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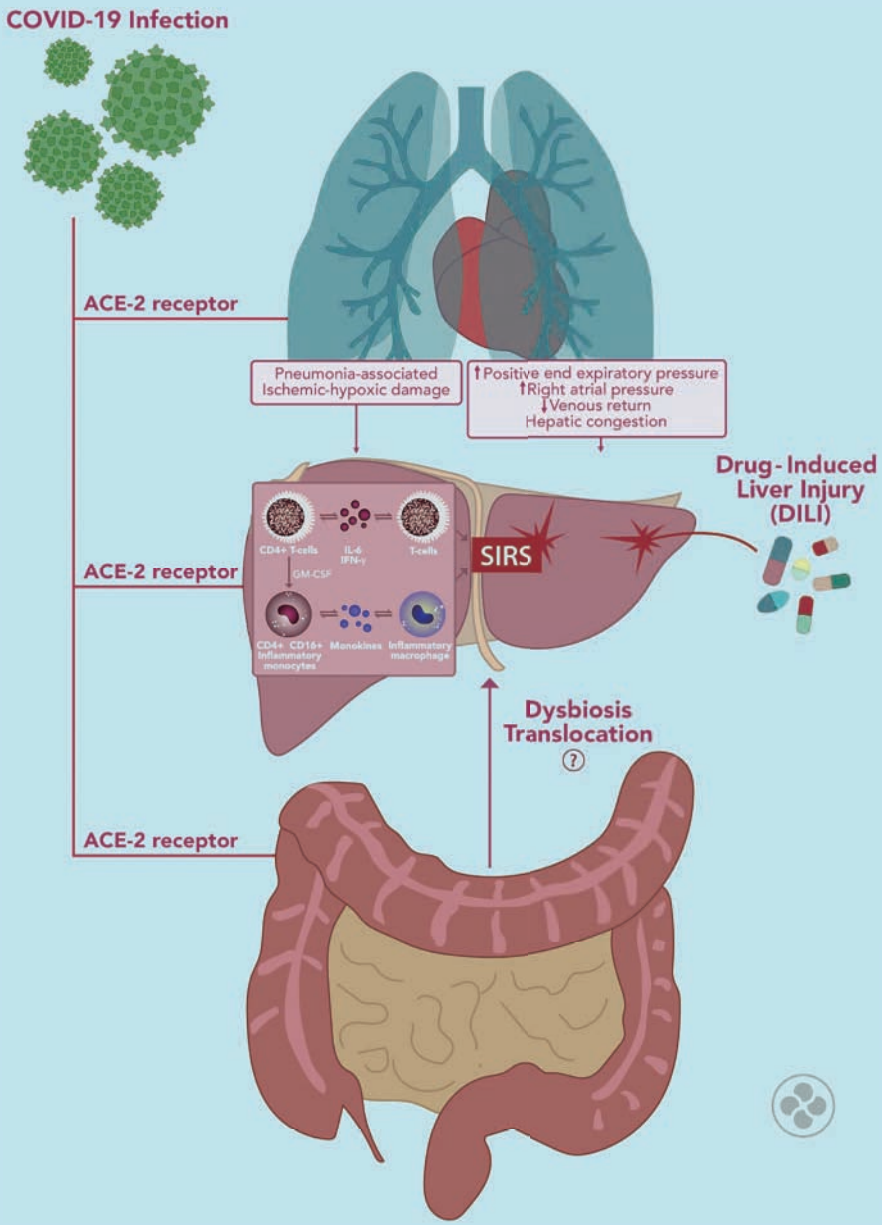

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Aims and Scope

Journal of Clinical and Translational Hepatology (JCTH, J Clin Transl Hepatol) publishes high quality, peer-reviewed studies in the clinical and basic human health sciences of liver diseases. *JCTH* welcomes submissions of articles within its topical scope including: novel discoveries in clinical and basic hepatology; liver disease mechanisms; novel techniques in research and management of liver diseases; epidemiological/environmental factors of liver diseases; role of immune system function in liver diseases; acute and chronic hepatitis; cirrhosis; genetic and metabolic liver diseases and their complications; hepatobiliary diseases; liver cancer; drug metabolism; biliary disease; peritoneal tuberculosis. *JCTH* publishes various types of articles, including original article, review, short communication, systematic review, meta-analysis, case report, methodology article, letter to the editor, and editorial.

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Editorial

One Year After First Spontaneous Bacterial Peritonitis: Who Survives?

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Spontaneous bacterial peritonitis (SBP) is defined as infection in ascites fluid without evidence of an intra-abdominal treatable source.[1] Diagnosis is confirmed by presence of ≥250 cells/mm^3 polymorphonuclear cells (PMNs).[2] Although SBP responds well to appropriate antibiotic treatment, in patients with underlying cirrhosis severe enough to develop SBP, long-term prognosis is poor. In-hospital non-infection-related mortality is 20–40% and 1- and 2-year mortality rates are 70% and 80%, respectively.[3]

In this issue of the journal, Wang *et al*.[4] report on development and validation of a prognostic model for 1-year survival in cirrhotic patients with first-ever SBP. In this study, SBP was defined based on Chinese guidelines on the management of ascites, 1) Patients must have at least one of the following: signs or symptoms of acute peritonitis; or signs or symptoms of acute inflammatory response syndrome, deteriorated liver function without obvious etiology, hepatic encephalopathy, shock, refractory ascites, sudden lack of response to diuretics, renal failure, or acute gastrointestinal bleeding. 2) Patients with at least one of following test abnormalities: ascitic fluid with PMNs ≥250 cells/mm^3; or positive ascites fluid culture; procalcitonin >0.5 ng/mL. It should be noted that this criterion is different from that of the American Association for the Study of Liver Diseases and European Association for the Study of the Liver guidelines.[2,5]

Etiology of the cirrhosis in both the derivation and validation cohorts was mainly hepatitis B infection. The goal of the study was to evaluate potential predictive variables that might be associated with long-term survival in cirrhosis with SBP and create a prediction model, which was then assessed in the validation cohort.

Independent predictors of mortality were hepatitis C, bilirubin, sodium, hypertension, and hepatic encephalopathy. These, along with age, were used to establish a nomogram. The nomogram was then used to estimate the probability of 1-year survival. Ultimately, this nomogram had a higher area under the curve (AUC) compared to Child-Turcotte-Pugh score or model for end-stage liver disease score in both the derivation and validation cohorts.

It is important to note that this study also included hypertension and diabetes as two very common comorbidities and assessed their effect on 1-year mortality. Although diabetes prevalence was only 22% in this cohort and information regarding presence of fatty liver, hyperlipidemia, obesity, and sleep apnea were not provided. These variables might be important, especially if this nomogram is going to be used in Western countries, which can have very different demographics and etiologies of liver disease. Other important risk factors that could affect mortality include cardiac function and presence of refractory ascites.[6]

It would be important to replicate such a study in a western cohort with mainly nonalcoholic steatohepatitis and alcohol-related liver disease as etiologies and significantly more prevalent risk factors, such as obesity, diabetes, and other features of metabolic syndrome.

Furthermore, it would be important to assess the relationship between certain types of bacteria and their resistance profile to mortality at 1 year. However, there were not many positive cultures and specifics on the type of bacteria in the current study. Given the rising incidence of multidrug-resistant bacteria in patients with SBP, consideration of resistance profiles and culture data might be helpful in decision-making for the empirical first-line treatment.[7]

Nevertheless, this was an important study to develop a prognostic tool for long-term survival of patients after first SBP and to include hypertension as a comorbidity affecting mortality.

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None to declare.

Conflict of interest

AR has been an editorial board member of *Journal of Clinical and Translational Hepatology* since January 2020.

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Abbreviations: PMNs, polymorphonuclear cells; SBP, Spontaneous bacterial peritonitis.

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Letter to the Editor

Characteristics and Outcome of Exertional Heatstroke Patients Complicated by Acute Hepatic Injury

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To the editor,

We have read the article titled "Characteristics and Outcome of Exertional Heatstroke Patients Complicated by Acute Hepatic Injury: A Cohort Study" by Ji *et al*.[1] We congratulate the authors for this insightful article. In this letter, we would like to raise several issues about the article to provide constructive criticisms.

Heatstroke involves a systemic inflammatory response, the full damage of which is not only limited to the hepatobiliary system. Instead, it can also cause damage to other organ systems, including the central nervous system, cardiovascular system, gastrointestinal system, hematological system, and immune system.[2] However, the authors do not seem to weigh multiple organ failure following heatstroke. This can be misleading to the readers, who may believe that development of acute liver failure (ALF) is common in patients with heatstroke. Bi *et al*.[3] reported that exertional heatstroke uncommonly progresses to ALF; therefore, ALF secondary to heatstroke is rare. Additionally, the authors define acute hepatic injury simply as a coagulopathy with the elevated international normalized ratio (INR), to which we cannot agree. Lack of sweat and impaired heat dissipation would represent hypovolemic conditions that increase blood viscosity and the risk of thrombosis leading to microvascular hypoperfusion.[4] During the coagulation response in heatstroke, hyperthermia typically occurs, damaging vascular endothelium and inducing microthrombosis and fibrin formation—the disseminated intravascular coagulation may clinically manifest.[5] Secondly, the authors seem to underestimate the significance of brain injury in the patients with severe heatstroke, evidenced by the use of the Glasgow Coma Scale only for evaluation in their study. According to Hifumi *et al*.,[2] brain injury in heatstroke can cause severe cerebral edema, an essential indicator of neurologic sequelae and death. Using an imaging modality, such as brain CT and MRI, should be considered for evaluating brain edema as a severity index of heatstroke.[6] Finally, we think that the criteria used for the infection group were inappropriate, i.e. procalcitonin elevation > 2 ng/mL and white blood cell count > 10×10^9 /L. Heatstroke is known as a systemic inflammatory syndrome that results in a sepsis-like condition.[7] The permeability of gastrointestinal epithelia may increase due to damaged tight junctions and cell membranes during the state of hyperthermia, splanchnic hypoperfusion, and hypoxia.[8] Subsequently, the barrier dysfunctions in the gastrointestinal tract tend to result in the translocation of bacterial endotoxin or complete microorganisms from the intestinal epithelium to the bloodstream that may favor the occurrence of a systemic inflammatory response.[9,10] These inflammatory mechanisms can lead to elevated serum procalcitonin levels in the patients but without evidence of bacterial infection.[11] However, among the typical heatstroke patients, the association between elevated serum procalcitonin level and bacterial infection is not clear. Therefore, the authors' criteria for the infection group are misleading in that elevated procalcitonin level alone does not indicate the infectious state and is not a risk factor for mortality in heatstroke.

We hope that our clarification would assist the readers in obtaining a correct understanding of the essential roles of the liver in the development of heatstroke.

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Conflict of interest

The authors have no conflict of interests related to this publication.

Author contributions

Study design (SC, HK), drafting of the manuscript (HY), revision of the manuscript (SC, HY). All authors approved the final version of the manuscript.

Abbreviations: ALF, acute liver failure.

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Original Article

Metabolic Disorders Combined with Noninvasive Tests to Screen Advanced Fibrosis in Nonalcoholic Fatty Liver Disease

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Abstract

Background and Aims: Nonalcoholic fatty liver disease (NAFLD) is associated with metabolic disorders. This study aimed to explore the role of metabolic disorders in screening advanced fibrosis in NAFLD patients. ***Methods:*** A total of 246 histologically-proven NAFLD patients were enrolled across 14 centers. We compared the severity of fibrosis in patients with different components of metabolic disorders. Based on standard noninvasive tests and metabolic disorders, we developed new algorithms to identify advanced fibrosis. ***Results:*** Metabolic syndrome (MetS) was frequent in NAFLD patients (133/246, 54%). Patients with MetS had a higher proportion of significant fibrosis (p=0.014) and higher LSM values (9.2 kPa, vs. 7.4 kPa, p=0.002) than those without MetS. Patients with more metabolic disorders had higher fibrosis stages (p=0.017). Reduced high-density lipoprotein cholesterol (odds ratio [OR]: 2.241, 95% confidence interval [CI]: 1.004–5.002, p=0.049) and raised fasting glucose (OR: 4.500, 95% CI: 2.083–9.725, p<0.001) were significantly associated with advanced fibrosis. Using these two metabolic disorders as a screening tool, a sensitivity, specificity and accuracy of 92%, 81% and 83% was achieved, respectively. With the new algorithms combining metabolic disorders with noninvasive measurements, the number of patients requiring liver biopsy was reduced, especially in combination with the Fibrosis-4 score and metabolic disorders (36% to 17%, p<0.001). In addition, this stepwise algorithm could achieve a high accuracy (85%) and high negative predictive value (93%). ***Conclusions:*** Metabolic disorders should be taken into consideration in the diagnosis of advanced fibrosis. With further validation and investigation, new algorithms could be recommended in primary care units to spare patients from unnecessary referral and liver biopsies.

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Keywords: Nonalcoholic fatty liver disease; Liver fibrosis; Metabolic syndrome; Noninvasive measurement.

Abbreviations: APRI, aspartate aminotransferase to platelet ratio index; AUROC, Area under the ROC curve; BMI, body mass index; CI, confidence interval; FIB-4, fibrosis-4 score; FPG, fasting plasma glucose; HDL-C, high-density lipoprotein cholesterol; HOMA-IR, homeostasis model assessment-insulin resistance; LSM, liver stiffness measurement; MAFLD, metabolic dysfunction-associated fatty liver disease; MetS, metabolic syndrome; NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; NFS, NAFLD fibrosis score; ORs, odds ratios; SAF, steatosis, activity score and fibrosis stages; T2DM, type 2 diabetes mellitus; TG, triglyceride.

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Introduction

Nonalcoholic fatty liver disease (NAFLD) is closely associated with the features of metabolic syndrome (MetS), including insulin resistance, hyperglycemia and dyslipidemia. Since the global epidemic of obesity has increased metabolic dysfunction, the health burden of NAFLD is becoming enormous.[1] A study from the Third National Health and Nutrition Examination Survey database reported that 95% of NAFLD patients had type 2 diabetes mellitus (T2DM), obesity or other metabolic abnormalities.[2] Approximately 43% of patients with MetS had NAFLD.[3] There were also opinions that NAFLD was a representation of MetS in the liver.

It has been reported that overweight/obese NAFLD patients have more severe histological features, including higher fibrosis scores, than lean patients.[4] Among these, metabolically unhealthy obese patients had a significantly higher prevalence of advanced liver fibrosis (F3–F4) than metabolically healthy obese ones.[5] Even in nonobese patients with NAFLD, metabolic-related diseases were also common. Nonobese NAFLD patients had impaired glucose tolerance, low adiponectin concentrations and a distinct metabolite profile compared with patients without steatosis.[6] NAFLD has a universal association with insulin resistance, which plays an essential role in the development of steatohepatitis and fibrosis.

Individuals with raised fasting glucose or T2DM and other metabolic abnormalities of MetS have an increased risk of advanced fibrosis.[3] We know that advanced fibrosis is directly associated with liver-related events.[7] Therefore, metabolic abnormalities could predict a worse long-term prognosis in NAFLD patients. Hence, metabolic disorders should be considered in screening fibrosis. Currently, the application of noninvasive diagnostic measurements in clinical practice is still insufficient. The diagnostic performance, accessibility and cost-effectiveness all need to be improved. Thus, this study aimed to explore the role of metabolic disorders in screening for liver fibrosis.

Methods

Study design and population

This was an observational, multicenter, cross-sectional registry study that enrolled patients with liver biopsy-proven NAFLD in 14 participating sites across mainland China from July 4, 2016 to August 9, 2018. The procedures of this study were in accordance with the ethical standards of the responsible committee on human experimentation and conformed to the ethical guidelines of the latest version of the Declaration of Helsinki. This retrospective study did not involve any sensitive patient data, so informed consent was not required. The protocol of the study was registered at http://www.chictr.org.cn (ChiCTR-OOC-16007902).

Patients were included if they (a) were aged 18–65 years, (b) had received liver biopsy within 6 months before enrollment and biopsy sample could be collected for re-evaluation, and (c) had ≥5% hepatic steatosis on liver biopsy and were diagnosed with NAFLD. Patients were excluded if they (a) had other chronic liver diseases, including viral hepatitis, alcoholic liver diseases, toxic liver damage, autoimmune liver disease, drug-induced liver injury, Wilson's disease and other genetic liver diseases, (b) had significant alcohol consumption of >140 g/week for men or >70 g/week for women within the past 12 months, (c) had end-stage liver disease, such as decompensated cirrhosis or liver cancer, or (d) were pregnant or breastfeeding.

Data collection, laboratory, imaging and liver biopsy examination

Demographic and anthropometric characteristics, medical history and metabolic disorders were collected at enrollment. Weight and height were used to calculate body mass index (BMI=weight/height^2). Medical history was recorded in detail, including comorbid diseases, alcohol consumption, smoking status and comedications. Clinical and laboratory information of the patients, including blood biochemical parameters, was obtained within 1 week before or after liver biopsy. The cardiovascular disease risk score was calculated according to the Framingham general risk score algorithm (2008). The controlled attenuation parameter and liver stiffness measurement (LSM) were performed within 1 week of biopsy using the FibroScan 502 instrument (Echosens, Paris, France).

Liver biopsy samples of eligible patients were collected for histopathological rereading. The biopsy specimens were stained with hematoxylin and eosin, reticulin, and Masson's trichrome. Pathologists at each site read biopsy slices in terms of steatosis, activity score and fibrosis stages (SAF) and provided a standard report according to the Fatty Liver Inhibition of Progression (commonly known as FLIP) Algorithm.[8] The diagnosis of NAFLD was based on the EASL-EASD-EASO Clinical Practice Guidelines for the management of NAFLD (2016).[9] Nonalcoholic steatohepatitis (NASH) was defined as the presence of steatosis with inflammation and ballooning. Significant fibrosis was defined as fibrosis stage ≥F2, while advanced fibrosis was defined as stage F3 and F4.[8] Liver biopsies were also used to differentiate other liver diseases in patients. The study protocol was published previously.[10] Qualified researchers may request access to patient-level data and related study documents.

Definitions of metabolic disorders and noninvasive fibrosis tests

MetS consisted of central obesity plus any two of the following metabolic disorders: elevated triglyceride (TG), reduced high-density lipoprotein cholesterol (HDL-C), elevated blood pressure and raised fasting glucose, according to the guideline from the International Diabetes Foundation (2005).[11] Central obesity was defined as increased waist circumference, with thresholds of ≥90 cm in men and ≥80 cm in women. Elevated TG was defined as fasting TG ≥150 mg/dL or being on TG therapy. Reduced HDL-C was defined as <40 mg/dL in men and <50 mg/dL in women or being on HDL-C therapy. Elevated blood pressure was defined as ≥130/85 mm Hg or being on hypertension therapy. Raised fasting glucose was defined as ≥100 mg/dL or previously diagnosed T2DM. Insulin resistance was defined as homeostasis model assessment-insulin resistance (HOMA-IR) score ≥2.5, calculated as fasting insulin (mU/L) × fasting glucose (mmol/L)/22.5.

NAFLD fibrosis score (NFS) was calculated as: −1.675+0.037×age (years)+0.094×BMI (kg/m^2) +1.13×impaired fasting glycemia or diabetes (yes=1, no=0) +0.99×aspartate aminotransferase/alanine aminotransferase ratio-0.013×platelet (×10^9/L)−0.66×albumin (g/dL). The cut-off value of −1.455 was used to rule-out advanced fibrosis with 90% sensitivity, and 0.676 was used to rule-in advanced fibrosis with 90% specificity.[12] Fibrosis-4 (FIB-4) was calculated as age×aspartate aminotransferase (U/L)/platelet (×10^9/L) ×alanine aminotransferase$^{1/2}$ (U/L). Two diagnostic cut-offs, namely 1.30 and 3.25, corresponding to the 90% sensitivity and 90% specificity thresholds, were also used to diagnose advanced fibrosis.[13] We used 7.9 kPa as a cut-off

of LSM to exclude advanced fibrosis and 9.6 kPa to diagnose advanced fibrosis according to the published data.[14]

Statistical analysis

The database for final analysis was locked on December 5, 2018. Statistical analyses were performed using SPSS software (version 25.0; IBM Corp., Armonk, NY, USA). The comparison of LSM values between groups with different numbers of metabolic disorders was carried out by one-way ANOVA. Univariate and multivariate binary logistic regression analyses were applied to define risk factors for advanced fibrosis. All related factors were calculated in the multivariate model using the forward stepwise (conditional) method. Odds ratios (ORs) and 95% confidence intervals (CIs) were calculated to evaluate the effect.

We used receiver operating characteristic curves (commonly known as ROCs) to assess the accuracy and to identify optimal cut-offs. The area under the ROC curve (commonly known as AUROC), diagnostic OR and diagnostic accuracy were calculated to assess the overall diagnostic performance. In the new stepwise algorithms, two cut-off values of each noninvasive assessment measurement were applied to determine advanced fibrosis. The second step was applied for the patients in the "gray zone" of the first step, and the patients in the final "gray zone" would then be recommended for a liver biopsy. Overall diagnostic indexes, including sensitivity, specificity, positive predictive value, negative predictive value, positive likelihood ratio and negative likelihood ratio, were calculated.

Results

Demographic and clinical characteristics of NAFLD patients

Eligible biopsy samples were obtained from 250 patients enrolled in the study. Two patients were excluded for alcohol abuse, and another two patients were excluded for insufficient steatosis of less than 5% during histological re-examination. Finally, 246 patients with histologically-proven significant steatosis were diagnosed with NAFLD and included in the final analysis. Approximately 61% (151/246) of patients had moderate or severe steatosis, and 84% (207/246) of patients had NASH. The median SAF score was 5 (interquartile range: 4, 7). A total of 76 (31%) patients had significant fibrosis, and 38 (15%) patients had advanced fibrosis.

Metabolic disorders were frequent among the patients in this study. Approximately 76% (178/234) of patients had central obesity, and more than half (133/246, 54%) of the patients met the criteria for MetS. Patients with MetS had higher BMI, higher waist circumference and higher hip circumference (all $p<0.001$). These patients also had a higher proportion of hypertension and T2DM ($p<0.001$ and $p=0.001$, respectively). It was also not surprising that patients with MetS appeared to have worse metabolic status, including fasting plasma glucose (FPG), glycosylated hemoglobin, HOMA-IR, TG and HDL-C (Table 1).

Metabolic disorders were associated with liver fibrosis

NAFLD patients with MetS showed more severe fibrosis. Patients in the MetS group had a significantly higher proportion of significant fibrosis than those without MetS (50 cases, 38% vs. 26 cases, 23%, $p=0.014$). The proportion of advanced fibrosis in patients with MetS (23/133, 17%) was also higher than that in patients without MetS (15/113, 13%) but there was no significant difference ($p=0.245$). Patients with MetS had higher overall LSM values than patients without MetS (9.2, 7.2–13.2 kPa vs. 7.4, 5.5–10.1 kPa, $p=0.002$) and similar IQR values (1.2, 0.7–2.2 vs. 0.8, 0.6–1.5, $p=0.174$).

