	2 (18.2)	6 (35.3)	0.419
Etiology of liver transplant			
Hepatocellular carcinoma	13 (31.0)	7 (25.0)	0.419
Hepatitis B virus	20 (47.6)	13 (46.4)	0.934
BSI source, episodes			
Primary	0	2 (11.8)	0.505
Respiratory tract	3 (27.3)	2 (11.8)	0.353
Intra-abdominal	4 (36.4)	5 (29.4)	0.7
Biliary tract	5 (45.5)	2 (11.8)	0.076
Urinary tract	1 (9.1)	5 (29.4)	0.355
Management of infection			
Empiric therapy	3 (27.3)	11 (64.7)	0.053
Target therapy	7 (63.6)	5 (29.4)	0.074
Source control	1 (9.1)	1 (5.9)	0.747
Patient outcome			
Suspected rejection[1]	6 (54.5)	1 (5.9)	0.007
Pathogen			
Acinetobacter baumannii	5 (45.5)	2 (11.1)	0.044
Klebsiella *pneumoniae*	6 (54.5)	5 (29.4)	0.184
Management of immunosuppressive therapy			
IS reduction	1 (9.1)	7 (41.2)	0.099
Complete IS withdrawal	10 (90.9)	5 (29.4)	0.002

[1]Within 90 d after liver transplantation. BSI: bloodstream infection; IS: Immunosuppression; SD: Standard deviation.

Table 4 Univariate and multivariate Cox regression analysis of risk factors for 30 d mortality after Gram-negative bacterial infections in liver transplant recipients

Variable	Univariate analysis		Multivariate analysis	
	HR (95%CI)	*P* value	aHR (95%CI)	*P* value
Age	1.025 (0.969-1.085)	0.392		
Sex	0.778 (0.157-3.857)	0.759		
Etiology of liver transplant				
Hepatocellular carcinoma	0.839 (0.169-4.157)	0.829		
Hepatitis B virus	0.666 (0.159-2.79)	0.578		
Rejection[1]	13.89 (2.741-70.376)	0.001	7.021 (1.581-31.188)	0.01
BSI source				
Primary	0.044 (0.00-3.849)	0.581		
Respiratory tract	1.987 (0.448-8.81)	0.366		
Intra-abdominal	1.343 (0.391-4.611)	0.639		
Biliary tract	2.376 (0.72-7.843)	0.156		

Urinary tract	0.51 (0.063-4.146)	0.529		
Pathogen				
Klebsiella pneumoniae	5.165 (1.22-21.87)	0.026		0.47
Acinetobacter baumannii	0.038 (0.00-125.635)	0.428		
Management of infection				
Empiric therapy	0.545 (0.13-2.282)	0.406		
Target therapy	1.539 (0.384-6.163)	0.543		
Source control	1.6 (0.197-13.018)	0.661		
Management of immunosuppressive therapy				
IS reduction	0.026 (0.0-12.782)	0.249		
Complete IS withdrawal	14.362 (1.818-113.46)	0.012	12.65 (1.51-105.965)	0.019

[1]Within 90 d after bloodstream infection. aHR: Adjusted hazard ratio; BSI: Bloodstream infection; CI: Confidence interval; HR: Hazard ratio; IS: Immunosuppression.

ARTICLE HIGHLIGHTS

Research background

Bacterial infections continue to be the most common infectious complication after liver transplantation (LT), usually within 2 mo after LT. Immunosuppression (IS) is the single most important factor contributing to the incidence of infections in transplant recipients.

Research motivation

The management of IS therapy during infection after LT is highly controversial, although IS reduction (partially discontinue or reduce the dosage of at least one IS agent) or complete withdrawal may be a generally accepted option in life-threatening infections. Few studies are available on the management of IS treatment in the LT recipients complicated with infection.

Research objectives

To describe our experience in the management of IS treatment during bacterial bloodstream infection (BSI) in LT recipients and assess the effect of temporary IS withdrawal on 30 d mortality of recipients presenting with severe infection.

Research methods

A retrospective study was conducted with the patients diagnosed with BSI after LT in the Department of Liver Surgery, Renji Hospital from January 1, 2016 through December 31, 2017. All recipients diagnosed with BSI infections after LT were included in this study. Univariate and multivariate Cox regression analysis of risk factors for 30 d mortality was conducted in LT patients with Gram-negative bacterial (GNB) infections.

Research results

Seventy-four episodes of BSI were identified in 70 LT recipients, including 45 episodes of Gram-positive bacterial (GPB) infections in 42 patients and 29 episodes of Gram-negative bacterial infections in 28 patients. Overall, IS reduction (at least 50% dose reduction or cessation of one or more immunosuppressive agent) was made in 28 (41.2%) cases, specifically, in 5 (11.9%) cases with GPB infections and 23 (82.1%) cases with GNB infection. The 180 d all-cause mortality rate was 18.5% (13/70). The mortality rate in GNB group (39.3%, 11/28) was significantly higher than that in GPB group (4.8%, 2/42) (P = 0.001). All the deaths in GNB group were attributed to worsening infection secondary to IS withdrawal but the deaths in GPB group were all due to graft-versus-host disease. GNB group was associated with significantly higher incidence of intra-abdominal infection, IS reduction, and complete IS withdrawal than GPB group (P < 0.05). Cox regression showed that rejection (adjusted hazard ratio 7.021, P = 0.001) and complete IS withdrawal (adjusted hazard ratio 12.65, P = 0.019) were independent risk factors for 30 d mortality in patients with GNB infections after

LT.

Research conclusions

IS reduction is more frequently associated with GNB infection than GPB infection in LT recipients. Complete IS withdrawal should be cautious due to increased risk of mortality in the LT recipients complicated with BSI.

Research perspectives

IS reduction may be a generally accepted option in life-threatening infections after LT. However, this practice must be discussed thoroughly, as it seems to be associated with worse outcome in patients with BSI. A multidisciplinary approach, timely and appropriate successful antimicrobial therapy, and source control, when necessary, may be safer and more effective than IS reduction therapy in recipients with BSI after LT.

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ORIGINAL ARTICLE

Retrospective Study

Liver fibrosis index-based nomograms for identifying esophageal varices in patients with chronic hepatitis B related cirrhosis

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Author contributions: Xu SH and Wu F carried out the ultrasonography and endoscopy in the study, performed the data collection, and drafted the manuscript; Guo LH and Zhang WB helped to draft the manuscript and performed the statistical analysis; Xu HX conceived the study, participated in its coordination, and gave final approval of the version to be published; all authors read and approved the final manuscript.

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Abstract

BACKGROUND

Esophageal varices (EV) are the most fatal complication of chronic hepatitis B (CHB) related cirrhosis. The prognosis is poor, especially after the first upper gastrointestinal hemorrhage.

AIM

To construct nomograms to predict the risk and severity of EV in patients with CHB related cirrhosis.

METHODS

Between 2016 and 2018, the patients with CHB related cirrhosis were recruited and divided into a training or validation cohort at The First Affiliated Hospital of Wenzhou Medical University. Clinical and ultrasonic parameters that were closely related to EV risk and severity were screened out by univariate and multivariate logistic regression analyses, and integrated into two nomograms, respectively. Both nomograms were internally and externally validated by calibration, concordance index (C-index), receiver operating characteristic curve, and decision curve analyses (DCA).

RESULTS

A total of 307 patients with CHB related cirrhosis were recruited. The independent risk factors for EV included Child-Pugh class [odds ratio (OR) = 7.705, 95% confidence interval (CI) = 2.169-27.370, P = 0.002], platelet count (OR = 0.992, 95%CI = 0.984-1.000, P = 0.044), splenic portal index (SPI) (OR = 3.895,

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95%CI = 1.630-9.308, P = 0.002), and liver fibrosis index (LFI) (OR = 3.603, 95%CI = 1.336-9.719, P = 0.011); those of EV severity included Child-Pugh class (OR = 5.436, 95%CI = 2.112-13.990, P < 0.001), mean portal vein velocity (OR = 1.479, 95%CI = 1.043-2.098, P = 0.028), portal vein diameter (OR = 1.397, 95%CI = 1.021-1.912, P = 0.037), SPI (OR = 1.463, 95%CI = 1.030-2.079, P = 0.034), and LFI (OR = 3.089, 95%CI = 1.442-6.617, P = 0.004). Two nomograms (predicting EV risk and severity, respectively) were well-calibrated and had a favorable discriminative ability, with C-indexes of 0.916 and 0.846 in the training cohort, respectively, higher than those of other predictive indexes, like LFI (C-indexes = 0.781 and 0.738), SPI (C-indexes = 0.805 and 0.714), ratio of platelet count to spleen diameter (PSR) (C-indexes = 0.822 and 0.726), King's score (C-indexes = 0.694 and 0.609), and Lok index (C-indexes = 0.788 and 0.700). The areas under the curves (AUCs) of the two nomograms were 0.916 and 0.846 in the training cohort, respectively, higher than those of LFI (AUCs = 0.781 and 0.738), SPI (AUCs = 0.805 and 0.714), PSR (AUCs = 0.822 and 0.726), King's score (AUCs = 0.694 and 0.609), and Lok index (AUCs = 0.788 and 0.700). Better net benefits were shown in the DCA. The results were validated in the validation cohort.

CONCLUSION

Nomograms incorporating clinical and ultrasonic variables are efficient in noninvasively predicting the risk and severity of EV.

Key Words: Real-time tissue elastography; Chronic hepatitis B; Cirrhosis; Esophageal varices; Nomogram; Decision curve analysis

Core Tip: In this study, we constructed two liver fibrosis index (LFI)-based nomograms for predicting the risk and severity of esophageal varices (EV) in patients with chronic hepatitis B related cirrhosis, through incorporating the clinical and ultrasonic variables screened out by univariate and multivariate logistic regression analyses. The nomograms were well-calibrated and had a good discriminative ability that was validated by receiver operating characteristic curve, concordance index, and decision curve analyses. Both nomograms were more efficient than LFI, splenic portal index, ratio of platelet count to spleen diameter, King's score, and Lok index in the training and validation cohorts, and can be clinically used for diagnosing EV and making clinical interventions.

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INTRODUCTION

Chronic hepatitis B (CHB) causes considerable liver-related morbidity and mortality worldwide[1]. CHB is victimizing over 290 million people, and killed 1.34 million in 2015 (96% died from CHB complications)[2]. When CHB progresses to liver cirrhosis, poor prognosis is always suspected for its serious complications, including esophageal varices (EV) that could lead to upper gastrointestinal bleeding[3]. Current primary and secondary treatment strategies cannot prevent the advent of poor prognosis after the first upper gastrointestinal hemorrhage[4].

Hepatic venous pressure gradient and surveillance endoscopy are required to monitor EV in patients with CHB related cirrhosis[5,6]. However, both techniques are invasive and costly[7]. Therefore, there is an urgent need for an effective novel non-invasive tool.

Some clinical indicators are associated with EV, and EV prediction models based on

ultrasound imaging have been established[8]. Doppler ultrasonography has been considered an ideal tool for diagnosing portal hypertension. Several studies have evaluated the predictive efficiency of Doppler ultrasound for EV, but the results remain contradictory[9,10]. Compared with portal vein velocity or splenic index alone, splenic portal index (SPI) demonstrated superior diagnostic accuracy [cutoff 3.0, area under the curve (AUC) 0.96], with higher sensitivity, specificity, positive predictive value, and negative predictive value[11]. Supranormal spleen diameter (SD) is regarded as an independent risk marker in diagnosing large esophageal varices for cirrhotic patients[12,13]. Real-time tissue elastography (RTE) is a relatively new non-invasive method for measuring liver tissue elasticity[14-16]. RTE makes the elasticity of the target area visual by capturing secondary echo signals arising from repetitive compression caused by heartbeat[17]. Liver fibrosis index (LFI) is quantitated by RTE and has a good predictive efficiency for liver fibrosis and cirrhosis. As EV always develops as liver cirrhosis progresses, cirrhosis indexes assessed by ultrasonic diagnostic methods were used to evaluate EV in this study.

Nomograms can visualize the complicated predictive models and make the results more readable[18]. In this study, we constructed two nomograms integrating clinical and ultrasonic indicators for determining the risk and severity of EV in patients with CHB related cirrhosis.

MATERIALS AND METHODS

Patient selection

A total of 123 consecutive patients were recruited as a training cohort from January 1, 2016 to December 31, 2016, and 184 consecutive patients as a validation cohort from January 1, 2017 to December 31, 2018 at The First Affiliated Hospital of Wenzhou Medical University. The inclusion criteria were: (1) Clear etiological evidence of hepatitis B virus (HBV) infection (*i.e.*, defined as positive HBV surface antigen and HBV DNA ≥ 30 IU/mL); (2) Clinical manifestations and laboratory results of cirrhosis; and (3) Cirrhosis confirmed by liver biopsy or two among ultrasound, computed tomography, and magnetic resonance. The exclusion criteria were: (1) Diagnosed with other liver diseases, such as chronic viral hepatitis C (HCV, defined as positive anti-HCV antibodies and HCV RNA > 1000 IU/mL), autoimmune hepatitis (based on serum autoantibodies or histology), drug-induced hepatic disease, alcoholic liver disease, and cholestatic liver disease; (2) Evidence of hepatic carcinoma, or other malignant or benign tumors; (3) History of endoscopy to determine the condition of EV; and (4) Previous diagnosis of EV and related treatment.

Patient characteristics

The following variables were collected from each patient within 1 wk before endoscopy: Age, sex, body mass index, systolic blood pressure, diastolic blood pressure, and Child-Pugh class. Also recorded were laboratory results: Total bilirubin, direct bilirubin, total protein, albumin, alanine transaminase (ALT), aspartic transaminase (AST), alkaline phosphatase, gamma-glutamyl transpeptidase, international normalized ratio (INR), platelet count (PLT), mean platelet volume (MPV), and platelet distribution width. All patients underwent endoscopies to determine the EV condition. EV were classified into Degrees 0-3 according to the published criteria[19], with Degrees 2 and 3 defined as severe varices.

Ultrasound parameter assessment

After overnight fasting, the patients underwent Doppler ultrasonography operated by two experienced ultrasonographers at The First Affiliated Hospital of Wenzhou Medical University. The patient was placed in a lateral position through a HI-VISION Avius ultrasound system (Hitachi Medical Corporation, Tokyo, Japan) and EUP-C715 phased-array electronic probe (1-5MHz; Hitachi Medical Corporation, Tokyo, Japan). The following indicators were detected: Hepatic artery diameter, hepatic artery peak velocity, portal vein diameter (PVD), mean portal vein velocity (MPVV), splenic artery diameter, splenic artery peak velocity, splenic vein diameters, mean splenic vein velocity, spleen thick (ST), and SD.

RTE was performed using ultrasonography (HI-VISION Avius; Hitachi Medical Corporation, Tokyo, Japan) and an EUP-L52 linear array probe (3-7 MHz; Hitachi Medical Corporation, Tokyo, Japan). The patient was placed in a supine position with maximum right-arm abduction, and required to hold their breath. The right lobe of the liver (the sixth-to-ninth intercostal space) was examined as the examiner gripped the

transducer without exerting pressure on the skin. The region of interest (ROI) on the strain image was localized about 1 cm below the margin of the liver, with a size of 2.5 cm × 2.5 cm. Additionally, large blood vessels, lower lobe of the right lung, and ribs should not be included into the ROI, in order that its image quality was not impaired. The LFI was calculated according to a multiple regression equation using nine parameters of each RTE image, including the mean of relative strain value (MEAN), standard deviation of relative strain value (SDV), area ratio of low-strain region (%AREA), complexity of low-strain region (COMP), angular second moment (ASM), entropy (ENT), inverse difference moment (IDM), kurtosis (KURT), and skewness (SKEW)[20].

Nomogram construction

After the variables with multicollinearity were excluded, those with $P < 0.05$ in univariate regression analysis were selected for multivariate regression analysis. Then independent clinical and ultrasonic predictive factors were estimated and used to construct the nomograms for assessing the risk and severity of EV, respectively.

Prediction of other indexes

Cirrhosis and EV were also predicted with current predictive indexes: LFI[20], SPI[11], ratio of platelet count to spleen diameter (PSR)[21,22], King's score[23], and Lok index[24]; LFI = −0.009 × MEAN − 0.005 × SDV + 0.023 × %AREA + 0.025 × COMP + 0.775 × SKEW − 0.281 × KURT + 2.083 × ENT + 3.042 × IDM + 39.979 × ASM − 5.542, SPI = ST × SD/MPVV, PSR = PLT/SD, King's score = Age × AST × INR/PLT, and Lok index = −5.56 − 0.0089 × PLT + 1.26 × AST/ALT + 5.27 × INR.

Predictive performance of nomograms

The receiver operating characteristic (ROC) curve and concordance index (C-index) analyses were used to evaluate the accuracy of nomograms for predicting the risk and severity of EV. The predicted and observed probabilities of the nomograms were illustrated with calibration curves. The potential net benefits of the nomograms were demonstrated by decision curve analysis (DCA). The discrimination performances of LFI, SPI, PSR, King's score, and Lok index were compared with those of the nomograms.

Statistical analysis

Statistical analyses were performed using R version 3.6.1. Continuous variables in a normal distribution are expressed as the mean ± SD and were compared using the Student's *t*-test, and those in a skewed distribution are presented as median (interquartile range) and were analyzed by the nonparametric Mann-Whitney U test. Categorical variables are expressed as frequencies (%) and were compared using the chi-square test. Univariate and multivariate logistic regression analyses were completed with SPSS statistics version 26. R software was used for building the nomogram using "rms" package. Packages of "pROC" and "rmda" were used in ROC and DCA analyses. The values of AUCs were compared using the DeLong method with MedCalc version 18.11.3. All *P* values were two-sided.

RESULTS

Clinical and ultrasonic characteristics of patients

From 2016 to 2018, we recruited 307 eligible patients. All of them were divided into either a training cohort ($n = 123$) or a validation cohort ($n = 184$) based on recruitment time. Figure 1 shows the workflow of our study. Table 1 shows the clinical and ultrasonic characteristics of patients. All clinical, laboratory, and ultrasonic characteristics of both cohorts were comparable ($P > 0.05$). The mean age of the patients was 54.05 years in the training cohort and 54.54 years in the validation cohort. The majority of patients were men (78.05% *vs* 79.89%) and had Child-Pugh class A liver function (47.15% *vs* 46.74%). The percentages of patients suffering from EV (Degrees 0-3) were 26.02%, 13.01%, 17.07%, and 43.90% in the training cohort, and 26.09%, 14.13%, 13.59%, and 46.19% in the validation cohort, respectively.

Risk factors

Univariate logistic regression analysis in the training cohort indicated that age [odds ratio (OR) = 1.038, 95% confidence interval (CI) = 1.001-1.076, $P = 0.043$)], Child-Pugh

Table 1 Patient's clinical characteristics of the training and validation cohort

Variable	Training cohort (*n* = 123)	Validation cohort (*n* = 184)	*P* value
Clinical parameter			
Age (yr)	54.05 ± 12.02	54.54 ± 12.25	0.727
Male (%)	96 (78.05)	147 (79.89)	0.697
BMI (kg/m^2)	22.97 ± 3.24	22.91 ± 3.41	0.877
SBP (mmHg)	129.29 ± 20.62	129.03 ± 20.56	0.914
DBP (mmHg)	71.77 ± 12.16	71.41 ± 11.76	0.796
Child-Pugh class A (%)	58 (47.15)	86 (46.74)	0.943
Degree of EV (Q1, Q3)	2 (0, 3)	2 (0, 3)	0.873
0 (%)	32 (26.02)	48 (26.09)	
1 (%)	16 (13.01)	26 (14.13)	
2 (%)	21 (17.07)	25 (13.59)	
3 (%)	54 (43.90)	85 (46.19)	
Laboratory test			
TBil (μmol/L)	19.00 (12.00, 31.00)	18.50 (12.00, 31.00)	0.943
DBil (μmol/L)	9.00 (5.00, 16.00)	9.00 (5.00, 17.00)	0.766
TP (g/L)	65.80 ± 10.03	66.05 ± 9.95	0.831
Albumin (g/L)	32.85 ± 7.11	33.14 ± 7.14	0.726
ALT (IU/L)	27.00 (20.00, 47.00)	27.50 (20.25, 47.00)	0.850
AST (IU/L)	38.00 (28.00, 70.00)	41.00 (28.00, 70.75)	0.734
ALP (IU/L)	94.00 (74.00, 131.00)	99.00 (73.00, 140.00)	0.728
GGT (IU/L)	51.00 (24.00, 126.00)	50.00 (29.25, 126.75)	0.865
INR	1.36 (1.17, 1.54)	1.36 (1.17, 1.54)	0.994
PLT (× 10^9/L)	82.00 (53.00, 140.00)	84.00 (56.00, 139.00)	0.760
MPV (fl)	11.50 (10.50, 12.50)	11.30 (10.40, 12.30)	0.301
PDW (fl)	14.90 (12.60, 17.30)	14.65 (12.70, 17.18)	0.760
Ultrasound parameter			
HAD (mm)	3.46 ± 0.56	3.47 ± 0.57	0.914
HAPV (cm/s)	50.35 ± 12.23	50.51 ± 13.33	0.915
PVD (mm)	12.30 ± 1.54	12.05 ± 1.61	0.186
MPVV (cm/s)	19.11 ± 5.28	19.93 ± 5.59	0.196
SAD (mm)	5.05 ± 1.00	5.00 ± 0.98	0.678
SAPV (cm/s)	55.89 ± 15.38	55.91 ± 16.03	0.990
SVD (mm)	9.61 ± 1.82	9.59 ± 1.70	0.899
MSVV (cm/s)	22.22 ± 4.93	22.69 ± 5.20	0.428
ST (mm)	44.47 ± 9.36	44.61 ± 9.68	0.900
SD (mm)	110.88 ± 23.73	112.27 ± 25.39	0.629
SPI	2.96 ± 1.75	2.92 ± 1.96	0.871
LFI	3.74 ± 0.69	3.72 ± 0.68	0.886

BMI: Body mass index; SBP: Systolic blood pressure; DBP: Diastolic blood pressure; EV: Esophageal varices; Q1: 1st quartile; Q3: 3rd quartile; TBil: Total bilirubin; DBil: Direct bilirubin; TP: Total protein; ALT: Alanine transaminase; AST: Aspartic transaminase; ALP: Alkaline phosphatase; GGT: Gamma-glutamyl transpeptidase; INR: International normalized ratio; PLT: Platelet count; MPV: Mean platelet volume; PDW: Platelet distribution width; HAD:

Hepatic artery diameter; HAPV: Hepatic artery peak velocity; PVD: Portal vein diameter; MPVV: Mean portal vein velocity; SAD: Splenic artery diameter; SAPV: Splenic artery peak velocity; SVD: Splenic vein diameters; MSVV: Mean splenic vein velocity; ST: Spleen thick; SD: Spleen diameter; SPI: Splenic portal index; LFI: Liver fibrosis index.