When compared from the perspective of the numbers of metabolic disorders included within MetS (central obesity, elevated TG, reduced HDL-C, elevated blood pressure and raised FPG according to the diagnosis criteria), patients with more disorders had significantly more severe fibrosis ($p=0.017$; Fig. 1). In patients without any of the metabolic disorders, no patients had advanced fibrosis. In patients with only one metabolic disorder, there were no cirrhotic patients. In patients with five metabolic disorders, 36% had advanced fibrosis. We also compared the LSM values among patients with different numbers of metabolic disorders. NAFLD patients with more metabolic disorders had significantly higher LSM values ($p<0.001$; Fig. 1). In addition, the number of disorders showed a linear correlation with the LSM values ($p=0.005$).

Reduced HDL-C levels and raised fasting glucose were risk factors for fibrosis

To determine which of the metabolic disorders were significant risk factors for advanced fibrosis, we performed univariate analysis and multivariate analysis among the five components of MetS. Raised FPG (OR: 4.500, 95% CI: 2.083–9.725, $p<0.001$) and reduced HDL-C (OR: 2.241, 95% CI: 1.004–5.002, $p=0.049$) were the most important risk factors (Table 2). Approximately 28% (27/98) of patients with raised FPG and 19% (27/141) of patients with reduced HDL-C had advanced fibrosis.

Next, we used the two metabolic disorders as risk factors to screen advanced fibrosis. A total of 66 patients had neither of these metabolic disorders, 121 patients had either reduced HDL-C levels or raised fasting glucose, and 59 patients had both. The proportion of significant fibrosis and advanced fibrosis was significantly different among the three groups (both $p<0.001$). There was also a trend towards a higher proportion of cirrhosis in patients with more metabolic disorders (1 case vs. 2 cases vs. 3 cases, $p=0.319$). Patients with more metabolic disorders also had higher LSM values (7.3, 5.3–9.5 kPa; 9.0, 6.6–12.1 kPa; and 10.0, 7.0–14.2 kPa, respectively, $p<0.001$; Table 3).

New algorithms improved the diagnostic performance of advanced fibrosis

We then used these metabolic disorders as a screening tool for advanced fibrosis. Patients with both raised FPG plus reduced HDL-C were ruled-in to the consideration of a diagnosis of advanced fibrosis; patients with neither of these were ruled out and patients with either of these were considered in the gray zone. This new diagnostic tool (MetDis) could achieve a sensitivity, specificity and accuracy of 92%, 81% and 83%, respectively. We also evaluated the diagnostic performance of three standard noninvasive tests at their best Youden's index to identify advanced fibrosis (Supplementary Table 1). This new algorithm had significantly better diagnostic performance than LSM ($p<0.001$; Table 4).

We also combined metabolic disorders with standard noninvasive tests using their published cut-offs to form several new stepwise algorithms (Supplementary Table 2). The specificity of the new algorithms was also improved com-

Table 1. Clinical characteristics between patients with and without metabolic syndromes

Variable	Patients with MetS	Patients without MetS	*p*
Number of patients	133	113	–
Age in years	42±13	38±12	0.037
Male, %	89, 67%	88, 78%	0.057
Body mass index in kg/m^2	28.9±4.2	25.6±3.2	<0.001
Smoking, %	20,15 %	10, 9%	0.146
Alcohol intake, %	48, 36%	46, 41%	0.458
Hypertension, %	42, 32%	14, 12%	<0.001
Type 2 diabetes mellitus, %	33, 25%	10, 9%	0.001
Dyslipidemia, %	35, 26%	22, 19%	0.205
Coronary heart disease, %	6, 5%	0, 0%	0.022
Cerebrovascular disease, %	1, 1%	1, 1%	0.908
Chronic kidney diseases, %	5, 4%	1, 1%	0.145
Waist circumference in cm	99.4±10.0	89.0±9.9	<0.001
Hip circumference in cm	104.9±9.3	98.3±11.2	<0.001
Platelets as 10^9/L	237±68	231±70	0.508
ALT in U/L	55 (31, 102)	61 (32, 109)	0.972
FPG, mmol/L	5.9±1.8	5.1±0.9	<0.001
HbA1c, %	6.37±1.69	5.64±0.70	<0.001
TG in mmol/L	1.90 (1.44, 2.62)	1.42 (1.06, 2.05)	<0.001
Total cholesterol in mmol/L	4.91±1.12	4.91±1.01	0.943
HDL-C in mmol/L	1.01±0.22	1.13±0.24	<0.001
Low-density lipoprotein-cholesterol in mmol/L	3.10±0.81	3.18±0.84	0.497
HOMA-IR	3.8 (2.6, 5.6)	2.4 (1.7, 3.5)	<0.001
eGFR in mL/min per 1.73m^2	107±17	108±17	0.702
CVD risk score	9 (5, 14)	4 (0, 10)	<0.001

ALT, alanine aminotransferase; CVD, cardiovascular disease; eGFR, estimated glomerular filtration rate; FPG, fasting plasma glucose; HbA1c, glycated hemoglobin; HOMA-IR, homeostasis model assessment-insulin resistance; MetS, metabolic syndrome.

pared to the use of only one noninvasive measurement. Using these stepwise algorithms, the number of patients requiring liver biopsy was significantly reduced (Fig. 2), and liver biopsy could be reduced from 36% to 17% (p<0.001). Among these, FIB-4-MetDis had better diagnostic performance than FIB-4 alone (66%, p<0.001). It provided the highest accuracy (85%), highest positive likelihood ratio (5.92) and a high negative predictive value, which could avoid unnecessary liver biopsy (Table 4). Therefore, we recommend evaluating metabolic disorders after calculating the FIB-4 score for patients with hepatic steatosis found incidentally in the primary care unit (Fig. 3).

Discussion

In this study, we demonstrated the association between metabolic disorders and the severity of liver fibrosis in patients with NAFLD, and developed new algorithms combining metabolic disorders with noninvasive measurements to improve the diagnostic performance of advanced fibrosis. It is well known that liver biopsy is invasive, with an accompanying 1% risk of serious complications and an approximately 0.2% risk of mortality. FIB-4-MetDis could reduce the need for liver biopsy due to its high negative predictive value at the current study. Therefore, we recommend FIB-4-MetDis to screen advanced fibrosis in patients with NAFLD, which is available in most primary care units. The combination of metabolic disorders and noninvasive assessment is simple for clinicians to use to make a quick judgment.

The liver is the main organ that handles the excess burden of energy overload. NAFLD was even recognized as being among the spectrum of MetS. Recently, an international panel of experts suggested the nomenclature of metabolic dysfunction-associated fatty liver disease (i.e. MAFLD)[15] and issued guidelines[16] to characterize the disease and call attention to metabolic dysfunctions. In the current study, most of the patients (239/246, 97%) met the criteria of MAFLD. Compared to obesity or increased BMI, metabolic disorders may represent the most significant characteristic of NAFLD. We speculate that the severity of NAFLD, including clinical characteristics and pathological stages, reflects the severity of metabolic status in the liver. Previous studies revealed that as the number of metabolic abnormalities increased, the hepatic steatosis grades also increased in NAFLD,[3] which was similar to our findings. Thus, these metabolic disorders may act as predictors of the severity of NAFLD.

Raised FPG and insulin resistance are the most important features of metabolically unhealthy individuals and are

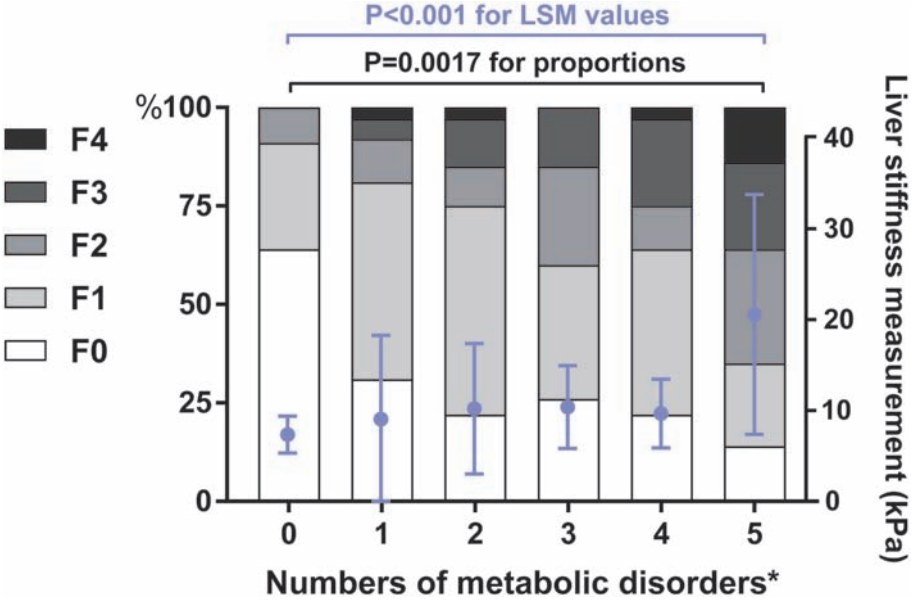

Fig. 1. Association between fibrosis stages and LSM with numbers of metabolic disorders. *Metabolic disorders referred to five components of metabolic disorders (IDF 2005): central obesity, raised blood pressure, reduced high-density lipoprotein cholesterol, raised triglyceride and raised fasting plasma glucose. LSM, liver stiffness measurement.

widespread in NAFLD patients.[17,18] NAFLD patients with diabetes and insulin resistance are at higher risk of developing advanced fibrosis and liver complications.[19,20] Insulin resistance also interacts with advanced fibrosis. Reduced glycogen synthesis and defects in glucose oxidation in cirrhotic patients further promote the development of impaired glucose tolerance and insulin resistance.[21] Among the current noninvasive models, other related indexes, such as blood glucose, hyperglycemia or diabetes, were also used to diagnose fibrosis according to the BARD score, NFS, FibroMeter and FIB-C3. Another risk factor for advanced fibrosis is reduced serum levels of HDL-C. Dyslipidemia is rather common in NAFLD patients.[22] The majority of hepatic fatty acids are from adipose tissue lipolysis, which is promoted by insulin resistance. HDL-C levels are also related to the severity of fibrosis.[23] Abnormal cholesterol metabolism could directly drive hepatic stellate cell activation, which promotes collagen secretion and fibrogenesis.[24] In addition, several lipid-regulating agents have been shown to improve liver fibrosis.[25] Although HDL-C is not often included in noninvasive tests, dyslipidemia also provides a clue for detecting fibrosis.

With the pandemic of metabolic-associated diseases, the prevalence of NAFLD is rapidly increasing in the past dec-

Table 2. Metabolic factors associated with advanced fibrosis

Variable	Univariate analysis		Multivariate analysis	
	OR, 95% CI	*p*	OR, 95% CI	*p*
Central obesity	0.705 [0.291, 1.706]	0.438		
Raised FPG	4.736 [2.221, 10.101]	<0.001	4.500 [2.083, 9.725]	<0.001
Raised BP	0.906 [0.452, 1.817]	0.781		
Raised TG	0.548 [0.271, 1.110]	0.095		
Reduced HDL-C	2.204 [0.954, 4.295]	0.066	2.241 [1.004, 5.002]	0.049

BP, blood pressure; CI, confidence interval; FPG, fasting plasma glucose; HDL-C, high density lipoprotein cholesterol-C; OR, odds ratio; TG, elevated triglyceride.

Table 3. Distribution of fibrosis stages in patients with different metabolic disorders

Metabolic disorders*	None	Either	Both	*p*
n	66	121	59	–
Significant fibrosis, %	11, 17%	34, 28%	31, 53%	<0.001
Advanced fibrosis, %	3, 5%	16, 13%	19, 32%	<0.001
Cirrhosis, %	1, 2%	2, 2%	3, 5%	0.319
LSM in kPa	7.3 (5.3, 9.5)	9.0 (6.6, 12.1)	10.0 (7.0, 14.2)	<0.001

*Reduced high-density lipoprotein cholesterol or raised fasting plasma glucose. LSM, liver stiffness measurement.

Table 4. Diagnostic performance of new algorithms combined with metabolic factors

Diagnostic algorithm	Accuracy, %	Sensitivity, %	Specificity, %	PPV, %	NPV, %	LR+	LR-	DOR	*p*
MetDis	82.52	92.11	80.77	46.67	98.25	4.79	0.10	49.0	<0.001[a]
MetDis-LSM	65.42	95.49	62.25	62.25	95.49	2.53	0.07	8.2	0.692[a]
LSM-MetDis	69.66	86.11	66.67	31.96	96.35	2.58	0.21	12.4	0.170[a]
MetDis-NFS	79.27	71.05	80.77	40.30	93.85	3.69	0.36	10.3	0.101
NFS-MetDis	84.02	48.78	91.13	52.63	89.81	5.5	0.56	9.8	0.003[b]
MetDis-FIB-4	80.08	81.58	79.81	42.47	95.95	4.04	0.23	17.5	<0.001[c]
FIB-4- MetDis	85.31	65.79	88.89	52.08	93.40	5.92	0.38	15.4	<0.001[c]

[a]Comparison of accuracy with LSM; [b]Comparison with NFS; [c]Comparison with FIB-4. MetDis-LSM, evaluating metabolic disorders (reduced HDL-C or raised FPG) as a first step, and use LSM as second step; LSM-MetDis is opposite. MetDis-NFS, evaluating metabolic disorders as a first step, and use NFS as second step; NFS-MetDis is opposite. MetDis-FIB-4, evaluating metabolic disorders as a first step, and use FIB-4 as second step; FIB-4- MetDis is opposite. DOR, diagnostic odds ratio; LR+, positive likelihood ratio; LR-, negative likelihood ratio; NPV, negative predictive value; PPV, positive predictive value.

ades. An important issue concerning clinical practice is the diagnostic performance of noninvasive measurements for advanced fibrosis. Serum-based models are more available in primary medical centers. The aspartate aminotransferase to platelet ratio index (commonly known as APRI), FIB-4 and BARD score indexes could be collected from routine tests and are easily calculated. However, these models lack sufficient sensitivity to rule in advanced fibrosis.[26] Other serum-based models consisting of special indicators (i.e. Pro-C3, PIIINP, or TIMP-1) and patents (e.g., FibroMeter) have limitations in their application due to accessibility. Image-based noninvasive measurements are more sensitive in detecting advanced fibrosis. These tests have a rather high negative predictive value for ruling out advanced fibrosis.[27] In this situation, sequential combinations of noninvasive measurements could provide a solution.

Our study has the strengths of a well-established design with all the data monitored by experienced groups. The tissue biopsy slices from each patient were reread by pathologists, which guaranteed an accurate evaluation of the characteristics of NAFLD and exclusion of other liver diseases. However, there were still several limitations. First, the sample size was relatively small. Patients in this study were all collected from tertiary medical centers. The information of the patients was collected mainly from in-patient medical reports within 6 months to reduce recall bias. Complete data ensured the quality of the research. Given an expanded sample size, the power of the conclusion could be enhanced. Second, we did not follow-up with the patients to observe long-term prognosis and liver-related events. It

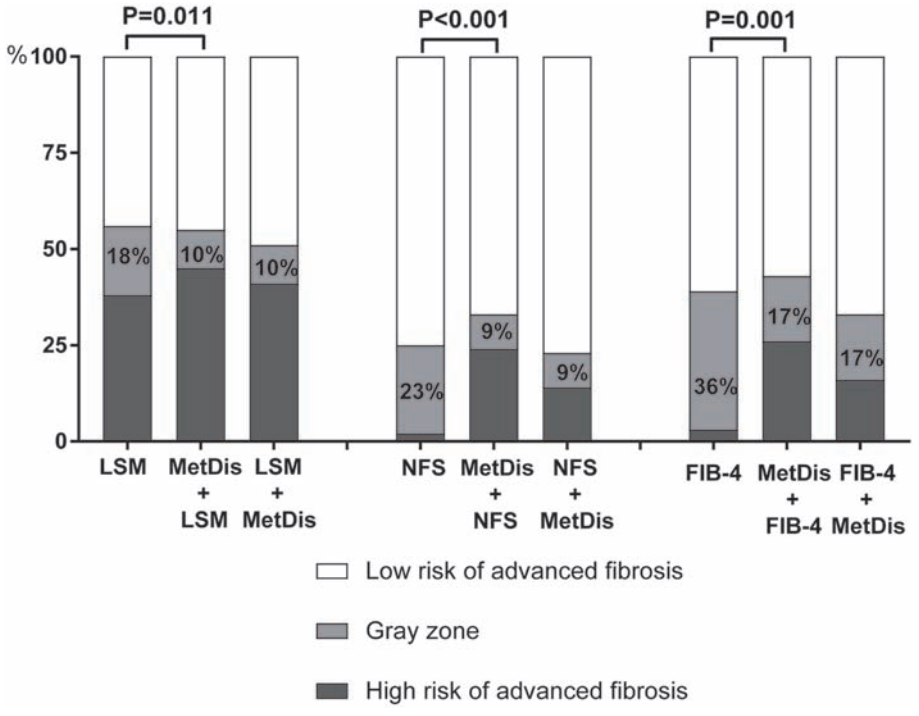

Fig. 2. Distribution of patients when using different diagnostic algorithms. Rule-in, patients met the criteria to diagnose advanced fibrosis according to published cut-off; Rule-out, patients met the criteria to exclude advanced fibrosis according to published cut-off; Gray zone, undiagnosed patients in the middle of the criteria, needed to be further examined, for instance, liver fibrosis. FIB-4, fibrosis-4 score; LSM, liver stiffness measurement; MetDis, metabolic disorders; NFS, NAFLD fibrosis socre.

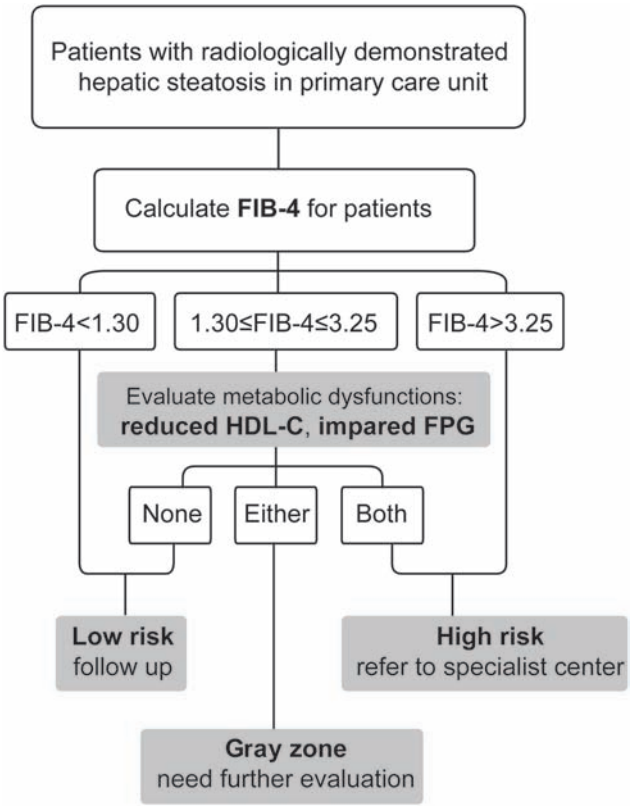

Fig. 3. Diagnostic flow-chart to monitor risk of advanced fibrosis. Combining FIB-4 test and metabolic disorders in monitoring advanced fibrosis. Low risk: follow-up every 2 years, according to EASL guideline; Gray zone: further evaluation included other non-invasive tests, liver stiffness measurement, magnetic resonance elastography, even liver biopsy, or specialist referral; High risk: refer to specialist to evaluate disease severity and identify other potential liver diseases. FIB-4, fibrosis-4 score; FPG, fasting plasma glucose; HDL-C, high-density lipoprotein cholesterol.

has been reported that overweight status and T2DM are key determinants of fibrosis progression.[28] Thus, in further studies, we could focus on the role of raised FPG and reduced HDL-C in the development of liver fibrosis and cirrhosis.[29]

In conclusion, metabolic disorders contributed to the severity of fibrosis in NAFLD patients, which should be taken into consideration during diagnosis and management. New combinations of metabolic disorders with noninvasive measurements provided a more accurate diagnosis for advanced fibrosis. With further validation in external cohorts, this algorithm could be recommended as a first-line screening of advanced fibrosis in primary care units.

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Conflict of interest

BP is an employee of Sanofi. None of the other authors have any potential or real conflicts of interest to declare. The authors did not receive any payment for authoring this publication.

Author contributions

Study concept and design (BP, JGF), acquisition of data (FPH, JJC, HD, JPS, CYZ, YQM, ZSZ, YJZ, FSD, RDZ, QD, JS, RXY, BHZ, JGF), analysis and interpretation of data (YWS, JGF), drafting of the manuscript (YWS, JGF), critical revision of the manuscript for important intellectual content (JGF). All authors confirmed critical revision of the manuscript for important intellectual content.

Data sharing statement

No additional data are available.

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Original Article

Significant Histologic Changes Are Not Rare in Treatment-naive Hepatitis B Patients with Normal Alanine Aminotransferase Level: A Meta-analysis

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Abstract

Background and Aims: Chronic hepatitis B is the main cause of liver cancer. However, the most neglected group has been treatment-naive chronic hepatitis B patients with normal alanine aminotransferase (ALT). People have tended to subjectively assume that the liver lesions of these patients are not serious and do not need antiviral treatment. However, the truth is not as optimistic as we thought. We aimed in this study to analyze the proportion of significant inflammation or fibrosis in aforementioned patients. ***Methods:*** Medline, Embase, and Cochrane Library were searched up to January 10th 2020, to identify studies of these patients with liver biopsy. The double arcsine method was used with a random-effect model to combine the proportion of significant inflammation or fibrosis. Potential heterogeneity was explored by subgroup analysis and meta-regression. Outcome of interests included the proportion of significant inflammation or fibrosis and cirrhosis. The secondary outcome was to find the risk factors of significant histological changes. ***Results:*** Nineteen eligible studies, with 2,771 participants, were included. The pooled proportion of significant inflammation or fibrosis was 35% [95% confidence interval (CI): 27 to 43] and 30% (95% CI: 25 to 36), respectively. The pooled proportion of cirrhosis was 3% [95% CI: 1 to 5, (12 studies; 1,755 participants)]. In subgroup analysis, old age [vs. young (<40 years-old), 44% vs. 26%, p=0.012] was significantly associated with higher fibrosis stage as well as cirrhosis [vs. young (<40 years-old), 4.8% vs. 1.8%, p<0.001]. ***Conclusions:*** About 1/3 of the treatment-naive chronic hepatitis B patients with normal ALT show significant histological changes, and some even have cirrhosis.

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Introduction

Chronic hepatitis B CHB) infection remains an important global public health problem. Hepatitis B surface antigen (HBsAg) seroprevalence is about 3.61% all over the world, of which about 240 million people are chronically infected.[1]

Current CHB practice guidelines from the American Association for the Study of Liver Diseases (AASLD), European Association for the Study of the Liver (EASL) and Asian Pacific Association for the Study of the Liver (commonly known as the APASL) stratify patients using serum tests for alanine aminotransferase (ALT), HBV DNA and hepatitis B e antigen (HBeAg) to evaluate the need for liver biopsy or antiviral therapy.[2–4] According to the current recommendations of the aforementioned guidelines,[2–4] treatment and liver biopsy are not recommended in CHB patients with normal ALT (except for special cases, such as liver cirrhosis, hepatitis C or human immunodeficiency virus infection, tumor chemotherapy, etc.), regardless of HBeAg status and HBV DNA level. However, recently, numerous studies have shown that there are varying degrees of moderate and severe inflammation or significant fibrosis, and even liver cirrhosis in patients with CHB whose ALT remains normal. The proportion of severe inflammation ranges from 4% (6/140)[5] to 63% (60/95),[6] while the proportion of significant fibrosis ranges from 9% (10/113)[7] to 56% (63/113)[8] and the proportion of liver cirrhosis ranges from 0% (0/140)[5] to 19% (22/113).[8]

All aforementioned guidelines suggest the need for antiviral treatment for moderate and severe inflammation or fibrosis. Therefore, it is necessary to summarize the proportion of significant histological changes in CHB patients with normal ALT, so as to adjust the indications for antiviral therapy and liver biopsy. In addition, an American population-based study (including 39,206 people)[9] found that

Keywords: Significant histologic changes; Chronic hepatitis B; Normal ALT; Meta-analysis.

Abbreviations: AASLD, American Association for the Study of Liver Diseases; ALT, alanine aminotransferase; APASL, Asian Pacific Association for the Study of the Liver; AST, aspartate aminotransferase; BMI, body mass index; CHB, chronic hepatitis B; CI, confidence interval; EASL, European Association for the Study of the Liver; HBeAg, hepatitis B e antigen; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; NOS, Newcastle–Ottawa scale; PLT, platelet; Tbil, total bilirubin; ULN, upper limit of normal.

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the mortality of adults with CHB was still higher than that of uninfected patients, despite improved treatment. Those with chronic infection had 1.9-fold [95% confidence interval (CI): 1.1 to 3.3] and 13.3-fold (95% CI: 3.9 to 45.5) increased hazard of all-cause mortality and liver-related mortality compared to uninfected patients. In order to improve the survival rate of patients with CHB, it is necessary to start antiviral therapy in eligible patients.

The primary goal of this study was to identify the proportion of significant hepatic inflammation or fibrosis and cirrhosis in CHB patients with normal serum ALT levels. The secondary goal was to identify possible indications of significant histological changes.

Methods

This systematic review and meta-analysis was reported in accordance with the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) statement[10] and MOOSE check list (Supplementary Tables 7, 8), and was registered at International Prospective Register of Systematic Reviews (PROSPERO number: CRD42020164923).

Search strategy and selection criteria

Medline, Embase, and the Cochrane Central Register of Controlled Trials databases were searched from inception to January 10th, 2020 using the following keywords: "chronic hepatitis B", "liver biopsy", and "alanine aminotransferase". Two reviewers independently screened the potential publication titles and abstracts, and reviewed the full-text of the eligible articles. In addition, if two or more studies were published based on the same data, the article with the highest quality was included.

The selected studies met the following inclusion and exclusion criteria:

Inclusion criteria were definite diagnosis of treatment-naive chronic hepatitis B. CHB patients with normal ALT, and available liver biopsy data (inflammation grade or fibrosis stage).

Exclusion criteria were sample size less than 50 CHB patients, patients with other forms of chronic viral hepatitis (hepatitis C virus, hepatitis D virus, or human immunodeficiency virus co-infection) and other chronic liver diseases (autoimmune, genetic, drug-induced etc.), patients with liver cancer or liver transplantation, reviews, editorials, letters, guidelines, and protocol type publications, or language other than English (Supplementary Table 1).

Data extraction

Two authors (CZ and ZW) independently reviewed each included paper using a standardized form for extraction of data including basic patient information [e.g., author's name, publication year, study design, country, age, sex, sample size, body mass index (BMI)], clinical data [e.g., HBV DNA, HBeAg status, ALT, aspartate aminotransferase (AST), γ-glutamyl transpeptidase, albumin, total bilirubin (Tbil), platelet (PLT)], and pathological data (e.g., inflammation grade, fibrosis stage, pathological scoring system). Any discrepancies were resolved by discussion by the senior investigators (HZ, GQW).

Quantitative variables were expressed as the mean±standard deviation and categorical variables were demonstrated with number and percentage. If the quantitative variables in the original study were expressed as median and interquartile range or median with maximum and minimum, they were converted to mean±standard deviation by means of mathematical statistics.[11–13] Furthermore, singularities were handled by adding one to all cell frequencies of studies with a zero cell count.

According to the standards of the EASL2017 guidelines,[3,4] we defined 40 U/L as the normal ALT upper limit of normal (commonly referred to as ULN). The pathological scoring system was converted to Scheuer's scoring system.[14] In the Scheuer's score system, the inflammation or fibrosis score was more than 2 points, which was considered as moderate to severe inflammation (G ≥2) or significant fibrosis (S ≥2). According to Zachary D. Goodman's liver puncture pathology score conversion method, if using the other scoring system of inflammation, histological activity index (HAI) ≥5 (Ishak[15] or Knodell[16] scoring system) or A ≥2 (Metavir scoring system)[17] were also defined as moderate to severe inflammation.[18] The fibrosis scoring system used >2 points to indicate significant fibrosis. Scheuer's or Metavir fibrosis scoring system score of 4 (G4 or F4) and Ishak fibrosis scoring system score of 5 to 6 (F5–6) were considered to indicate liver cirrhosis.

Quality assessment

Two independent investigators (CZ, ZW) assessed study quality using the Newcastle-Ottawa scale (commonly referred to as NOS)[19] for all the prospective and retrospective studies, including eight items (Supplementary Table 3). Studies with a score of ≤4, 5–6, and >6 were considered as having high, moderate, and low risk of bias, respectively.

Outcome measure

The primary outcome of interests were the proportion of significant histological changes (moderate to server inflammation or significant fibrosis) and the proportion of cirrhosis in CHB patients with normal ALT. The secondary outcome of interest was to find the risk factors of significant histological changes.

Statistical analysis

Considering the low incidence of interest events, the double arcsine transformation was used to calculate the proportion of significant histological changes and cirrhosis.[20] Q-statistics and Cochrane Q-test were used to assess heterogeneity between studies, where $p<0.10$ was regarded as statistically significant.[21,22] The I^2 statistic was calculated to describe the percent of observed variation across studies caused by heterogeneity, with an I^2 statistic of >75%, 25–75%, and <25% considered as high, moderate, and low heterogeneity, respectively.[21] Heterogeneity was expected, so all analyses were performed with a random-effects model. Subgroup analysis and meta-regression analysis were performed to explore potential sources of heterogeneity. Factors examined included study design (prospective vs. retrospective), region (Asian vs. Europe vs. Middle East vs. North America), age (<40 years vs. ≥40 years), BMI (<24 kg/m^2 vs. ≥24 kg/m^2), HBV DNA (<6 log10 IU/mL vs. ≥6 log10 IU/mL), Tbil (<17.1 μmol/L vs. ≥17.1 μmol/L), PLT (<200 ×10^9/L vs. ≥200 ×10^9/L), ALT (<25 U/L vs. ≥25 U/L), and AST (<25 U/L vs. ≥25 U/L). In subgroup analyses, we examined differences between groups with the chi-square test. In addition, to examine the impact of a single study on total effect, sensitivity analysis was carried out by leaving out one study each time.

Funnel plot (and trim-and-fill analysis,[23] which yields an effect adjusted for funnel plot asymmetry), Begg's test and

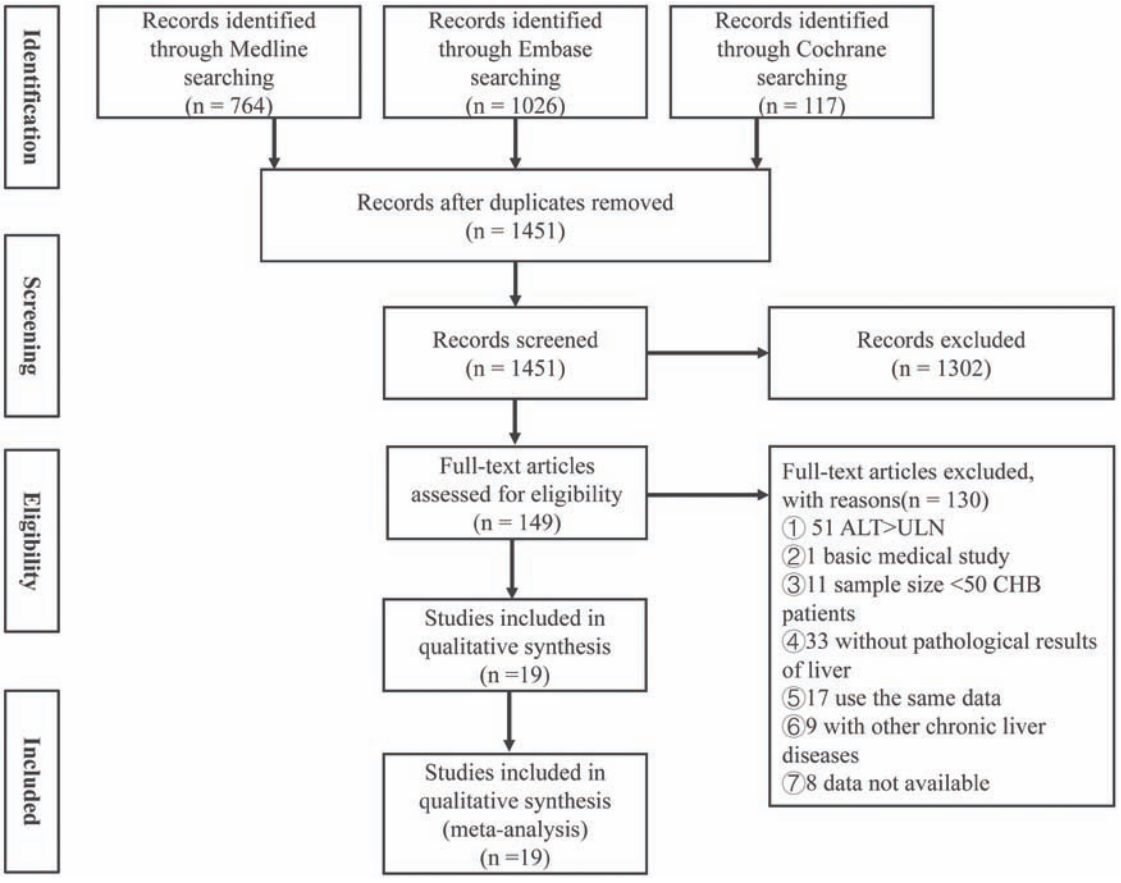

Fig. 1. Flowchart for study selection in the meta-analysis.

Egger's test were used to examine potential publication bias. A *p*-value of <0.05 was considered to be statistically significant. Analyses were done with Stata 15.0 (StataCorp LLC, College Station, TX, USA) and R version 3.6.2 using the meta and metafor packages.

Results

Search results and study characteristics

A total of 1,907 citations were retrieved from the Medline, Embase, and Cochrane Library database search. After screening of titles and abstracts for relevant publications and removal of duplicates, 149 potential articles were eligible for full-text screening, of which 19 studies[5–8,24–38] (including 2,771 participants; Supplementary Table 2) met our inclusion criteria and were included in the meta-analysis (Fig. 1).

The characteristics of the included studies are summarized in Table 1,[5–8,24–38] Supplementary Tables 4 and 5. Among the 19 studies published from 2007 to 2018, there were 7 prospective studies and 13 studies from the Asian region. The total number of people included in each study was quite different, with a median of 120 (ranging from 59 to 455). The youngest mean age was 23.8±6.7 years-old and the oldest was 50.0±15.0 years-old. The median male-to-female ratio was 1.9. Only one study did not report HBV DNA data, and 72.2% (13/18) of the remaining 18 studies had HBV DNA average of >6 log10 IU/mL. For other clinical data (such as ALT, AST, HBeAg status, etc.), please see Supplementary Table 4.

Four different scoring systems were used in the evaluation of liver pathology, including Scheuer's, Ishak, Knodell and Metavir scoring systems. The proportion of moderate to severe inflammation ranged from 4% (6/140) to 63% (60/95), with a median of 36%. The proportion of significant fibrosis ranged from 9% (10/113) to 56% (63/113), with a median of 30%. Twelve studies reported on cirrhosis; in most (11/12), the proportion of cirrhosis was <5%, but in one study, the proportion of liver cirrhosis was as high as 19% (22/113).

Methodological quality assessment

All of the selected studies were assessed for methodological quality by NOS. The NOS score of each study is presented in Supplementary Table 3. Ten studies[5,6,8,24,30,31,33,34,38] were of high quality and 9 studies[7,25–29,32,35–37] were of moderate quality. There were no studies with low quality.

Proportion of moderate to severe inflammation, significant fibrosis and liver cirrhosis

As shown in Figure 2A, the pooled proportion of moderate to severe inflammation was 35% (95% CI: 27 to 43). In the HBeAg-positive patients and the HBeAg-negative patients (Supplementary Fig. 2A) the rate of severe inflammation

Table 1. Characteristics of studies included in the meta-analysis

First author (Year)	Study design	Country	Age	Total	Male/ Female	Moderate to severe inflammation	Significant fibrosis	Cirrhosis	Histology assessment
Lai M[32] (2007)	Retrospective	USA	36.7±5.3	59	24/35	20	11	2	Scheuer's
Papatheodoridis G[8] (2008)	Prospective	Greece	50.0±15.0	113	74/39	61	63	21	Ishak
Kumar M[33] (2008)	Prospective	India	27.7±15.3*/34.6±14.5#	131	102/29	69	37	2	Knodell and Metavir
Nguyen MH[30] (2009)	Retrospective	USA	44.8±11.4	101	52/49	22	30	0	Scheuer's
Chen EQ[36] (2010)	Retrospective	China	33.0±10.1	141	82/59	67	47	NA	Scheuer's
Gui HL[34] (2010)	Retrospective	China	33.6±10.4	252	176/76	55	40	NA	Ishak
Montazeri G[38] (2010)	Prospective	Iran	36.7±12.0	132	80/52	53	40	NA	Knodell and metavir
Sanai FM[28] (2011)	Prospective	KSA	35.0±11.5	108	69/39	37	32	1	Metavir
Lesmana CR[31] (2011)	Prospective	Indonesia	41.5±10.7	103	58/45	57	56	NA	Metavir
Alam S[37] (2011)	Retrospective	Bangladesh	26.8±7.9	181	151/30	95	36	2	Knodell and metavir
Liao B[5] (2013)	Retrospective	China	23.8±6.7*/35.4±7.2#	140	73/67	6	59	0	Metavir
Wan R[27] (2015)	Retrospective	China	33.8±8.9	125	82/43	46	38	3	Scheuer's
Gong X[35] (2015)	Retrospective	China	32.0±12.2*/41.8±9.6	100	70/30	13	37	NA	Scheuer's
Tan Y[7] (2015)	Retrospective	China	32.4±13.2	113	77/36	66	10	0	Knodell
Ormeci A[29] (2016)	Retrospective	Turkey	42.8±11.32	120	58/62	18	43	0	Ishak
Zhou J[24] (2017)	Prospective	China	37.6±10.1*/42.3±10.6#	193	134/59	70	63	NA	Ishak
Tan YW[6] (2017)	Retrospective	China	34.5±11.2	95	70/25	60	23	NA	Knodell
Xing YF[26] (2018)	Prospective	China	34.9±6.4	455	287/168	137	182	6	Ishak
Xu Z[25] (2018)	Retrospective	China	33.3±8.3	109	91/18	13	23	3	Scheuer's

*HBeAg-positive; #HBeAg-negative. Based on the Ishak scoring system, the definition of moderate to severe inflammation by Gui HL (2010) was HAI ≥4, by Papatheodoridis G (2008), Zhou J (2017), and Xing YF (2018) was HAI ≥5, and by Ormeci A (2016) was HAI ≥6. Based on the Knodell scoring system, Kumar M (2008), Montazeri G (2010), Alam S (2011), Tan Y (2015) and Tan YW (2017) defined moderate to severe inflammation as HAI ≥4.

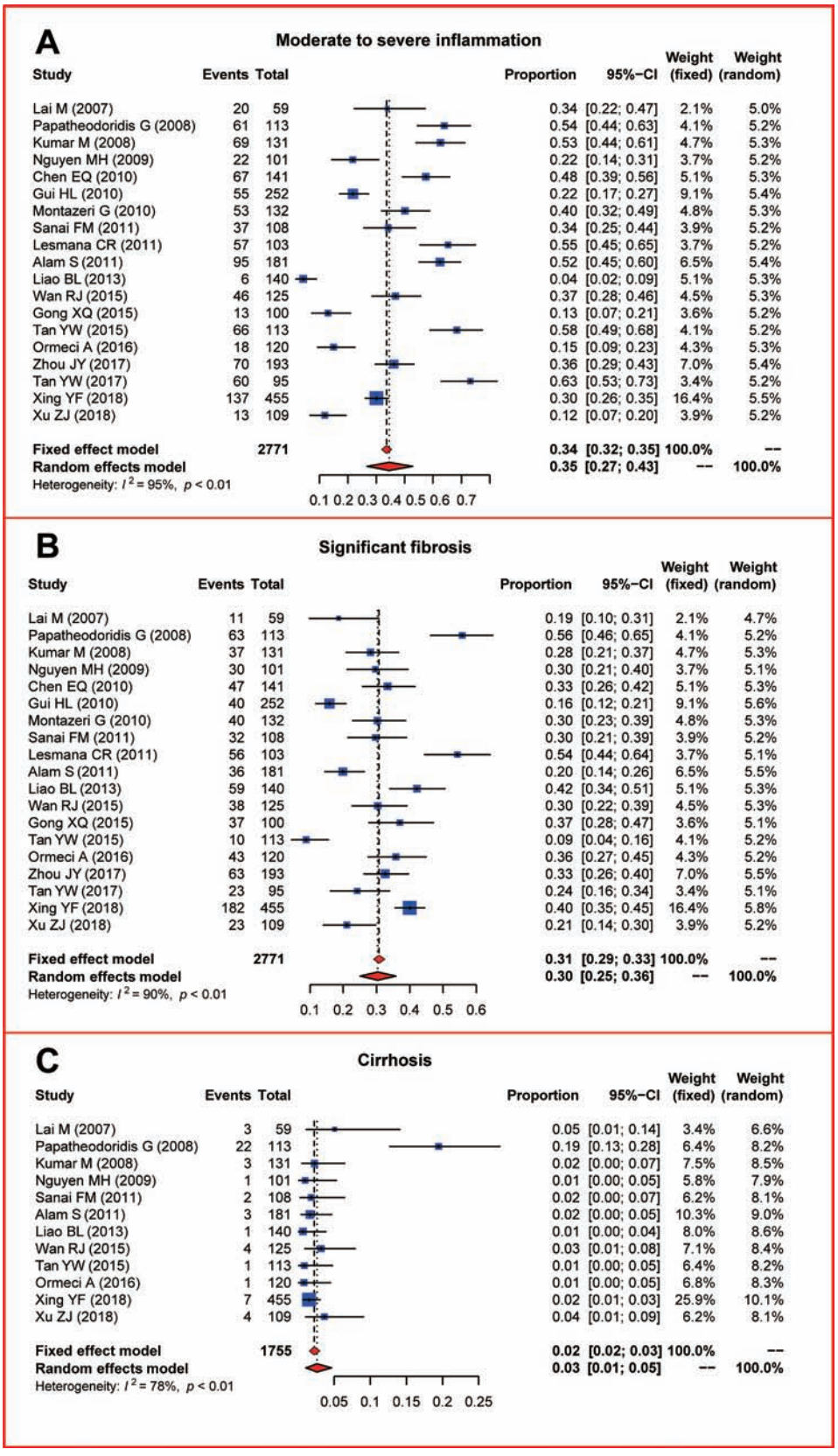

Fig. 2. Proportion of significant pathological changes in patients with CHB and normal ALT. (A) Inflammation grade ≥2. (B) Fibrosis stage ≥2. (C) Cirrhosis.

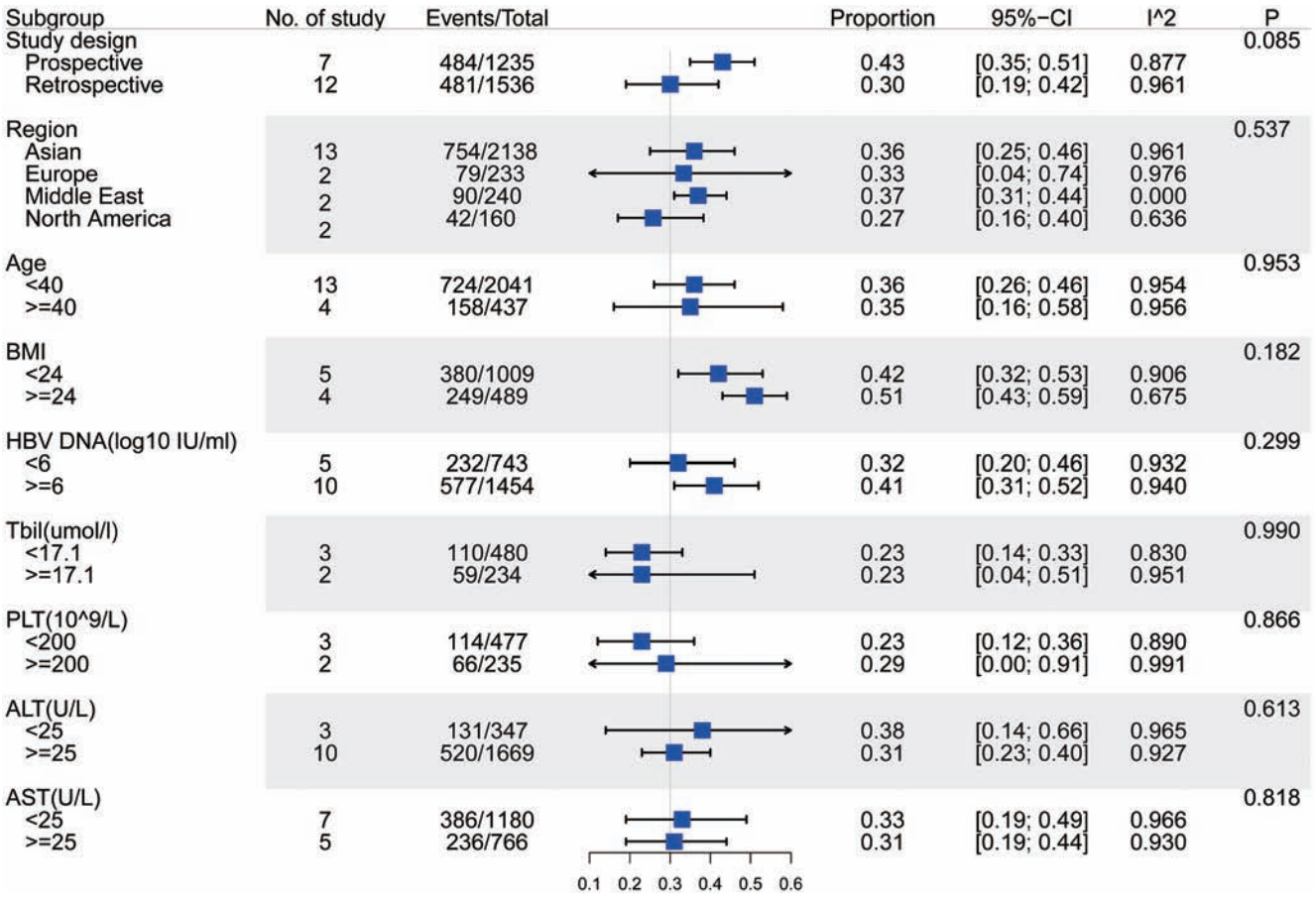

Fig. 3. Summary of the proportion of moderate to severe inflammation in different subgroups.

was 34% (95% CI: 19 to 50) and 32% (95% CI: 21 to 43), respectively, but the difference between the two was not statistically significant (p=0.806). The pooled proportion of significant fibrosis (Fig. 2B) was 30% (95% CI: 25 to 36), 27% (95% CI: 18 to 36) in the HBeAg-positive patients and 34% (95% CI: 26 to 42) in the HBeAg-negative patients; again, the between-group difference was not statistically significant (p=0.255; Supplementary Fig. 2B). The proportion of liver cirrhosis (Fig. 2C) accounted for 3% (95% CI: 1 to 5), and there was no significant difference between the HBeAg-positive and HBeAg-negative patients [2% (95% CI: 1 to 4) vs. 3% (95% CI: 0 to 8), p=0.571; Supplementary Fig. 2C].