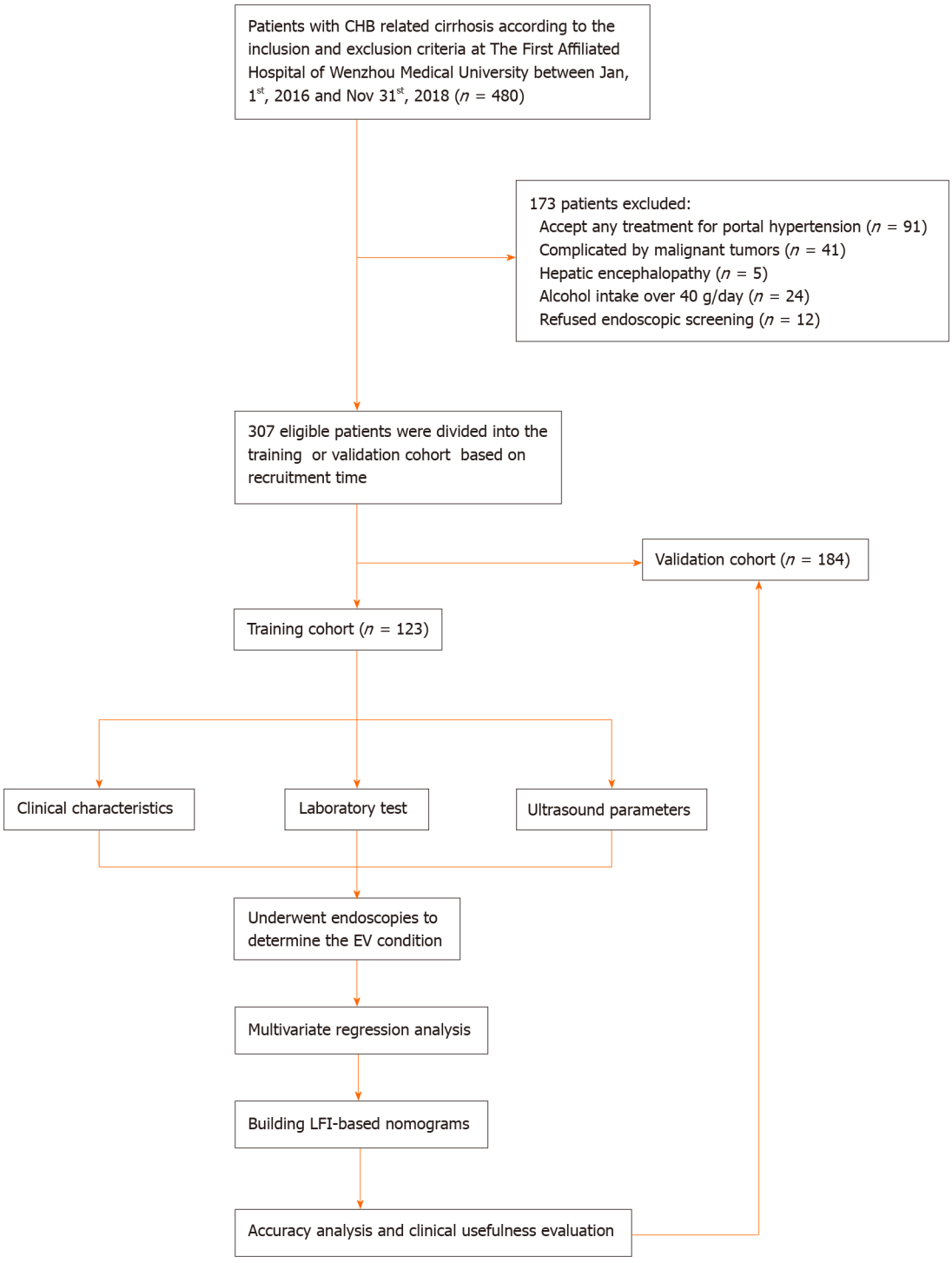

Figure 1 Study workflow. CHB: Chronic hepatitis B; EV: Esophageal varices; LFI: Liver fibrosis index.

class (OR = 7.990, 95%CI = 2.979-21.426, $P < 0.001$), albumin (OR = 0.932, 95%CI = 0.877-0.990, $P = 0.022$), INR (OR = 20.331, 95%CI = 2.870-144.037, $P = 0.003$), PLT (OR = 0.990, 95%CI = 0.985-0.995, $P < 0.001$), PVD (OR = 1.505, 95%CI = 1.121-2.021, $P = 0.006$), MPVV (OR = 0.915, 95%CI = 0.845-0.991, $P = 0.029$), ST (OR = 1.312, 95%CI = 1.178-1.462, $P < 0.001$), SD (OR = 1.036, 95%CI = 1.012-1.059, $P = 0.003$), SPI (OR =

4.183, 95%CI = 2.044-8.559, $P < 0.001$), and LFI (OR = 7.067, 95%CI = 2.938-16.995, $P < 0.001$) were risk factors for EV. To reduce multiple collinearity, albumin was excluded in the subsequent multivariate logistic regression analysis, because it was an objective indicator in the Child-Pugh system. Similarly, ST, SD, and MPVV were excluded since they were calculated with SPI formula. On this basis, the multivariate logistic regression analysis identified Child-Pugh class (OR = 7.705, 95%CI = 2.169-27.370, $P = 0.002$), PLT (OR = 0.992, 95%CI = 0.984-1.000, $P = 0.044$), SPI (OR = 3.895, 95%CI = 1.630-9.308, $P = 0.002$), and LFI (OR = 3.603, 95%CI = 1.336-9.719, $P = 0.011$) as independent indicators for the risk of EV (Table 2).

As shown in Table 3, univariate logistic regression analysis indicated that Child-Pugh class (OR = 5.161, 95%CI = 2.344-11.361, $P < 0.001$), INR (OR = 10.764, 95%CI = 2.342-49.469, $P = 0.002$), PLT (OR = 0.994, 95%CI = 0.989-0.999, $P = 0.016$), MPV (OR = 1.399, 95%CI = 1.050-1.865, $P = 0.022$), PVD (OR = 1.465, 95%CI = 1.126-1.906, $P = 0.004$), ST (OR = 1.146, 95%CI = 1.077-1.218, $P < 0.001$), SD (OR = 1.030, 95%CI = 1.010-1.049, $P = 0.003$), SPI (OR = 1.644, 95%CI = 1.193-2.265, $P = 0.002$), and LFI (OR = 4.184, 95%CI = 2.080-8.416, $P < 0.001$) were statistically associated with EV severity in the training cohort. Indicators of ST and SD were also excluded from multivariate logistic regression analysis, since they were calculated with SPI formula. The multivariate logistic regression analysis identified Child-Pugh class (OR = 5.436, 95%CI = 2.112-13.990, $P < 0.001$), MPV (OR = 1.479, 95%CI = 1.043-2.098, $P = 0.028$), PVD (OR = 1.397, 95%CI =1.021-1.912, $P = 0..037$), SPI (OR = 1.463, 95%CI = 1.030-2.079, $P = 0.034$), and LFI (OR = 3.089, 95%CI = 1.442-6.617, $P = 0.004$) as independent indicators for EV severity.

Nomograms and clinical usage

Based on the univariate and multivariate logistic regression analyses, variables that achieved a value of $P < 0.05$ in multivariate analysis were selected and incorporated into the nomograms for predicting the probability and severity of EV (Figure 2).

The nomograms were used to predict the EV in a patient (PLT, 60 × 10^9/L; MPV, 10 fl; and Child-Pugh class A). After Doppler ultrasonography and RTE examinations, ultrasound showed PVD of 10 mm, SPI of 3.5, and LFI of 3. In the nomogram predicting the risk of EV, 0 was given to Child-Pugh class A, 18.5 to PLT, 32.5 to SPI, and 18 to LFI. Moreover, in the nomogram predicting the severity of EV, 0 was given to Child-Pugh class A, 15 to MPV, 13 to PVD, 26 to SPI, and 44 to LFI. By summing up all the points, he scored 69 points in the EV risk prediction nomogram and 98 points in the EV severity prediction nomogram. Eventually, his estimated risk of EV occurrence was over 80%, and that of severe EV was slightly lower than 10%.

Comparison between nomograms and other indexes

As depicted in Figure 3, the calibration curves of both nomograms were close to the standard curves in the training cohort and validation cohort, which suggested that the nomograms were well-calibrated.

In the training cohort, the C-index values to predict EV risk were 0.916, 0.781, 0.805, 0.822, 0.694, and 0.788 for EV risk prediction nomogram, LFI, SPI, PSR, King's score, and Lok index, respectively. In the validation cohort, these values were 0.907, 0.731, 0.810, 0.844, 0.702, and 0.782, respectively. In the training cohort, the C-index values to predict EV severity were 0.846, 0.738, 0.714, 0.726, 0.609, and 0.700 for EV severity prediction nomogram, LFI, SPI, PSR, King's score, and Lok index, respectively. In the validation cohort, these values were 0.835, 0.747, 0.705, 0.754, 0.621, and 0.721, respectively.

AUC of EV risk prediction nomogram was 0.916 in the training cohort and 0.907 in the validation cohort, while AUC of EV severity prediction nomogram was 0.846 in the training cohort and 0.835 in the validation cohort. In the training cohort, the AUCs of LFI, SPI, PSR, King's score, and Lok index for predicting EV risk were 0.781, 0.805, 0.822, 0.694, and 0.788, respectively. The AUCs of LFI, SPI, PSR, King's score, and Lok index for predicting EV severity were 0.738, 0.714, 0.726, 0.609, and 0.700, respectively. In the validation cohort, these values were 0.731, 0.810, 0.844, 0.702, and 0.782 in the prediction of EV risk, and 0.747, 0.705, 0.754, 0.621, and 0.721 in the prediction of EV severity, respectively. A pairwise comparison of each index with the nomogram showed statistical significance ($P < 0.05$), as shown in Figure 4.

DCA, a novel prediction tool, was also used to evaluate the efficiency of both nomograms. The decision curves of both nomograms and other indexes in the training and the validation cohorts are shown in Figure 5. The results indicated that nomograms provided better clinical net benefits within most thresholds.

Table 2 Univariate and multivariate logistic regression analyses for esophageal varices risk in patients with chronic hepatitis B related cirrhosis in the training cohort

Variable	Univariate analysis			Multivariate analysis		
	OR	95%CI	*P* value	OR	95%CI	*P* value
Clinical parameter						
Age (yr)	1.038	1.001-1.076	**0.043**			
Gender (Female = 0, Male = 1)	0.994	0.375-2.633	0.990			
BMI (kg/m^2)	1.031	0.907-1.171	0.643			
SBP (mmHg)	1.005	0.985-1.025	0.657			
DBP (mmHg)	0.985	0.953-1.018	0.367			
Child-Pugh class	7.990	2.979-21.426	**< 0.001**	7.705	2.169-27.370	**0.002**
(Child A = 1, Child B/C = 2)						
Laboratory test						
TBil (μmol/L)	1.001	0.988-1.013	0.930			
DBil (μmol/L)	0.998	0.981-1.016	0.831			
TP (g/L)	0.976	0.936-1.018	0.255			
Albumin (g/L)	0.932	0.877-0.990	**0.022**			
ALT (IU/L)	0.992	0.984-1.000	0.052			
AST (IU/L)	0.997	0.990-1.003	0.294			
ALP (IU/L)	1.009	0.999-1.018	0.065			
GGT (IU/L)	0.998	0.995-1.000	0.081			
INR	20.331	2.870-144.037	**0.003**			
PLT (× 10^9/L)	0.990	0.985-0.995	**< 0.001**	0.992	0.984-1.000	**0.044**
MPV (fl)	1.373	0.999-1.887	0.051			
PDW (fl)	1.128	0.981-1.297	0.092			
Ultrasound parameter						
HAD (mm)	1.619	0.786-3.335	0.192			
HAPV (cm/s)	1.017	0.984-1.051	0.318			
PVD (mm)	1.505	1.121-2.021	**0.006**			
MPVV (cm/s)	0.915	0.845-0.991	**0.029**			
SAD (mm)	0.935	0.629-1.390	0.740			
SAPV (cm/s)	0.995	0.970-1.021	0.711			
SVD (mm)	1.086	0.860-1.372	0.489			
MSVV (cm/s)	1.073	0.977-1.180	0.142			
ST (mm)	1.312	1.178-1.462	**< 0.001**			
SD (mm)	1.036	1.012-1.059	**0.003**			
SPI	4.183	2.044-8.559	**< 0.001**	3.895	1.630-9.308	**0.002**
LFI	7.067	2.938-16.995	**< 0.001**	3.603	1.336-9.719	**0.011**

The bold *P* values represent $P < 0.05$. OR: Odds ratio; 95%CI: 95% Confidence interval; BMI: Body mass index; SBP: Systolic blood pressure; DBP: Diastolic blood pressure; TBil: Total bilirubin; DBil: Direct bilirubin; TP: Total protein; ALT: Alanine transaminase; AST: Aspartic transaminase; ALP: Alkaline phosphatase; GGT: Gamma-glutamyl transpeptidase; INR: International normalized ratio; PLT: Platelet count; MPV: Mean platelet volume; PDW: Platelet distribution width; HAD: Hepatic artery diameter; HAPV: Hepatic artery peak velocity; PVD: Portal vein diameter; MPVV: Mean portal vein velocity; SAD: Splenic artery diameter; SAPV: Splenic artery peak velocity; SVD: Splenic vein diameters; MSVV: Mean splenic vein velocity; ST: Spleen thick; SD:

Spleen diameter; SPI: Splenic portal index; LFI: Liver fibrosis index.

DISCUSSION

In this study, nomograms based on clinical and ultrasonic variables showed favorable efficiency in predicting the risk and severity of EV in patients with CHB related cirrhosis.

At present, the diagnosis of EV still relies on endoscopic examination. Since the patients with no varices or mild varices have a low risk for hemorrhage, nonselective beta blocker (NSBB) is not recommended for preventive treatment. Several studies[7,25] have shown that NSBB failed to prevent or delay the progression of EV, and exerted more side effects and complications than the control group. Endoscopic follow-up must be performed every 1-2 years to exclude the occurrence of EV in the low-risk hemorrhagic cirrhotic population[26], while this operation is invasive, expensive, and time-consuming, and some patients even cannot tolerate it. The widespread application of painless endoscopy brings comfortable experience to patients, but anesthesia also increases the risk. The Baveno VI criteria indicated that liver stiffness measurement (LSM) < 20 kPa (an index of transient elastography) and PLT > 150000/mm^3 predicted a low risk of varices[26]. Although LSM and PLT cut-offs have been proposed to screen patients with a risk of EV, esophagogastroduodenoscopy still finds no varices in a certain fraction of patients[27,28]. Given the high variance in the results (cut-offs, positive predictive values, and negative predictive values) of LSM, more efficient and non-invasive ways are urgently needed to stratify EV risk. RTE depends on the relative strain within liver tissue caused by external pressure arising from rhythmic heartbeats. Therefore, RTE can reduce intra- and inter-observer variations since this external pressure is stable[29].

After univariate and multivariate logistic regression analyses, LFI, SPI, PVD, PLT, MPV, and Child-Pugh class were found as independent indicators for the risk or severity of EV. This finding was consistent with the result of prior studies that PVD, as a portal hemodynamic indicator, could independently predict EV occurrence and severity[30,31]. PLT decreases in portal hypertension and related hypersplenia, and has been identified as a noninvasive predictive index for the risk of EV in a cross-sectional and longitudinal study[32]. Accordingly, MPV rises as a result of compensatory production of platelets in the peripheral blood[33]. Our outcomes were in line with the finding of prior studies that SPI Doppler index can be used to predict the probability of EV[34,35]. According to our literature review, it was the first time to report the favorable efficiency of SPI in predicting EV severity. Chinese researchers have utilized RTE to examine 71 patients with post-hepatitis B cirrhosis, finding that LFI is positively correlated with EV severity, which is also consistent with our findings. Child-Pugh class is well recognized to evaluate liver function, but in the present study, it is also associated with EV risk and severity. However, what brings forth this association needs to be interpreted.

Various non-invasive and integrated prediction models have been developed. Giannini *et al*[21] found that PSR, with a cutoff of 909, could keep 27.4% of patients without EV from being screened by endoscopy. However, a meta-analysis containing 1275 patients yielded a pooled positive likelihood ratio of 3.5 and a negative likelihood ratio of 0.1 for predicting EV in cirrhosis of various etiologies, suggesting that PSR could not replace endoscopic screening[36]. According to a study for CHB population, the AUC of PSR for prediction of EV was 0.7095, also indicating that it was better to incorporate PSR with other clinical characteristic[37]. In our study, PSR was efficient for most cases in both the training and the validation cohorts. King's score has been demonstrated to be a simple and non-invasive model to predict the presence of cirrhosis[38]. However, a study including 39 newly diagnosed cirrhotic patients found that King's score showed a low specificity of 44% in predicting esophageal variceal bleeding, and no association with EV was verified[39]. Similarly, considering the poor AUC and C-index in this study, King's score was not a preferred tool for the prediction of EV and its severity in CHB related cirrhosis patients. Additionally, Lok index, as a noninvasive predictive alternative index for liver cirrhosis, was also applied to predict EV in cirrhosis in previous studies. Lok index was proved to be associated with portal hypertension[40]. In a retrospective study including 132 patients, Zhou *et al*[41] found that in patients who did not meet Baveno VI criteria, Lok index could spare 24.2% of gastroscopy screening without missing high-risk varices which were defined as EV with red wale signs[42]. However, none of PSR, King's score, or Lok index can take full advantage of imaging examination in the prediction of EV. Our ROC and DCA

Table 3 Univariate and multivariate logistic regression analyses for esophageal varices severity in patients with chronic hepatitis B related cirrhosis in the training cohort

Variable	Univariate analysis			Multivariate analysis		
	OR	95%CI	*P* value	OR	95%CI	*P* value
Clinical parameter						
Age (yr)	0.997	0.967-1.027	0.833			
Gender (Female = 0, Male = 1)	0.898	0.372-2.168	0.811			
BMI (kg/m^2)	1.011	0.904-1.132	0.844			
SBP (mmHg)	1.001	0.983-1.019	0.928			
DBP (mmHg)	0.992	0.962-1.022	0.584			
Child-Pugh class	5.161	2.344-11.361	**< 0.001**	5.436	2.112-13.990	**< 0.001**
(Child A = 1, Child B/C = 2)						
Laboratory test						
TBil (μmol/L)	1.000	0.989-1.012	0.937			
DBil (μmol/L)	0.996	0.980-1.012	0.643			
TP (g/L)	0.978	0.942-1.015	0.246			
Albumin (g/L)	0.987	0.937-1.038	0.605			
ALT (IU/L)	0.994	0.986-1.001	0.109			
AST (IU/L)	0.997	0.991-1.003	0.282			
ALP (IU/L)	1.001	0.995-1.007	0.637			
GGT (IU/L)	0.997	0.995-1.000	0.064			
INR	10.764	2.342-49.469	**0.002**			
PLT (× 10^9/L)	0.994	0.989-0.999	**0.016**			
MPV (fl)	1.399	1.050-1.865	**0.022**	1.479	1.043-2.098	**0.028**
PDW (fl)	1.036	0.920-1.166	0.564			
Ultrasound parameter						
HAD (mm)	1.327	0.693-2.541	0.393			
HAPV (cm/s)	1.025	0.995-1.057	0.108			
PVD (mm)	1.465	1.126-1.906	**0.004**	1.397	1.021-1.912	**0.037**
MPVV (cm/s)	0.964	0.899-1.033	0.301			
SAD (mm)	0.973	0.678-1.396	0.880			
SAPV (cm/s)	1.000	0.977-1.024	0.985			
SVD (mm)	1.109	0.900-1.366	0.332			
MSVV (cm/s)	1.062	0.979-1.151	0.148			
ST (mm)	1.146	1.077-1.218	**< 0.001**			
SD (mm)	1.030	1.010-1.049	**0.003**			
SPI	1.644	1.193-2.265	**0.002**	1.463	1.030-2.079	**0.034**
LFI	4.184	2.080-8.416	**< 0.001**	3.089	1.442-6.617	**0.004**

The bold *P* values represent $P < 0.05$. OR: Odds ratio; 95%CI: 95% Confidence interval; BMI: Body mass index; SBP: Systolic blood pressure; DBP: Diastolic blood pressure; TBil: Total bilirubin; DBil: Direct bilirubin; TP: Total protein; ALT: Alanine transaminase; AST: Aspartic transaminase; ALP: Alkaline phosphatase; GGT: Gamma-glutamyl transpeptidase; INR: International normalized ratio; PLT: Platelet count; MPV: Mean platelet volume; PDW: Platelet distribution width; HAD: Hepatic artery diameter; HAPV: Hepatic artery peak velocity; PVD: Portal vein diameter; MPVV: Mean portal vein velocity; SAD: Splenic artery diameter; SAPV: Splenic artery peak velocity; SVD: Splenic vein diameters; MSVV: Mean splenic vein velocity; ST: Spleen thick; SD:

Spleen diameter; SPI: Splenic portal index; LFI: Liver fibrosis index.

analyses indicated that our nomograms were superior to PSR, King's score, and Lok index, which was verified in the validation cohort.

To our knowledge, this is the first study to construct nomograms integrating clinical and ultrasonic parameters to predict the risk and severity of EV. Our nomograms showed a strong discriminative ability and a clinical net benefit compared with other indexes. These predictive nomograms are useful for clinicians to make preventive and therapeutic measures.

Inevitably, the study has several limitations. Intra-observer and inter-observer variations may discount the efficiency of nomograms[43,44]. Next, our study is a single center retrospective study, and needs to be improved into a multi-center prospective study.

CONCLUSION

The nomograms incorporating clinical and ultrasonic variables are efficient in noninvasively predicting the probability and severity of EV, and can be used in individualized treatment and follow-ups.

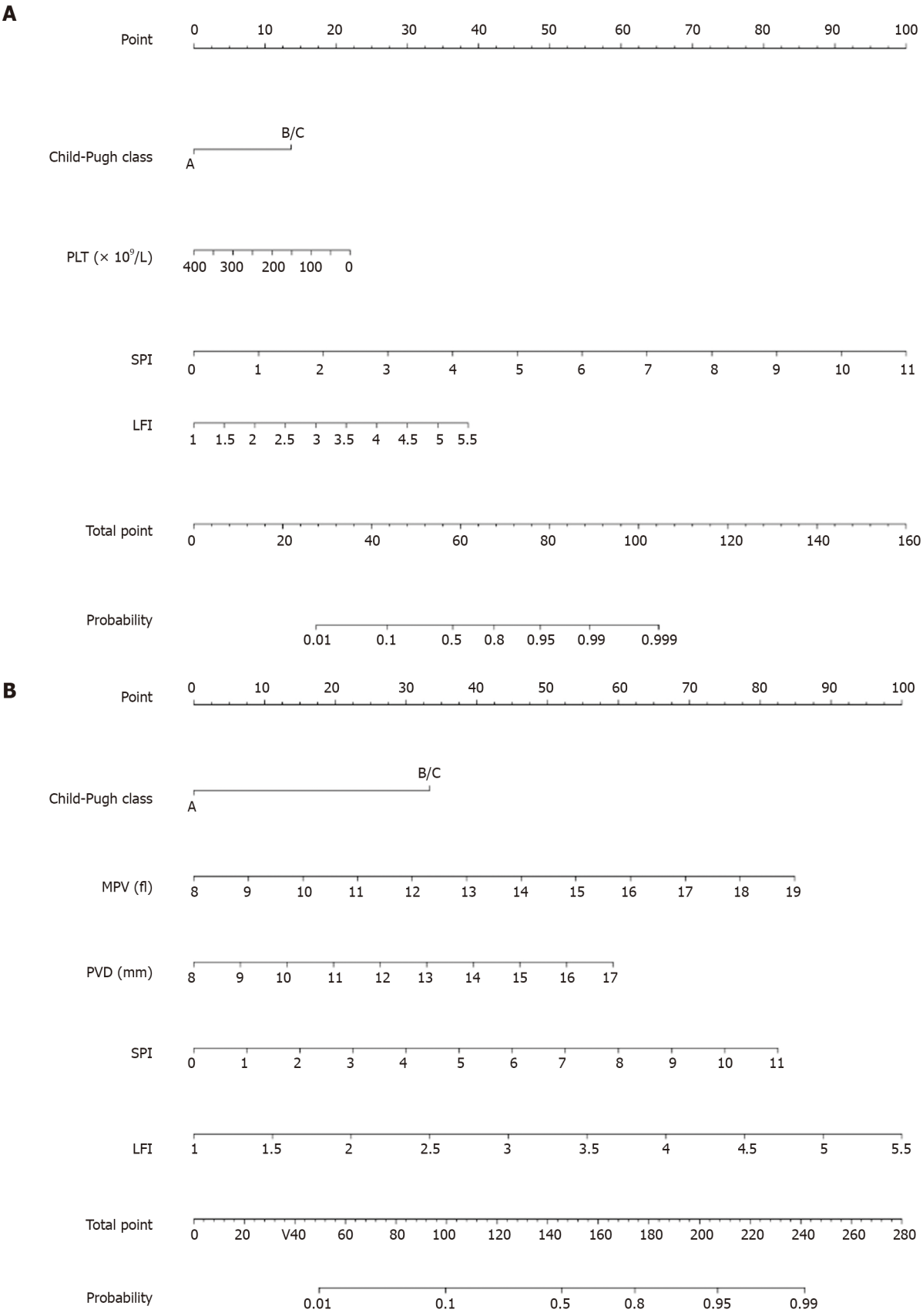

Figure 2 Nomograms for predicting the risk and severity of esophageal varices in patients with chronic hepatitis B related cirrhosis. A: Nomogram for predicting esophageal varices (EV) risk; B: Nomogram for predicting EV severity. PLT: Platelet count; SPI: Splenic portal index; LFI: Liver fibrosis index; MPV: Mean platelet volume; PVD: Portal vein diameter.