Subgroup analysis and meta-regression

Proportion of moderate to severe inflammation: Figure 3 and Supplementary Table 6 shows the proportion of moderate to severe inflammation in different subgroups and meta-regression results. Prospective studies (n=7) seemed to have a higher proportion of moderate to severe inflammation than retrospective studies (n=12), but the difference was not statistically significant [43% (95% CI: 35 to 51) vs. 30% (95% CI: 19 to 42), p=0.087] nor by meta-regression (p=0.126). There was no statistical difference in age (<40 years vs. ≥40 years), BMI (<24 kg/m^2 vs. ≥24 kg/m^2), HBV DNA (<6 log10 IU/mL vs. ≥6 log10 IU/mL), Tbil (<17.1 μmol/L vs. ≥17.1 μmol/L), PLT (<200×10^9/L vs. ≥200×10^9/L), ALT (<25 U/L vs. ≥25 U/L) and AST (<25 U/L vs. ≥25 U/L). Similarly, there was no statistical difference by meta-regression.

Proportion of significant fibrosis: The results of subgroup analysis and meta-regression of significant fibrosis ratio are shown in Figure 4 and Supplementary Table 6. Similar to the proportion of moderate to severe inflammation, the proportion of significant fibrosis in prospective studies was higher than that in retrospective studies, and the difference was statistically significant [38% (95% CI: 31 to 46) vs. 26% (95% CI: 20 to 32), p=0.011]. The result by meta-regression was also significant (p=0.013). The proportion of significant fibrosis in people >40 years-old [44% (95% CI: 31 to 57)] was almost twice as high as that in people <40 years-old [26% (95% CI: 20 to 32)]. There were significant differences in subgroup analysis (p=0.012) and meta regression (p=0.009). The remaining seven subgroups (region, BMI, HBV DNA, Tbil, PLT, ALT, and AST) were also analyzed, and no statistical difference was found in either subgroup analysis or meta-regression.

Proportion of liver cirrhosis: Figure 5 and Supplementary Table 6 show the proportion of liver cirrhosis. In the subgroup analysis, only the factor of age (<40 years or ≥40 years) showed statistically significant difference [1.8% (95% CI: 1.1 to 2.6) vs. 4.8% (95% CI: 0 to 19.2), p<0.001]. No statistical difference was found in the other nine subgroups (study design, region, BMI, HBV DNA, Tbil, PLT, ALT, and AST). However, there was statistical significance in AST and region by meta-regression, probably because the range of 95% CI in subgroups with AST was so large that there was no statistical difference in subgroup

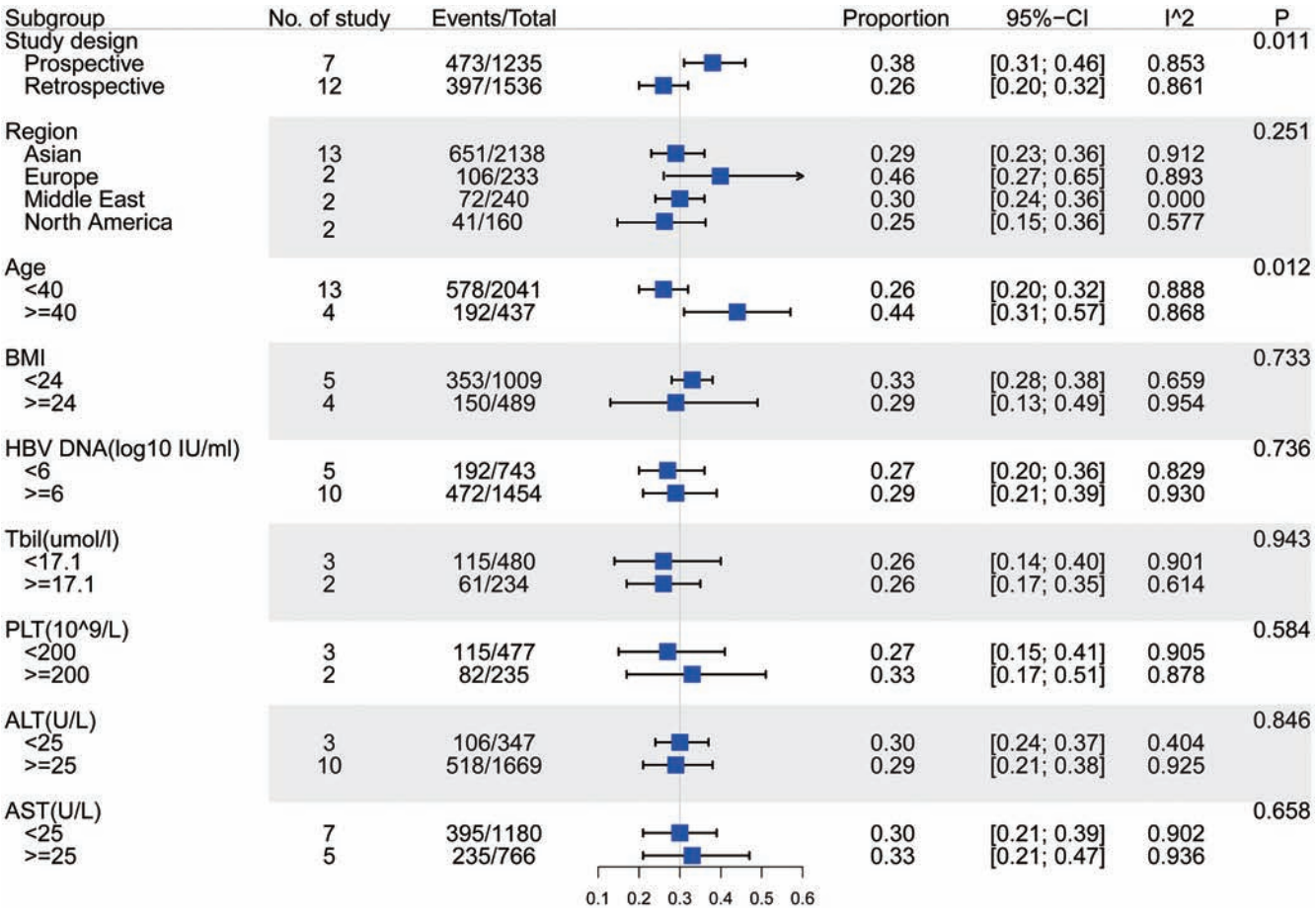

Fig. 4. Summary of the proportion of significant fibrosis in different subgroups.

analysis. For region subgroup analysis, only one study was included in two subgroups, so it was necessary to be cautious in explaining the proportion of liver cirrhosis in different subgroups.

Publication bias and sensitivity analysis

We drew a funnel plot and conducted a trim-and-fill analysis (Fig. 6). For moderate to severe inflammation, funnel plot (Fig. 6A) showed a slight asymmetry. However, both Begg's test (p=0.834) and Egger's test (p=0.573) did not indicate publication bias. Two studies were added to the trim-and-fill analysis (Fig. 6D) but there was no significant change in the proportion of moderate to severe inflammation [adjusted value: 32% (95% CI: 24 to 40)].

In the aspect of significant fibrosis, funnel plot (Fig. 6B), Begg's test (p=0.779) and Egger's test (p=0.672) were also applied, and the findings indicated that there was no publication bias. Trim-and-fill analysis (Fig. 6E) added six studies but did not significantly change the proportion of significant fibrosis [adjusted value: 37% (95% CI: 31 to 43)].

For proportion of cirrhosis, the aforementioned analysis was also carried out. The funnel plot (Fig. 6C) was symmetrical, without any study added or deleted in the trim-and-fill analysis (Fig. 6F). The Begg's test (p=0.063) and Egger's test (p=0.298) also showed no publication bias.

Sensitivity analysis was carried out on moderate to severe inflammation, significant fibrosis and cirrhosis, and the results were robust. We excluded each study in turn, and the results did not change much (see Supplementary Fig. 1 for details).

Discussion

The findings of our systematic review and meta-analysis show that significant histologic changes are not rare among the treatment-naive CHB patients with normal ALT. Among them, the proportion of moderate to severe inflammation or significant fibrosis was about one-third, and the proportion of cirrhosis was about 3%.

A previous study[39] has reported the proportion of significant fibrosis. On the basis of this, we have added several new research results in recent years to supplement the data of significant fibrosis. What is more important, we have improved the data of the proportion of moderate to severe inflammation and cirrhosis, which are as important as fibrosis evaluation in histological evaluation. Moreover, AASLD2018, EASL2017 and APASL2016 guidelines have recognized noninvasive alternatives for the evaluation of liver fibrosis, such as liver stiffness measurement (transient elastography).[2–4,40] However, there was no recognized evaluation method for liver histological inflammation, except liver biopsy. To some extent, the progression of inflammation was more hidden than fibrosis, and our data show that the proportion of moderate and severe inflammation [35% (95% CI: 27 to 43)] was higher than that of sig-

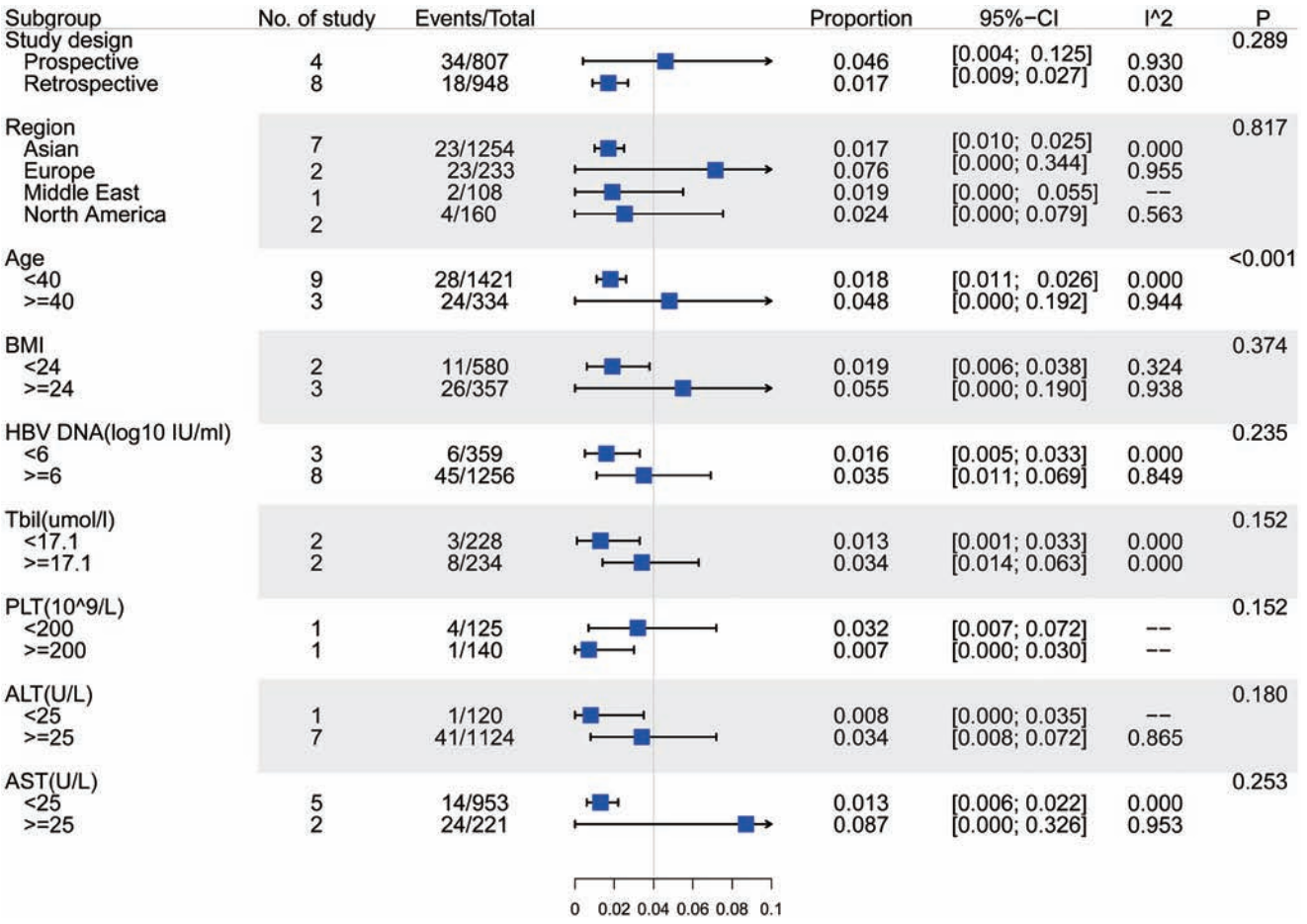

Fig. 5. Summary of the proportion of cirrhosis in different subgroups.

nificant fibrosis [30% (95% CI: 25 to 36)]. Previously, it has been believed that CHB patients with normal ALT did not need special treatment, mainly observation, and only a clear family history of liver cancer or other special circumstances need to be paid attention to. But our study gives a different answer. Although ALT has its simple and rapid advantages in the evaluation of chronic liver disease, there are too many factors that affect the concentration of ALT in serum, so the specificity of reflecting liver inflammation is not high. Especially, when other liver diseases or systemic diseases were involved in the liver, the limitations of ALT became more obvious.

If CHB patients do not start antiviral therapy in time, the disease can progress to liver cirrhosis or even liver cancer. At present, first-line antiviral drugs (i.e. entecavir, tenofovir disoproxil fumarate, and tenofovir alafenamide) have a good effect on inhibiting virus and improving liver histological inflammation and fibrosis.[41] Therefore, it is necessary to make clear the proportion of significant histological changes (including inflammation and fibrosis) in CHB patients with normal ALT. Meanwhile, identifying possible signs in people with significant histological changes is also momentous.

Our study found that there were obvious differences in significant fibrosis among different age subgroups (>40 years-old or not), suggesting that age was as an important sign of significant fibrosis. Similarly, age also showed value in cirrhosis. Previously, there have been some small sample studies, ranging from 10s to 100s, that support our conclusions. Research findings by Xing *et al*.[26] and Tan *et al*.[7] also support this view, but authors of the former believed that the age of 50 needed special attention. Sanai *et al*.[28] held that serum HBV DNA levels are predictive of liver fibrosis in CHB but found it to be in the mildly elevated ALT population. However, our results did not suggest the role of HBV DNA in the differential diagnosis of significant fibrosis. We also used HBV DNA level of 7 log10 IU/mL and 8 log 10 IU/mL as cutoff values, and found no statistical difference (data not shown). There are other indicators (collagen 4, laminin, procollagen III N-terminal peptide, hyaluronic acid, etc.) and models (APRI, FIB-4, etc.) that suggest significant fibrosis which need further study.

Unfortunately, no distinguishing indication of moderate and severe inflammation can be found. Considering the studies by Park *et al*.[42] and Kumar *et al*.,[33] persistent high ALT (0.5–1 of ULN) may be an indicator of liver histological inflammation. However, our research showed a lack of statistical significance for ALT differences among groups (<25 U/L vs. ≥25 U/L, p=0.613) and the possible reason was that some of the included studies did not provide the original ALT mean in the original text; thus, we could only use mathematical statistics to estimate the possible mean, and this approach may have caused some errors. Therefore, it may patients with high normal of ALT for a long time may still be worthy of our attention. In addition, our team[24,43] and Xia *et al*.[44] have shown that quantitative anti-hepatitis B core antibody measures have good application value in reflecting liver inflammation and natural history of hepatitis B. The quantitative anti-hepatitis B core antibody measure in the

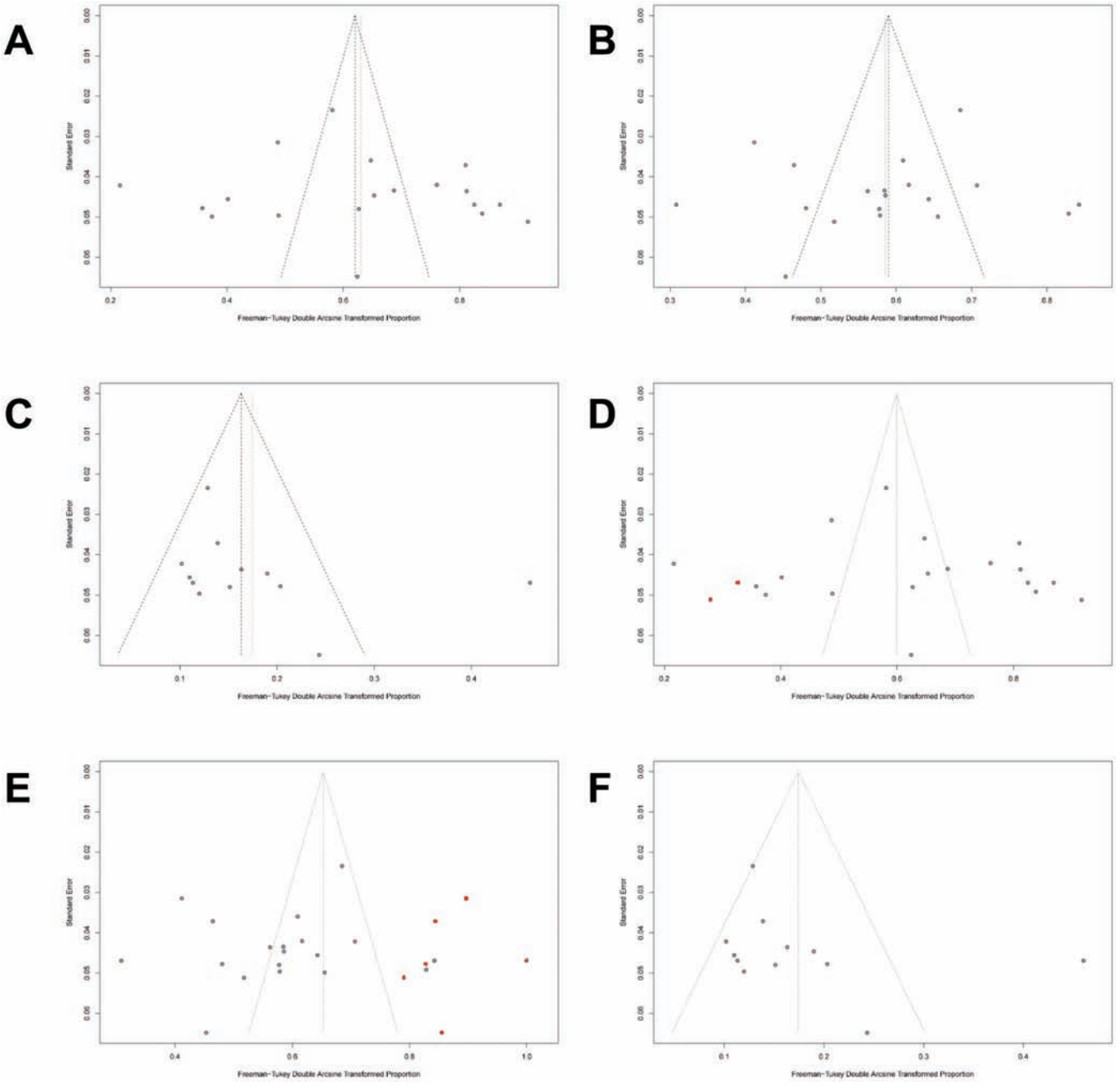

Fig. 6. Funnel plot and trim-and-fill analysis plot. (A) Funnel plot of the proportion of moderate to severe inflammation. (B) Funnel plot of the proportion of significant fibrosis. (C) Funnel plot of the proportion of cirrhosis. (D) Trim-and-fill plot of the proportion of moderate to severe inflammation (two studies were added, as shown by the red points in the figure). (E) Trim-and-fill plot of the proportion of significant fibrosis (six studies were added, as shown by the red points in the figure). (F) Trim-and-fill plot of the proportion of cirrhosis (no studies were added).

immune tolerance stage was significantly lower than that in the immune clearance stage.

Although subgroup analysis and meta-regression were carried out as far as possible, there was still some heterogeneity implication for outcomes. According to the results of proportion of significant fibrosis subgroup analysis, different ALT levels may represent the main source of heterogeneity (I^2: 40.4% vs. 92.5% in ALT <25 U/L and ≥25 U/L, respectively). Several factors can explain the source of heterogeneity in proportion of cirrhosis. Among them, prospective studies had greater heterogeneity than regression studies (I^2: 93.0% vs. 3.0%), and older age had greater heterogeneity than younger age (I^2: 94.4% vs. 0.0%). Unfortunately, the source of moderate and severe inflammatory heterogeneity has not been found. We speculate that the first reason may be that there was no recognized value for the normal upper limit of ALT, which was considered by the APASL and EASL guidelines as 40 U/L but by the AASLD guidelines as 35 U/L for male and 25 U/L for female. Second, compared with the pathological evaluation of fibrosis, the evaluation of inflammation was more easily affected by the scoring system and pathologists, especially upon the

application of Ishak and Knodell scoring systems, as the items were too detailed to form a unified consensus.

Our study has several limitations. First, there may be a patient selection bias in this study. For the CHB patients with normal ALT, both the patients and doctors were reluctant to carry out invasive liver biopsy due to its inherent risks, which reduced the implementation of liver biopsy to a certain extent. Therefore, the proportion of significant histological changes may be higher in actuality than this study found. Second, there were non-randomized controlled trials among the included studies. Although the results of publication bias were negative, their inclusion inevitably reduced the overall quality of the study.

Conclusions

In summary, significant histologic changes present in approximately one-third of treatment-naive CHB patients with normal ALT levels, and about 3% of patients even progressed to cirrhosis. It is worth noting that the proportion of significant fibrosis and cirrhosis in people >40 years-old are more than twice as high as those in younger people. The management of treatment-naive CHB patients with normal ALT remains a challenge and requires an individualized approach, in addition to the standardized paradigms recommended by current guidelines.

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Conflict of interest

The authors have no conflict of interests related to this publication.

Author contributions

Search of the literature and data extraction (CZ, ZW), drafted the manuscript (CZ), creation of figures and table (CZ, ZW, JWL), methodological guidance (HZ), and provision of the overall principle and oversight of the direction of the study (HZ, GQW).

Data sharing statement

All data are available upon request.

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Original Article

Clinical Course and Outcome Patterns of Acute-on-chronic Liver Failure: A Multicenter Retrospective Cohort Study

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Abstract

Background and Aims: Acute-on-chronic liver failure (ACLF) is acute decompensation of liver function in the setting of chronic liver disease, and characterized by high short-term mortality. In this study, we sought to investigate the clinical course of patients at specific time points, and to propose dynamic prognostic criteria. ***Methods:*** We assessed the clinical course of 453 patients with ACLF during a 12-week follow-up period in this retrospective multicenter study. The clinical course of patients was defined as disease recovery, improvement, worsening or steady patterns based on the variation tendency in prothrombin activity (PTA) and total bilirubin (TB) at different time points. ***Results:*** Resolution of PTA was observed in 231 patients (51%) at 12 weeks after the diagnosis of ACLF. Among the remaining patients, 66 (14.6%) showed improvement and 156 (34.4%) showed a steady or worsening course. In patients with resolved PTA, the clinical course of TB exhibited resolved pattern in 95.2%, improved in 3.9%, and steady or worse in 0.8%. Correspondingly, in patients with improved PTA, these values for TB were 28.8%, 27.3%, and 43.9%, respectively. In patients with steady or worsening PTA, these values for TB were 5.7%, 32.3%, and 65.6%, respectively. Dynamic prognostic criteria were developed by combining the clinical course of PTA/TB and the clinical outcomes at 4 and 12 weeks after diagnosis in ACLF patients. ***Conclusions:*** We propose the following dynamic prognostic criteria: rapid progression, slow progression, rapid recovery, slow recovery, and slow persistence, which lay the foundation for precise prediction of prognosis and the improvement of ACLF therapy.

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Introduction

Acute-on-chronic liver failure (ACLF) represents acute decompensation of liver function in the setting of chronic liver disease, and is characterized by high short-term mortality.[1] In view of the different etiological compositions of chronic liver disease in Eastern and Western countries, the definition and diagnostic criteria of ACLF are also diverse. The European definition of ACLF was proposed by the Chronic Liver Failure ACLF in Cirrhosis (referred to as CANONIC) study,[2] which means acute decompensation of cirrhosis associated with organ/system failure(s) (including extrahepatic organ failure), and the severity of ACLF is graded according to the number of organ/system failures.

The dynamic clinical course of ACLF can be divided into disease resolution, improvement, worsening, and steady or fluctuating course,[3] which is evaluated by the variation in ACLF grades at different time points. In Eastern countries, ACLF is defined as acute decompensation in the setting of chronic liver disease or compensated rather than decompensated cirrhosis by the Asian Pacific Association for the Study of the Liver (commonly known as APASL).[4] This definition only includes hepatic failure, and extrahepatic insults are considered as complications of this syndrome. Furthermore, ACLF under this definition is considered reversible, defined as improvement in coagulation and jaundice, and without hepatic encephalopathy, but its clinical course pat-

Keywords: Acute-on-chronic liver failure; Clinical course; Outcome patterns; Retrospective cohort study.

Abbreviations: ACLF, acute-on-chronic liver failure; AKI, acute kidney injury; APASL, Asian Pacific Association for the Study of the Liver; CANONIC, Chronic Liver Failure Acute-on-Chronic Liver Failure in Cirrhosis; WGO, World Gastroenterology Organization; CTP, Child-Turcotte-Pugh; d3-7 ACLF, third and seventh day after ACLF diagnosis; GIB, gastrointestinal hemorrhage; LT, liver transplantation; MELD, model for end-stage liver disease; PTA, prothrombin activity; TB, total bilirubin.

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tern has not been determined.

Although definitions and diagnostic criteria of ACLF differ, it is generally accepted that ACLF has a dynamically changing course, with high mortality, and requires organ support therapy or liver transplantation (LT). Timely and dynamic assessment on clinical course of ACLF patients is essential to avoid futile treatment and to reasonably choose LT. Many prognostic models have been proposed for evaluating the outcomes of ACLF patients, but they are not universally accepted. Specially, most of the models belong to a single time-point assessment based on short-term mortality.[5–7] In recent years, although the application of dynamic scoring models to assess the prognosis of ACLF patients has aroused extensive attention,[8–10] a single time-point outcome (death or LT) is still utilized as a prognostic variable. As is known, the clinical outcome of liver failure exhibits a dynamic pattern whether the final outcome is recovery or death, and it can be divided into rapid and slow processes. Thus, accurate assessment of prognosis will contribute to the improvement in ACLF management.

To evaluate the clinical course of ACLF patients more precisely, we formulated new dynamic prognostic criteria based on the dynamic alterations in key clinical indicators and outcomes, and analyzed the potential predictors of clinical course. The patients in our study were from an Asian population, the main cause was hepatitis B virus infection, and the main clinical manifestation was intrahepatic injury,[4,11] so liver function (total bilirubin) and coagulation index [prothrombin activity (PTA) or international normalized ratio] were used to evaluate the progression of ACLF. These new prognostic criteria will help develop a more practical predictive scoring model, determine the factors potentially influencing progression, and lay the foundation for making appropriate treatment strategies (intensive care unit treatment, organ support treatment, liver transplantation treatment, or hospice care treatment).

Methods

Patients

In this observational study, we retrospectively collected the data of ACLF patients from the Department of Hepatology in three hospitals in China. The patients included had been admitted to the Tianjin Third Central Hospital and the Fifth Medical Center of PLA General Hospital between November 1, 2012 and June 30, 2019, and to Beijing You'an Hospital Affiliated to Capital Medical University between January 1, 2015 and June 30, 2019.

The diagnosis of ACLF was made according to the APASL recommendations, as follows:[4] an acute hepatic insult that occurs in patients with chronic liver disease, manifested by jaundice (serum total bilirubin [TB] ≥5 mg/dL) and coagulation dysfunction (PTA≤40%), and complicated within 4 weeks by ascites and/or encephalopathy. Patients were divided into three types according to the severity of chronic liver diseases, as follows: type-A for patients without cirrhosis, type-B for patients with well-compensated cirrhosis, and type-C for patients with previous decompensated cirrhosis.[12–15]

All patient data were retrieved from electronic medical records. All treatments that were performed, mainly including etiological and comprehensive treatment, complied with the guidelines for ACLF, which is accredited by the Chinese Medical Association.[13]

The study procedures conformed to the ethical guidelines of the Declaration of Helsinki, and were approved by the ethics committees of Beijing You'an Hospital Affiliated to Capital Medical University, the Tianjin Third Central Hospital, and the Fifth Medical Center of PLA General Hospital. Due to the retrospective nature of this study, informed consent was waived.

Data collection

We collected information on patients who met ACLF diagnostic criteria during in-hospital stay and at 12-week post-discharge follow-up visit. This information included demographic data, complications, and laboratory measurements (e.g., TB, PTA, international normalized ratio). The outcome information, such as LT or death after enrollment, was also collected.

Exclusion criteria included: a) liver cancer or other malignant tumors; b) severe underlying diseases, such as severe chronic obstructive pulmonary disease with respiratory failure, severe coronary heart disease with heart failure, diabetes mellitus with severe complications; and c) chronic kidney disease and renal failure. In addition, we also excluded patients whose bilirubin and coagulation indicators were missing. The specific screening flowchart is detailed in Figure 1.

Definitions of clinical course pattern in ACLF patients

The clinical course pattern of ACLF patients was determined according to the variation tendency of PTA/TB, which was assessed at diagnosis, during the 12-week follow-up period, until death or LT. ACLF was diagnosed at admission or after admission. The variation tendency of PTA/TB (Fig. 2) was defined as resolution, improvement, and steady or worsening, respectively. Resolution of PTA was considered when PTA was increased to >40%, and TB resolution was defined as a 50% decrease in TB from its peak. Improvement indicated a decrease in TB and an increase in PTA, but it did not meet resolution. Steady course referred to the absence of variation in PTA/TB during follow-up. Worsening course indicated an increase in TB and a decrease in PTA. In assessing the variation tendency of PTA and TB, we excluded the effects of artificial liver therapy and blood transfusion on the transient variation of these two indicators.

Dynamic stratification criteria for clinical outcome

The stratification criteria for dynamic prognosis were developed by the combination of clinical course pattern and final outcome of ACLF patients. Clinical course pattern was assessed at 12 weeks after diagnosis, or before death or LT. The 12-week ACLF outcomes were divided into three categories: recovery, death (including LT), and still in a state of liver failure (persistence). The time course of outcomes was designated as rapid or slow recovery, progression, or persistence in accordance with the variation tendency in PTA/TB at 4 weeks and 12 weeks after diagnosis, respectively.

Statistical analysis

Continuous variables were presented as mean±standard deviation or median (interquartile range), and categorical variables as *n* (%). Dynamic prognostic stratification criteria were formulated according to the distribution of 12-week outcomes under different clinical course patterns of PTA/TB. Univariate analyses using Chi-square, one-way analyses of variance or Kruskal-Wallis test were performed to assess the association between patients' characteristics and dynamic stratification criteria of clinical outcome. A two-

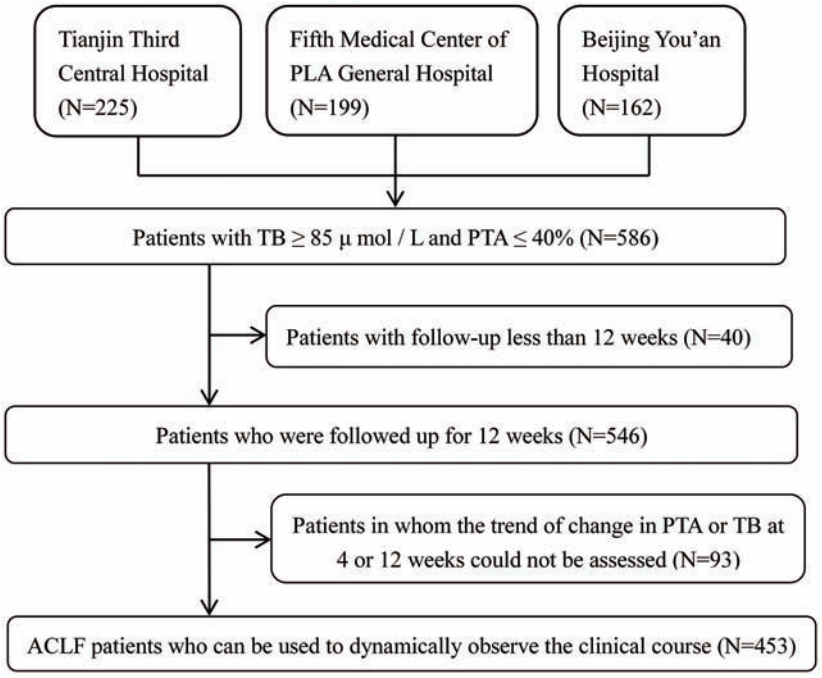

Fig. 1. Flowchart of patient enrollment. Patients whose variation tendency in PTA or TB at 4 or 12 weeks could not be assessed (*n*=93). ACLF patients whose clinical course can be dynamically observed (*n*=453). ACLF, acute-on-chronic liver failure; PTA, prothrombin activity; TB, total bilirubin.

sided *p*-value of less than 0.05 was considered statistically significant. All statistical analyses were performed with the Statistical Package for Social Sciences version 23.0 (IBM Corp., Armonk, NY, USA).

Results

Patient summary

Demographic and clinical characteristics of the patients are shown in Table 1. Four hundred and fifty-three patients were enrolled into the study. The mean age of patients was 48.3±11.5 years, and male patients accounted for 75.9%. The etiologies were as follows: hepatitis B virus (*n*=290, 64.0%), alcohol (*n*=67, 14.8%), hepatitis B virus+alcohol (*n*=47, 10.4%), and other (*n*=49, 10.8%). The World Gastroenterology Organization (WGO) type of all enrolled patients included type A in 144 (31.8%), type B in 146 (32.2%) and type C in 163 (36.0%). The occurrence rate of complications in these patients was 70.6% for ascites, 87.4% for bacterial infection, 12.1% for fungal infection, 7.3% for gastrointestinal hemorrhage (referred to here as GIB), 18.5% for hepatic encephalopathy, and 28.9% for acute kidney injury (AKI). Overall, the 4-week and 12-week LT-free survival rate in this study was 74.4% and 57.6%, respectively.

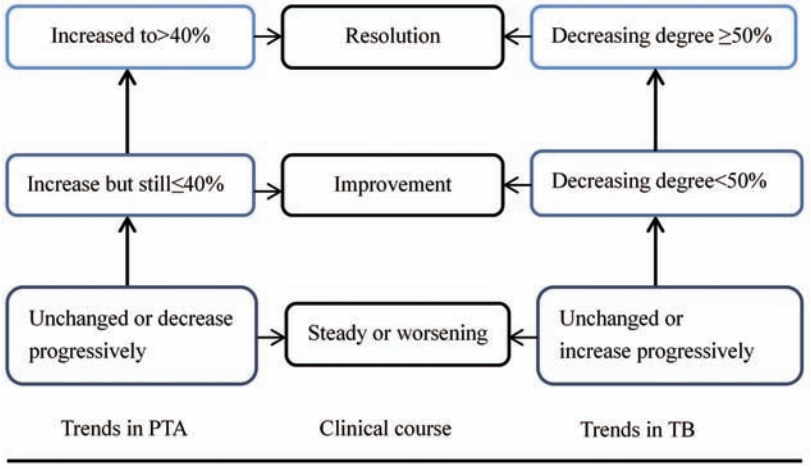

Fig. 2. Clinical course of ACLF patients assessed by variation tendency in PTA and TB. If international normalized ratio ≥1.5 is taken as the criterion of coagulation abnormality in the diagnosis of ACLF, INR and PTA show the opposite trend and can be used to evaluate the clinical process. ACLF, acute-on-chronic liver failure; PTA, prothrombin activity; TB, total bilirubin.

Table 1. Clinical characteristics of the patients with ACLF

Characteristics	*n*=453
Age in years, mean±SD	48.3±11.5
Male sex, *n* (%)	198 (75.9)
Underlying liver disease, *n* (%)	
Without cirrhosis	144 (31.8)
Compensated cirrhosis	146 (32.2)
Decompensated cirrhosis	163 (36.0)
Etiology of liver disease, *n* (%)	
Hepatitis B virus	290 (64.0)
Alcohol	67 (14.8)
Hepatitis B virus and alcohol	47 (10.4)
Other etiologies	49 (10.8)
Precipitating events, *n* (%)	
Reactivation of HBV	59 (13.0)
Alcohol	24 (5.3)
Bacterial infection	48 (10.6)
Drugs or poisons	34 (7.5)
Other	42 (9.3)
Unclear	246 (54.3)
Complications, *n* (%)	
Ascites	320 (70.6)
Bacterial infection	396 (87.4)
Fungal infection	55 (12.1)
Gastrointestinal hemorrhage	33 (7.3)
Hepatic encephalopathy	84 (18.5)
AKI	131 (28.9)
Laboratory data and scores, mean±SD	
Serum total bilirubin in mg/dL	17.9±9.2
Prothrombin activity, %	32.5±10.1
International normalized ratio	2.4±0.8
Serum creatinine in μmol/L	82.2±44.2
Blood sodium in mmol/L	134.2±5.1
White blood cell count as $\times 10^9$/L	8.1±6.9
Platelet count as $\times 10^9$/L	102.9±54.4
MELD score	24.6±5.7
CTP score	11.4±1.3
Survival rates, *n* (%)	
4-week LT-free survival	337 (74.4)
12-week LT-free survival	261 (57.6)

ACLF, acute-on-chronic liver failure; AKI, acute kidney injury; CTP, Child-Turcotte-Pugh; HBV, hepatitis B virus; MELD, model for end-stage liver disease; SD, standard deviation.

Clinical course pattern and its relationship with 4-week and 12-week mortality in ACLF

At 4 weeks after ACLF diagnosis, PTA was found to be resolved in 191 patients (42.2%) (Table 2), steady or worsening in 155 (34.2%), and improved in 107 (23.6%). For 191 patients with resolved PTA, resolution pattern was most frequent (72.8%), followed by an improved pattern (23.6%) and a steady or worsening pattern (3.7%; Table 2). For 107 patients with improved PTA, TB improvement was most frequent (38.3%), followed by TB resolution (30.8%) and steady or worsening pattern (30.8%) (Table 2). For patients with steady or worsening PTA (34.2%), resolution was found in 8 (5.2%), improvement in 50 (32.3%), and a steady or worsening course in 97 (65.6%).

We also assessed the clinical course of PTA and TB at 12 weeks after ACLF diagnosis (Table 2). Overall, PTA resolution was observed in 231 patients (51%). Among the remaining patients, 66 (14.6%) showed improvement and 156 (34.4%) showed a steady or worsening course. For patients with resolved PTA, the clinical course of TB was as follows: resolution in 95.2%, improvement in 3.9%, and steady or worsening in 0.8%. For patients with improved PTA, the corresponding proportion of TB clinical course with resolution, improvement and steady or worsening pattern was 28.8%, 27.3%, and 43.9%, respectively. Similar to the 4-week data, the distribution of TB clinical course in patients with a steady or worsening PTA was resolved in 5.7%, improved in 32.3%, and steady or worsening in 65.6%.

Consistency was found in the clinical course of TB and PTA. The frequency of TB resolution was high in patients with resolved PTA and low in those with steady or worsening PTA, and vice versa (Table 2).

The 4-week and 12-week mortality rate was low in patients with resolved PTA and TB (0%), moderate in those with improved PTA and TB (14.6% and 55.6%), and high in those with steady or worsening PTA and TB (63.9% and 98%). Of note, the 12-week mortality rate was very high in those with steady or worsening PTA, regardless of TB course (Table 2).

Dynamic stratification criteria for clinical outcome

Dynamic prognostic criteria were developed by combining the clinical course of PTA/TB and the clinical outcomes of ACLF patients at 4 and 12 weeks after diagnosis. Patients were divided into three categories according to these criteria: recovery, progression, and persistence (Fig. 3). Rapid recovery was considered when both PTA and TB in ACLF patients were resolved within 4 weeks after diagnosis and the patients survived, and slow recovery was defined if both PTA and TB were resolved at 12 weeks after diagnosis. Progression could be categorized into rapid progression and slow progression, which were considered when ACLF patients had worsening PTA and TB or they did not achieve resolution and died within 4 and 12 weeks after diagnosis. Persistence was designated when ACLF patients had PTA less than 40% and/or worsened TB or the patients did not achieve resolution at 12 weeks after diagnosis. In addition, we defined the clinical course of two ACLF patients as slow persistence because they had resolved PTA and TB at 4 weeks but decreased PTA (<40%) at 12 weeks.

Clinical characteristics of ACLF patients stratified by the dynamic criteria for clinical outcome

According to dynamic prognostic criteria, 116 (25.6%) patients with ACLF were classified as rapid progression, 76 (16.8%) as slow progression, 137 (30.2%) as rapid recovery, 83 (18.3%) as slow recovery, and 41 (9.1%) as persistence (Table 3). The age of ACLF patients with recovery pattern was significantly lower than that of patients with

Table 2. Clinical course patterns in ACLF patients within 4 weeks and 12 weeks after diagnosis.

	Variation tendency in TB					
	4 weeks			12 weeks		
Variation tendency in PTA	Recovery (n=180)	Improvement (n=136)	Steady or worsening (n=137)	Recovery (n=248)	Improvement (n=72)	Steady or worsening (n=133)
Recovery, *n* (%)						
Prevalence	139 (72.8)	45 (23.6)	7 (3.7)	220 (95.2)	9 (3.9)	2 (0.8)
Mortality, *n*/total	0/139 (0)	1/45 (2.3)	1/7 (14.3)	0/220 (0)	2/9 (22.2)	1/2 (50)
Improvement, *n* (%)						
Prevalence	33 (30.8)	41 (38.3)	33 (30.8)	19 (28.8)	18 (27.3)	29 (43.9)
Mortality, *n*/total	0/33 (0)	6/41 (14.6)	9/33 (27.3)	0/19 (0)	10/18 (55.6)	29/29 (100)
Steady or worsening, *n* (%)						
Prevalence	8 (5.2)	50 (32.3)	97 (65.6)	9 (5.7)	45 (32.3)	102 (65.6)
Mortality, *n*/total	1/8 (12.5)	23/50 (46.0)	62/97 (63.9)	7/9 (77.8)	43/45 (95.6)	100/102 (98.0)

ACLF, acute-on-chronic liver failure; PTA, prothrombin activity; TB, total bilirubin.

progressive and persistent patterns (p=0.011). To be specific, the ages of ACLF patients with rapid and slow recovery patterns were 46.5±12 and 45.7±10.9 years, while the ages of patients with rapid progression, slow progression and persistence patterns were 50.3±11.7, 50.4±9.4 and 50.3±11.7 years, respectively.

The proportion of WGO type-C in patients with rapid and slow progression was 42.2% and 44.7%, which was significantly higher than that in patients with recovery pattern (24.1% and 31.3%, respectively), as the highest proportion of WGO type-C was 51.2% in patients with persistent pattern. Moreover, the proportion of complications (ascites, bacterial infection, fungal infection, and GIB) was significantly higher in patients with progression pattern compared to that in patients with recovery pattern; however, there was no significant difference between progression and persistence patterns (Table 3). With regard to other complications, the occurrence rate of hepatic encephalopathy was highest in patients with rapid and slow progression (34.5% and 21.1%), followed by slow persistence (19.5%), but it was low in patients with rapid and slow recovery patterns (6.6% and 13.3%). The rate of AKI was 50.9%, 35.5%, 14.6%, 21.7%, and 17.1% in patients with rapid progression, slow progression, rapid recovery, slow recovery, and persistence patterns, respectively (p=0.000).

However, outcome pattern cannot be distinguished accurately by a single complication. Thus, we classified patients into three categories according to the number of complications, namely, a: 0–1 complication, b: 2 complications, and c: 3 or more complications. The number of complications was significantly different among the five prognostic patterns. For patients with rapid progression, slow progression, slow persistence, slow recovery, and rapid recovery patterns, the percentage with 0–1 complication was 7%, 14%, 20%, 28%, and 42%, respectively (Fig. 4). Similarly, the percentage with two complications was 18%, 37%, 49%, 41%, and 42%, and the percentage with 3 or more complications was 75%, 49%, 32%, 31%, and 16%, respectively (p=0.000).

The baseline PTA in ACLF patients with rapid progression was significantly lower than that in patients with other outcome patterns; conversely, TB in these patients was remarkably higher than that in patients with other patterns. There was no significant difference in PTA and TB among the other patterns (p>0.05). Similarly, baseline model for end-stage liver disease (MELD) score and Child-Turcotte-Pugh (commonly referred to as CTP) score were notably higher in ACLF patients with rapid progression than in those with other patterns; however, they were not significantly different among the other patterns.