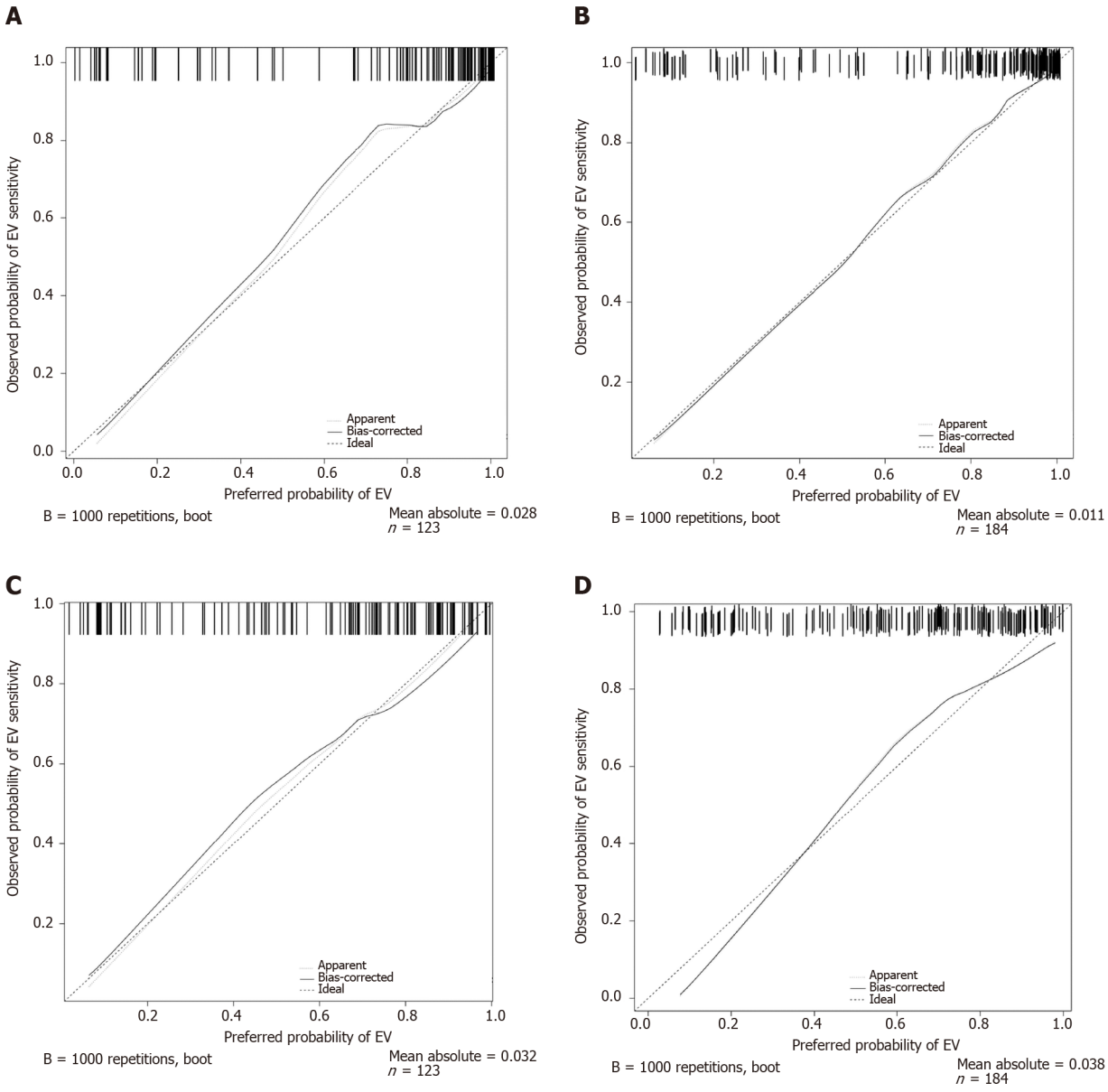

Figure 3 Calibration plots. A: The calibration curve of nomogram for esophageal varices (EV) risk in the training cohort; B: The calibration curve of nomogram for EV risk in the validation cohort; C: The calibration curve of nomogram for EV severity in the training cohort; D: The calibration curve of nomogram for EV severity in the validation cohort. EV: Esophageal varices.

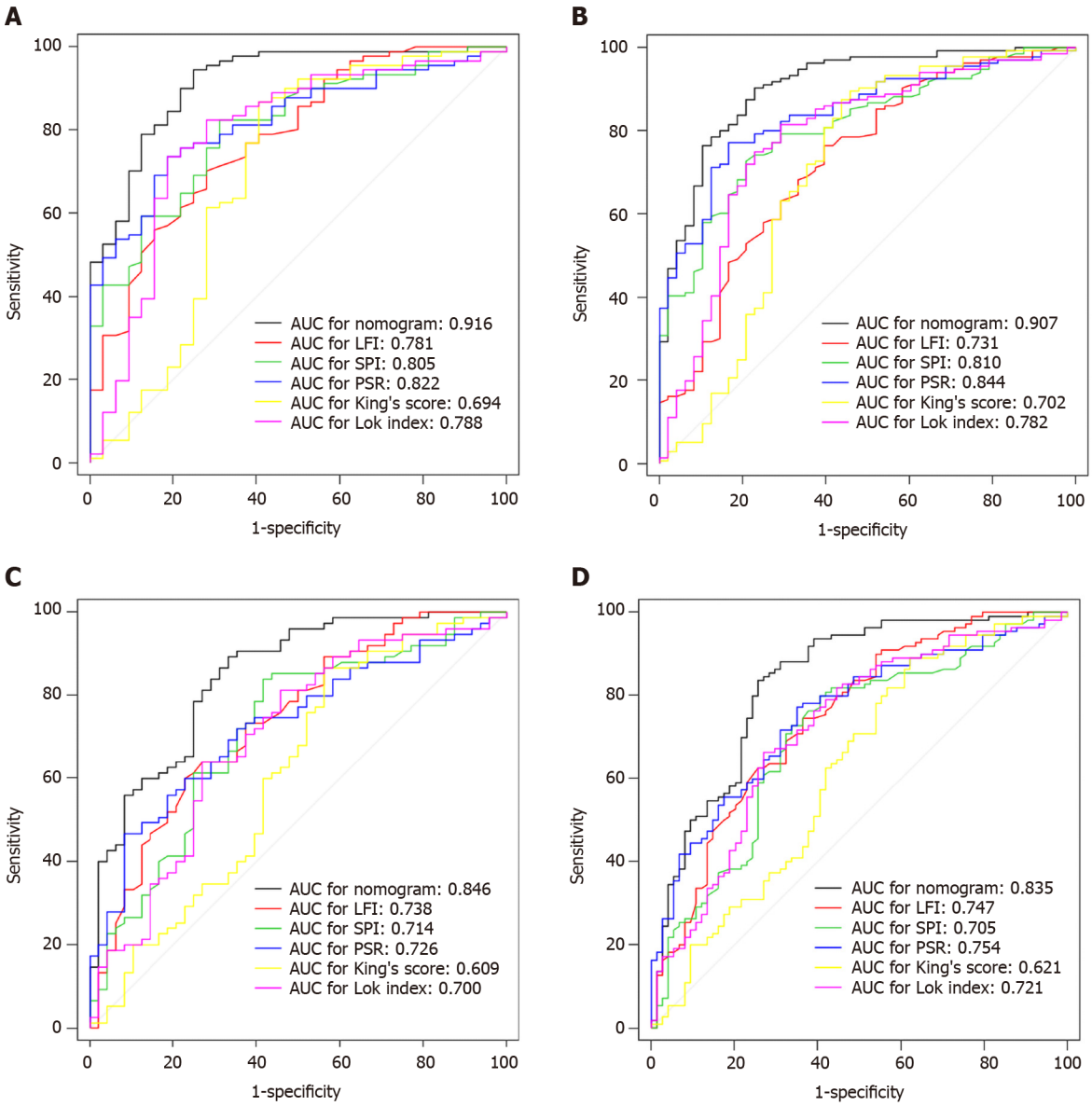

Figure 4 The areas under the curves of the nomograms, liver fibrosis index, splenic portal index, ratio of platelet count to spleen diameter, King's score, and Lok index to predict the risk or severity of esophageal varices in the training and validation cohorts. A: The areas under the curves (AUCs) of esophageal varices (EV) risk prediction nomogram, liver fibrosis index (LFI), splenic portal index (SPI), ratio of platelet count to spleen diameter (PSR), King's score, and Lok index in the training cohort; B: The AUCs of EV risk prediction nomogram, LFI, SPI, PSR, King's score, and Lok index in the validation cohort; C: The AUCs of EV severity prediction nomogram, LFI, SPI, PSR, King's score, and Lok index in the training cohort; D: The AUCs of EV severity prediction nomogram, LFI, SPI, PSR, King's score, and Lok index in the validation cohort. AUC: Area under the curve; LFI: Liver fibrosis index; SPI: Splenic portal index; PSR: Ratio of platelet count to spleen diameter.

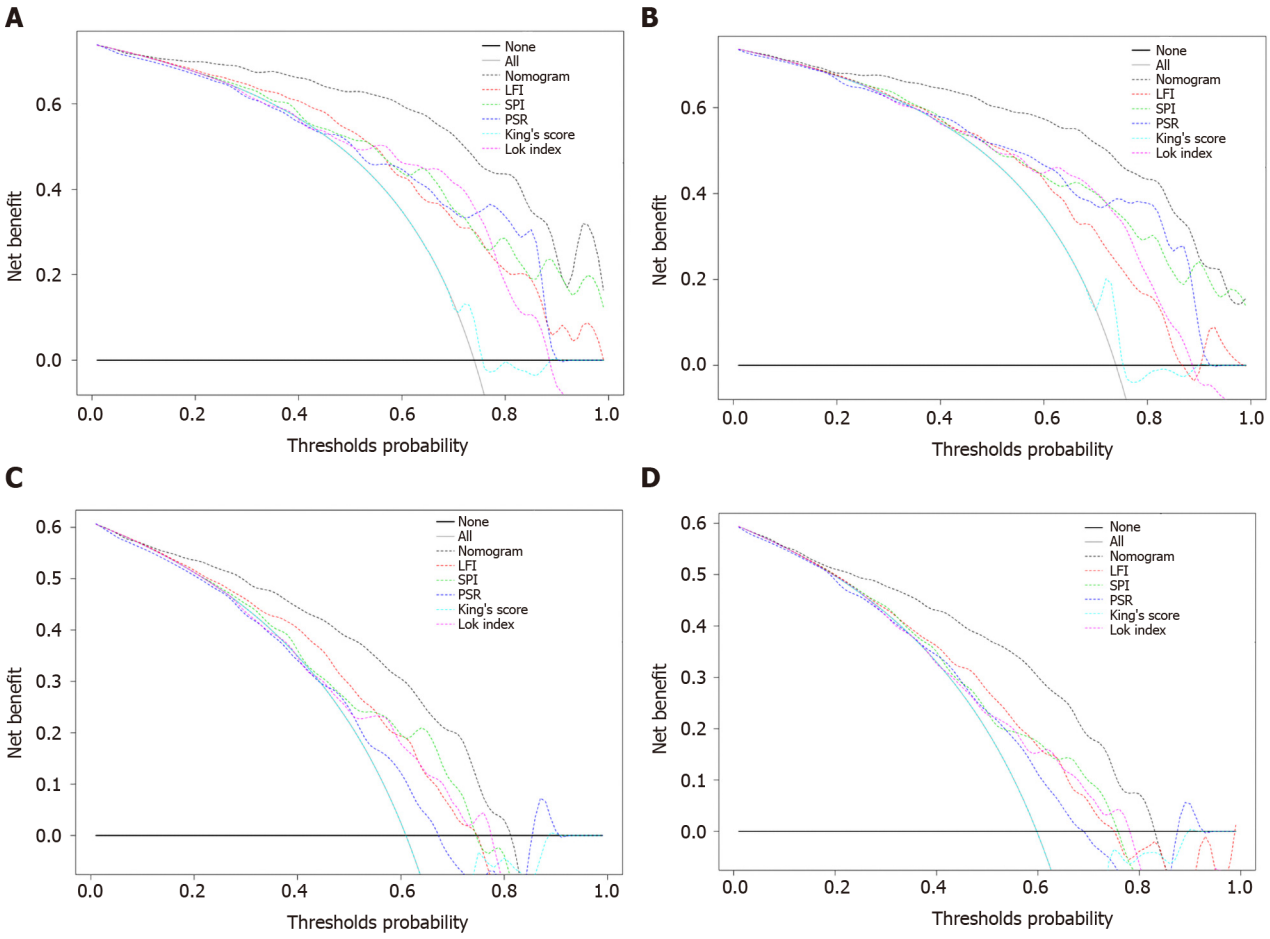

Figure 5 Decision curves of the nomograms, liver fibrosis index, splenic portal index, ratio of platelet count to spleen diameter, King's score, and Lok index to predict the risk or severity of esophageal varices in the training and validation cohorts. A: The decision curve of esophageal varices (EV) risk prediction nomogram, liver fibrosis index (LFI), splenic portal index (SPI), ratio of platelet count to spleen diameter (PSR), King's score, and Lok index in the training cohort; B: The decision curve of EV risk prediction nomogram, LFI, SPI, PSR, King's score, and Lok index in the validation cohort; C: The decision curve of EV severity prediction nomogram, LFI, SPI, PSR, King's score, and Lok index in the training cohort; D: The decision curve of EV severity prediction nomogram, LFI, SPI, PSR, King's score, and Lok index in the validation cohort. LFI: Liver fibrosis index; SPI: Splenic portal index; PSR: Ratio of platelet count to spleen diameter.

ARTICLE HIGHLIGHTS

Research background

Esophageal varices (EV) are an important cause of mortality for patients with chronic hepatitis B (CHB) related cirrhosis.

Research motivation

There is no reliable and non-invasive tool to monitor EV, predict the clinical outcome, and adjust the follow-up strategy.

Research objectives

This study aimed to develop nomogram models including non-invasive and clinically accessible indicators to assess the risk and severity of EV.

Research methods

Patients with CHB related cirrhosis were retrospectively included and divided into a training or validation cohort. Ultrasound parameters and blood indexes were applied to construct the nomograms, which were subsequently evaluated by receiver operating characteristic, concordance index, and decision curve analyses, and tested in the validation cohort.

Research results

The novel nomograms composed of clinical and ultrasonic variables were constructed and proved better than liver fibrosis index, splenic portal index, ratio of platelet count to spleen diameter, King's score, and Lok index for predicting the risk and severity of EV.

Research conclusions

The established novel nomograms are reliable and convenient for clinicians to predict EV in a non-invasive way and make preventive and therapeutic measurements.

Research perspectives

The novel models need to be tested by multi-center prospective studies and adjusted for particular groups, such as patients complicated with other liver diseases.

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ORIGINAL ARTICLE

Clinical Trials Study

Relationship between the incidence of non-hepatic hyperammonemia and the prognosis of patients in the intensive care unit

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Institutional review board statement: The study was approved by The Ethics Committee of The Second Affiliated Hospital of Harbin Medicine University (Nangang, China).

Clinical trial registration statement: This study is registered at Chinese Clinical Trial Register (ChiCTR), registration No. ChiCTR1900026632.

Informed consent statement: All patients in our study provided informed consent.

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Abstract

BACKGROUND

Ammonia is a normal constituent of body fluids and is found mainly through the formation of urea in the liver. Blood levels of ammonia must remain low as even slightly elevated concentrations (hyperammonemia) are toxic to the central nervous system.

AIM

To examine the relationship between the incidence of non-hepatic hyperammonemia (NHH) and the prognosis of patients who were admitted to the intensive care unit (ICU).

METHODS

This is a prospective, observational and single-center study. A total of 364 patients who were admitted to the ICU from November 2019 to February 2020 were initially enrolled. Changes in the levels of blood ammonia at the time of ICU admission and after ICU admission were continuously monitored. In addition, factors influencing the prognosis of NHH patients were analyzed.

RESULTS

A total of 204 patients who met the inclusion criteria were enrolled in this study, including 155 NHH patients and 44 severe-NHH patients. The incidence of NHH and severe-NHH was 75.98% and 21.57%, respectively. Patients with severe-NHH exhibited longer length of ICU stay and higher Acute Physiologic Assessment and Chronic Health Evaluation and Sequential Organ Failure Assessment scores compared to those with mild-NHH and non-NHH. Glasgow Coma Scale scores of patients with severe-NHH were than those of non-NHH patients. In addition, the

interest.

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mean and initial levels of ammonia in the blood might be helpful in predicting the prognosis of NHH.

CONCLUSION

High blood ammonia level is frequent among NHH patients admitted to the ICU, which is related to the clinical characteristics of patients. Furthermore, the level of blood ammonia may be helpful for prognosis prediction.

Key Words: Non-hepatic hyperammonemia; Intestinal absorption; Blood ammonia level; Metabolism of amino acid; Severe patients; Intensive care unit

Core Tip: Ammonia is a normal constituent of body fluids, and the concentration of blood ammonia must remain low. Herein, a prospective and single-center study was conducted to investigate the relationship between the incidence of non-hepatic hyperammonemia and the prognosis of patients admitted to the intensive care unit. Furthermore, the level of blood ammonia may be helpful for prognosis prediction of critically ill patients.

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INTRODUCTION

The human body metabolizes three substances, including sugar, fat and protein. Ammonia in the human body is mainly produced as a byproduct of protein digestion and bacterial metabolism in the gut. The majority of ammonia is either reutilized for the biosynthesis of nitrogenous compounds (such as amino acids), or converted to urea by the urea cycle, with only a small amount of ammonia released into the blood[1-3]. As the main metabolite of amino acids, ammonia can enter the brain through the blood-brain barrier when blood ammonia increases. This may cause glial cell edema, changes in consciousness, increased intracranial pressure, and even hepatic encephalopathy, seriously threatening the life of patients. Generally, hyperammonemia occurs due to a reduction in the metabolic capacity of the liver urea cycle. However, it may occur in the absence of hepatic diseases, namely non-hepatic hyperammonemia (NHH)[4-6]. In recent years, the importance of blood ammonia and NHH in the treatment and prognosis of patients is often neglected in clinical practice.

The occurrence of NHH in severe patients is easily ignored. As NHH patients have no history of liver diseases, this disorder is easily missed. In addition, critically ill patients often encounter various risk factors leading to high blood ammonia levels, including increased ammonia production and reduced clearance[7]. Increased ammonia production is common in microbial infections, such as urease production, pneumonia and fever, and other stress-related diseases, eventually resulting in high blood ammonia level. Catabolic states induced by seizures, extreme exercise, trauma, steroid use and gastrointestinal bleeding can also increase the production of ammonia[8-10]. Multiple studies have demonstrated that probiotics reduce inflammation and oxidative stress in liver cells, thereby leading to increased hepatic clearance of ammonia and reduced uptake of other toxins. Urea cycle disorders are inborn errors of ammonia detoxification/arginine synthesis caused by mutations in one of five core enzymes, one activating enzyme, or one of two mitochondrial anti-porters. Acute kidney injury may result in a large number of complications, including metabolic acidosis, high potassium level, uremia, and changes in fluid balance[11-13]. Therefore, blood ammonia level not only reflects the status of liver function and energy supply, but also affects the tricarboxylic acid cycle[14]. High blood ammonia mainly affects severe patients with insufficient energy supply or metabolism[15-17]. Therefore,

monitoring blood ammonia is the first step of disease assessment in clinical practice. In addition, high blood ammonia is associated with the development and mortality of severe diseases.

The understanding of serum ammonia should not be limited to traditional liver diseases or hepatic encephalopathy, and NHH should also be given sufficient attention. In clinical practice, the first thing is to actively monitor blood ammonia and timely detect the increase of blood ammonia caused by various reasons. In addition, early intervention and treatment may play a key role in improving the prognosis of severe patients. Herein, a prospective and single-center study was conducted to investigate the relationship between the incidence of NHH and the prognosis of patients admitted to the intensive care unit (ICU).

MATERIALS AND METHODS

Study design

This is a prospective, observational and single-center study. A total of 364 patients who were admitted to the ICU from November 2019 to February 2020 were initially enrolled. Inclusion criteria were as follows: (1) Patients who were admitted to the ICU during the study; and (2) Patients who were aged > 18 years. Exclusion criteria were as follows: (1) Patients with acute liver failure (ALF); (2) Patients with chronic liver disease (CLD); (3) Patients who were re-admitted to the ICU; or (4) Patients who did not sign the written informed consent form. Arterial blood was collected on ICU admission and at 09:00 a.m. each day after ICU admission.

HNN is defined as high blood ammonia level (> 35 μmol/L) in patients without liver diseases. According to the changes in blood ammonia level, the severity of NHH is classified into two stages: 36-99 μmol/L and ≥ 100 μmol/L representing mild-NHH and severe-NHH, respectively[18]. Blood samples were stored at 4 °C or wrapped in ice packs immediately after collection. All samples were analyzed within 30 min after collection. The study was approved by The Ethics Committee of The Second Affiliated Hospital of Harbin Medicine University (Nangang, China). Informed consent was obtained from patients or their families before the study.

Statistical data included patients' demographic characteristics, history of other diseases, patients' current conditions, and patients' Acute Physiologic Assessment and Chronic Health Evaluation II (APACHE-II) score at ICU admission[19]. Changes in blood ammonia levels, Glasgow Coma Scale (GCS) score, and Sequential Organ Failure Assessment (SOFA) score were recorded each day after ICU admission[20].

Statistical analysis

SPSS 20.0 software (IBM, Armonk, NY, United States) was used for all statistical analysis. Experimental data were expressed as mean ± SD. Continuous variables were analyzed using the Student's *t*-test, and categorical variables were analyzed by the χ^2 test. Mann-Whitney *U* test was used to compare the differences between two groups. One-way analysis of variance (ANOVA) was used to compare the differences among different groups. The correlation of blood ammonia level with length of ICU stay, APACHE-II, SOFA, and GCS scores was evaluated using the *t*-test. Pearson's correlation coefficient was used to assess the relationship between blood ammonia level and related indicators. Logistic regression analysis revealed that the difference between mean blood ammonia level and initial blood ammonia level was statistically significant for predicting prognosis. A receiver operating characteristic (ROC) curve was plotted, and the area under curve (AUC) was calculated to evaluate the statistical significance of high-, mean-, and initial-levels of ammonia in the blood. $P < 0.05$ was considered statistically significant.

RESULTS

From May 2019 to August 2019, 364 patients were recruited, of whom 28 patients who were aged < 18 years, 19 patients with CLD, 28 patients with ALF, 10 patients with ICU re-admission, 61 patients with incomplete blood ammonia test data, and 14 patients who did not sign the written informed consent form were excluded (Figure 1). Finally, a total of 204 patients were enrolled, including 101 (49.51%) male patients and 103 (50.49%) female patients. The median age of the enrolled patients was 51.94 ± 18.34 years old. The mean length of ICU stay was 3.38 ± 2.96 d, and the mean APACHE-II

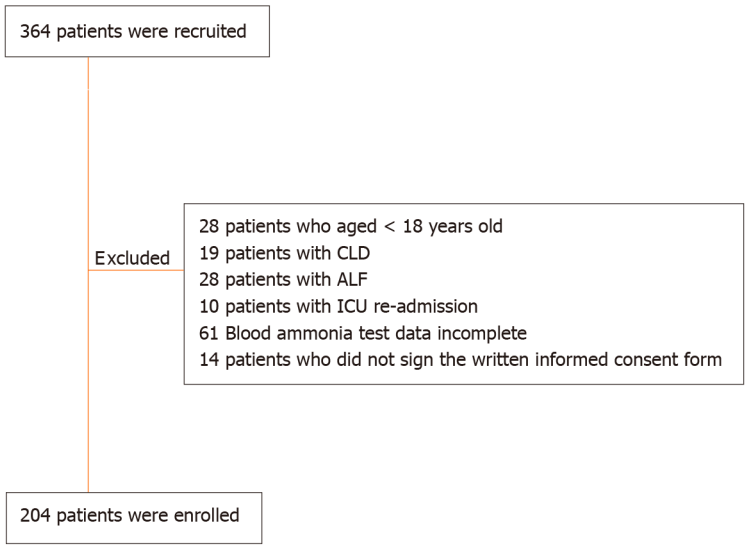

Figure 1 Enrollment of patients. CLD: Chronic liver disease; ALF: Acute liver failure; ICU: Intensive care unit.

score was 14.68 ± 9.29.