Discussion

This study analyzed the clinical course of ACLF patients using jaundice and coagulation function as key diagnostic indicators. We found that the death or LT rate was 42.4% at 12 weeks after diagnosis. In the remaining patients, PTA and TB were both resolved in 48.6% of patients, and liver failure was persistent in 9.1% of patients. In view of the dynamics of the ACLF process, we proposed dynamic prognostic criteria based on the different clinical outcomes at 4 and 12 weeks after ACLF diagnosis, and found that the percentage of ACLF patients who exhibit rapid progression, slow progression, rapid recovery, slow recovery, and slow persistence was 25.6%, 16.8%, 30.2%, 18.3%, and 9.1%, respectively. We then preliminarily analyzed the clinical factors potentially affecting the dynamic outcome of ACLF patients. We also observed that an increasing number of complications not only accelerated death in ACLF patients but also deferred possible recovery. However, indicators in-

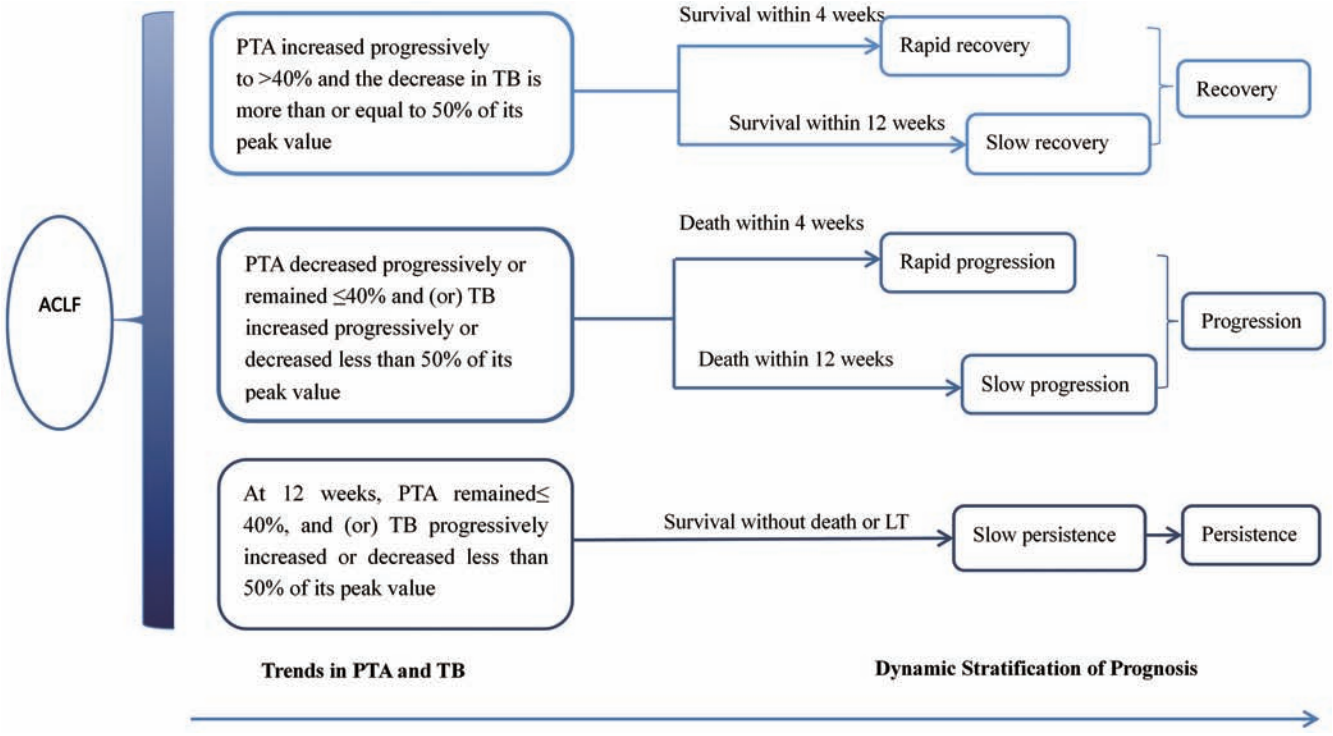

Fig. 3. Dynamic stratification criteria for clinical outcomes in ACLF patients. If INR ≥1.5 is taken as the criterion of coagulation abnormality in the diagnosis of ACLF, INR and PTA show the opposite trend and can be used to evaluate the clinical process. ACLF, acute-on-chronic liver failure; INR, international normalized ratio; PTA, prothrombin activity; TB, total bilirubin.

cluding baseline PTA, TB, MELD score and CTP score have limited power to predict dynamic prognosis.

Our findings suggest that the assessment on ACLF prognosis should be dynamically stratified in order to develop a more precise and individualized prognostic scoring model. Timely assessment of the clinical course of PTA and TB and monitoring of the complications during ACLF treatment can help to formulate subsequent treatment strategies: intensive care unit management and LT, or discontinuation due to futility.

Our data showed that the percentages of resolved PTA in ACLF patients at 4 and 12 weeks after diagnosis were 42.2% and 51.0%, respectively; for resolved TB, they were 39.7% and 54.7%, respectively and for resolved PTA and TB, they were 30.7% and 48.7%, respectively. These results are similar to those reported by APASL.[4] According to the consensus on ACLF, approximately 70% of ACLF patients who survived 90 days gradually recover, and the coagulation index returns to normal earlier than TB, which is consistent with our data. The European CANONIC study[3] used the change in ACLF grade of patients within 4 weeks after diagnosis to define the disease outcome, which showed that resolution (no-ACLF) is observed in 42.5% of patients within 4 weeks after diagnosis, arguing that the best period to define the clinical course of ACLF is between the third and seventh day after ACLF diagnosis (referred to as d3-7 ACLF). This viewpoint is based on its ability to predict 28-day and 90-day mortality. However, our study demonstrated that within 12 weeks after diagnosis, apart from survivors with a recovery pattern, 9.1% of survivors have a persistence pattern, excepting death or LT. Therefore, the predictive value of the d3-7 ACLF clinical course is limited.

To construct a more precise and comprehensive prognostic model, we proposed the following dynamic prognostic criteria: rapid progression, slow progression, rapid recovery, slow recovery, and slow persistence. We preliminarily analyzed the clinical factors potentially affecting the dynamic outcome of ACLF patients. The results showed that baseline PTA, TB, MELD and CTP scores are obviously different between patients with rapid progression and those with other prognostic patterns, but they are not remarkably different among other prognostic patterns. Thus, the MELD score, which is currently most commonly used to allocate liver resources,[16] can rapidly identify progressive patients, but it has restricted predictive value for other prognostic patterns. Nevertheless, patients with rapid progression die within 4 weeks after onset and have more complications, they are often in the late stage of liver failure, and have poor prognosis, even after LT. Studies[17–19] have shown that ACLF-3 LT has a lower survival rate than ACLF-1, 2 and a short transplantation window. Therefore, for patients with rapid progression, treatment decisions need to be made quickly to avoid salvage LT, and for patients with slow progression, LT can be delayed. At the same time, dispensable LT should be avoided in patients with potential recovery. Therefore, new scoring criteria should be derived from the dynamic outcome model in order to reasonably allocate scarce donor livers.

In addition, dynamic prognostic classification is beneficial for identifying patients who are receiving futile treatment and to adjust treatment strategies in a timely manner. In the present study, the mortality rate of patients with non-resolution of PTA and TB within 4 and 12 weeks after ACLF diagnosis was 63.9% and 98%, respectively. For these patients, whether emergency LT or termination of futile organ support treatment, such as artificial liver therapy, needs to be performed requires further study to prove.

As reported in many previous studies, hepatic encephalopathy,[20] infection,[21] GIB[22] and AKI[23] have predictive value for death in ACLF patients. They can be used to dis-

Table 3. Clinical characteristics of ACLF patients with dynamic stratification based on different clinical outcomes

Characteristics	Rapid progression	Slow progression	Rapid recovery	Slow recovery	Slow persistence	*p*
	n=116 (25.6%)	*n*=76 (16.8%)	*n*=137 (30.2%)	*n*=83 (18.3%)	*n*=41 (9.1%)	
Age in years, mean±SD	50.3±11.7	50.4±9.4	46.5±12	45.7±10.9	50.3±11.7	0.011
Male sex, *n* (%)	94 (81.0)	61 (80.3)	104 (75.9)	68 (81.9)	30 (73.2)	0.664
Underlying liver disease, *n* (%)						0.001
Without cirrhosis	28 (24.1)	18 (23.7)	63 (46)	27 (32.5)	8 (19.5)	
Compensated cirrhosis	39 (33.6)	24 (31.6)	41 (29.9)	30 (36.1)	12 (29.3)	
Decompensated cirrhosis	49 (42.2)	34 (44.7)	33 (24.1)	26 (31.3)	21 (51.2)	
Ascites, *n* (%)	96 (82.8)	55 (72.4)	84 (61.3)	54 (65.1)	31 (75.6)	0.003
Bacterial infection, *n* (%)	108 (93.1)	70 (92.1)	106 (77.4)	74 (89.2)	38 (92.7)	0.001
Fungal infection, *n* (%)	23 (19.8)	11 (14.5)	7 (5.1)	10 (12)	4 (9.8)	0.010
GIB, *n* (%)	12 (10.3)	11 (14.5)	3 (2.2)	6 (7.2)	1 (2.4)	0.007
Hepatic encephalopathy, *n* (%)	40 (34.5)	16 (21.1)	9 (6.6)	11 (13.3)	8 (19.5)	0.000
AKI, *n* (%)	59 (50.9)	27 (35.5)	20 (14.6)	18 (21.7)	7 (17.1)	0.000
ALT in U/L, median (IQR)	150 (65–548)	169.8 (54.9–405)	257 (75.8–797.7)	181.7 (62–546.5)	85 (31–247.4)	0.008
AST in U/L, median (IQR)	189 (93.6–189)	166 (106.6–451.6)	244 (119.6–542.8)	193.1 (107.3–326.8)	115.5 (70.5–243)	0.010
ALB in g/L, mean±SD	29.2±4.7	28.0±5.7	29.1±5.4	29.4±4.8	27.4±4.9	0.165
TB in mg/dL, mean±SD	22±9.7	18.1±9.2	15.9±8.1	17.2±9.6	13.9±6.5	0.000
PTA in %, mean±SD	27.7±10.6	34.2±11.5	35.3±9.2	32.4±7.9	33.5±7.8	0.000
INR, mean±SD	2.8±1.0	2.3±0.6	2.2±0.6	2.3±0.7	2.3±0.7	0.000
Cr in µmol/L, mean±SD	91±51.1	79.4±40.1	75.9±33.2	82.3±51	83.2±46.1	0.084
Na in mmol/L, mean±SD	132.7±5.1	133.9±4.9	135.7±4.6	134.5±4.9	132.8±5.9	0.000
WBC as $\times 10^9$/L), mean±SD	7.8±3.6	7.5±4.1	8.1±8.0	8.5±4.8	9.3±14.3	0.114
PLT as $\times 10^9$/L), mean±SD	92.1±49.1	100±51.2	113.9±57.9	109.6±54.7	87.8±54.6	0.004
MELD score, mean±SD	27.8±6.2	23.8±6.0	22.7±4.7	24.3±4.6	23.4±5.6	0.044
Child-Pugh score, mean±SD	11.7±1.3	11.4±1.6	11.1±1.3	11.5±1.2	11.6±1.3	0.028

ACLF, acute-on-chronic liver failure; AKI, acute kidney injury; ALB, albumin; ALT, alanine aminotransferase; AST, aspartate aminotransferase; Cr, creatinine; GIB, gastrointestinal bleeding; INR, international normalized ratio; MELD, model for end-stage liver disease; PLT, platelet; PTA, prothrombin activity; SD, standard deviation; TB, total bilirubin; WBC, white blood cell count.

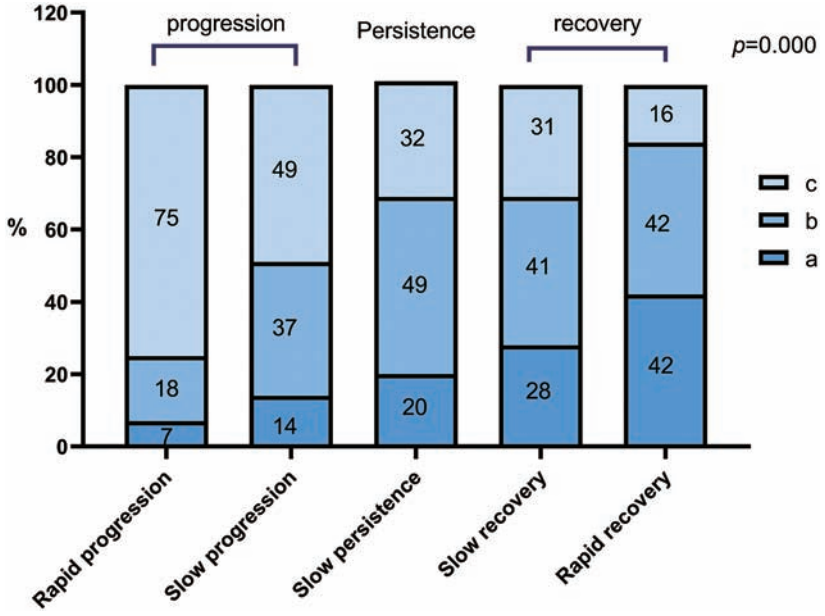

Fig. 4. Distribution of complications in ACLF patients with different clinical outcomes according to the dynamic stratification criteria. (a) 0–1 complication; (b) 2 complications; (c) 3 or more complications. ACLF, acute-on-chronic liver failure.

tinguish patients with recovery from those with progression but cannot distinguish patients with progression from those with persistence. It is well known that mortality will increase cumulatively as the number of dysfunctional or failed organs increases. Undoubtedly, these events can be utilized to predict outcomes and to calculate SOFA scores.[2] Meanwhile, a higher proportion of patients with three or more complications are observed in those with the aggravated dynamic prognostic classification, which is consistent with another study.[24] Thus, preventing complications is important to improve the dynamic outcome of ACLF patients.

There are limitations in this study. This is a retrospective cohort with insufficient information on the treatment of liver failure. Also, the impact of treatment options such as artificial liver therapy on dynamic prognosis was not analyzed. In addition, bacterial infection was judged according to the use of antibiotics, which, since it is often related to the diagnosis and treatment experience of clinicians, may have led to an overestimation of the bacterial infection rate among our patients. Moreover, ACLF patients were enrolled in this study when experiencing different disease courses, which may lead to misjudgment and ensuing uncertain results affecting the dynamic prognosis. Furthermore, multivariate analysis could not be performed in this study, due to the limited sample size. Hence, it is imperative to conduct a prospective cohort study for analyzing factors potentially affecting dynamic prognosis and developing new prognostic models.

In conclusion, we propose a more refined dynamic prognostic classification, which lays the foundation for developing a new accurate prognostic model for ACLF. Prediction of dynamic prognosis is helpful for making the optimal treatment strategy for ACLF patients and utilizing medical resources reasonably.

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Conflict of interest

The authors have no conflict of interests related to this publication.

Author contributions

Study conception and design (MMX, MK, YC, ZPD), acquisition of data (MMX, MK, PFY, YC, FL, YYC), analysis and interpretation of data (MMX, MK, YC, SLY, YZZ, WL, HBZ, BWD), drafting of the manuscript (MMX, MK), critical revision of the manuscript for important intellectual content (YC, ZPD, SJX, TH, HBZ, BWD), administrative, technical, or material support and study supervision (YC, ZPD).

Data sharing statement

No additional data are available.

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Original Article

Development and Validation of an RNA Binding Protein-associated Prognostic Model for Hepatocellular Carcinoma

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Abstract

Background and Aims: The survival rate of patients with hepatocellular carcinoma is variable. The abnormal expression of RNA-binding proteins (RBPs) is closely related to the occurrence and development of malignant tumors. The primary aim of this study was to identify RBPs related to the prognosis of liver cancer and to construct a prognostic model of liver cancer. ***Methods:*** We downloaded the hepatocellular carcinoma gene sequencing data from The Cancer Genome Atlas (cancergenome.nih.gov/) database, constructed a protein-protein interaction network, and used Cytoscape to realize the visualization. From among 325 abnormally expressed genes for RBPs, 9 (XPO5, enhancer of zeste 2 polycomb repressive complex 2 subunit [EZH2], CSTF2, BRCA1, RRP12, MRPL54, EIF2AK4, PPARGC1A, and SEPSECS) were selected for construction of the prognostic model. Then, we further verified the results through the Gene Expression Omnibus (www.ncbi.nlm.nih.gov/geo/) database and *in vitro* experiments. ***Results:*** A prognostic model was constructed, which determined that the survival time of patients in the high-risk group was significantly shorter than that of the low-risk group ($p<0.01$). Univariate and multivariate Cox regression analysis suggested that the risk score was an independent prognostic factor ($p<0.01$). We also constructed a nomogram based on the risk score, survival time, and survival status. At the same time, we verified the high expression and cancer-promoting effects of EZH2 in tumors. ***Conclusions:*** Survival, receiver operating characteristic curve and independent prognostic analyses demonstrated that we constructed a good prognostic model, which might be useful for estimating the survival of patients with hepatocellular carcinoma.

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Keywords: Hepatocellular carcinoma; RNA binding protein; Prognostic model; Nomogram.

Abbreviations: AUC, area under the curve; BP, biological processes; CC, cellular components; DERs, differentially-expressed RBPs; EZH2, enhancer of zeste 2 polycomb repressive complex 2 subunit; FC, fold-change; FDR, false discovery rate; GEO, Gene Expression Omnibus (www.ncbi.nlm.nih.gov/geo/); GO, gene ontology; HCC, hepatocellular carcinoma; ICC, intrahepatic cholangiocarcinoma; KEGG, Kyoto Encyclopedia of Genes and Genomes; MF, molecular functions; PI, propidium Iodide; PPI, protein-protein interactions network; qRT-PCR, quantitative real-time PCR; RBPs, RNA-binding proteins; ROC, receiver operating characteristic; si, small interfering; TCGA, The Cancer Genome Atlas (cancergenome.nih.gov/).

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Introduction

Worldwide, liver cancer is the fourth leading cause of cancer-related deaths and has the sixth highest incidence.[1] It is estimated that 840,000 new cases of liver cancer are diagnosed and at least 780,000 people die of liver cancer every year, with China accounting for 47% of the total number of liver cancer cases as well as the related mortality.[2,3] Hepatocellular carcinoma (HCC) is the predominant type of primary liver cancer, accounting for approximately 90% of all liver cancer cases.[4] Although great progress has been made in the diagnosis and treatment of liver cancer, the 5-year survival rate of patients with advanced liver cancer is still less than 20%.[5] In China, liver cancer ranks third in cancer-related mortality due to the large number of liver cancer patients, delayed diagnosis, and limited treatment options.[6] Therefore, it is important to study the molecular mechanism of tumorigenesis and development, to find new targets of drug therapy, to identify new tumor markers, and to achieve an earlier diagnosis of liver cancer.

RNA-binding proteins (RBPs) are a group of proteins associated with RNA regulation and metabolism. Their main role is to mediate the maturation, transport, localization and translation of RNA, and their abnormal expression can cause a variety of diseases. At present, there are approximately 1,542 known human RBPs, accounting for approximately 7.5% of all protein-coding genes. It is now clear that RBPs are dysregulated in different types of cancer, affecting the expression and function of oncoproteins and tumor suppressors. For example, IGF-II mRNA-binding proteins (IMPs) are involved in the progression of tumors and the establishment and maintenance of tumor cell hierarchies.[7] Therefore, studying the complex interaction network between RBPs and their cancer-related RNA targets will help in understanding the molecular mechanisms of RBPs in cancer progression, and may also enable the discovery of new cancer treatment targets. Although RBPs are known to be involved in the occurrence and development of a variety of tumors, we still know very little about the molecular mechanism of RBPs in tumor progression.

Because there are few studies on the role of RBPs in the occurrence and development of liver cancer, we designed this study to screen-out RBPs related to the prognosis of patients. We aimed to construct a predictive model that would be able to provide some help to clinical work and direction

for future research, including of molecular mechanisms and the identification of molecular targets for treatment.

Methods

Clinical data and RNA sequencing data of the patients

We downloaded the RNA high-throughput sequencing data from The Cancer Genome Atlas (cancergenome.nih.gov/; TCGA) database, including 374 tumor tissue samples and 50 normal liver tissue samples (www.cancer.gov/about-nci/organization/ccg/research/structural-genomics/tcga). We also downloaded the clinical data of 377 HCC patients from the TCGA. After excluding six patients with incomplete data, a total of three hundred and seventy-one patients were finally included in the study, with each having data on follow-up time, survival status, disease stage, etc. The RNA high-throughput sequencing data included the expression data of 60,483 genes, and we extracted the expression levels of 1,473 RBPs from such. As this research did not involve human participants, no research ethics review was necessary.

Identification of the differentially-expressed RBPs (DERs) in HCC patients

We used R software to analyze expression of the extracted 1,473 RBPs. A total of 325 RBPs showed expression differences between the tumor tissues and normal tissues, including 203 up-regulated and 122 down-regulated DERs. The threshold for the DERs was set as |log fold-change (FC)|>1 and false discovery rate (FDR) <0.05.

Enrichment analysis and protein-protein interaction (PPI) network of the DERs

We divided the DERs into an up-regulated group and a down-regulated group, and then performed gene ontology (GO) function analysis on the two groups of RBPs for three GO domains: molecular functions (MF), biological processes (BP) and cellular components (CC). Next, we performed Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis on the two groups of patients. We used the clusterProfiler package in R software for the GO function enrichment analysis and the KEGG pathway enrichment analysis. Then, we uploaded these DERs to the online tool STRING (https://string-db.org) to construct the PPI network. We deleted the disconnected nodes, and the remaining RBPs were used for the next analysis. To further study the role of the DERs in HCC, we used Cytoscape software to create a PPI network that incorporated the nodes from the STRING database. At the same time, we also used the MCODE tool in the Cytoscape software to create a PPI subnet. Then, enrichment analysis and coexpression analysis were performed on the sub-network.

Screening of prognostic RBPs, construction and testing of the prognostic models

We used univariate Cox regression analysis to screen out 29 RBPs related to the patients' outcomes, from among all of the DERs. The threshold of the Cox regression analysis was set to 0.001. All HCC patients were divided into a training group and a test group. Then, in the training group, a multivariate Cox regression analysis was performed to screen out nine RBPs. A prognostic model was constructed based on the relationship between the expression levels of these nine RBPs and the patients' outcomes in the training group. The risk score of the HCC patients in the test group was calculated according to the prognostic model, and the patients in the test group were divided into high-risk and low-risk groups according to the risk scores. Then, we used survival curves, receiver operating characteristic (ROC) curves, risk curves and independent prognostic analysis to test the predictive power of the prognostic model. In addition, we still used the data GSE76427 in the Gene Expression Omnibus (GEO) database to verify our model externally.