In this study, 155 NHH patients and 44 severe-NHH patients were enrolled. The incidence rate of NHH patients and severe-NHH patients was 75.98% and 21.57%, respectively. Patients with severe-NHH had a longer length of ICU stay and higher APACHE-II and SOFA scores compared to those with mild-NHH and non-NHH. Additionally, patients with severe-NHH had lower GCS scores than non-NHH patients. However, no statistically significant differences were observed in the length of ICU stay, APACHE-II, SOFA, and GCS scores between patients with mild-NHH and non-NHH (Figure 2).

Subsequent results demonstrated that the highest level of ammonia in the blood was closely correlated with the length of ICU stay, APACHE-II score, and the highest SOFA score, and was negatively correlated with the lowest GCS score and the median age of patients. Mean-level of ammonia (M-ammonia) and initial-level of ammonia (I-ammonia) in the blood showed no significant correlation with the median age of patients and the length of ICU stay (Table 1).

The patients were divided into the following two groups: good-prognosis group and poor-prognosis group, and their basic data and blood ammonia levels were assessed. The results showed that patients in the poor-prognosis group had higher APACHE-II scores and blood ammonia levels compared with those in the good-prognosis group (Table 2). Logistic regression analysis revealed that M-ammonia and I-ammonia in the blood were statistically significant for predicting prognosis (Table 3). The ROC curve showed that the AUC value of M-ammonia was 0.591 (P = 0.041). With consideration of M-ammonia equal to 69.5 μmol/L, critical values could be achieved to predict good-prognosis and poor-prognosis, with a sensitivity and specificity of 30.5% and 86.2%, respectively. In addition, the AUC value of I-ammonia was 0.612 (P = 0.012). With consideration of I-ammonia equal to 62.00 μmol/L, critical values could be attained to predict good-prognosis and poor-prognosis, with a sensitivity and specificity of 39% and 80%, respectively.

Similarly, we grouped patients according to central nervous system (CNS) disorders, circulatory system diseases, respiratory diseases, and multiple injuries. The results found that no statistically significant differences were observed in blood ammonia level among the groups. In addition, the patients were categorized into the drug poisoning group and non-drug poisoning group. Subsequent results revealed that blood ammonia level in the drug poisoning group was higher than that in the non-drug poisoning group (P < 0.05) (Table 4).

However, there was no statistically significant difference in the area under the ROC curve for H-, M- and I- between the drug poisoning group and the non-drug poisoning group.

Table 1 Linear relationship between blood ammonia and various indicators

	H-Ammonia		M-Ammonia		I-Ammonia	
	r	*P* value	*r*	*P* value	*r*	*P* value
Age	-0.161	0.022	0.136	0.052	0.008	0.236
Days in ICU	0.314	0.000	0.004	0.572	0.004	0.573
APACH-II	0.240	0.000	0.221	0.002	0.263	0.000
H-SOFA	0.312	0.000	-	-	-	-
L-GCS	-0.205	0.004	-	-	-	-
M-SOFA	-	-	0.279	0.000	-	-
M-GCS	-	-	-0.183	0.009	-	-
I-SOFA	-	-	-	-	0.278	0.000
I-GCS	-	-	-	-	-0.174	0.013

$P < 0.05$ was considered statistically significant. M-Ammonia: Mean-level of ammonia; I-Ammonia: Initial-level of ammonia; ICU: Intensive care unit; APACHE-II: Acute Physiologic Assessment and Chronic Health Evaluation II; H-SOFA: The high level of Sequential Organ Failure Assessment score; L-GCS: The low level of Glasgow Coma Scale score; M-SOFA: The mean level of Sequential Organ Failure Assessment score; M-GCS: The mean level of Glasgow Coma Scale score; I-SOFA: The initial level of Sequential Organ Failure Assessment score; I-GCS: The initial level of Glasgow Coma Scale score.

Table 2 Comparison of prognosis between groups

Variables, mean ± SD	Good	Poor	*t*/*Z*	*P* value
Age (yr)	51.58 ± 18.49	52.51 ± 18.24	-0.434	0.665
Sex (M/F)	69/76	32/27	0.742	0.389
Time in ICU (d)	3.32 ± 2.57	3.53 ± 3.81	-0.999	0.318
APACHE-II	12.36 ± 7.53	20.39 ± 10.77	-4.933	0.000
H-Ammonia, μmol/L	67.06 ± 48.24	81.69 ± 59.63	-1.776	0.076
M-Ammonia, μmol/L	47.70 ± 23.79	63.85 ± 46.35	-2.039	0.041
I-Ammonia, μmol/L	48.43 ± 30.97	65.17 ± 46.26	-2.516	0.012

Good means cure or transferred to the ward; Poor means unhealed or died. $P < 0.05$ was considered statistically significant. ICU: Intensive care unit; APACHE-II: Acute Physiologic Assessment and Chronic Health Evaluation II; H-Ammonia: High-level of ammonia; I-Ammonia: Initial-level of ammonia.

Table 3 The logistic regression analysis of ammonia

	B	S.E.	Wald	*P* value	Exp (B)	95%CI for Exp (B)	
						Lower	Upper
H-Ammonia	-0.0051	0.0028	3.2123	0.0731	0.9949	0.9894	1.0005
M-Ammonia	-0.0144	0.0049	8.6664	0.0032	0.9857	0.9762	0.9952
I-Ammonia	-0.0116	0.0041	7.8614	0.0051	0.9885	0.9805	0.9965

$P < 0.05$ was considered statistically significant. Exp: Expedition; H-Ammonia: High-level of ammonia; M-Ammonia: Mean-level of ammonia; I-Ammonia: Initial-level of ammonia.

DISCUSSION

In the present study, we investigated blood ammonia level in patients with NHH who were admitted to the ICU for the first time. The results showed that patients with severe-NHH had a longer length of ICU stay and a lower level of consciousness. The mean and initial levels of ammonia in the blood might be helpful in predicting

Table 4 Comparison between drug poisoning and non-drug poisoning

Variables, mean ± SD	Drug poisoning *n* (20)	Non-drug poisoning *n* (184)	*P* value
H-Ammonia, μmol/L	86.70 ± 46.54	69.61 ± 52.47	**0.032**
M-Ammonia, μmol/L	68.11 ± 36.88	50.66 ± 31.85	**0.016**
I-Ammonia, μmol/L	73.10 ± 46.37	51.12 ± 35.01	**0.024**

$P < 0.05$ was considered statistically significant. H-Ammonia: High-level of ammonia; M-Ammonia: Mean-level of ammonia; I-Ammonia: Initial-level of ammonia.

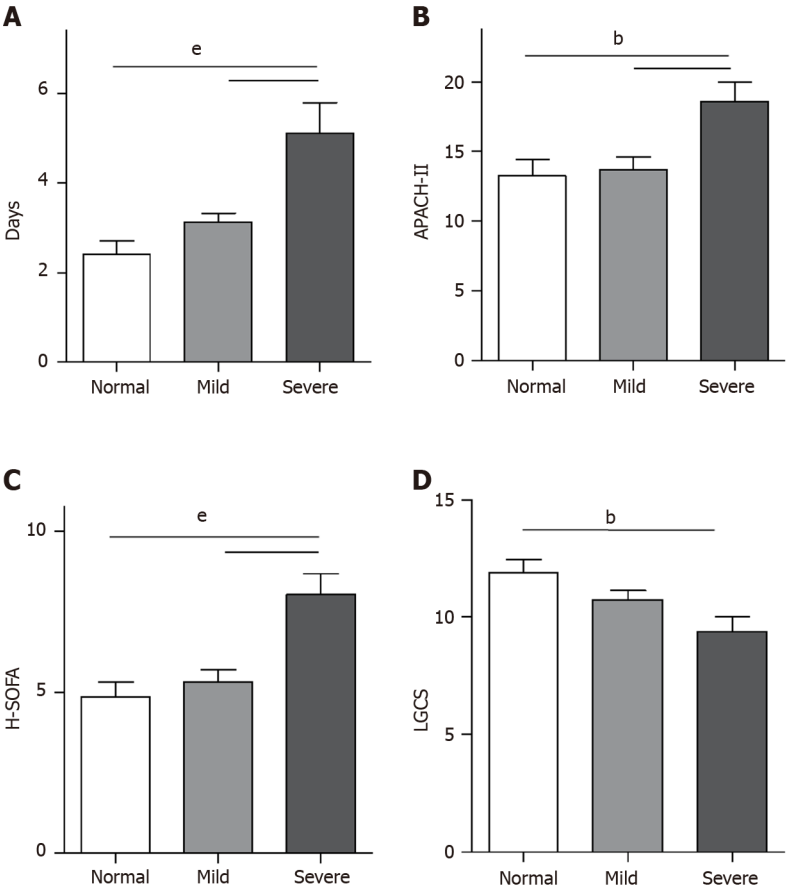

Figure 2 Comparison of clinical characteristics of normal (non-hepatic hyperammonemia) patients with mild and severe hepatic hyperammonemia patients. A: The length of stay at intensive care unit; B: The Acute Physiologic Assessment and Chronic Health Evaluation II score; C: The high level of Sequential Organ Failure Assessment score; D: The low level of Glasgow Coma Scale score. $^{b}P < 0.01$, $^{e}P < 0.001$. APACHE-II: Acute Physiologic Assessment and Chronic Health Evaluation II; H-SOFA: The high level of Sequential Organ Failure Assessment score; L-GCS: The low level of Glasgow Coma Scale score.

prognosis. In addition, patients in the drug poisoning group had higher blood ammonia levels compared with those in the non-drug poisoning group.

Currently, it is well-known that hyperammonemia often occurs in patients with liver diseases. However, there is limited research on whether blood ammonia level increases in patients without liver diseases. A previous study reported that elevated blood ammonia level is common in critically ill patients (nearly 70%)[21]. When liver capacity is surpassed due to increased ammonia production and/or reduced ammonia degradation, kidneys, muscles and the CNS increase their participation in ammonia detoxification. In this study, our results confirmed this previously overlooked clinical phenomenon. This indicated that the majority of patients had excessive accumulation of ammonia in the blood, thereby resulting in hyperammonemia in the ICU.

Subsequently, we divided NHH patients into mild and severe disease, and found no statistically significant difference between mild-NHH and non-NHH patients. However, patients with severe-NHH had a longer length of ICU stay and a lower level

of consciousness. These results suggested that patients with blood ammonia level > 100 μmol/L might be accompanied by severe diseases and physical conditions. A large number of studies have demonstrated that increased blood ammonia level is considered the most important factor in the pathogenesis of hepatic encephalopathy. The present study demonstrated that the higher the blood ammonia level, the worse the consciousness of patients. This may be due to the high blood ammonia level caused by cerebral edema and poor consciousness. In addition, poor consciousness may be related to the patient's poor body condition. Thus, we suggest that further attention should be paid to patients with severe-NHH who are at high risk of infection. However, further experiments are required to explore the pathogenic mechanism.

Additionally, significant differences were observed in the APACHE-II score, mean blood ammonia level and initial blood ammonia level between the poor-prognosis and good-prognosis groups. Logistic regression analysis showed that high levels of mean blood ammonia and initial blood ammonia, but not high levels of ammonia, could reflect the poor prognosis of patients. Initial blood ammonia level reflects the untreated level of the disease. The duration of ammonia clearance is very short (about 7.7 h). Thus, the mean blood ammonia level reflects the continued level of the disease after treatment, which may have a greater impact on the prognosis of severely ill patients. Hyperammonia is only a transient metabolic abnormality, which, if treated in a timely manner, is reversible. It may be that hyperammonia is not statistically significant for disease prognosis. Therefore, real-time and continuous monitoring of blood ammonia level is highly essential for critically ill patients. Current studies have indicated that there are numerous factors influencing patients' prognosis. However, whether increased blood ammonia level can be used as an independent prognostic indicator still requires large-scale experiments.

We further assessed the relationship between blood ammonia level with CNS disorders, circulatory system diseases, respiratory diseases and multiple injuries. However, no significant correlation was noted, which is consistent with the results of previous studies[21-31]. Our results also revealed that high-, mean-, and initial-level of ammonia in the blood increased markedly in the drug poisoning group compared with those in the non-drug poisoning group. However, there was no significant difference between the two groups due to the small number of patients in the poisoning group.

The limitation of our study is that it is a single-center study with a small sample size. Hence, further multi-center, large-scale, clinical studies should be conducted on NHH patients with elevated blood ammonia level admitted to the ICU.

At present, only guidelines released by the Middle East countries indicate that patients with blood ammonia level > 50 μmol/L require dietary treatment, and those with blood ammonia level > 100 μmol/L require medication. The current study revealed that patients with blood ammonia level > 100 μmol/L had more severe clinical symptoms and required urgent treatment. However, it is still unknown whether hyperammonia caused by severe non-hepatic diseases requires conventional ammonia-lowering treatments. In addition, the majority of ammonia-lowering drugs are only appropriate for patients with liver diseases. Hence, further research is needed to indicate whether these drugs can be applied in NHH patients with elevated blood ammonia levels. Besides, blood purification therapy may be an appropriate option for patients with increased blood ammonia level. Further studies are needed to confirm this hypothesis in the future.

CONCLUSION

High blood ammonia level is frequent among NHH patients admitted to the ICU, which is related to the clinical characteristics of patients. Furthermore, the level of blood ammonia may be helpful for prognosis prediction.

ARTICLE HIGHLIGHTS

Research background

Ammonia is a normal constituent of body fluids, and the concentration of blood ammonia must remain low.

Research motivation

Ammonia is a normal constituent of body fluids and is treated mainly through the formation of urea in the liver. Blood levels of ammonia must remain low as even slightly elevated concentrations (hyperammonemia) are toxic to the central nervous system.

Research objectives

The aim of this study was to determine the relationship between the incidence of non-hepatic hyperammonemia (NHH) and the prognosis of patients who were admitted to the intensive care unit (ICU).

Research methods

This is a prospective, observational and single-center study. A total of 204 patients who were admitted to ICU from November 2019 to February 2020 were finally enrolled. Changes in the levels of blood ammonia at the time of ICU admission and after ICU admission were continuously monitored. In addition, factors influencing the prognosis of NHH patients were analyzed.

Research results

A total of 204 patients who met the inclusion criteria were enrolled in this study, including 155 NHH patients and 44 severe-NHH patients. The incidence of NHH and severe-NHH was 75.98% and 21.57%, respectively. Patients with severe-NHH exhibited a longer length of ICU stay and higher Acute Physiologic Assessment and Chronic Health Evaluation and Sequential Organ Failure Assessment scores compared to those with mild-NHH and non-NHH. Glasgow Coma Scale scores of patients with severe-NHH were lower than those of non-NHH patients. In addition, the mean and initial levels of ammonia in the blood might be helpful in predicting the prognosis of NHH.

Research conclusions

High blood ammonia level is frequent among NHH patients admitted to the ICU, which is related to the clinical characteristics of patients. Furthermore, the level of blood ammonia may be helpful for prognosis prediction.

Research perspectives

It is necessary to explore the relationship between the incidence of NHH and the prognosis of patients in the ICU. Early intervention and treatment may be the key to improving the prognosis of critically ill patients, a hypothesis that needs to be confirmed by further studies in the future.

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ORIGINAL ARTICLE

Observational Study

Association between ADAMTS13 activity–VWF antigen imbalance and the therapeutic effect of HAIC in patients with hepatocellular carcinoma

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Institutional review board statement: Informed consent for the use of resected tissue was obtained from all patients. The

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Abstract

BACKGROUND

Prediction of HAIC treatment response is important for improving the prognosis in patients with hepatocellular carcinoma (HCC). The progression of HCC is related to hypercoagulability and angiogenesis. It is known that ADAMTS13 and von Willebrand factor (VWF) are related to hypercoagulability. In addition, previous study reported that the association between ADAMTS13 and VWF, and angiogenesis *via* vascular endothelial growth factor (VEGF). Recently, ADAMTS13 and VWF have been associated with the prognosis in patients with various kinds of cancer undergoing chemotherapy.

AIM

To investigate whether ADAMTS13 and VWF become useful biomarkers of treatment response in HCC patients before the initiation of HAIC treatment.

METHODS

Seventy-two patients were enrolled in this study. ADAMTS13 activity (ADAMTS13:AC), VWF antigen (VWF:Ag) and VEGF levels were determined *via* enzyme-linked immunosorbent assay. Univariable and multivariable analyses were performed to determine the predictive factors of treatment response in patients with HCC undergoing HAIC treatment.

RESULTS

ADAMTS13:AC levels in HCC patients with stable disease (SD) + partial response (PR) of HAIC treatment were significantly higher than those with progressive disease (PD) ($P < 0.05$). In contrast, VWF:Ag/ADAMTS13:AC ratio and VEGF levels in HCC patients with SD + PR were significantly lower than those with PD (both $P < 0.05$). Patients with high VWF:Ag/ADAMTS13:AC ratio (> 2.7) had higher VEGF levels than those with low ratio (≤ 2.7). Multivariable analysis revealed that VWF:Ag/ADAMTS13:AC ratio was a predictive factor of HAIC treatment response.

CONCLUSION

VWF:Ag/ADAMTS13:AC ratio may become a useful biomarker of treatment response in HCC patients before the initiation of HAIC treatment.

Key Words: ADAMTS13; Von Willebrand factor; Vascular endothelial growth factor; Biomarkers; Hepatocellular carcinoma; HAIC

Core Tip: The prediction of HAIC treatment response is needed to improve the prognosis in patients with hepatocellular carcinoma (HCC). Von Willebrand factor antigen (VWF:Ag)/ADAMTS13 activity (ADAMTS13:AC) ratio was significantly lower in HCC patients with stable disease + partial response than those with progressive disease. VWF:Ag/ADAMTS13:AC ratio become a useful biomarker to predict HAIC treatment response.

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INTRODUCTION

Hepatocellular carcinoma (HCC) has one of the highest mortality rates of any cancer[1,2]. In Japan, HCC management follows the consensus-based clinical practice guidelines of the Japan Society of Hepatology (JSH)[3]. JSH recommends that advanced HCC patients with vascular invasion or more than four tumors should undergo chemotherapy such as HAIC or molecular-targeted drugs[3]. However, it is important to predict HAIC response before deciding on the appropriate chemotherapy protocol for improving the prognosis in patients with HCC.

ADAMTS13 is a metalloproteinase that is exclusively produced from hepatic stellate cells adjacent to endothelial cells (ECs)[4-8]. It specifically cleaves multimeric von Willebrand factor (VWF) between the Tyr1605 and Met1606 residues in the A2 domain[4-7]. VWF is synthesized in vascular ECs and released into the plasma as unusually large multimers[9]. During ADAMTS13 enzyme–VWF substrate imbalance, VWF is improperly cleaved, resulting in the accumulation of multimers and the induction of platelet thrombi formation in the microvasculature under high shear-stress conditions[10]. In other words, ADAMTS13 enzyme–VWF substrate balance is related to hypercoagulability. Furthermore, the blood coagulation cascade is related to cancer progression[11,12], and our previous study has reported that ADAMTS13 enzyme–VWF substrate imbalance becomes worse based on HCC progression[13,14].

Angiogenesis plays an important role in HCC progression[15]. A recent study has reported that ADAMTS13 enzyme–VWF substrate imbalance is related to angiogenesis[16] as well as hypercoagulability and is associated with the prognosis in patients with various kinds of cancer undergoing chemotherapy[14,17].

In the present study, we investigated the relationship between ADAMTS13

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enzyme–VWF substrate balance and HCC in patients undergoing HAIC treatment. In addition, we sought to determine whether ADAMTS13 and VWF may become predictive biomarkers of treatment response in HCC patients before starting HAIC treatment.

MATERIALS AND METHODS

Patients

This retrospective observational study included patients with HCC who underwent HAIC treatment from December 2009 to March 2019. Patients with HCC had no vascular invasion or less than four tumors were excluded. A total of 72 patients with HCC were included in this study. HAIC treatment was performed according to the Moriya method[18,19], which features a bi-monthly protocol that is simple and easy to manage. The patients underwent dynamic computed tomographic scanning or dynamic magnetic resonance imaging at various points, namely before starting HAIC treatment, 1 mo after commencement of the treatment, and every 2 mo thereafter. HAIC treatment responses were evaluated according to modified response evaluation criteria in solid tumors. This study had no patient with infection, uncontrolled hepatic encephalopathy, ascites, or gastroesophageal varices. This study was approved by the local ethics committee in Nara Medical University and was performed according to the ethical standards laid down in the Declaration of Helsinki. Informed consent was obtained from all patients included in the study.

Determination of ADAMTS13 activity and VWF antigen levels

We collected blood samples from each patient at the time of admission, during their hospital stay, or during regular outpatient treatment before starting HAIC treatment. The plastic tubes with 0.38% sodium citrate was used to store these samples. We centrifuged these samples at 3000 × *g* at 4 ℃ for 15 min to prepare the plasma and stored the plasma at -80 ℃ until analysis. Plasma ADAMTS13 activity (ADAMTS13:AC) was determined using a sensitive chromogenic enzyme-linked immunosorbent assay (ELISA) (Kainos Laboratories Inc., Tokyo, Japan)[20] to show a normal value of 99% ± 22%. Plasma VWF antigen (VWF:Ag) levels were measured *via* sandwich ELISA using a rabbit anti-human VWF polyclonal antiserum (Dako, Glostrup, Denmark). The normal VWF:Ag value is 102% ± 33%[21].

VEGF measurements

VEGF levels were determined using a commercially available kit (Immunoassay Kits, RayBiotech Inc., United States). The detection limit of VEGF was < 10 pg/mL.

Statistical analysis

The Mann–Whitney *U*-test and the Fisher's exact test were performed to analyze differences between study groups and categorical data, respectively. Univariable and multivariable analysis were performed to evaluate HAIC response for HCC. Logistic regression analysis was performed to determine independent response factors, and data were expressed as median (interquartile range). A two-tailed *P* value of < 0.05 was considered significant. Analyses were conducted using EZR (Saitama Medical Center, Jichi Medical University, Japan), a graphical user interface of R version 2.13.0 (The R Foundation for Statistical Computing, Vienna, Austria), and a modified version of R commander (version 1.6-3) that includes statistical functions that are frequently used in biostatistics[22].

RESULTS

Clinical characteristics of the HCC patients

Table 1 showed the clinical characteristics of HCC patients. The median period of HAIC treatment was 121 (range 41-218) d and the median age of HCC patients was 70.5 (range 64.2-76.1) years. Of the study population (57 males, 15 females), 17 patients had hepatitis B virus infection, 36 had hepatitis C virus infection, 10 had alcohol abuse, 4 had non-alcoholic steatohepatitis, and 5 had others. The median maximum tumor size was 3.3 (range 2.2-5.0) cm. Tumors numbering 1, 2, 3, 4, or > 4 were 9, 5, 6, 2, and 50, respectively. Thirty-one patients had vascular invasion and no patient had distant metastasis. Serum alpha-fetoprotein (AFP), des-γ-carboxy prothrombin, and lens

Table 1 Hepatocellular carcinoma patients' characteristics between stable disease + partial response and progressive disease with hepatic arterial infusion chemotherapy

Variable	Total (*n* = 72)	SD + PR (*n* = 41)	PD (*n* = 31)	*P* value
Age (yr)	70.5 (64.2–76.1)	72.0 (66.4–76.4)	67.8 (58.9–75.1)	NS
Sex (male/female)	57/15	32/9	25/6	NS
Etiology (HBV/HCV/alcohol/NASH/others)	17/36/10/4/5	6/23/5/4/3	11/13/5/0/2	NS
Albumin (g/dL)	3.2 (3.0–3.6)	3.3 (3.0–3.6)	3.2 (3.0–3.7)	NS
Prothrombin time (%)	76.0 (60.5–85.3)	78.0 (57.0–86.0)	73.0 (63.5–83.0)	NS
Total bilirubin (mg/dL)	1.2 (0.8–2.1)	1.2 (0.7–2.1)	1.2 (0.8–2.2)	NS
Platelet count ($\times 10^4$/mm^3)	10.4 (7.1–14.9)	10.8 (7.0–13.2)	10.1 (7.6–17.1)	NS
AFP (ng/mL)	95.3 (17.9–1162.5)	101.0 (21.2–931.5)	87.1 (13.2–1349.2)	NS
DCP (mAU/mL)	359.5 (58.0–5277.5)	348 (42.3–1542.8)	609.0 (88.2–279.4)	NS
AFP-L3% (%)	33.7 (7.7–73.4)	34.3 (6.9–73.4)	22.2 (8.1–68.8)	NS
Maximum tumor size (cm)	3.3 (2.2–5.0)	3.0 (2.0–5.0)	3.5 (2.9–3.5)	NS
Tumor number (1/2/3/4/> 4)	9/5/6/2/50	5/4/3/2/27	4/1/3/0/23	NS
Vascular invasion (present/absent)	31/41	20/21	11/20	NS
Treatment period (days)	121 (41–218)	191 (120–311)	40.0 (26–61)	< 0.05

Data are expressed as median (interquartile range). *P* values represent comparisons between SD + PR and PD with hepatic arterial infusion chemotherapy. SD: Stable disease; PR: Partial response; PD: Progressive disease; HBV: Hepatitis B virus; HCV: Hepatitis C virus; NASH: Non-alcoholic steatohepatitis; AFP: Alpha-fetoprotein; DCP: Des-γ-carboxy prothrombin; AFP-L3%: Lens culinaris agglutinin-reactive alpha-fetoprotein.

culinaris agglutinin-reactive fraction of AFP levels were 95.3 (17.9–1162.5) ng/mL, 359.5 (58.0–5277.5) mAU/mL, and 33.7% (7.7%–73.4%), respectively. We investigated the HAIC treatment response between stable disease (SD) + partial response (PR) and progressive disease (PD). No significant differences were observed in HCC patients' characteristics between SD + PR and PD, except for treatment periods.