Construction of the nomogram

The coefficients obtained by the Cox regression model were used to construct the nomogram of overall survival. To construct the nomogram, we first determined a scale axis of 0–100 to represent the score (in order to make the expression of the RBPs correspond to the scores on the scale axis) and we calculated the score of each RBP. We added the scores of each RBP to obtain the total score. Based on the correspondence between the total score axis and the survival rate, the 1-, 2-, 3- and 5-year survival rates of the patients could be predicted.

Cell culture, RNA isolation and quantitative real-time PCR (qRT-PCR) analysis

Because the risk ratio of enhancer of zeste 2 polycomb repressive complex 2 subunit (EZH2) is greater and the coefficient in the risk score calculation formula is greater, we choose EZH2 for further verification. A total of 20 HCC patient samples and paired non-tumor liver tissue samples were collected from the patients hospitalized at the Zhongnan Hospital, Wuhan University (Hubei Province, China). All patients provided written informed consent to the use of tissues for scientific research in the Department of Hepatobiliary and Pancreatic Surgery. All cell lines were purchased from the Chinese Type Culture Collection. All the cell lines were maintained in Dulbecco's modified Eagle's medium/high glucose (GE, USA) containing 10% fetal bovine serum (Gibco, USA). RNA was extracted using TRIzol reagent (TaKaRa, Japan) according to the manufacturer instructions. cDNAs were generated by the reverse transcription synthesis kit (TaKaRa) and the SYBR Green PCR Kit (TaKaRa) was used for qRT-PCR analysis. The primers used for EZH2 were 5′-GAGTTGGTGAATGCCCTTGGT-3′ and 5′-CATCTCGGTGATCCTCCAGATC-3′.

Gene knock-down

For generation of EZH2 knock-down cells, small interfering (si)RNA transfections were performed using the siRNA transfection reagent. The following siRNA sequences were used: EZH2-siRNA, CGGCUUCCCAAUAACAGUATT, UACUGUUAUUGGGAAGCCGTT.

Cell proliferation, migration, invasion, apoptosis, and cell cycle analyses

A CCK8 Kit (Dojindo, China) was used to measure cell viability. Transwell assay was used to assess cell migration. Cell migration was assessed by *in vitro* scratch wound assay. A BioCoat Matrigel Invasion Chamber (BD Biosciences, USA) was used to assess cell invasion; the number of cells

migrating and invading was counted in three random areas. Apoptosis was assayed using the Annexin V-FITC Apoptosis Detection Kit (Invitrogen, USA), according to the manufacturer's instructions, and the percentage of apoptotic cells was verified by flow cytometry (Beckman-Coulter, USA). To detect the cell cycle, 48 hours after transfection, the cells were stained with PI (propidium iodide) and assessed.

Statistical analysis

The datasets generated and analyzed during the current study are available in TCGA (https://cancergenome.nih.gov/) and GEO (www.ncbi.nlm.nih.gov/geo). Difference analysis and regression analysis of the data were processed in the R3.6.3 software (https://www.r-project.org). Statistical analysis of clinical data was performed using SPSS v.23.0 (IBM Corp., USA). Variables were compared by *t*-test or chi-square test.

Results

DERs in HCC patients

A flowchart showing the steps of data processing and integrative data analysis is provided in Figure 1A. The original data downloaded from TCGA included the RNA sequencing data of 50 normal tissues and 374 liver cancer tissue samples. The sequencing data itself comprised 60,483 sets of RNA expression data, including that of 1,473 RBPs. After data processing, it was found that 325 RBPs had expression differences (Fig. 1B), including 203 up-regulated and 122 down-regulated RBPs (Fig. 1C). The results of the DERs are presented in the form of heat maps and volcano maps.

GO functional and KEGG pathway enrichment analysis

The GO function enrichment analysis was performed on the obtained up-regulated RBPs and down-regulated RBPs. The GO terms included BP, MF, and CC. For BP, the up-regulated RBPs were mainly enriched in non-coding RNA metabolic process, RNA splicing, and non-coding RNA processing. For CC, the up-regulated RBPs were mainly enriched in spliceosomal complex, cytoplasmic ribonucleoprotein granules, and ribonucleoprotein granules. For MF, the RBPs were mainly enriched in catalytic activity and acting on RNA (Fig. 1D). Down-regulated RBPs were mainly enriched in regulation of translation, regulation of cellular amide metabolic processes, cytoplasmic ribonucleoprotein granules, ribonucleoprotein granules, and catalytic activity (Fig. 1E). KEGG pathway enrichment analysis of up-regulated RBPs showed that they were mainly enriched in spliceosome, RNA transport, and mRNA surveillance pathways (Fig. 1F). Down-regulated RBPs were enriched in herpes simplex virus 1 infection, influenza A, and RNA degradation (Fig. 1G). The results of the enrichment analysis are also displayed in the form of tables (Table 1 and Table 2). The results in the chart are sorted by *p*-values, and the top 15 results of the GO enrichment analysis are shown.

PPI network and the coexpression network of RBPs

To study the interactions among the 325 RBPs, we used STRING to construct a PPI network. The connections between the molecules represent the possible interactions between two protein molecules, and the different colors of the lines represent different levels of evidence. After deleting 21 disconnected RBPs, there were a total of 304 nodes and 2,794 connections in the PPI diagram (Fig. 2A). Then, we used Cytoscape software to create a coexpression network of interactions among all nodes in the PPI. Among them, the block of proliferation 1 (i.e. BOP1) had the most interactive RBPs, and it had confirmed or potential interactions with 50 RBPs (Fig. 2B). We applied the MCODE tool in Cytoscape to construct a sub-network of the coexpression network (Fig. 2D–H), and then we selected the first five sub-networks according to their association score to identify the first important module, including 115 nodes and 1,295 edges (Fig. 2C).

Screening of prognosis-related RBPs and construction of a risk scoring model

A total of 305 RBPs were included in the PPI network. To screen-out the RBPs related to prognosis, we used univariate Cox regression analysis to screen-out a total of 29 RBPs, and we calculated their hazard ratios (Fig. 3A). Among them, 19 RBPs had a hazard ratio greater than 1, which means they had a negative impact on patient prognosis, and 10 RBPs had a positive impact on the prognosis. We then divided the patients into a training group and a test group at a ratio of 7:3, and we performed multivariate Cox regression analysis on the expression of the selected RBPs of patients in the training group (Fig. 3B). Then, we built a prediction model based on the relationship between the expression of RBPs in the training group of patients and the patient's survival and survival status. The formula for calculating the patient risk score was as follows:

$$\text{Risk score} = (-0.4520 \times \text{ExpXPO5}) + (0.7493 \times \text{ExpEZH2}) + (0.3913 \times \text{ExpCSTF2}) + (-0.6586 \times \text{ExpBRCA1}) + (0.4145 \times \text{ExpRRP12}) + (-0.3734 \times \text{ExpMRPL54}) + (-0.4652 \times \text{ExpEIF2AK4}) + (-0.2380 \times \text{ExpPPARGC1A}) + (0.3293 \times \text{ExpSEPSECS})$$

To test the effectiveness of the predictive model, we used the survival curve method to evaluate the prognostic model. In the training group, patients with high-risk scores had a worse prognosis than patients with low-risk scores ($p<0.01$; Fig. 3C). In the test group, patients were divided into a high-scoring group and a low-scoring group according to the risk score model, and the survival rates of the two groups were also significantly different ($p<0.01$; Fig. 3D). In addition, we adopted the ROC test method and calculated the area under the ROC curve (AUC). The AUC of the training group was 0.735 and the AUC of the test group was 0.740, indicating that the risk scoring model we constructed has good diagnostic performance (Fig. 3F, G). Patients in the GEO cohort were also divided into high-risk groups and low-risk groups based on this model. There were also significant differences in survival rates between the two groups ($p<0.05$), and the AUC of the GEO cohort was 0.740 (Fig. 3E, H). This shows that the risk scoring formula we established can accurately divide patients into a high-risk group with a poor prognosis and a low-risk group with a good prognosis.

Independent prognostic analysis of the risk score

To evaluate whether the risk score is an independent prognosis-related factor, we conducted an independent prognostic analysis of risk scores for patients in the training group and test group. The prognostic analysis used univariate and multivariate Cox regressions. The univariate prognostic analysis of the training group showed that the clinical stage and risk score of the disease were independent factors affecting the prognosis ($p<0.001$; Fig. 3I). The multivariate prognostic analysis showed that the risk score ($p<0.001$) and clinical stage ($p<0.05$) were independent factors affecting the prog-

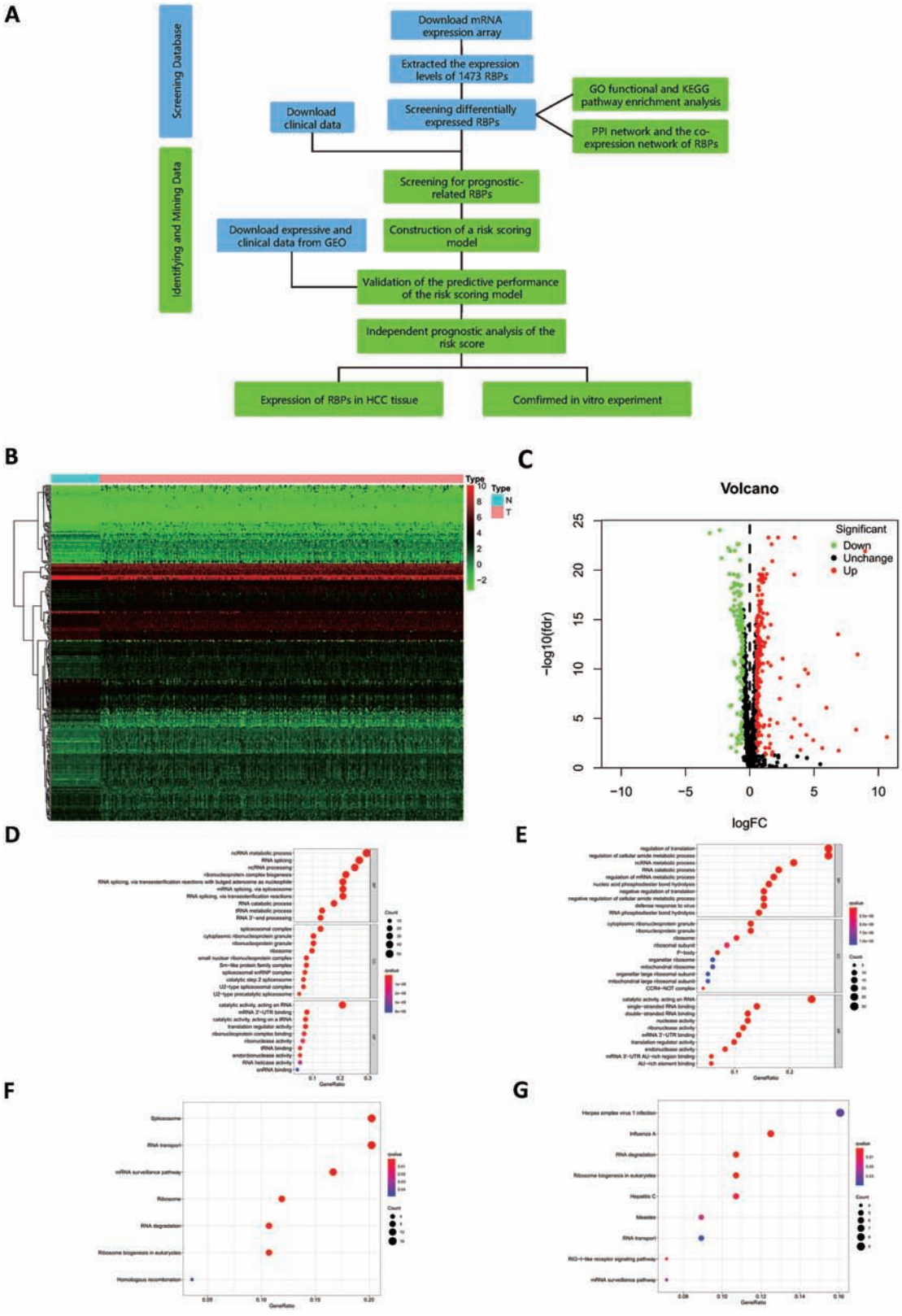

Fig. 1. Flow chart of heat map, volcano plot, GO enrichment analysis and KEGG pathway enrichment of DERs. (A) Flow chart. (B) Heat map of DERs. (C) Volcano plot of DERs. (D) GO enrichment analysis of up-regulated RBPs. (E) GO enrichment analysis of down-regulated RBPs. (F) KEGG pathway enrichment of up-regulated RBPs. (G) KEGG pathway enrichment of down-regulated RBPs.

Table 1. GO functional enrichment analyses

ID	Description	GO term	*p*	*q*
Up-regulated RBPs				
GO:0034660	ncRNA metabolic process	BP	3.17E-43	4.16E-40
GO:0034470	ncRNA processing	BP	7.52E-38	4.93E-35
GO:0008380	RNA splicing	BP	2.34E-37	1.02E-34
GO:0140098	catalytic activity, acting on RNA	MF	3.85E-29	6.17E-27
GO:0000377	RNA splicing, via transesterification reactions with bulged adenosine as nucleophile	BP	2.08E-28	5.45E-26
GO:0000398	mRNA splicing, via spliceosome	BP	2.08E-28	5.45E-26
GO:0000375	RNA splicing, via transesterification reactions	BP	2.81E-28	6.14E-26
GO:0022613	ribonucleoprotein complex biogenesis	BP	4.21E-27	7.87E-25
GO:0031123	RNA 3′-end processing	BP	2.65E-22	4.34E-20
GO:0005681	spliceosomal complex	CC	2.01E-21	2.70E-19
GO:0006399	tRNA metabolic process	BP	2.53E-21	3.68E-19
GO:0006401	RNA catabolic process	BP	3.97E-21	5.21E-19
GO:0008033	tRNA processing	BP	4.46E-21	5.31E-19
GO:0006402	mRNA catabolic process	BP	3.91E-18	4.27E-16
GO:1903311	regulation of mRNA metabolic process	BP	1.97E-17	1.99E-15
Down-regulated RBPs				
GO:0006417	regulation of translation	BP	3.73E-24	5.23E-21
GO:0140098	catalytic activity, acting on RNA	MF	2.76E-22	4.31E-20
GO:0034248	regulation of cellular amide metabolic process	BP	1.08E-22	7.55E-20
GO:0003727	single-stranded RNA binding	MF	8.03E-20	6.26E-18
GO:0003725	double-stranded RNA binding	MF	2.21E-18	1.15E-16
GO:0090501	RNA phosphodiester bond hydrolysis	BP	8.66E-16	4.04E-13
GO:0004540	ribonuclease activity	MF	3.98E-14	1.41E-12
GO:0003730	mRNA 3′-UTR binding	MF	4.52E-14	1.41E-12
GO:0034660	ncRNA metabolic process	BP	5.83E-15	1.79E-12
GO:0017148	negative regulation of translation	BP	6.39E-15	1.79E-12
GO:0034249	negative regulation of cellular amide metabolic process	BP	2.34E-14	5.47E-12
GO:0051607	defense response to virus	BP	5.50E-14	1.01E-11
GO:1903311	regulation of mRNA metabolic process	BP	5.75E-14	1.01E-11
GO:0006401	RNA catabolic process	BP	2.03E-13	2.98E-11
GO:0090305	nucleic acid phosphodiester bond hydrolysis	BP	2.13E-13	2.98E-11

nc, non-coding.

nosis, and the risk score had a higher hazard ratio (Fig. 3J). In the test group of patients, both univariate and multivariate prognostic analysis showed that the risk score was an independent factor affecting the prognosis ($p<0.01$). This showed that the risk scoring model we built has good predictive ability.

Validation of the predictive performance of the risk scoring model

The expression levels of the nine RBPs in the training group and the test group were significantly different between the high-risk group and the low-risk group (Fig. 4A, B). Fig. 4C and D show the distribution of the patients' risk scores. Fig. 4E and F show the relationship between the patient's survival status, survival time, and risk score. The red dots represent high-risk patients, and the green dots represent low-risk patients. It can be seen that the higher the risk score, the higher the proportion of patients whose follow-up outcome is death and the shorter the follow-up time.

Clinical features of the high-risk group and low-risk group

We obtained the clinical characteristics of the two groups of

Table 2. KEGG enrichment analysis

ID	Description	*p*	*q*
Up-regulated RBPs			
hsa03040	spliceosome	1.24E-13	4.85E-12
hsa03015	mRNA surveillance pathway	3.33E-13	6.49E-12
hsa03013	RNA transport	2.86E-12	3.71E-11
hsa03018	RNA degradation	1.08E-07	1.05E-06
hsa03008	ribosome biogenesis in eukaryotes	2.18E-06	1.70E-05
hsa03010	ribosome	5.08E-06	3.30E-05
hsa03440	homologous recombination	0.008791	0.048915
Down-regulated RBPs			
hsa03018	RNA degradation	1.62E-05	0.000718
hsa03008	ribosome biogenesis in eukaryotes	0.000117	0.002460
hsa05164	influenza A	0.000167	0.002460
hsa05160	hepatitis C	0.000703	0.007773
hsa04622	RIG-I-like receptor signaling pathway	0.001372	0.012129
hsa05162	measles	0.002696	0.019863
hsa03015	mRNA surveillance pathway	0.003590	0.022672
hsa05168	herpes simplex virus 1 infection	0.006320	0.034924
hsa03013	RNA transport	0.008050	0.039545

patients, and performed a statistical analysis of the surgical methods, alpha-fetoprotein values, degree of liver cirrhosis, and other adjuvant treatments. The above-mentioned and other features of the two groups of patients found no significant difference, indicating risk score is an independent predictor of prognosis (Table 3).

Nomogram construction

To better establish the relationship among RBPs' expression, risk score and patient survival, we developed a nomogram. According to the nomogram, the expression levels of 9 RBPs can be converted into corresponding scores, and then the scores can be added to obtain the total risk score of the patient. The risk score corresponds to the estimated survival rate, including 1-, 2-, 3- and 5-year survival rates. According to the nomogram, the prognostic model can be applied in the clinic, and the long-term survival rate of a single patient can be predicted based on the expression of RBPs (Fig. 4G).

Expression of RBPs in HCC tissue

To further determine the expression of RBPs in liver cancer tissues for constructing this prognostic model, we used the immunohistochemical staining results in the database to show that BRCA1, CSTF2, EZH2 and XPO5 are highly expressed in liver cancer tissues. EIF2AK4 and MRPL54 are expressed at low levels in liver cancer tissues (Fig. 4H).

Verification of expression of EZH2 in tissues

The primers for EZH2 mRNA were designed, and the expression of 20 pairs of HCC and adjacent tissues was further verified by qRT-PCR. EZH2 showed a significant increase in liver cancer (Fig. 5A). In addition to the mRNA level, at the protein level, we also verified the high expression of EZH2 in tumors by western blotting (Fig. 5D). In addition, we also tested the data of EZH2 expression in normal liver cell lines and various HCC cell lines (Fig. 5B). Among them, the Hep3B cell line showed the highest expression of EZH2. We chose the Hep3B cell line for subsequent *in vitro* experiments.

Effect of EZH2 on the malignant behaviors of liver cancer cells

After transfection with siRNA, the mRNA and protein levels of EZH2 decreased significantly (Fig. 5C, E). We used the scratch test and the Transwell assay to determine whether reducing EZH2 affects the invasion and migration of HCC cells. Compared with the control group (siRNA-control), the migration ability of the HCC cells at the edge of the scratch in the siRNA-EZH2 group was significantly reduced ($p<0.05$; Fig. 5F). In addition, the number of HCC cells in the si-EZH2 group that passed through the Transwell chamber was decreased significantly ($p<0.05$; Fig. 5G). CCK8 experiment demonstrated that knock-down of EZH2 reduced the proliferation ability of cells (Fig. 5H). However, flow cytometry did not find a significant effect of EZH2 on cell apoptosis and cell cycle (Fig. 5I, J).

Discussion

RBPs are involved in almost all steps of RNA post-transcription regulation, regulating RNA splicing, polyadenylation, stability, localization, translation, and degradation.[8,9] Studies have shown that the abnormal expression of certain RBPs is related to the HCC transcriptome and tumorigenicity and is related to the poor prognosis of liver cancer patients.[10,11] However, due to the large number of RBPs, their diverse functions and complex mechanisms, there are still many RBPs

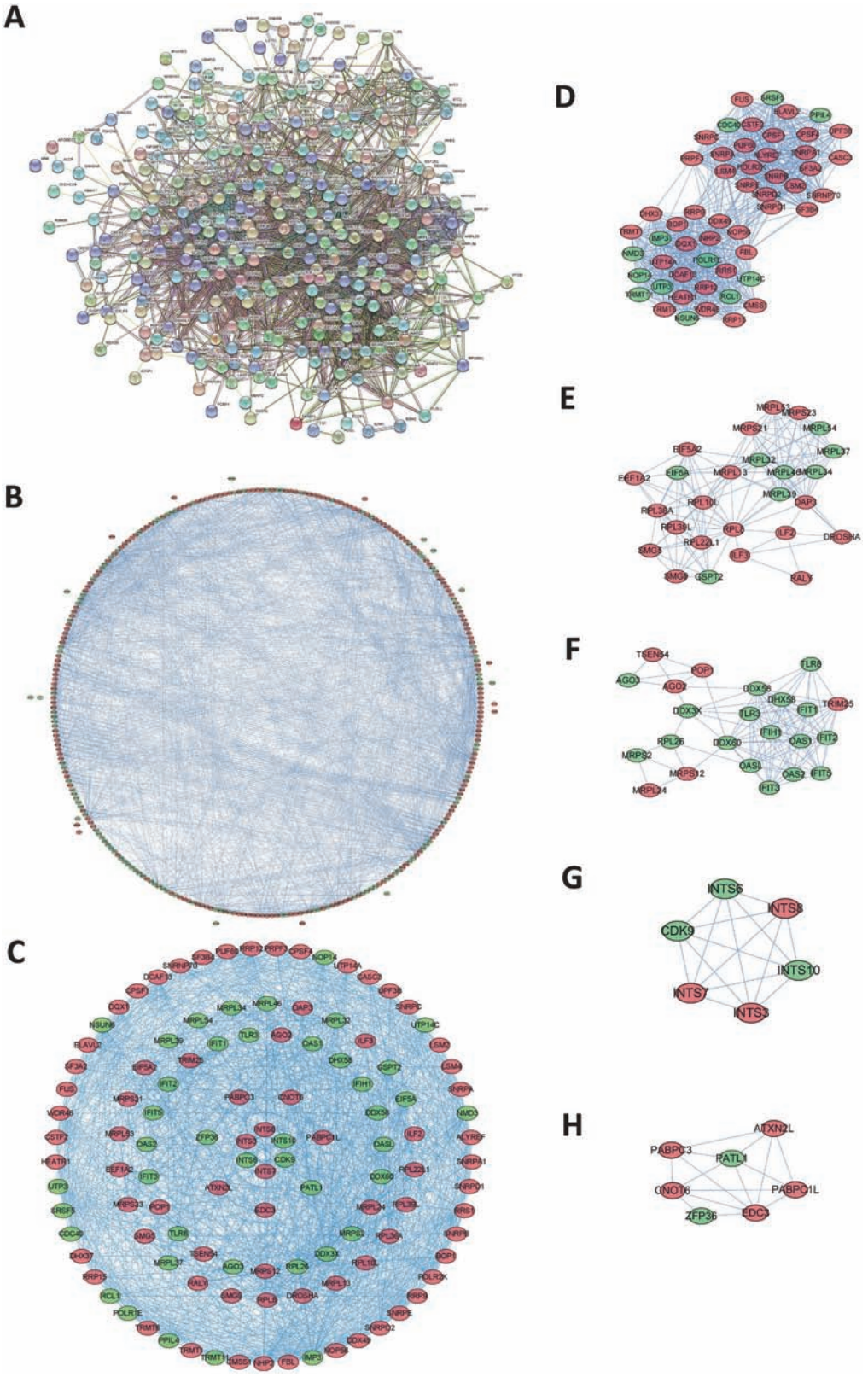

Fig. 2. Interaction network of DERs. (A) PPI network. (B) Network visualized using Cytoscape. (C) Network of important modules. (D-H) Important subnetworks. The light blue and purple lines, respectively, indicate known interactions from curated databases and experimentally determinations. Green, red and dark blue represent predicted interactions, including gene neighborhood, gene fusions and gene co-occurrences in Fig. 2A. Red circles: up-regulated RBPs; Green circles: down-regulated RBPs in Fig. 2B-H.