Plasma ADAMTS13:AC and VWF:Ag levels

ADAMTS13:AC levels in HCC patients with SD + PR were significantly higher than those with PD ($P < 0.05$) (Figure 1A). VWF:Ag levels were no different between patients with SD + PR and PD (Figure 1B). The ratio of VWF:Ag to ADAMTS13:AC (VWF:Ag/ADAMTS13:AC ratio) in patients with SD + PR was significantly lower than those with PD ($P < 0.05$) (Figure 1C).

Plasma VEGF levels

VEGF levels in HCC patients with SD + PR were significantly lower than those with PD ($P < 0.05$) (Figure 2A). Patients were categorized into two groups according to receiver operating characteristic (ROC) cut-off VEGF: Low, ≤ 100 and high, > 100. Patients with high VEGF levels also had higher platelet levels than those with low VEGF (Figure 2B). Patients were categorized into two groups according to ROC cut-off VWF:Ag/ADAMTS13:AC ratio: Low, ≤ 2.7 and high, > 2.7. Patients with high VWF:Ag/ADAMTS13:AC ratio had higher VEGF levels than those with low ratio (Figure 2C).

Predictive factors for HAIC response

Patients were categorized into two groups according to ROC cut-off. Univariable analysis showed that HAIC treatment response is associated with prothrombin time (PT), VEGF, and VWF:Ag/ADAMTS13:AC ratio (Table 2). To determine the predictive factors of HAIC response, multivariable analysis was performed using PT, VEGF, and VWF:Ag/ADAMTS13:AC ratio, with these factors showing $P < 0.05$ in univariable analysis. VWF:Ag/ADAMTS13:AC ratio was significantly associated with HAIC treatment response *via* multivariable analysis (Table 2). ROC analysis showed that VWF:Ag/ADAMTS13:AC ratio is sensitivity of 53.7%, specificity of 87.1%, and area under the curve of 0.715.

Table 2 Predictive factors for response of hepatic arterial infusion chemotherapy in patients with hepatocellular carcinoma

Variable	Univariable analysis		Multivariable analysis	
	OR (95%CI)	*P* value	OR (95%CI)	*P* value
Age > 65 yr	2.61 (0.905–7.5)	0.076		
Sex (male *vs* female)	0.853 (0.268–2.72)	0.788		
Albumin > 2.8 g/dL	1.40 (0.404–4.85)	0.596		
Prothrombin time > 80%	2.68 (1.010–7.10)	0.0469	1.98 (0.654–5.96)	0.227
Total bilirubin > 2 mg/dL	0.896 (0.317–2.53)	0.836		
Platelet count > 20 × 10^4/μL	1.57 (0.268–9.16)	0.618		
AFP > 50 ng/mL	0.523 (0.194–1.41)	0.199		
DCP > 20 mAU/mL	0.381 (0.0938–1.55)	0.177		
AFP-L3% > 20%	1.33 (0.491–3.62)	0.572		
VEGF > 100 pg/mL	0.223 (0.0802–0.618)	0.0039	0.370 (0.127–1.07)	0.0677
Maximum tumor size > 2.3 cm	0.414 (0.130–1.32)	0.137		
Tumor number > 2	1.07 (0.261–4.35)	0.928		
Vascular invasion (present/absent)	1.73 (0.665–4.51)	0.261		
ADAMTS13:AC > 75%	1.25 (0.355–4.41)	0.727		
VWF:Ag > 260%	0.711 (0.279–1.81)	0.476		
VWF:Ag/ADAMTS13:AC > 2.7	0.141 (0.0418–0.476)	0.0016	0.176 (0.0493–0.631)	0.00766

OR: Odds ratio; CI: Confidence interval; AFP: Alpha-fetoprotein; DCP: Des-γ-carboxy prothrombin; AFP-L3%: Lens culinaris agglutinin-reactive alpha-fetoprotein; VEGF: Vascular endothelial growth factor; ADAMTS13: A disintegrin-like and metalloproteinase with thrombospondin type 1 motifs 13; ADAMTS13:AC: ADAMTS13 activity; VWF: Von Willebrand factor; VWF:Ag: VWF antigen; VWF:Ag/ADAMTS13:AC ratio: Ratio of VWF:Ag to ADAMTS13:AC.

DISCUSSION

We suggests that VWF:Ag/ADAMTS13:AC ratio is a potential biomarker for HAIC treatment response in the present study. It is well-known that this ratio is related to the coagulation cascade[10], which in turn plays an important role in the cancer development, including HCC[11,12]. Previous studies have reported that ADAMTS13 enzyme–VWF substrate imbalance is associated with cancer progression, prognosis of patients with various kinds of cancer, and response to chemotherapy[17,23]. Our previous study reported that VWF:Ag[7] and VWF:Ag/ADAMTS13:AC ratio[13] are predictive and detective factors of HCC in patients with cirrhosis, respectively. Moreover, a study has reported that the association between ADAMTS13:AC and VWF:Ag, and the treatment efficiency of molecular-targeted drugs[14].

It is well-known that angiogenesis is related to the pathophysiology of HCC development[15] and that VEGF plays an important role in angiogenesis[15]. Recently, studies have reported that VWF reduces VEGF-dependent angiogenesis *via* multiple intracellular and extracellular pathways involving integrin avβ3 and angiopoietin-2[16,24,25] and that ADAMTS13 cleaves VWF and promotes VEGFR-2 phosphorylation, as the result, induces angiogenesis. This in turn results in enhancement of VEGF expression[26]. Xu have reported that the important role of ADAMTS13 enzyme–VWF substrate balance in the regulation of blood vessel formation[16]. A previous study has reported that HAIC treatment decreases VEGF levels in patients with advanced HCC[27]. Therefore, VWF:Ag/ADAMTS13:AC ratio may be associated with HAIC treatment response *via* VEGF and angiogenesis.

Furthermore, anti-platelet therapy inhibits VEGF that induces HCC development[28]. A recent study has reported that anti-platelet therapy for cirrhotic patients prevents HCC development[29] and prolongs survival time in hepatitis B virus mouse model of chronic liver disease[28]. ADAMTS13 enzyme–VWF substrate imbalance induces platelet thrombi formation[10]. In other words, ADAMTS13 enzyme–VWF substrate imbalance, VEGF, angiogenesis, and hypercoagulability are closely related to the cancer progression, including HCC. A previous study has found that VEGF is

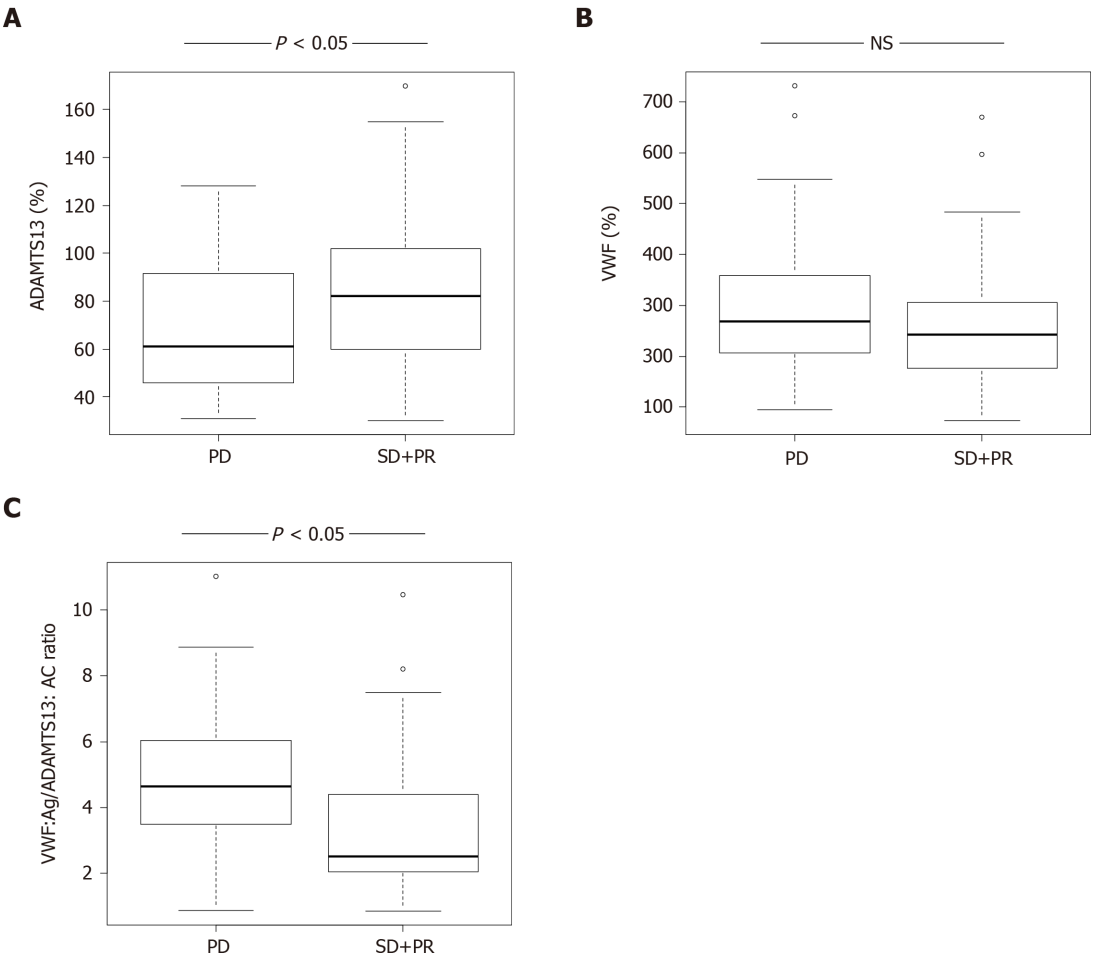

Figure 1 Plasma ADAMTS13:AC and VWF:Ag levels in patients with hepatocellular carcinoma receiving hepatic arterial infusion chemotherapy treatment. A: ADAMTS13 activity (ADAMTS13:AC) levels were significantly higher in hepatocellular carcinoma patients receiving hepatic arterial infusion chemotherapy treatment with stable disease (SD) + partial response (PR) than in those with progressive disease (PD) ($P < 0.05$); B: VWF antigen (VWF:Ag) levels were not different between patients with SD + PR and PD; C: VWF:Ag/ADAMTS13:AC ratio was significantly lower in patients with SD + PR than in those with PD ($P < 0.05$). SD: Stable disease; PR: Partial response; PD: Progressive disease; ADAMTS13: A disintegrin-like and metalloproteinase with thrombospondin type 1 motifs 13; ADAMTS13:AC: ADAMTS13 activity; VWF: Von Willebrand factor; VWF:Ag: VWF antigen; VWF:Ag/ADAMTS13:AC ratio: Ratio of VWF:Ag to ADAMTS13:AC.

associated with HAIC treatment response and prognosis[30]. Our study reported the association between VWF:Ag/ADAMTS13:AC ratio and HAIC treatment response; however, our analysis indicated that VEGF is not a predictive factor of HAIC treatment response. VWF:Ag/ADAMTS13:AC ratio may become a more useful to predict HAIC treatment response than VEGF.

Our study has some limitations that include a small sample size and short observation. Cirrhotic patients with advanced HCC occasionally develop thrombosis or inflammation (*e.g.*, portal thrombosis, and bacterial overgrowth and translocation). When VWF:Ag/ADAMTS13:AC ratio is used as a biomarker of HAIC treatment response, thrombosis and inflammation may affect the values[4,23,31]. In addition, VWF:Ag/ADAMTS13:AC ratio has high specificity but moderate sensitivity to predict HAIC treatment response. Therefore, we should continue to investigate high-sensitivity biomarkers.

CONCLUSION

In summary, VWF:Ag/ADAMTS13:AC ratio is an independent predictive factor for response in patients with HCC undergoing HAIC treatment.

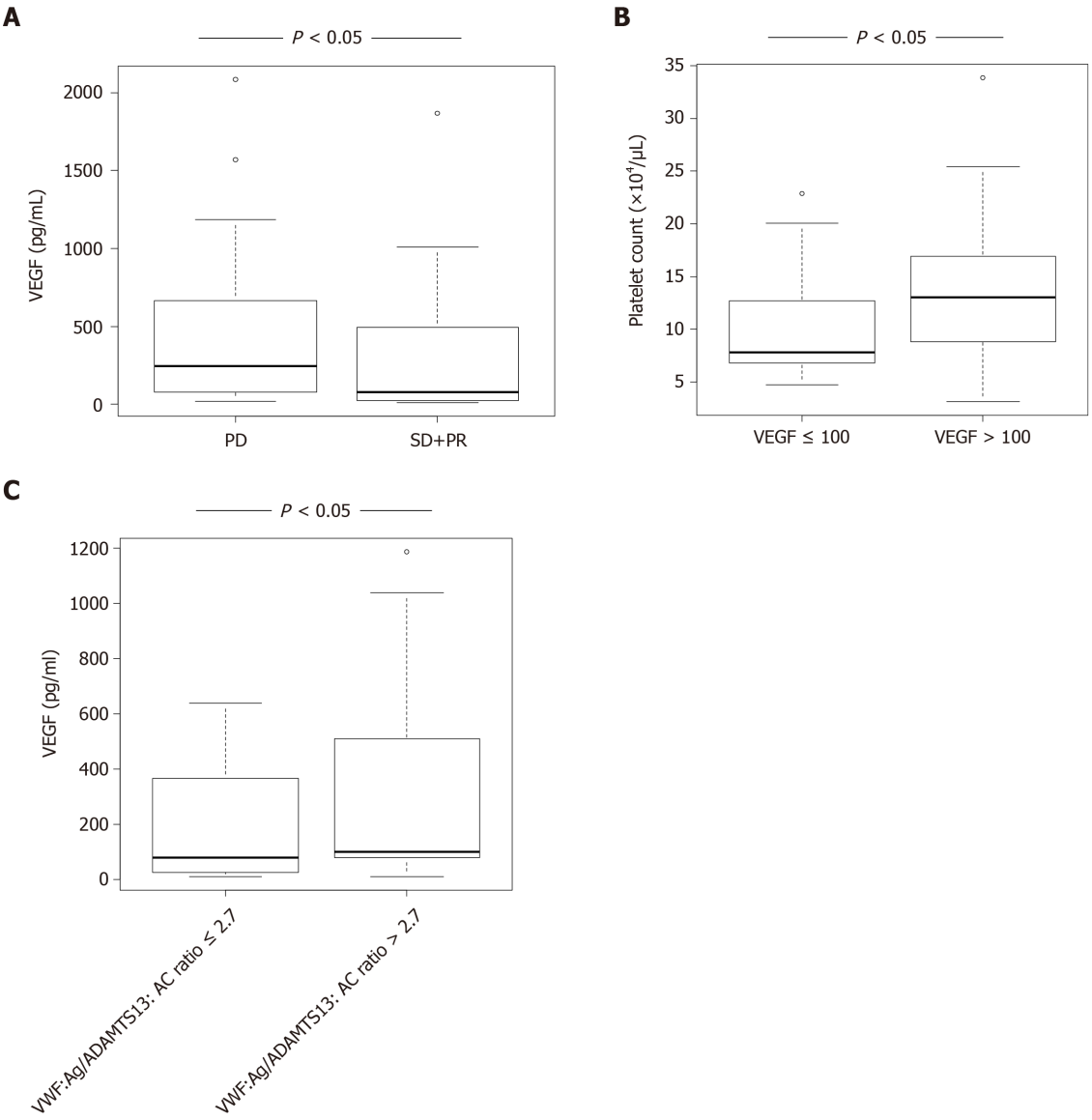

Figure 2 Plasma vascular endothelial growth factor levels in patients with hepatocellular carcinoma receiving hepatic arterial infusion chemotherapy treatment. A: Vascular endothelial growth factor (VEGF) levels were significantly lower in hepatocellular carcinoma patients with stable disease + partial response than in those with progressive disease ($P < 0.05$); B: Patients with high VEGF levels (> 100) had higher platelet levels than those with low VEGF levels (≤ 100) ($P < 0.05$); C: Patients with high Von Willebrand factor antigen (VWF:Ag)/ADAMTS13 activity (ADAMTS13:AC) ratio (> 2.7) had higher VEGF levels than those with low VWF:Ag/ADAMTS13:AC ratio (≤ 2.7). VEGF: Vascular endothelial growth factor; SD: Stable disease; PR: Partial response; PD: Progressive disease; ADAMTS13: A disintegrin-like and metalloproteinase with thrombospondin type 1 motifs 13; ADAMTS13:AC: ADAMTS13 activity; VWF: Von Willebrand factor; VWF:Ag: VWF antigen; VWF:Ag/ADAMTS13:AC ratio: Ratio of VWF:Ag to ADAMTS13:AC.

ARTICLE HIGHLIGHTS

Research background

Predicting HAIC treatment response is important for improving the prognosis of hepatocellular carcinoma (HCC) patients.

Research motivation

ADAMTS13 and von Willebrand factor (VWF) have been associated with the prognosis in patients with various kinds of cancer receiving chemotherapy.

Research objectives

The present study was investigated whether ADAMTS13 and VWF become useful biomarkers of treatment response in HCC patients before the initiation of HAIC treatment.

Research methods
Multivariable analysis was performed to determine the predictive factors of HAIC treatment response in patients with HCC.

Research results
VWF antigen (VWF:Ag)/ADAMTS13 activity (ADAMTS13:AC) ratio predicted HAIC treatment response in multivariable analysis.

Research conclusions
VWF:Ag/ADAMTS13:AC ratio may be a useful biomarker of treatment response in patients with HCC before HAIC treatment.

Research perspectives
VWF:Ag/ADAMTS13:AC ratio has high specificity to predict HAIC treatment response. On the other hand, this biomarker has moderate sensitivity. Therefore, we should continue to investigate high-sensitivity biomarkers.

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SYSTEMATIC REVIEWS

Diagnosis and treatment of iron-deficiency anemia in gastrointestinal bleeding: A systematic review

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Abstract

BACKGROUND

Anemia is considered a public health issue and is often caused by iron deficiency. Iron-deficiency anemia (IDA) often originates from blood loss from lesions in the gastrointestinal tract in men and postmenopausal women, and its prevalence among patients with gastrointestinal bleeding has been estimated to be 61%. However, few guidelines regarding the appropriate investigation of patients with IDA due to gastrointestinal bleeding have been published.

AIM

To review current evidence and guidelines concerning IDA management in gastrointestinal bleeding patients to develop recommendations for its diagnosis and therapy.

METHODS

Five gastroenterology experts formed the Digestive Bleeding and Anemia Workgroup and conducted a systematic literature search in PubMed and professional association websites. MEDLINE (*via* PubMed) searches combined medical subject headings (MeSH) terms and the keywords "gastrointestinal bleeding" with "iron-deficiency anemia" and "diagnosis" or "treatment" or "management" or "prognosis" or "prevalence" or "safety" or "iron" or "transfusion" or "quality of life", or other terms to identify relevant articles reporting the management of IDA in patients over the age of 18 years with gastrointestinal bleeding; retrieved studies were published in English between January 2003 and April 2019. Worldwide professional association websites were searched for clinical practice guidelines. Reference lists from guidelines were reviewed to identify additional relevant articles. The recommendations were developed by consensus during two meetings and were supported by the published literature identified during the systematic search.

RESULTS

From 494 Literature citations found during the initial literature search, 17 original articles, one meta-analysis, and 13 clinical practice guidelines were analyzed. Based on the published evidence and clinical experience, the workgroup developed the following ten recommendations for the management of IDA in patients with gastrointestinal bleeding: (1) Evaluation of hemoglobin and iron status; (2) Laboratory testing; (3) Target treatment population identification; (4) Indications for erythrocyte transfusion; (5) Treatment targets for erythrocyte transfusion; (6) Indications for intravenous iron; (7) Dosages; (8) Monitoring; (9) Indications for intravenous ferric carboxymaltose treatment; and (10) Treatment targets and monitoring of patients. The workgroup also proposed a summary algorithm for the diagnosis and treatment of IDA in patients with acute or chronic gastrointestinal bleeding, which should be implemented during the hospital stay and follow-up visits after patient discharge.

CONCLUSION

These recommendations may serve as a starting point for clinicians to better diagnose and treat IDA in patients with gastrointestinal bleeding, which ultimately may improve health outcomes in these patients.

Key Words: Anemia iron-deficiency; Erythrocyte transfusion; Ferric carboxymaltose; Gastrointestinal hemorrhage; Iron; Practice guidelines as topic

Core Tip: Iron-deficiency anemia (IDA) is a public health issue often caused by gastrointestinal bleeding. Few clinical practice guidelines regarding the appropriate investigation of IDA due to gastrointestinal bleeding were published. Therefore, five gastroenterology experts conducted a systematic search in PubMed and medical

association websites to analyze the current evidence and guidelines on IDA management in patients with gastrointestinal bleeding. From 494 search results, 13 clinical practice guidelines, 17 original articles, and one meta-analysis were analyzed. Ten recommendations were developed for screening, treatment indications, appropriate therapies, and treatment goals, being a starting point for diagnosing and treating IDA in gastrointestinal bleeding patients.

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INTRODUCTION

Anemia is a public health issue affecting approximately 25% of the world's population[1]. Several anemia etiologies exist, and of these, iron deficiency is the most widespread and is estimated to cause up to 50% of all anemia cases[1]. Iron-deficiency anemia (IDA) often originates from blood loss from lesions in the gastrointestinal tract in men and postmenopausal women[2,3]. The prevalence of IDA among patients with gastrointestinal bleeding has been estimated to be 61%[4]. However, studies have shown that IDA is often underdiagnosed, underrecognized, and undertreated in hospitalized patients with gastrointestinal bleeding[5-7]. Moreover, evidence suggests that therapeutic approaches for iron-deficiency correction have been poorly implemented[5-7], and clinical practice guidelines are not being followed[6].

Treatment options for correcting IDA in patients with gastrointestinal bleeding include the administration of oral or intravenous iron therapy and transfusion. Oral iron is often considered a first-line treatment because it is safe, inexpensive, and convenient[8]. However, many patients with gastrointestinal bleeding have a poor response to oral iron therapeutics because of gastrointestinal side effects, malabsorption, or requirements of higher supplemental iron doses to correct iron deficiency that consequently aggravate side effects[9]. In these situations, intravenous iron formulations may be a more effective and better tolerated therapeutic alternative than oral formulations[10-13]. Proper treatment of IDA alleviates symptoms of iron deficiency, such as fatigue[14], and improves quality of life[14-16].

Clinical practice recommendations and guidelines on the management of IDA in gastrointestinal bleeding patients are still scarce[12,13,17], and there is no standardization on the management of these patients[13], in which different strategies have been used in daily clinical practice. Therefore, it is urgent to develop evidence-based recommendations to better diagnose and treat IDA in patients with gastrointestinal bleeding. The main purpose of this systematic review, developed by the Digestive Bleeding and Anemia Workgroup, is to provide recommendations for simple and uniform diagnostic and therapeutic approaches for IDA in patients with gastrointestinal bleeding.

MATERIALS AND METHODS

Consensus meetings

The Digestive Bleeding and Anemia Workgroup was formed in 2016 by five key opinion leaders in gastroenterology in Portugal. The Workgroup members have significant experience in the management of gastroenterology departments and clinical practice in gastroenterology emergencies. Two meetings were held in Coimbra (Portugal) in March 2017, with the sole purpose of reaching a consensus among the five experts regarding the diagnosis and treatment of IDA in patients with gastrointestinal bleeding. A consensus was reached through discussions during the meetings and was further supported by a systematic literature search.

Search strategy and details

We followed the preferred reporting items for systematic reviews and meta-analyses (PRISMA) statement to report the results of this review[18]. Key research questions were established and approved a priori by the authors.

One reviewer performed a literature search in MEDLINE (PubMed) for studies published from January 1, 2003, to the present. The last search was conducted on April 2, 2019. Search strategies combined MeSH terms and the free text terms "gastrointestinal bleeding" crossed with "iron-deficiency anemia" and "diagnosis" or "treatment" or "management" or "prognosis" or "prevalence" or "safety" or "iron" or "transfusion" or "quality of life" or other terms. Complete search strategies are available in supplemental material SM1. The electronic database search was supplemented by searching for clinical practice guidelines on the websites of worldwide professional associations and reviewing the reference lists of the guidelines for relevant articles. Additional references were included after the peer review process.

Inclusion and exclusion criteria

The inclusion criteria were as follows: (1) Studies that included adults ≥ 18 years of age; (2) Studies that included patients with gastrointestinal bleeding (all etiologies); (3) Studies that included patients with IDA; (4) Systematic reviews with or without meta-analyses, clinical trials, registry-based studies, cohort studies, population-based studies, and clinical practice guidelines; (5) Studies that were written in English; and (6) Studies that were published after January 1, 2003.

The exclusion criteria were as follows: (1) Studies including children < 18 years of age; (2) Studies including animals; (3) Studies in which abstracts or full-text articles were not available; (4) Studies that included patients under critical care (emergency); (5) Studies that included patients who refuse transfusion treatment; and (6) Review articles, surveys, case reports, case series, case-control studies, comments, letters, conference abstracts or posters, or economic evaluations.

Study selection

One reviewer screened all titles and abstracts retrieved from the electronic searches to identify potentially eligible articles. Full texts of the potentially eligible articles were retrieved, and the same reviewer classified the articles as eligible, potentially eligible, unclear, or not eligible as well as the reason for exclusion. A second reviewer screened all full-text articles and reviewed the classifications. The second reviewer also screened potentially eligible or unclear full-text articles, determined whether they were eligible or not eligible and recorded the reason for exclusion. Disagreements were resolved by consensus.

Data collection

One reviewer extracted the relevant data. The extracted data included article or guideline characteristics (author or organization, year of publication, study type, number of patients, gastrointestinal etiology, subject sex, subject age, study period, country or region), incidence of IDA, mortality, rebleeding, rate of screening, rate of diagnosis, rate of treatment, recommended tests and thresholds for hemoglobin and/or serum ferritin for the diagnosis of IDA, target population for treatment, indications for erythrocyte transfusion, indications for intravenous iron treatment, recommendations for ferric carboxymaltose treatment, and treatment targets and timepoints.

RESULTS

Study and guideline characteristics

The results of the screening and selection process are shown in the flowchart in Figure 1. Our initial search yielded 494 literature citations, of which 51 were duplicates and were removed. The remaining 443 studies were screened by title and abstract. After excluding irrelevant studies, 69 studies were further assessed by reading the full texts. Of these, 38 were excluded; the remaining 31 studies were included in our analysis and comprised 17 original articles, 1 meta-analysis (Table 1), and 13 clinical practice guidelines (Table 2). Ten additional references regarding the differential diagnosis of IDA and anemia caused by inflammation, the potential role of ascorbic acid in increasing iron absorption, and the characteristics and advantages and disadvantages of oral and intravenous iron preparations were included after the peer

Table 1 Characteristics of included original articles and meta-analyses

Ref.	Year	Study type	No of patients	GI etiology	No of males/females	Age	Study period
Bager *et al*[5]	2013	Retrospective	169	Nonvariceal AUGIB	86/83	70 (22-95)[1]	2009
Bager *et al*[44]	2014	Double-blind, randomized, placebo-control	97	Nonvariceal AUGIB	51/46	70 (23-95)[2]	2010-2013
Bager *et al*[50]	2014	Double-blind, randomized, placebo-control	97	Nonvariceal AUGIB	51/46	70 (67.4-73.1)[3]	-
Ballester *et al*[45]	2019	Retrospective, single-center	84	Acute GIB	58/26	68.0 (16.9)[2]	2012-2015
Bosch *et al*[51]	2017	Prospective cohort	2818	GI diseases known to cause GIB	1398/1420	63.4 (15.7)[2]	2015-2016
Bosch *et al*[29]	2017	Prospective cohort	4552	Occult bleeding	2266/2286	63.7 (17.6)[2]	2005-2015
Brooklyn *et al*[6]	2003	N/A	153	Occult bleeding	51/102	66 (45-96)[2]	2000
Cheng *et al*[52]	2010	Prospective	390	Ulcers	263/127	63 (16)[2]	-
El-Halabi *et al*[7]	2016	Retrospective, chart review, single-center	307	Any GIB	130/177	66.2 (18.6)[2]	2011-2012
Geisser *et al*[46]	2010	Phase I/II, multicenter, open-label, multiple-dose	46	Bleeding due to GI disorder	10/36	42.9 (11.0)[2]	2003-2004
Jairath *et al*[35]	2010	Meta-analysis	-	AUGIB	2731/1710	Early RBC 67.9 (16.51)[2]; no early RBC 63.4 (19.19)[2]	2007
Jairath *et al*[36]	2015	Pragmatic, multicentric, open-label, randomized feasibility trial	936	AUGIB	566/370	Liberal 60.4 (20.0)[2]; restrictive 58.0 (20.3)[2]	2012-2013
Restellini *et al*[31]	2013	Observational, registry-based	1677	Nonvariceal AUGIB	1035/642	66.2 (16.8)[2]	1999-2002
Rockey *et al*[32]	2017	Prospective cohort	1460	Acute or chronic GIB	899/561	53 (14)[2]	2006-2011
Salvadori *et al*[47]	2016	Retrospective	38	GI chronic blood loss	22/16	78 (54-94)[4]	2014-2015
Schröder *et al*[48]	2004	N/A	31	GI blood loss	12/19	43.8 (18.0)[2]	-
Subramaniam *et al*[30]	2016	Retrospective cohort	2360	Nonvariceal AUGIB	1505/852	70 (56-81)[4]	2008-2010
Villanueva *et al*[37]	2013	RCT	889	Severe AUGIB	-	-	2003-2009

[1]The median (range).
[2]The mean (range) or mean (standard deviation).
[3]Mean (95% confidence interval).
[4]The median (Interquartile range). AUGIB: Acute upper gastrointestinal bleeding; GI: Gastrointestinal; GIB: Gastrointestinal bleeding; RBC: Red blood cell; RCT: Randomized controlled trial.

review process [Northrop-Clewes[19], World Health Organization (WHO)[20], Shin *et al*[21], Infusino *et al*[22], Shubham *et al*[23], Koulaouzidis *et al*[24], Muñoz *et al*[25], Drozd *et al*[26], Jimenez *et al*[27], McDonagh *et al*[28]].

Recommendations for the management of IDA in patients with gastrointestinal bleeding

Diagnosis: Hemoglobin and iron status should be routinely evaluated in all patients with gastrointestinal bleeding.

Although IDA is common in patients with gastrointestinal bleeding, the rate of IDA screening is generally low[6,7]. Moreover, patients with IDA are less likely to be investigated than patients with iron deficiency according to published guidelines[6].

The recommendation that all patients with gastrointestinal bleeding should be assessed for hemoglobin and iron status is based on data from studies reporting a high prevalence of anemia and IDA and a high incidence of mortality among patients with gastrointestinal bleeding. Two retrospective studies assessed the prevalence of IDA in

Table 2 Characteristics of the included guidelines

Guideline organization/society/authors	Year	GI etiology	Origin	Level of development
Iron-Deficiency Anemia Working Group Consensus Report[34]	2017	IBD and GIB	Turkey	Scientific committee/expert group
The International Consensus Upper Gastrointestinal Bleeding Conference Group[38]	2010	Nonvariceal UGIB	International	Scientific committee/expert group
Dahlerup *et al*[17] Guideline approved by the Danish Society of Gastroenterology and Hepatology	2014	GIB, various etiologies	Denmark	Independent authors and approved by a professional organization/society
Baveno IV Consensus Workshop[43]	2015	Variceal bleeding	International	Scientific committee/expert group
European Crohn's and Colitis Organization[10]	2015	IBD	Europe	Professional organization/society
Gasche *et al*[11]	2007	IBD	Europe	Scientific committee/expert group
British Society of Gastroenterology[12]	2011	-	United Kingdom	Professional organization/society
Hong Kong Society of Gastroenterology, the Hong Kong IBD Society, the Hong Kong Society of Digestive Endoscopy, and the Hong Kong Red Cross Blood Transfusion Service[13]	2018	Acute and chronic GIB	Hong Kong	Professional organization
The 2018 Patient Blood Management International Consensus Conference[39]	2019	Acute GIB	Germany	Scientific committee/expert group
British Society of Gastroenterology[40]	2019	ALGIB	United Kingdom	Professional organization
National Institute for Health and Care Excellence[41]	2015	-	United Kingdom	Professional organization
Strate *et al*[42]	2016	ALGIB	United States and Israel	Independent authors
World Health Organization[33]	2001	-	International	Professional organization

ALGIB: Acute lower gastrointestinal bleeding; GIB: Gastrointestinal bleeding; IBD: Inflammatory bowel disease; UGIB: Upper gastrointestinal bleeding.

gastrointestinal bleeding. The first study from Bager *et al*[5] included 169 patients with nonvariceal acute upper gastrointestinal bleeding (AUGIB) and found that 82% of the patients had anemia at hospital discharge. The second study was a single-center study by El-Halabi *et al*[7],which included 307 patients with any gastrointestinal bleeding and reported that 47.4% of the patients had IDA.

Moreover, mortality among patients with gastrointestinal bleeding was reported in two observational studies[29,30]. Among patients with nonvariceal upper gastrointestinal bleeding (UGIB), 30 d mortality ranged from 4.9% to 5.4%[30,31], 1-year mortality was 13.9%, and 2-year mortality was 19.6%[30]. For patients with occult gastrointestinal bleeding, 10-year mortality was 13%[29]. In addition, a study by Rockey *et al*[32] found that 30 d mortality was higher among patients with acute bleeding (7%) than among patients with chronic bleeding (0%).

Laboratory tests should include determining serum hemoglobin and ferritin levels and transferrin saturation percentage

The WHO defines anemia as a hemoglobin level below 13 g/dL in men and below 12 g/dL in nonpregnant women[33]. Hemoglobin together with serum ferritin are commonly recommended by international and national guidelines as markers for IDA, and most guidelines agree with the cutoff value for hemoglobin as defined by the WHO[10,11,17,34]. According to several guidelines, the recommended serum ferritin cutoff value for diagnosing IDA ranges from 12 to 30 μg/L in the absence of inflammation and from 30 to above 100 μg/L in the presence of inflammation[10-12,17,34]. However, as serum ferritin is an acute-phase reactant, additional markers, such as transferrin saturation, may be required to confirm IDA. Three guidelines recommend measuring transferrin saturation, and the suggested cutoff for diagnosing IDA is below 16%[11,34] or below 20% in the presence of inflammation[10]. To the differential diagnosis of IDA and anemia caused by inflammation, WHO recommends to assess both the concentration of serum transferrin receptor and the serum transferrin receptor/log ferritin ratio[19,20] or log (serum transferrin receptor/ferritin) ratio[19]. Shin and colleagues reported that serum transferrin receptor/log ferritin ratio enabled an

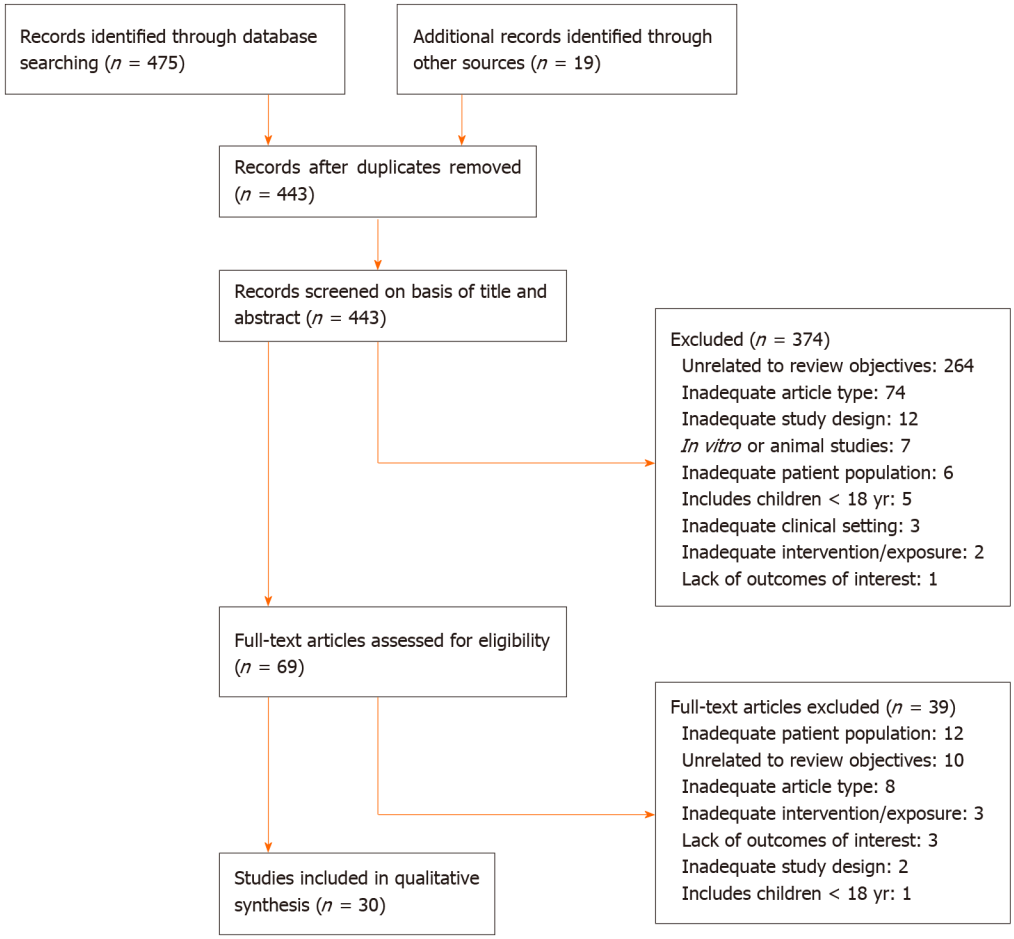

Figure 1 Flowchart of the selected articles. Adapted from Moher *et al*[18].

accurate diagnosis of IDA, as well as the differential diagnosis between IDA and anemia of chronic disease[21]. Infusino *et al*[22]. performed a meta-analysis and suggested that both serum transferrin receptor and the serum transferrin receptor/log ferritin ratio are useful to distinguish between patients with IDA and anemia of chronic disease, adding that serum transferrin receptor may be more efficient than the latter[22]. Koulaouzidis and colleagues critically reviewed the use of serum transferrin receptor as a marker for the evaluation of iron stores and suggested a cutoff value of 2.5 mg/L (29.5 nmoL/L) for the identification of IDA[24]. Nevertheless, serum transferrin receptor levels should not be used alone to distinguish between patients with or without iron deficiency in the presence of inflammation, because their levels are affected by the rate of erythropoiesis from any cause[20].

Target population for treatment

Treatment for IDA should be considered in patients with one or more of the following conditions: Evidence or clinical suspicion of gastrointestinal bleeding, *i.e.*, the presence of melena or hematochezia or hematemesis or if there was IDA (hemoglobin < 13 g/dL in men, < 12 g/dL in women) or a positive fecal occult blood test; previously diagnosed but untreated anemia; hemoglobin levels > 7 g/dL and ≤ 10 g/dL and no indication for erythrocyte transfusion (see indications for erythrocyte transfusion); hemodynamic stability, *i.e.*, the absence of visible active hemorrhaging, with systolic blood pressure > 100 mmHg and a heart rate < 100 beats *per* minute; comorbidities (for instance, heart or kidney disease); concomitant IDA with erythropoiesis-stimulating agents.

Iron deficiency should be corrected by iron treatment, with the goal of restoring hemoglobin levels, serum ferritin levels, and transferrin saturation to normal levels to avoid or reduce the need for erythrocyte transfusion. The decision to initiate iron treatment should be based on the patient's history and symptoms and should consider comorbidities, hemodynamic stability, hemoglobin level and additional treatments.

Gasche *et al*[11] suggested that the absolute indications for initiating intravenous iron treatment include hemoglobin levels below 10 g/dL, intolerance or inappropriate response to oral iron, severe intestinal disease activity, concomitant use of an erythropoietic agent, and patient preference. In patients who are not considered for iron treatment, other treatment options should be considered.

Indications for erythrocyte transfusion

The decision to transfuse erythrocytes should be individualized and based on multiple factors related to the patient's clinical status. In general, erythrocyte transfusion should be considered in patients with: Hemoglobin levels below 7 g/dL; hemoglobin levels above 7 g/dL and below 8 g/dL and comorbidities or under postoperative care; hemoglobin levels between 8 g/dL and below 10 g/dL and symptomatic anemia (*i.e.*, resulting in asthenia and a change in attention capacity), persistent bleeding, or heart disease; in exceptional cases, in patients with hemoglobin levels above 10 g/dL.

Treatment outcomes will depend on etiology, duration, and volume of blood loss. In general, a hemoglobin target of 7 to 9 g/dL should be considered in patients with levels below 7 g/dL and no comorbidities. For patients with comorbid illnesses (cardiovascular disease), hemoglobin target levels of 10 g/dL or above should be considered.

Current clinical practice restricts erythrocyte transfusion to special situations, such as severe anemia or anemia with comorbidities. Treatment with erythrocyte transfusion in patients with UGIB was associated with an increased risk of rebleeding in a meta-analysis of three randomized controlled trials (RCTs)[35] and one observational study[31]. Moreover, erythrocyte transfusion may also be linked to increased mortality, although the evidence is less conclusive than that for rebleeding. The abovementioned meta-analysis found an association between erythrocyte transfusion and increased mortality[35], whereas the observational study did not find an association[31]. Two large RCTs studied the hemoglobin threshold for initiating erythrocyte transfusion in patients with UGIB and its association with treatment complications. The study by Jairath *et al*[36] did not find any association between a restrictive transfusion strategy (8 g/dL) and rebleeding or mortality. The study by Villanueva *et al*[37] showed that a restrictive transfusion strategy (7 g/dL) was associated with better survival, a lower rate of further bleeding, and fewer complications than a more liberal transfusion strategy (9 g/dL). Similarly, an observational study by Subramaniam *et al*[30] found that for patients with hemoglobin levels above 9 g/dL, there was an association between the number of red blood cell units transfused and increased odds of rebleeding.

Most guidelines recommend a restrictive transfusion strategy for patients without comorbid illnesses, although the indications to initiate transfusion vary[10,13,38-42]. Most guidelines recommend transfusion when hemoglobin levels fall below 7 g/dL[10,38,40,41] and the target hemoglobin level is above 7 g/dL[40-43]. The recommended hemoglobin threshold and target levels for the included guidelines are shown in Table 3.

In addition to the recommendations mentioned above, there are exceptions in which a more liberal transfusion strategy can be adopted, such as in patients with comorbidities such as cardiovascular disease or massive bleeding[13,40-42]. However, the threshold differs between the guidelines, such as hemoglobin concentrations above 8 g/dL in patients with a history of cardiovascular disease[40] or acute coronary syndrome[41], above 9 g/dL in patients with cardiovascular ischemia or massive bleeding[42], or between 9 and 10 g/dL in patients with symptomatic coronary artery disease[13].

The decision to initiate erythrocyte transfusion should be decided on an individual basis after carefully weighing the potential benefits and risks. Patients with heart disease, symptomatic anemia, or persistent bleeding or those receiving postoperative care generally benefit from a less restrictive transfusion strategy. Moreover, the goal of erythrocyte transfusion should be to restore the hemoglobin concentration to a safe level and should generally be followed by iron supplementation to replenish iron stores.

Indications for intravenous iron

Intravenous iron should be considered for patients undergoing oral iron supplementation but not achieving IDA correction, reporting treatment-emergent adverse events, or reporting nonadherence to oral supplementation.

The goal of iron therapy is to normalize hemoglobin levels, serum ferritin levels, and transferrin saturation to avoid the need for erythrocyte transfusion. Two treatment approaches are available for restoring iron levels in patients with gastrointestinal bleeding: Oral and intravenous iron. Oral iron is the conventional treatment for IDA

Table 3 Erythrocyte transfusion: Guidelines for hemoglobin thresholds and targets

Professional association	GI etiology	Threshold Hb, g/dL	Threshold Hb cardiovascular disease, g/dL	Target Hb, g/dL	Target Hb cardiovascular disease, g/dL
The International Consensus Upper Gastrointestinal Bleeding Conference Group[38]	Nonvariceal UGIB	< 7	-	-	-
Baveno IV Consensus Workshop[43]	Variceal bleeding	7-8	-	-	-
European Crohn's and Colitis Organization[10]	IDA in IBD	< 7	-	-	-
Hong Kong Society of Gastroenterology, the Hong Kong IBD Society, the Hong Kong Society of Digestive Endoscopy, and the Hong Kong Red Cross Blood Transfusion Service[13]	Acute UGIB	7-8	9-10	-	-
The 2018 Patient Blood Management International Consensus Conference[39]	Acute GIB	7-8	-	-	-
British Society of Gastroenterology[40]	Acute LGIB	< 7	8	7-9	10
National Institute for Health and Care Excellence[41]	N/A	< 7	8	7-9	8-10
Strate *et al*[42]	Acute LGIB	-	9	> 7	> 9

GIB: Gastrointestinal bleeding; Hb: Hemoglobin; IBD: Intestinal bowel disease; IDA: Iron-deficiency anemia; LGIB: Lower gastrointestinal bleeding; UGIB: Upper gastrointestinal bleeding.

but has been associated with gastrointestinal side effects, malabsorption, and nonadherence. Intravenous iron, on the other hand, is generally well tolerated and considered safe[44-48]. One of the identified studies directly compared the effects of intravenous iron with oral iron in patients with nonvariceal AUGIB and found that both treatments were equally effective in restoring hemoglobin levels[44]. These findings were supported by clinical trials and retrospective studies showing that intravenous iron (ferric carboxymaltose or iron sucrose) was effective in restoring hemoglobin levels in patients with AUGIB and acute lower gastrointestinal bleeding[45], chronic gastrointestinal bleeding[47], and gastrointestinal disorder[46]. Intravenous iron (ferric carboxymaltose), however, has been shown to be more efficient in restoring ferritin levels and iron stores in patients with nonvariceal AUGIB than oral iron[44]. Although evidence is still scarce, in cases of suspected malabsorption, the co-administration of oral ascorbic acid may be considered to increase oral iron absorption[12,13,23]. Some pharmacological characteristics of worldwide available oral and intravenous iron preparations, as well as their advantages and disadvantages are shown in Table 4.

The decision to treat patients with intravenous iron should be based on the patient's clinical history and preference. Moreover, international guidelines recommend intravenous iron as the first-line therapy for patients with inflammatory bowel disease[10,11,13,17,34], intolerance to oral iron[10-13,17], or poor response to oral iron[13,17], or according to patient preference[11].

Recommendations for intravenous ferric carboxymaltose treatment

The recommended maximum cumulative dose of ferric carboxymaltose is 1000 mg of iron (20 mL of ferric carboxymaltose) *per* week. Ferric carboxymaltose treatment can be administered one or two times, with an interval of at least one week (in cases in which patient iron dose requirements are > 1000 mg).

Patients should be monitored during the infusion and for 30 min after each administration of intravenous ferric carboxymaltose.

Ferric carboxymaltose treatment should not be considered in patients with one or more of the following conditions: (1) active bleeding; (2) first-trimester pregnancy; (3) active bacterial infection; (4) hemochromatosis or hemosiderosis; (5) Evidence of iron overload (ferritin levels > 800 μg/L and transferrin saturation > 50%); (6) hypersensitivity to ferric carboxymaltose preparations or any of its excipients; and (7) known severe hypersensitivity to other intravenous iron formulations.

Concentrations of ferric carboxymaltose up to 1000 mg have been administered in clinical trials without serious adverse events[44-47]. The recommendations regarding the administration of ferric carboxymaltose are based on the Digestive Bleeding and

Table 4 Pharmacological characteristics, advantages and disadvantages of worldwide available oral and intravenous iron preparations

Type of preparation	Advantages	Disadvantages
Oral Ferric hydroxide polymaltose complex Sodium ferric gluconate Ferrous gluconate Ferrous sulfate Ferrous fumarate	Safe; readily available (does not require a prescription); administered at home; inexpensive; effective when intestinal absorption is not impaired; no need for venous access and infusion monitoring; eliminates the risk of infusion reactions	Slower repletion of iron stores; Intestinal absorption is relatively low, and may be impaired by concomitant food and medications; gastrointestinal adverse events, including constipation, dyspepsia, bloating, nausea, diarrhoea, heartburn, reducing tolerance and adherence to treatment; compliance difficulted by high pill burden (typically three tablets/day) and gastrointestinal intolerance; diminished efficacy when the uptake is impaired (*e.g.*, in celiac disease, autoimmune gastritis, anemia of chronic disease, or post–gastric or duodenal resection)
Intravenous Ferric gluconate Iron sucrose Low molecular weight iron dextran Ferric carboxymaltose Iron isomaltoside 1000 Ferumoxytol	Fast repletion of iron stores; safe when avoiding preparations with dextran; very effective; gastrointestinal adverse events less frequent; ferric carboxymaltose, iron isomaltoside 1000, and ferumoxytol are considered more stable	Administration by a health care professional, requiring clinic visits; increased costs per dose, but fewer doses required; risk of iron overload and transient increase in oxidative stress; risk of anaphylactic reactions with dextran-containing preparations; risk of hypersensitivity reactions

Adapted from Muñoz *et al*[25], Drozd *et al*[26], Jimenez *et al*[27], McDonagh *et al*[28].

Anemia Workgroup's clinical experience and the Summary of Product Characteristics[49]. In our opinion, the first step before administering ferric carboxymaltose is to evaluate the patients' iron requirements. Calculations according to patient body weight and hemoglobin level are shown in Table 5. The second step is to calculate the required dosage using the criteria in Table 5 while considering the following conditions: A maximum single dose of ferric carboxymaltose containing 1000 mg of iron (without exceeding 20 mg/kg of body weight) with an infusion duration of 15 min.

The recommendation to monitor patients for 30 min after administration reflects common clinical practice and the findings from a clinical trial showing that ferric carboxymaltose may induce adverse events during drug infusion[47].

Treatment targets and monitoring of patients

After administration of intravenous iron and in the absence of persistent bleeding, the goal of IDA treatment is to increase the hemoglobin level by 1 to 2 g/dL within 2 to 4 wk and maintain a serum ferritin level ≥ 50 ng/mL (in the absence of inflammatory conditions), increase the number of reticulocytes within 3 to 5 d, and maintain transferrin saturation ≥ 30% for 4 to 6 mo after normalization of hemoglobin levels and once the etiological cause of anemia has been corrected.

Clinical trials have shown that increasing hemoglobin levels by 1 to 2 g/dL within 4 wk can be achieved with the administration of intravenous iron (ferric carboxymaltose)[44,46]. Similarly, a retrospective study of 38 patients with chronic gastrointestinal bleeding reported a median hemoglobin increase of 2.4 g/dL at 5 wk after ferric carboxymaltose treatment[47]. Moreover, an 2 g/dL increase in hemoglobin levels within 4 wk is also considered an acceptable speed of response according to several guidelines[10,11,34].

Two guidelines recommend serum ferritin target levels. Gasche *et al*[11] recommend maintaining serum ferritin above 100 μg/L, whereas the European Crohn's and Colitis Organization guidelines recommend restoring serum ferritin to normal[10].

Reticulocytes are a relatively new biomarker for assessing response to iron treatment. Evidence supporting the monitoring of reticulocytes and their relevant target levels was therefore limited to one small clinical trial and recommendations in

Table 5 Calculation of iron requirement according to patient body weight and hemoglobin level

Hemoglobin (g/dL)	Hemoglobin (mmoL/L)	Patient body weight (below 35 kg)	Patient body weight (35 kg to 70 kg)	Patient body weight (70 kg and above)
< 10	< 6.2	500 mg	1500 mg	2000 mg
10 to 14	6.2 to 8.7	500 mg	1000 mg	1500 mg
≥ 14	≥ 8.7	500 mg	500 mg	500 mg

Adapted from Infarmed[49], 2015.

one guideline. Geisser *et al*[46] measured reticulocyte levels at baseline and 14 d after ferric carboxymaltose supplementation and found that the levels increased from 60 × 10^9/L to 89 × 10^9/L. Moreover, one guideline recommends measuring reticulocytes at one week after iron treatment to confirm an increase compared to the level before treatment[17]. Generally, reticulocyte levels increase within one week in response to intravenous treatment. We recommend measuring the number of reticulocytes after 3-5 d to assess whether a proper response to initial intravenous iron has been achieved to evaluate further treatment.

Only one guideline recommends measuring transferrin saturation to assess therapeutic response. This guideline recommends target levels of transferrin saturation between 16% and 50% after anemia treatment[11]. Transferrin saturation may temporarily increase in response to intravenous iron treatment and should therefore not be used for monitoring initial treatment response.

DISCUSSION

Summary algorithm for investigation and treatment

Figure 2 presents the algorithm for the diagnosis and treatment of IDA in patients with acute or chronic gastrointestinal bleeding. The algorithm is based on the literature and the experience of the members of the Digestive Bleeding and Anemia Workgroup. This algorithm should be implemented during the hospital stay and at follow-up visits after patient discharge and is not applicable for emergencies or critical care.

Limitations

This consensus recommendation had several limitations. First, the quality of the identified studies was generally low, and we found few RCTs on IDA and gastrointestinal bleeding. Therefore, the recommendations are mainly based on observational studies, guidelines, and clinical experience of the members of the Digestive Bleeding and Anemia Workgroup. Second, studies on lower gastrointestinal bleeding are scarce, and our recommendations are mainly based on studies on patients with UGIB. Third, the search strategy included only studies indexed in MEDLINE (PubMed), published from January 2003 until April 2019, and written in English.

CONCLUSION

Diagnosing and treating patients with IDA is challenging in clinical practice. IDA in patients with gastrointestinal bleeding should be diagnosed and treated promptly to improve the quality of life and reduce morbidity and, eventually, mortality. This consensus recommendation provides a starting point for diagnosing and treating IDA in patients with gastrointestinal bleeding by gastroenterologists and other physicians in daily clinical practice and should serve to optimize the decision-making process for the management of these patients. We believe that this guideline may facilitate improvements in the management of IDA in patients with gastrointestinal bleeding, which ultimately translates to improved health outcomes for these patients.

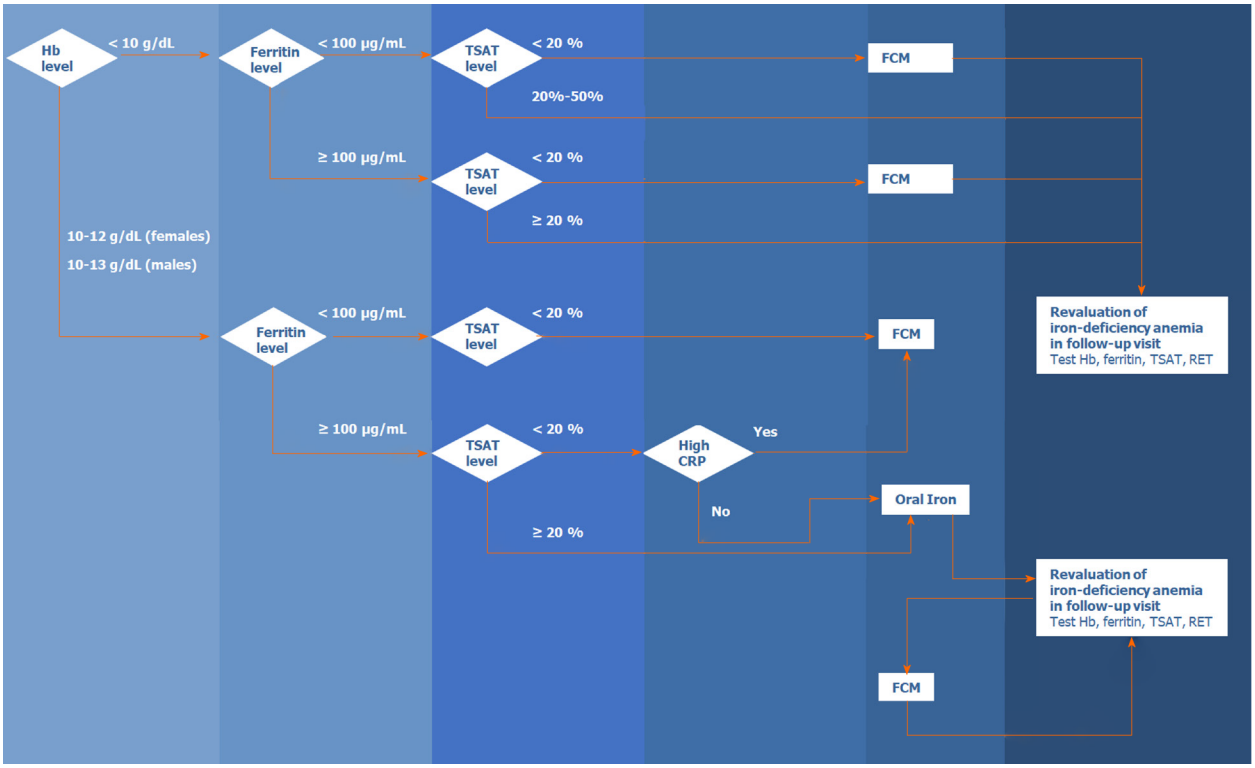

Figure 2 Algorithm for the diagnosis and treatment of iron-deficiency anemia in patients with acute or chronic gastrointestinal bleeding.
Hb: Hemoglobin; TSAT: Transferrin saturation; CRP: C-reactive protein; FCM: Ferric carboxy maltose; RET: Reticulocyte.

ARTICLE HIGHLIGHTS

Research background

Anemia is a public health issue affecting approximately 25% of the world's population, being often caused by iron deficiency. Iron-deficiency anemia (IDA) often originates from blood loss from lesions in the gastrointestinal tract in men and postmenopausal women, and its prevalence among patients with gastrointestinal bleeding has been estimated to be 61%. However, studies have shown that IDA is often underdiagnosed, underrecognized, and undertreated in hospitalized patients with gastrointestinal bleeding, and that therapeutic approaches for iron-deficiency correction have been poorly implemented and clinical practice guidelines are not being followed. Furthermore, clinical practice recommendations and guidelines on the management of IDA in gastrointestinal bleeding patients are still scarce and there is no standardization on the management of these patients. Therefore, standardized recommendations on the management of IDA in gastrointestinal bleeding patients, based on a systematic review of the current evidence, are needed.

Research motivation

Given the scarcity of clinical practice recommendations and guidelines on the management of IDA in gastrointestinal bleeding patients, and the need of standardization regarding the management of these patients, it is urgent to develop evidence-based standardized diagnostic and therapeutic approaches on the management of patients with IDA due to gastrointestinal bleeding.

Research objectives

With this study, we aimed to review the current evidence and guidelines concerning IDA management in gastrointestinal bleeding patients to develop recommendations for its diagnosis and therapy.

Research methods

Five gastroenterology experts formed the Digestive Bleeding and Anemia Workgroup and conducted a systematic literature search in PubMed and professional association websites. MEDLINE (*via* PubMed) searches combined MeSH terms and the keywords "gastrointestinal bleeding" with "iron-deficiency anemia" and "diagnosis" or

"treatment" or "management" or "prognosis" or "prevalence" or "safety" or "iron" or "transfusion" or "quality of life", or other terms to identify relevant articles reporting the management of IDA in patients over the age of 18 years with gastrointestinal bleeding; retrieved studies were published in English between January 2003 and April 2019. Worldwide professional association websites were searched for clinical practice guidelines. Reference lists from guidelines were reviewed to identify additional relevant articles. The recommendations were developed by consensus during two meetings and were supported by the published literature identified during the systematic search.

Research results

From 494 Literature citations found during the initial literature search, 17 original articles, one meta-analysis, and 13 clinical practice guidelines were analyzed. Ten additional references were included after the peer review process. Based on the published evidence and clinical experience, the workgroup developed the following ten recommendations for the management of IDA in patients with gastrointestinal bleeding: (1) evaluation of hemoglobin and iron status; (2) laboratory testing; (3) target treatment population identification; (4) indications for erythrocyte transfusion; (5) treatment targets for erythrocyte transfusion; (6) indications for intravenous iron; (7) dosages, (8) monitoring; (9) indications for intravenous ferric carboxymaltose treatment; and (10) treatment targets and monitoring of patients. The workgroup also proposed a summary algorithm for the diagnosis and treatment of IDA in patients with acute or chronic gastrointestinal bleeding, which should be implemented during the hospital stay and follow-up visits after patient discharge.

Research conclusions

Ten evidence-based recommendations were developed for screening, treatment indications, appropriate therapies, and treatment goals of IDA in patients with acute or chronic gastrointestinal bleeding. An algorithm for the diagnosis and treatment of these patients was also developed, based on the literature and on the experience of the members of the Digestive Bleeding and Anemia Workgroup. Therefore, this work serves as a starting point for diagnosing and treating IDA in patients with gastrointestinal bleeding by gastroenterologists and other physicians in daily clinical practice and should serve to optimize the decision-making process for the management of these patients. This guideline may facilitate improvements in the management of IDA in patients with gastrointestinal bleeding, which ultimately may improve health outcomes in these patients.

Research perspectives

This consensus recommendation provides a starting point for clinicians to better diagnose and treat IDA in patients with gastrointestinal bleeding. Nevertheless, more studies, specially RCTs on IDA and gastrointestinal bleeding are needed to further improve the management of these patients.

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CASE REPORT

Endoscopic mucosal ablation - an alternative treatment for colonic polyps: Three case reports

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Author contributions: Mendoza Ladd A performed all the procedures, edited the video, drafted, edited and approved the final manuscript; Espinoza J and Garcia C drafted, edited and approved the final manuscript.

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Abstract

BACKGROUND

Endoscopic resection of non-invasive lesions is now the standard of care for lesions in the GI tract. However, resection techniques require extensive training, are not available in all endoscopy centers and are prone to complications. Endoscopic mucosal ablation (EMA) is a combination of resection and ablation techniques and it may offer an alternative in the management of such lesions.

CASE SUMMARY

In this case series we report the successful treatment of three flat colonic polyps using the EMA technique. Two lesions were treatment naïve and 1 was a recurrence after an endoscopic mucosal resection. The sizes ranged from 2 to 4 cm. All three polyps were ablated successfully with no immediate or delayed complications. The recurrence rate at 1 year of follow up was 0%.

CONCLUSION

Based on this initial experience, we conclude that EMA is a safe and effective technique for the treatment of non-invasive colonic polyps when endoscopic resection techniques are not available.

Key Words: Endoscopy; Mucosal ablation; Colon polyp; Argon plasma coagulation; Alternative; Safe; Case report

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Core Tip: Endoscopic resection *via* endoscopic mucosal resection or endoscopic submucosal dissection is currently the standard of care for non-invasive colonic polyps. However, these resection techniques require extensive training, are not available in all endoscopy centers, and are prone to adverse events such as perforation and bleeding. Endoscopic mucosal ablation appears to be a safe and effective alternative in the treatment of colonic polyps without invasive features.

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INTRODUCTION

Endoscopic mucosal resection (EMR) and/or endoscopic submucosal dissection (ESD) is now the standard of care for non-dysplastic, dysplastic and early malignant lesions of the gastrointestinal tract. The *sine qua non* of these techniques is the adequate formation of a submucosal cushion prior to resection, in order to avoid damage of the muscularis propria and hence, perforation. However, these resection techniques require rigorous training, are prone to complications and are not always available at all centers. Recently the submucosal cushion principle has been adopted in the management of dysplastic Barrett's esophagus (BE) prior to ablation with argon plasma in a technique called Hybrid APC[1]. We explored this concept in the management of 3 flat colonic polyps.

CASE PRESENTATION

Chief complaints

All patients presented in this case series had colon polyps requiring treatment.

History of present illness

All three patients originally presented to their gastroenterology providers for routine colon cancer screening or surveillance and were referred to our endoscopy center.

History of past illness

Case 1: Hypertension (HTN), DM2.

Case 2: DM2, HTN, hypercholesterolemia and treated tuberculosis.

Case 3: Hypercholesterolemia and osteoarthritis.

Imaging examinations

Case 1: An 85 y/o M with previous medical history (PMH) described above was referred for evaluation of a cecal polyp. Colonoscopy revealed a 4 cm 0-IIa +Is lesion in the cecum that involved the IC valve (Figure 1A). Due to morphology of the lesion and the risk of incomplete resection *via* EMR (we did not perform ESD at our center at the time), removal was not attempted. The lesion was biopsied and pathological analysis established it was a tubular adenoma with no dysplasia. The patient was offered referral to an outside facility for ESD. However due to his lack of medical insurance, referral was not a possibility. Therefore, endoscopic mucosal ablation (EMA) was offered. After detailed explanation of the potential risks and benefits, he agreed to the procedure. The colonoscopy was repeated 2 mo later. The lesion was injected with a mix of O'rise gel™ (Boston Scientific, Natick, MA, United States) + epinephrine at 1:10000 to create the submucosal cushion. The lesion raised easily. Once raised, ablation of the lesion was performed using Argon Plasma Coagulation in forced

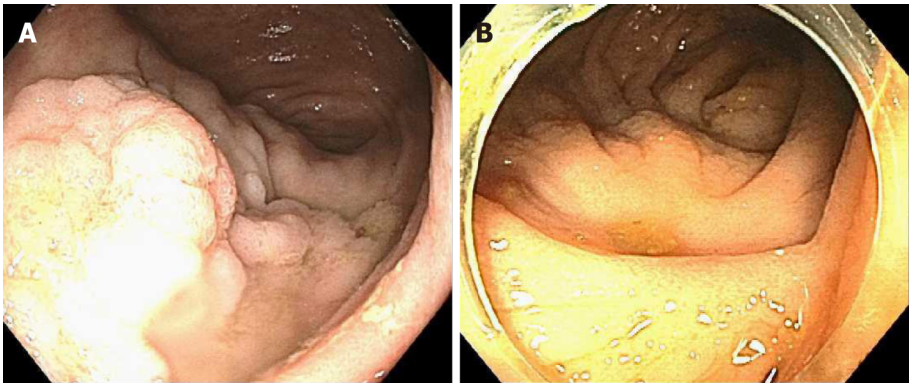

Figure 1 Colonoscopy. A: 0-IIa +Is lesion in the cecum involving the IC valve; B: Post endoscopic mucosal ablation scar.

coagulation mode at 1 L/min, 60 W, effect 2 (Video). The patient did well and was discharged the same day of the procedure. Colonoscopy 1 year later showed a scar with no evidence of polyp recurrence (Figure 1B). Biopsies of the scar confirmed eradication of the lesion.

Case 2: This was a 69 y/o M with PMH described above who was referred for removal of polyp recurrence after a previous EMR. Colonoscopy showed a 0-IIa lesion of approximately 3 cm at the previous EMR scar in the hepatic flexure (Figure 2A). The lesion was biopsied prior to ablation. After the biopsy, EMA was performed in a similar manner as in case 1 (video). The biopsy of the lesion revealed a tubular adenoma with no dysplasia. Colonoscopy at 11 mo showed a healthy scar with no signs of polyp recurrence (Figure 2B). Biopsy of the scar confirmed eradication.

Case 3: A 79 y/o F with a PMH described above was referred for evaluation of a flat polyp. Colonoscopy revealed a 2 cm 0-IIa lesion in the hepatic flexure (Figure 3A). The lesion was biopsied prior to ablation. Ablation ensued as described above (video). Biopsy of the lesion revealed tubular adenoma features with no dysplasia. Colonoscopy 13 mo later revealed a healthy scar with no polyp recurrence (Figure 3B). Biopsy of the scar confirmed eradication.

FINAL DIAGNOSIS

All patients were diagnosed with TA with no dysplasia.

TREATMENT

All patients were treated with the EMA technique.

OUTCOME AND FOLLOW-UP

All patients had follow-up colonoscopy at 1 year and biopsy of the ablation site revealed no evidence of recurrence.

DISCUSSION

EMA is a combination of established resection and ablation techniques already utilized in different sections of the GI tract. The EMA technique has been previously applied successfully in gastric lesions. Kothari *et al*[2] successfully treated a flat dysplastic lesion at the GJ anastomosis of a patient with a previous Roux-en-Y gastric bypass[2]. Estifan *et al*[3] also obtained adequate results using EMA to treat lesion recurrence after an ESD for intestinal metaplasia in the incisura[3]. The only available report of the application of this technique in the colon was published by Tsiamoulos *et al*[4]. In contrast to ours, this case series included only recurrent lesions after prior endoscopic resection. They

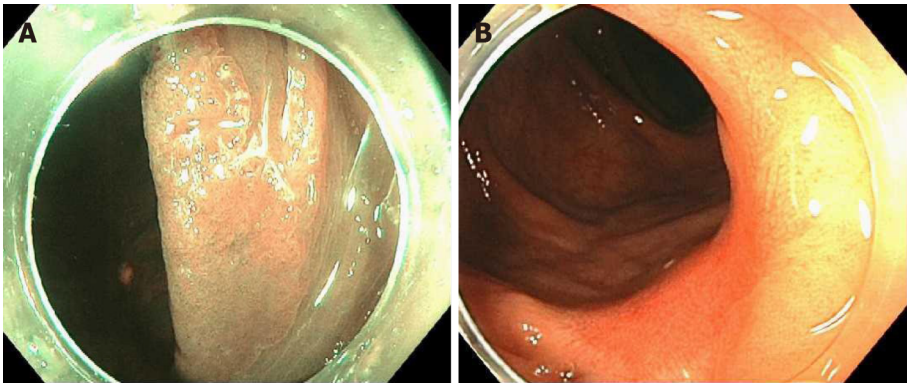

Figure 2 Colonoscopy. A: 0-IIa lesion in previous endoscopic mucosal resection site in hepatic flexure; B: Post endoscopic mucosal ablation scar.

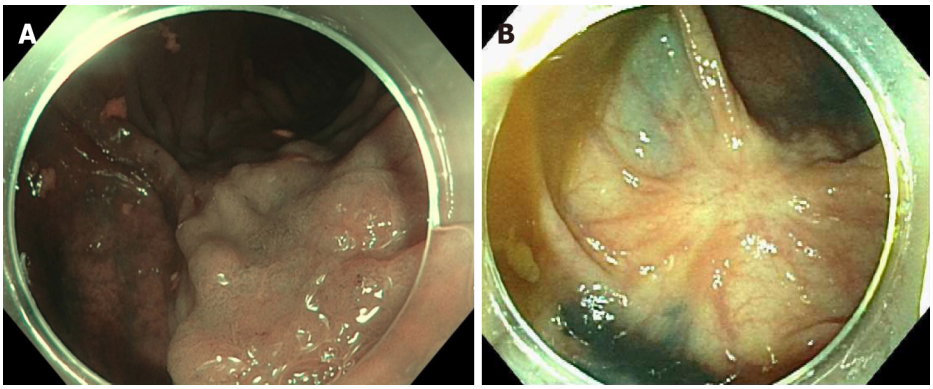

Figure 3 Colonoscopy. A: 0-IIa lesion in the hepatic flexure; B: Post endoscopic mucosal ablation scar.

reported no complications and a recurrence rate of 82% at one year.

When utilizing EMA, a natural concern is the risk of complications such as perforation. However, studies in porcine models have demonstrated that ablation with argon plasma does not cause injury to the musclularis propria when a submucosal cushion is created[5,6]. This finding led to the novel technique of Hybrid APC. In this technique, a submucosal cushion is created and the affected mucosa is ablated. This technique has been applied in the treatment of BE and has shown promising results[1].

The advantages of EMA include its simplicity, safety and its availability in most community endoscopy centers. The main disadvantage is that it does not produce a surgical specimen for pathological analysis. Although previous biopsy of the lesion can give preliminary information about the histology, it may not reflect that of the entire lesion. Therefore, endoscopists should only use EMA in colonic polyps when endoscopic resection *via* EMR or ESD is not available. Furthermore, this technique should not be utilized when the preparation of the colon is poor due to the risk of colonic explosion[7,8].

CONCLUSION

To our knowledge, this is the first report of the use of EMA in treatment naïve colonic lesions. We encountered no complications and our recurrence rate at 1 year was 0% with biopsies of the scars confirming eradication in all cases. Our results suggest that EMA is safe and effective in the treatment of colonic polyps when endoscopic resection is not possible or available.

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CASE REPORT

Tuberous sclerosis patient with neuroendocrine carcinoma of the esophagogastric junction: A case report

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Abstract

BACKGROUND

Tuberous sclerosis complex (TSC) is a rare inherited disease with non-cancerous tumor growths in the skin, brain, kidneys, heart, and lungs. The co-occurrence of neuroendocrine neoplasm (NEN) with TSC is even rarer. There have been few reports on the relationship between TSC and neuroendocrine tumors (NETs), and fewer on the relationship between TSC and neuroendocrine carcinoma (NEC), a subtype of NEN. This is the first reported case of NEC occurring at the esophagogastric junction in a patient with TSC.

CASE SUMMARY

A 46-year-old woman visiting our hospital for the treatment of TSC was admitted to the emergency department with tarry stools and dizziness. Computed tomography scans revealed thickness of the gastric cardia, multiple metastatic

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lesions of the liver, and enlarged lymph nodes near the lesser curvature of the stomach. Esophagogastroduodenoscopy revealed a type 3 tumor located from the esophagogastric junction to the fundus, and the pathological diagnosis by biopsy was NEC. The patient was treated with seven courses of cisplatin + irinotecan, followed by eight courses of ramucirumab + nab-paclitaxel, one course of nivolumab, and two courses of S-1 + oxaliplatin. Twenty-three months after the first treatment, the patient died because of disease progression and deterioration of the general condition.

CONCLUSION
This case of NEC occurring in a patient with TSC indicates a difference in the occurrence of NETs and NECs.

Key Words: Tuberous sclerosis complex; Neuroendocrine carcinoma; Neuroendocrine tumor; mTOR inhibitor; Esophagogastric junction; Chemotherapy; Case report

Core Tip: Although it has been reported that neuroendocrine tumors can merge with the tuberous sclerosis complex (TSC), the co-occurrence of neuroendocrine carcinoma (NEC) with TSC is rare. This is the first reported case of NEC occurring at the esophagogastric junction in a patient with TSC. This highly suggestive case indicates there is a difference in the occurrence of neuroendocrine tumors and NECs, which depends upon the pathogenesis of TSC developed despite inhibition of the AKT/mTOR pathway.

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INTRODUCTION

Tuberous sclerosis complex (TSC) is a rare, dominant inherited disease with non-cancerous tumor growths in the skin, brain, kidneys, heart, and lungs, and it is caused by abnormalities in the tumor suppressor genes, *TSC1* and *TSC2*[1]. *TSC1* and *TSC2* produce proteins that regulate the intracellular mTOR pathway activity, and the loss of mTOR regulation owing to the abnormalities in these genes is thought to cause many of the symptoms of TSC. In addition, the mTOR pathway is significantly involved in the development of neuroendocrine tumors (NETs), and the mTOR inhibitor, everolimus, is known to be a therapeutic agent for NET[2].

Neuroendocrine carcinoma (NEC) is a subtype of neuroendocrine neoplasm (NEN), and NEC in the digestive tract is rare. Furthermore, NEC that occurs in the esophagus accounts for only 0.4% of all esophageal carcinomas[3]. There are quite a few detailed case reports regarding NEC occurring at the esophagogastric junction, and there are no reported cases of NEC at the esophagogastric junction with TSC as the underlying disease[4-8].

We, therefore, report an extremely rare case of TSC, which is considered to have an abnormality in the mTOR pathway, co-occurring with NEC.

CASE PRESENTATION

Chief complaints

A 43-year-old woman visited the emergency room of our hospital complaining of tarry stool and dizziness.

History of present illness

The patient's symptoms started the previous day before visiting the emergency room.

History of past illness

The patient has a history of TSC, facial angiofibroma, seizure, renal angiofibroma, and lymphangioleiomyomatosis (LAM). She was administered sirolimus, which is an mTOR inhibitor, for the treatment of LAM.

Physical examination

On physical examination at the time of hospitalization, her heart rate was 113 beats per minute, and her blood pressure was 95/72 mmHg. The liver, spleen, and tumor were not palpable on physical examination.

Laboratory examinations

Laboratory examination revealed decreased hemoglobin levels (12.6 g/dL) and elevated carcinoembryonic antigen (41.2 ng/mL), carbohydrate antigen 19-9 (80 pg/mL), and neuron-specific enolase levels (56.0 ng/mL). Pro-gastrin-releasing peptide level was within the normal limits (40.7 pg/mL).

Imaging examinations

Computed tomography (CT) scans showed wall thickness from the esophagogastric junction to the gastric cardia. Enlarged lymph nodes near the lesser curvature of the stomach and multiple space-occupying, ring-enhancing metastatic lesions in the liver were observed (Figure 1). Gastric endoscopy revealed a type 3 tumor located from the esophagogastric junction to the gastric cardia (Figure 2). The bleeding from the lesion was the cause of the tarry stool, and the patient was then hospitalized and treated with a proton pump inhibitor.

FINAL DIAGNOSIS

The pathological diagnosis based on a biopsy of a mucosal lesion was NEC (Figure 3). Hematoxylin and eosin (H-E) staining showed atypical epithelial cells forming solid nests with a high nucleocytoplasmic ratio. Tests for immunohistological markers CD56, synaptophysin, and chromogranin A were positive, and the Ki 67 index was approximately 70%. Based on the above results, the patient was diagnosed with esophagogastric neuroendocrine carcinoma T3, N1, M1, Stage IVB (AJCC/UICC 8th edition[9]).

TREATMENT

Since the tumor was clinical stage IV and inoperable, cisplatin (CDDP) + irinotecan (CPT-11) therapy was selected for chemotherapy. The eligible regimen for this patient included cisplatin (60 mg/m^2, day 1) and irinotecan (60 mg/m^2, day 1, 8, and 15). However, the first cisplatin dose was reduced to 48 mg/m^2 (80% dose) because of renal failure due to renal angiomyolipoma. The patient experienced grade 2 neutropenia as a side effect after the first course of chemotherapy; irinotecan dose was then reduced to 48 mg/m^2 (80% dose). A CT scan after seven courses of chemotherapy revealed increased liver metastasis. Serum tumor markers were elevated and disease progression was observed (Figure 4). As a second-line regimen, ramucirumab (RAM) and nab-paclitaxel (nab-PTX) therapy were administered to this patient. The eligible regimen was ramucirumab (8 mg/kg, day 1 and 15) and paclitaxel (80 mg/m^2, day 1, 8, and 15). During the eight courses of the second-line chemotherapy, although the patient had no remarkable adverse events, another liver metastasis occurred. Nivolumab was administered as a third-line regimen. However, after one course of treatment, a further increase in tumor markers was observed and liver metastases worsened. As a fourth-line therapy, the S-1 + oxaliplatin regimen was administered to the patient.

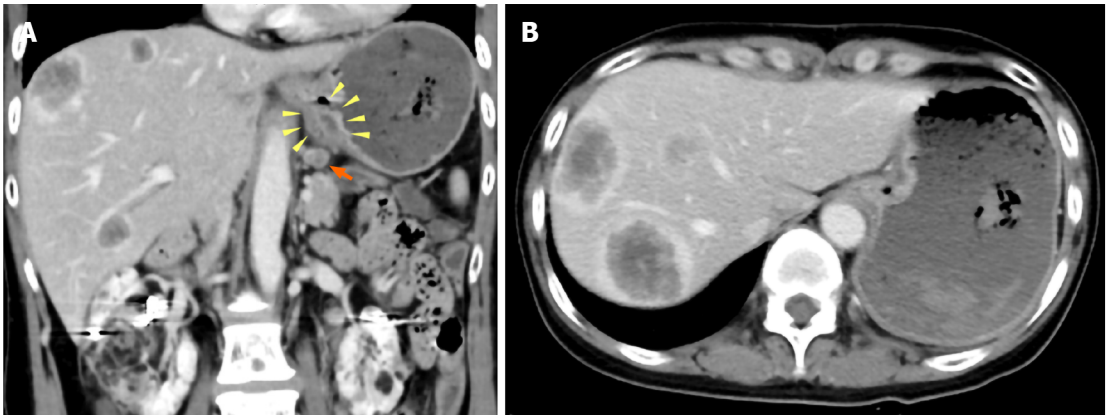

Figure 1 Abdominal contrast-enhanced computed tomography images. A: Wall thickness from the esophagogastric junction to the cardia (yellow arrowhead) and enlarged lymph nodes near the lesser curvature of the stomach (orange arrow); B: Multiple ring-enhanced tumors are observed in the liver.

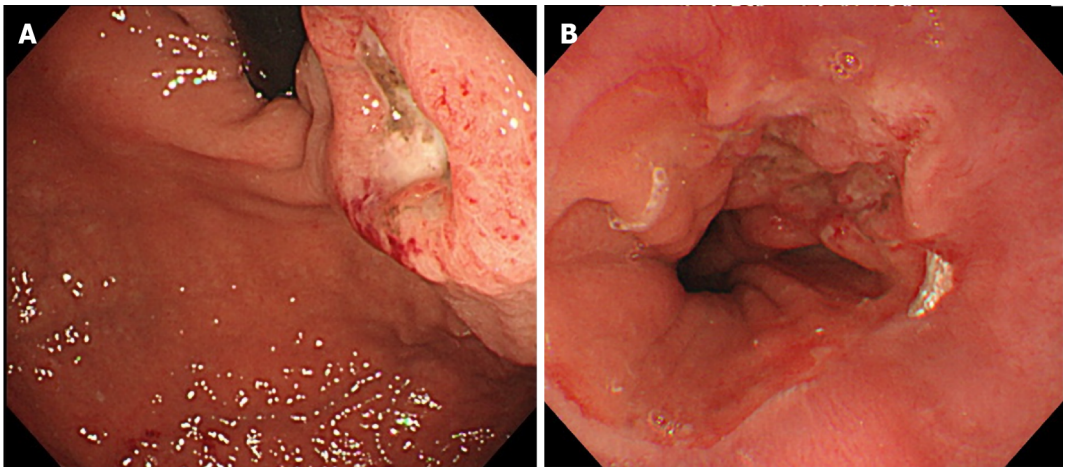

Figure 2 Esophagogastroduodenoscopy revealed type 3 tumor located from the esophagogastric junction to the cardia. A: Image from endoscopic examination of esophagus; B: Image from endoscopic examination of stomach.

OUTCOME AND FOLLOW-UP

After two courses of chemotherapy, owing to the exacerbation of NEC, the patient died 23 mo after the first diagnosis. The pathological autopsy was performed with the consent of the patient's family. In addition to multiple liver and lymph node metastases previously identified by CT examination, lung, adrenal, spleen, and kidney metastases, and peritoneal dissemination were observed.

DISCUSSION

The following three points are considered to have high clinical and academic value in this case: (1) NEC co-occurred with TSC, a rare disease; (2) Although the patient was administered sirolimus, an mTOR inhibitor, against LAM, she developed NEC, which is a type of NEN; and (3) The patient was treated with multiple chemotherapy regimens for small-cell lung cancer and gastric cancer, and she survived for 23 mo after the diagnosis.

TSC is an inherited, autosomal, dominant, multisystem disorder characterized by the development of multiple hamartomas in neuron organs. It occurs in 1 in 6000 individuals[10], and approximately 60% of TSCs are sporadic cases. In our case, none of the family members of the patient had TSC. TSC is caused by mutations in two tumor suppressor genes, *TSC1* on chromosome 9q4 and *TSC2* on chromosome 16p13.3, which encode hamartin and tuberin, respectively. The mutation type in the patient was not examined. Owing to these gene mutations, TSC may affect the skin, central nervous system, kidney, heart, eye, blood vessels, lung, bone, and gastrointestinal tract. The

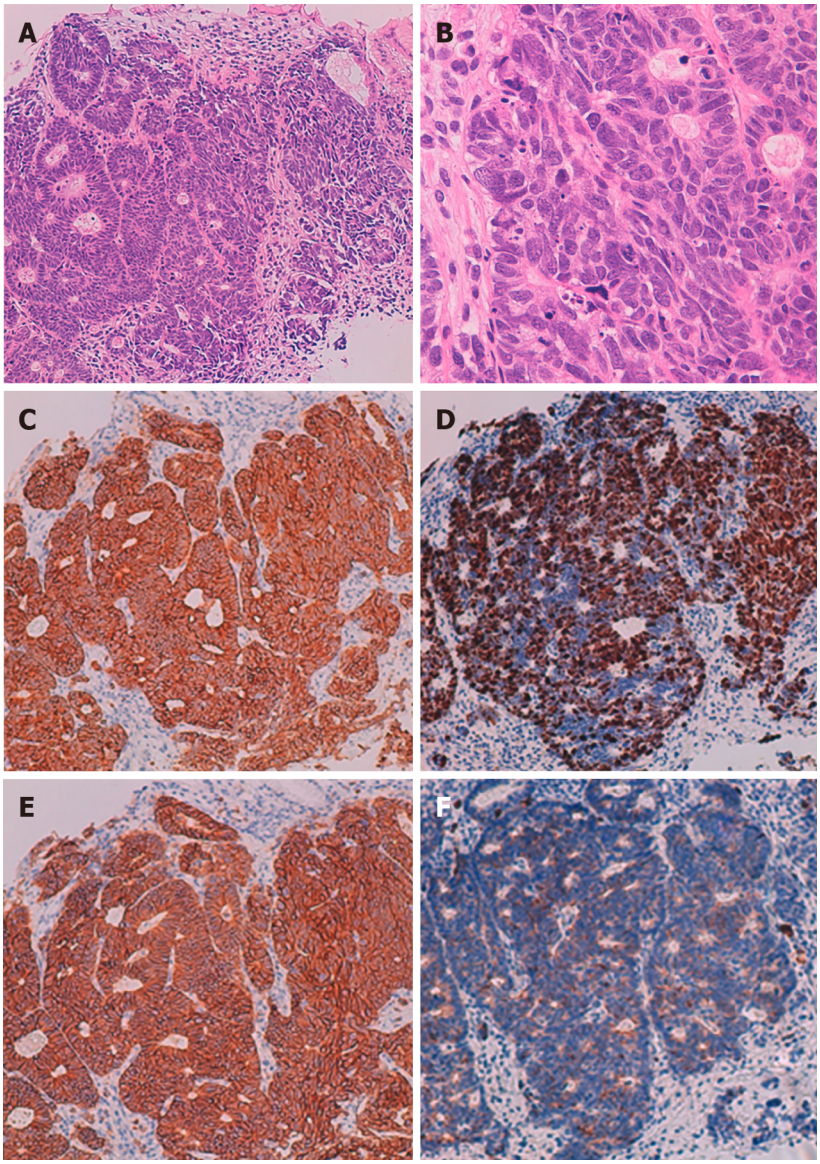

Figure 3 Histological findings of a mucosal lesion. A: Low-power histological view of Hematoxylin and eosin (H-E) stained specimens showing atypical epithelial cells with solid alveolar nests; B: High-power view of H-E stained specimens reveals poorly differentiated atypical cells with large nucleus-cytoplasm ratio; C: Synaptophysin positive; D: Ki 67 index is approximately 70%; E: CD56 positive; F: Chromogranin A positive.

TSC1 and *TSC2* genes are involved in the AKT-mTOR pathway activation and are, therefore, involved in the development and proliferation of NETs. Previous studies have reported a relationship between TSC and NETs[11-13]. Gastrointestinal NEC is a type of NEN and is a poorly differentiated cancer with neuroendocrine characteristics classified by the 2019 World Health Organization tumor classification[14]. NEC is known to have a high degree of malignancy and rapid progression. In our case, metastases to various organs were found by pathological autopsy after death.

There are few reported cases on the complications of NETs and TSCs, and there are few reports on the complications of NECs and TSCs. Most of the reported cases of TSCs complicated with NETs have grade 1 or 2 NET[13]. Arva *et al*[11] presented a case of well-differentiated NEC of the pancreas in a child with TSC[11]. However, there are no reports of TSC patients with gastrointestinal tract NEC listed in PubMed from 1950 to 2020.

LAM is a rare neoplastic disease in which abnormal LAM cells resembling smooth muscle cells grow relatively slowly in the lungs, lymph nodes, and kidneys. LAM diagnosis is age dependent and occurs in up to 80% of women with TSC by the age of 40[15]. Recently, the effect of the mTOR inhibitor, sirolimus, on LAM was reported[16], and our patient was also administered sirolimus for LAM. In the AKT-mTOR pathway, mTOR inhibitor is thought to reduce protein translation and suppresses subsequent neuroendocrine tumor development (Figure 5)[12]. However, in our case, NEC developed despite the administration of an mTOR inhibitor.

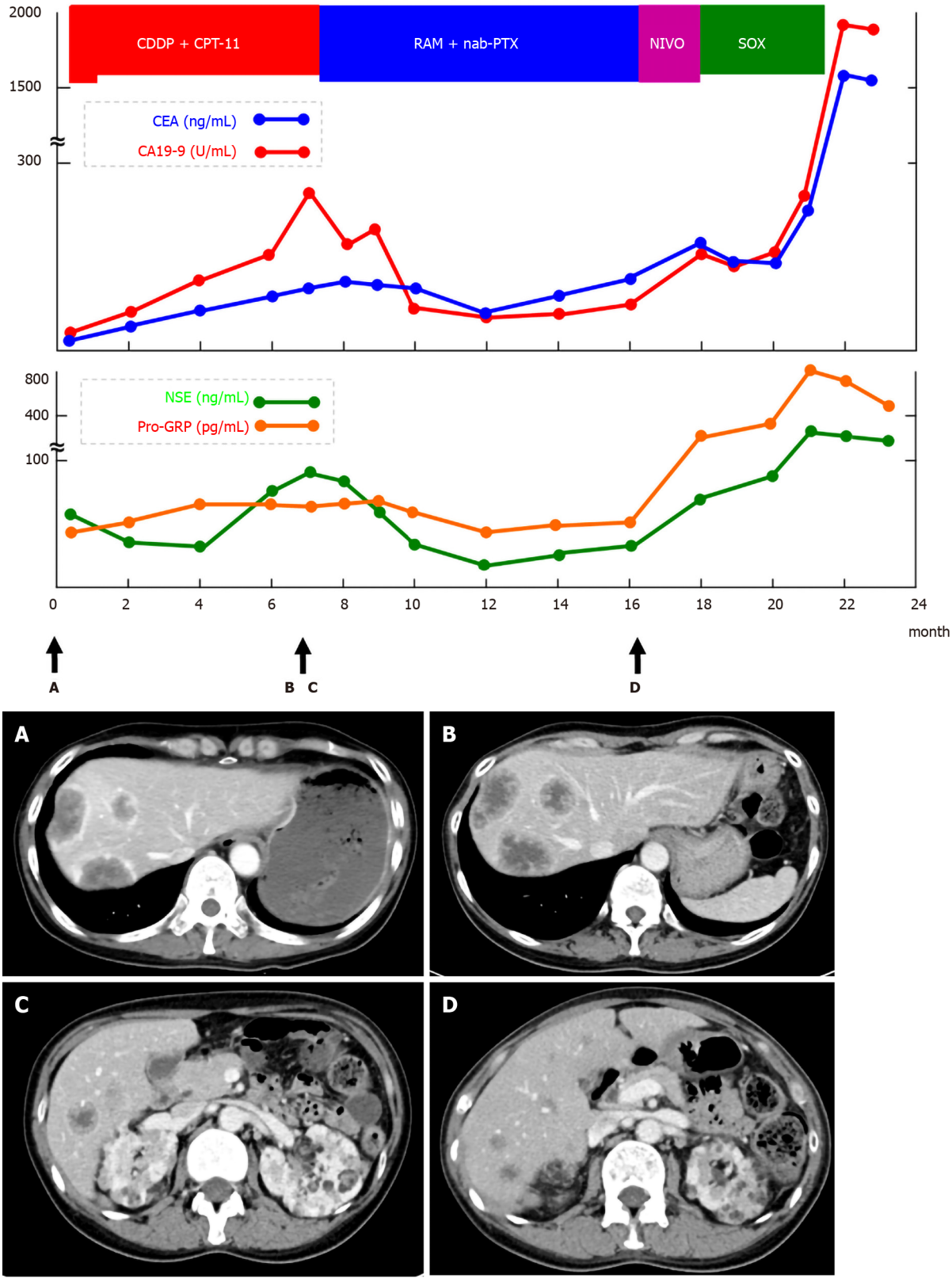

Figure 4 Clinical course of this case. A-D: Serum tumor markers were elevated. A CT scan after seven courses of chemotherapy revealed increased liver metastasis. CDDP: Cisplatin; CPT-11: Irinotecan; RAM: Ramucirumab; nab-PTX: Nab-Paclitaxel; NIVO: Nivolumab; SOX: S-1 and oxaliplatin; CEA: Carcinoembryonic antigen; CA19-9: Carbohydrate antigen 19-9; NSE: Neuron-specific enolase; Pro-GRP: Pro-gastrin-releasing peptide.

The carcinogenesis of NEC was once argued for the mechanism of stem cell development and development derived from NETs. However, there are genetic differences in NECs that are not observed in NETs such as the overexpression of p53 protein, diffuse deficiency of retinoblastoma protein, and diffuse overexpression of p16 protein in highly malignant tumors. Therefore, NETs and NECs have been

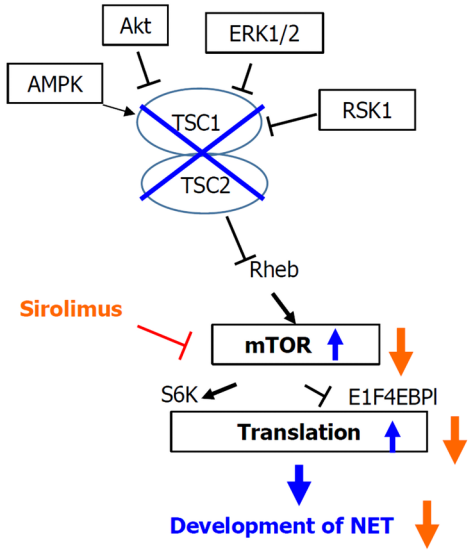

Figure 5 Diagram showing that the mammalian target of rapamycin inhibitor suppresses the formation of neuroendocrine tumor by suppressing the AKT-mTOR pathway. In the AKT-mTOR pathway, mutation of tuberous sclerosis complex *(TSC)1/TSC2* genes causes mTOR to proliferate and generate neuroendocrine tumor (NET). Sirolimus, an mTOR inhibitor, inhibits the abnormal growth of mTOR, which suppresses the generation of NET. TSC: Tuberous sclerosis complex; AMPK: Adenosine 5'-monophosphate (AMP)-activated protein kinase; ERK: Extracellular regulated protein kinases; RSK: Ribosomal S6 kinase; EBP: Evidence-based practice; NET: Neuroendocrine tumor.

reported to be genetically different[17-19]. In our case, NEC developed while mTOR was inhibited by sirolimus, so it is speculated that this case may have followed the clinical course supported by this theory, because sirolimus could inhibit the development of NEC only if NETs and NECs were genetically similar.

The European Neuroendocrine Tumor Society guidelines recommend that chemotherapy for NEC is based on a small-cell lung cancer regimen[20-21]. The first-line therapy for small-cell lung cancer regimens is CDDP + CPT-11 or CDDP + etoposide (VP16), and retrospective studies in Japan have reported that these platinum-based chemotherapies have equivalent effects for NEC[22]. In our case, the CDDP + CPT-11 regimen, which is frequently administered in Japan, was administered. Since the initial pathological diagnosis suggested that adenocarcinoma might coexist with NETs, chemotherapy of RAM + nab-PTX for adenocarcinoma was selected as the second-line treatment. Although this case was finally diagnosed with NEC, the second-line chemotherapy was successful since the patient was able to continue eight courses of RAM + nab-PTX therapy. Some previous cases have reported the effectiveness of RAM + nab-PTX treatment for gastrointestinal tract NEC[23,24]. In the future, accumulation of RAM + nab-PTX administration cases for gastrointestinal tract NEC is expected.

CONCLUSION

We have reported NEC occurring at the esophagogastric junction in a patient with TSC. Considering the pathogenesis of TSC developed despite the inhibition of the AKT/mTOR pathway, it was a highly suggestive case indicating a difference in the occurrence of NETs and NECs. We also supported the possibility that a RAM + nab-PTX regimen was effective against gastrointestinal NEC.

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