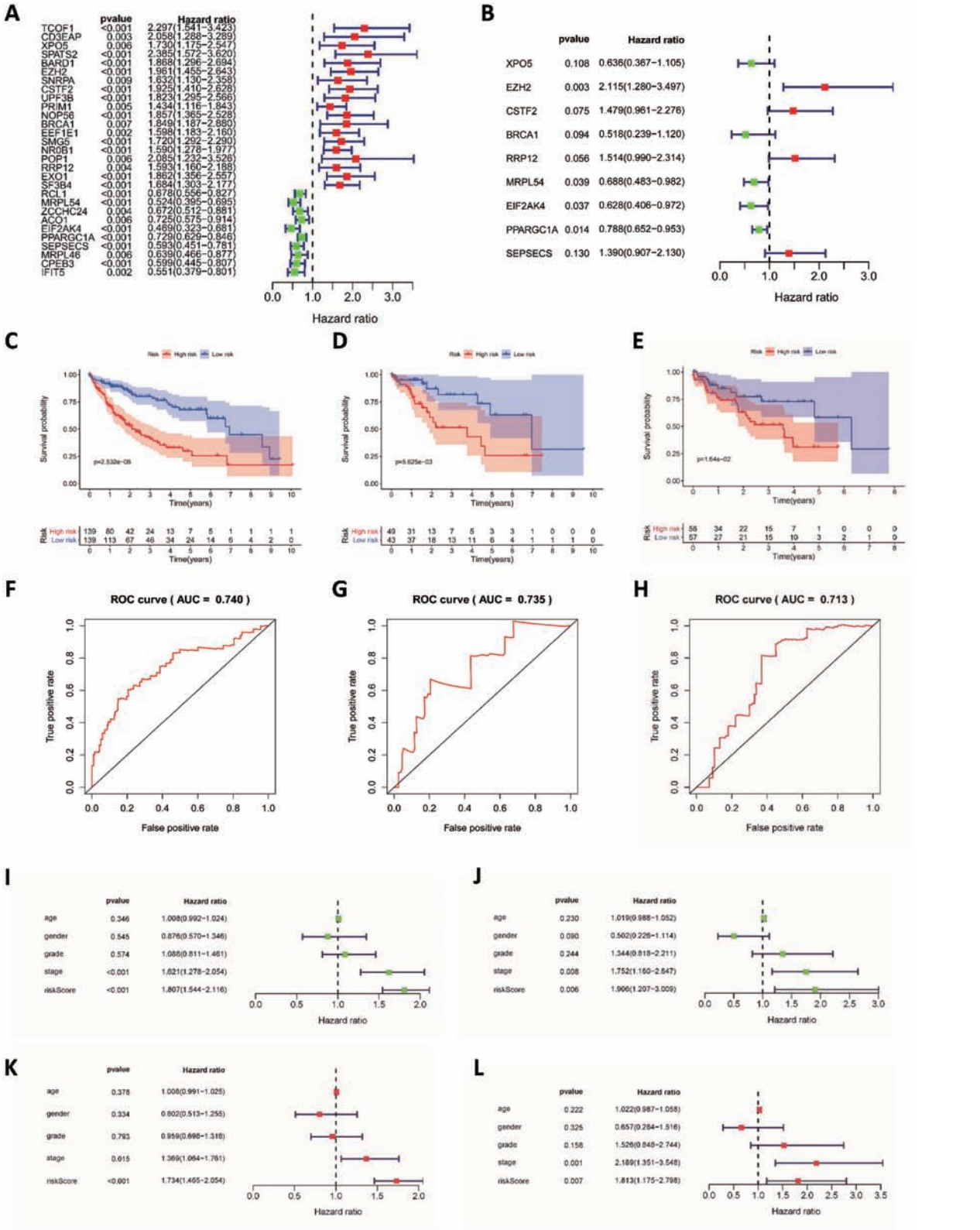

Fig. 3. Results of the Cox regression analysis, survival and ROC curves. (A) Univariate Cox regression analysis of DERs. (B) Multivariate Cox regression of DERs. (C) Survival curve of the training group. (D) Survival curve of the test group. (E) Survival curve of the GEO group. (F) ROC curve of the training group. (G) ROC curve of the test group. (H) ROC curve of the GEO group. (I) Univariate Cox regression analysis in the training group. (J) Univariate Cox regression analysis in the test group. (K) Multivariate Cox regression analysis in the training group. (L) Multivariate Cox regression analysis in the test group. Red and blue areas: 95% confidence interval.

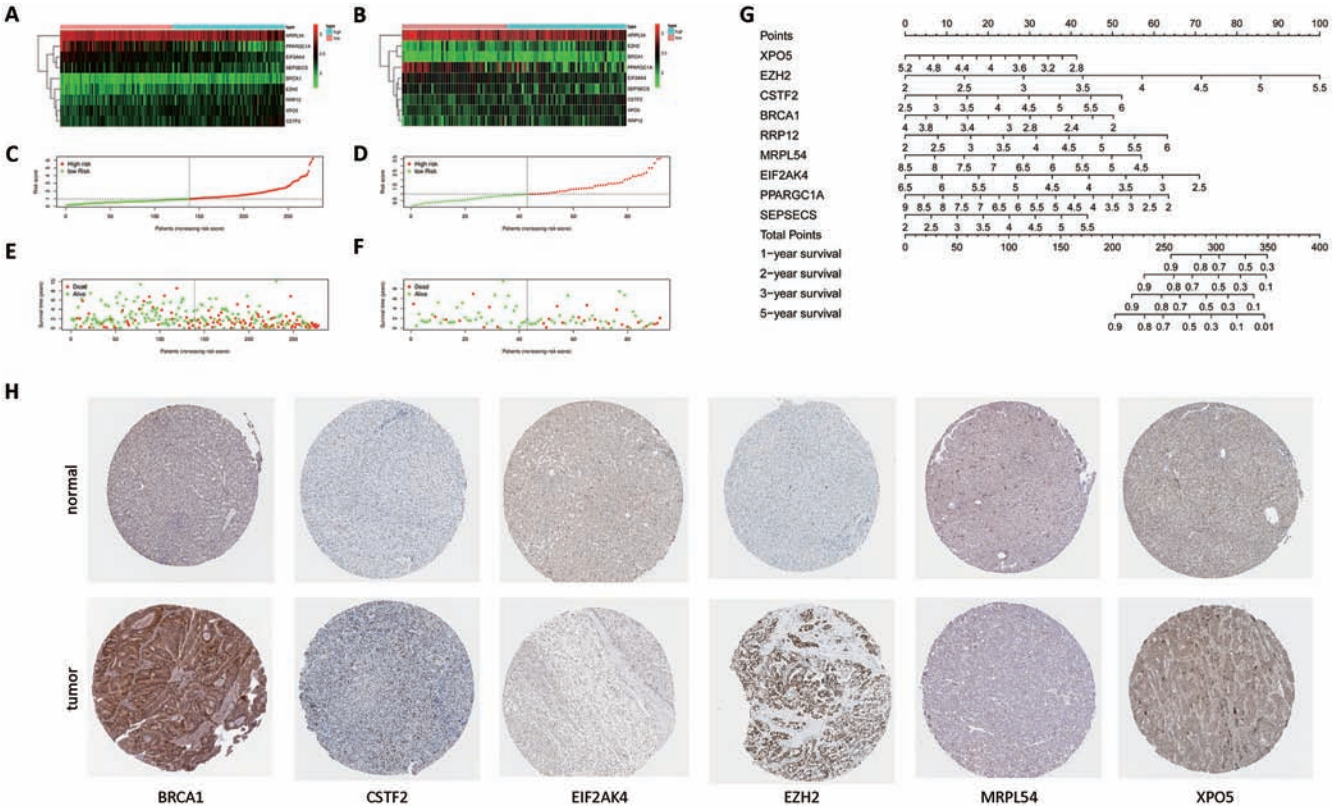

Fig. 4. Heat map and distribution of risk scores and survival status: nomogram and immunohistochemistry results. (A) Heat map of RBPs in the training group. (B) Heat map of RBPs in the test group. (C) Distribution of risk scores in the training group. (D) Distribution of risk scores in the test group. (E) Distribution of survival status of patients in the training group. (F) Distribution of survival status of patients in the test group. (G) Nomogram for predicting 1-, 2-, 3- and 5-year overall survival of patients with HCC. (H) Immunohistochemistry results.

whose mechanism of action has not been studied in depth.

To promote in-depth research of RBPs in HCC, we designed this study to screen-out the key RBPs that play a role in HCC. At the same time, we also developed a prognostic model of HCC patients based on the expression of RBPs.

To further study the interactions among the RBPs, we created a PPI network based on previous research, co-expression relationships, bioinformatics predictions, and gene-adjacent relationships. To study the relationships among the RBPs more intuitively, Cytoscape software was used to realize the visualization of the PPI network. In the graph created, we can see that some RBPs have a correlation with many RBPs, so we think these RBPs should have more biological functions and greater research value. In the network, BOP1 interacts with 50 RBPs. According to the results of enrichment analysis, it can be inferred that the research directions of RBPs mainly include ribonucleoprotein complex biogenesis, RNA splicing, non-coding RNA metabolic process, mRNA surveillance pathway, RNA transport, and others.

There are many studies on the mechanism of EZH2 in liver cancer. EZH2 is related to the prognosis of patients and can promote HCC progression by regulating the miR-22/galectin-9 axis or the expression of PD-L1 in hepatocellular carcinoma.[12] PPARGC1A can interact with MiR-93-5p to promote the proliferation of liver cancer cells, and it can also interact with MiR-30b-5p to regulate the lipid metabolism of liver cancer cells.[13,14] In addition, other mechanisms of PPARGC1A are also worthy of further study. There are few studies on EIF2AK4 and MRPL54 in HCC, but because these two RBPs are related to the prognosis of liver cancer in the results of the univariate and multivariate Cox regression analyses, they have great research value. EIF2AK4 and MRPL54 can be studied in terms of binding to RNA to affect the metabolism of RNA or to affect the variable shearing of RNA.

The nomogram makes the prognostic model used to predict the survival rate of patients at 1, 2, 3, and 5 years more intuitive and more convenient for clinical application. The cost of obtaining the expression level of nine RBPs is relatively low, and the survival rate calculated based on their expression level can help with clinical decision-making and selecting treatment options. For example, studies have shown that transarterial chemoembolization therapy for patients with poorly differentiated liver cancer and venous tumor thrombi after liver cancer resection can help prolong the survival of patients. However, for patients with early liver cancer and moderately differentiated liver cancer, whether to give interventional therapy is still controversial. According to our research, the treatment plan can be determined based on the risk score. If the risk score is high, it indicates a poor prognosis. It is thus recommended to give postoperative interventional chemotherapy, targeted therapy, and other treatment options.

In addition to the above-mentioned advantages, there are some shortcomings of this study. First, due to the need to construct the formula, not all RBPs included in the formula are prognosis-related RBPs in the multivariate Cox regression analysis, and some of the prognosis-related RBPs were not included in the prediction model. Second, in this study, the interaction and coexpression relationships among the RBPs were analyzed by an interaction network, but there is a lack of further research on the functions of these RBPs. In future research, the interactions among RBPs and mRNA or non-coding RNA need to be further studied, which

Table 3. Clinical features of the high-risk group and low-risk group

Feature	Variables	High-risk group	Low-risk group	t/χ^2	*p*
Sex					
	Male	135	124	0.040	0.442
	Female	53	58		
Stage					
	i	76	95	-0.052	0.321
	ii	50	35		
	iii	50	35		
	iv	1	4		
	Unknown	11	13		
	AFP	11,005.88±40,623.11	16,927.47±172,443.99	-0.391	0.696
Fibrosis					
	None	22	32	-0.074	0.155
	Portal fibrosis	18	13		
	Fibrous septa	12	16		
	Nodular formation	5	4		
	Established cirrhosis	33	36		
	Unknown	98	81		
Hepatitis					
	HBV	94	89	-0.031	0.550
	HCV	14	18		
	HBV+HCV	34	44		
	No hepatitis	46	31		
Radiation					
	Without	115	124	-0.074	0.157
	With	2	2		
	Unknown	71	56		
Surgical method					
	Lobectomy	77	64	0.042	0.421
	Single segmentectomy	42	45		
	Multiple segmentectomy	43	44		
	Extended Lobectomy	10	15		
	Other	16	14		
Ablation					
	Without	114	118	3.696	0.158
	With	4	9		
	Unknown	70	55		
Vascular invasion					
	Without	109	110	2.079	0.354
	With	50	53		
	Unknown	29	19		

AFP, alpha-fetoprotein; HBV, hepatitis B virus; HCV, hepatitis C virus.

can guide the functional research of these RBPs more effectively. Third, all data used in this research originated from public databases. In future studies, it will be more credible to collect single-center or multi-center clinical samples to

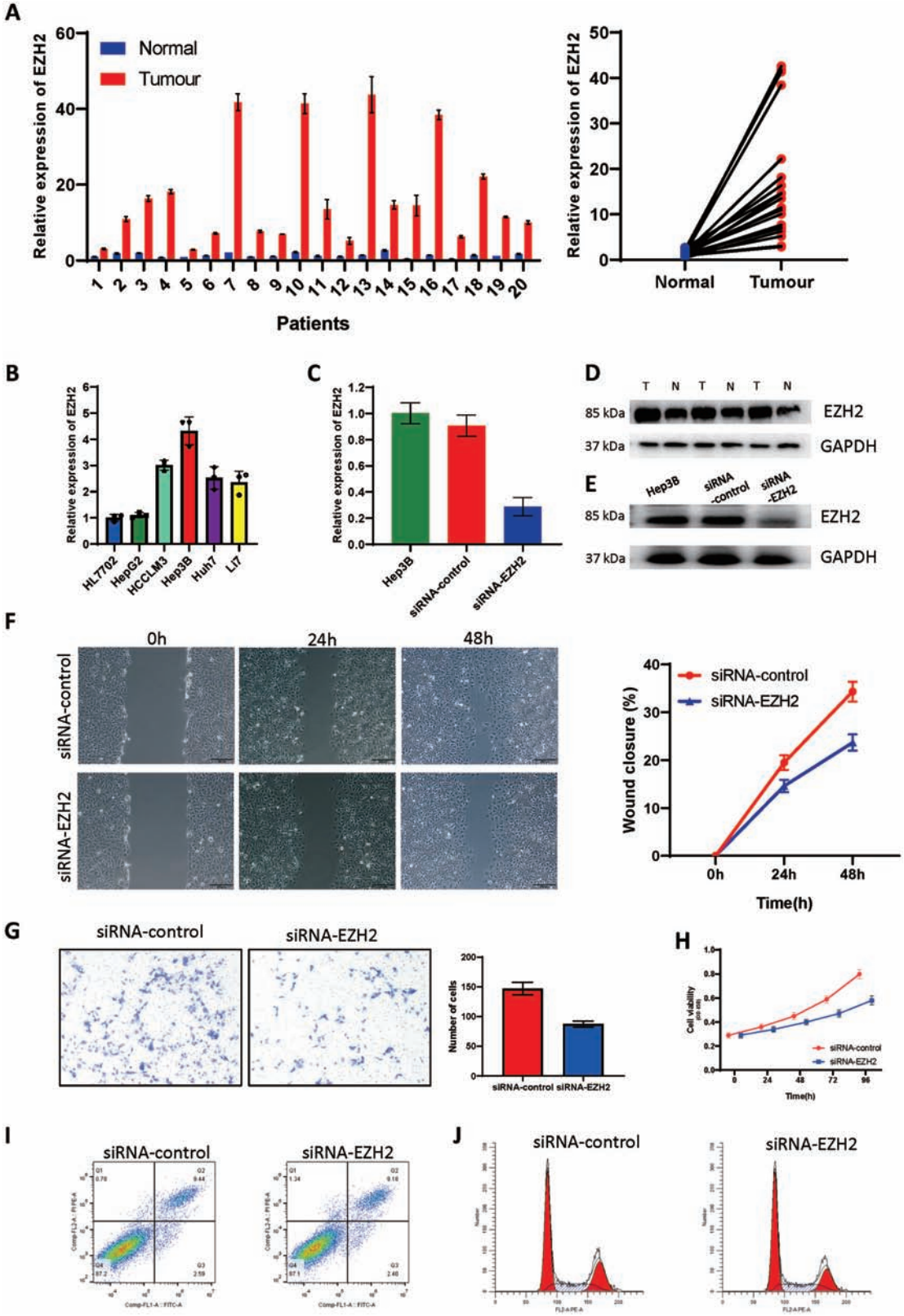

Fig. 5. Expression of EZH2 and its cancer-promoting effect. (A and D) EZH2 in tumor and paracarcinoma tissue for HCC patients. (B) EZH2 in different cell lines. (C and E) Regulatory effect of si-EZH2 transfection on the level of EZH2 in HCC cell lines. (F-H) Knock-down of EZH2 significantly inhibited invasion (F and G) and migration (H) of HCC cells. (H) Effect of EZH2 silencing on viability of HCC cell lines. (I) Knock-down of EZH2 had no effect on apoptosis of HCC cells. (J) Effects of EZH2 on the cell cycle.

test the predictive performance of the predictive model.

In short, we screened-out RBPs that are abnormally expressed in liver cancer and performed enrichment analysis and constructed a coexpression network. Some RBPs that play a role in the progression of liver cancer were identified, and some RBPs that need further research were highlighted. A prognostic model of liver cancer constructed based on the abnormal expression of RBPs has not been reported before. Our analysis results can provide certain guidance for studying the roles of RBPs in liver cancer. However, its actual predictive performance still needs to be verified with large clinical samples in the future. The constructed prediction model can be applied to clinical prognostication, and can also provide guidance for clinical work, drug treatment target selection and molecular marker research of liver cancer.

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Conflict of interest

The authors have no conflict of interests related to this publication.

Author contributions

Analyzed the data and wrote the manuscript (HZ), revised the paper (PX, WM), and designed the research and revised the paper (YY). All authors read and approved the final manuscript.

Data sharing statement

All data are available upon request.

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Original Article

Development and Validation of a Prognostic Model for One-year Survival of Cirrhosis Patients with First-ever Spontaneous Bacterial Peritonitis

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Abstract

Background and Aims: Spontaneous bacterial peritonitis (SBP) is one of the leading causes of death in patients with liver cirrhosis. We aimed to establish a prognostic model to evaluate the 1-year survival of cirrhosis patients after the first episode of SBP. ***Methods:*** A prognostic model was developed based on a retrospective derivation cohort of 309 cirrhosis patients with first-ever SBP and was validated in a separate validation cohort of 141 patients. We used Uno's concordance, calibration curve, and decision curve (DCA) analysis to evaluate the discrimination, calibration, and clinical net benefit of the model. ***Results:*** A total of 59 (19.1%) patients in the derivation cohort and 42 (29.8%) patients in the validation cohort died over the course of 1 year. A prognostic model in nomogram form was developed with predictors including age [hazard ratio (HR): 1.25; 95% confidence interval (CI): 0.92–1.71], total serum bilirubin (HR: 1.66; 95% CI: 1.28–2.14), serum sodium (HR: 0.94; 95% CI: 0.90–0.98), history of hypertension (HR: 2.52; 95% CI: 1.44–4.41) and hepatic encephalopathy (HR: 2.06; 95% CI: 1.13–3.73). The nomogram had a higher concordance (0.79) compared with the model end-stage liver disease (0.67) or Child-Turcotte-Pugh (0.71) score. The nomogram also showed acceptable calibration (calibration slope, 1.12; Bier score, 0.15±0.21) and optimal clinical net benefit in the validation cohort. ***Conclusions:*** This prediction model developed based on characteristics of first-ever SBP patients may benefit the prediction of patients' 1-year survival.

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Keywords: Spontaneous bacterial peritonitis; Liver cirrhosis; Bacterial infection; Nomogram; Prognostic model; Predictors; Long-term outcome.

Abbreviations: AASLD, American Association for the Study of Liver Diseases; ALB, albumin; ALT, alanine aminotransferase; AST, aspartate aminotransferase; Cr, serum creatinine; CRP, C-reactive protein; CTP, Child-Turcotte-Pugh; DCA, decision curve analyses; EASL, European Association for the Study of the Liver; HBV, hepatitis B virus; HE, hepatic encephalopathy; HIV, human immunodeficiency virus; HR, hazard ratio; IAUC, integrated time-dependent area under the curve; INR, international standardized ratio; MELD, model end-stage liver disease; NLR, neutrophil-lymphocyte ratio; PCT, procalcitonin; PLT, platelet; ROC, receiver operating characteristic; SBP, spontaneous bacterial peritonitis; TBIL, total serum bilirubin.

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Introduction

Spontaneous bacterial peritonitis (SBP) is one of the most common types of infections in patients with decompensated liver cirrhosis and a leading cause of acute-on-chronic liver failure in patients with cirrhosis.[1,2] Studies have reported an up to 20% mortality rate for patients after the first episode of SBP, and up to 70% after 1 year.[3] Bacterial infections account for 38% of mortality among patients with cirrhosis.[4] Previous reports on the prognosis for SBP have been focused on risk factors or prediction models for short-term or inpatient outcomes.[5–11] Detection of death within 1 year could aid in delivering proper care and optimizing use of limited resources for treatment. In this study, we aimed to identify independent predictors of 1-year survival in patients after the first episode of SBP in cirrhosis, in order to construct and validate a risk prediction score to assess individual prognosis.

Methods

Derivation and validation cohorts

A total of 2,821 SBP cases occurring between January 2013 and May 2018 were screened for this study. These definite SBP cases were diagnosed at Beijing Ditan Hospital in Chi-

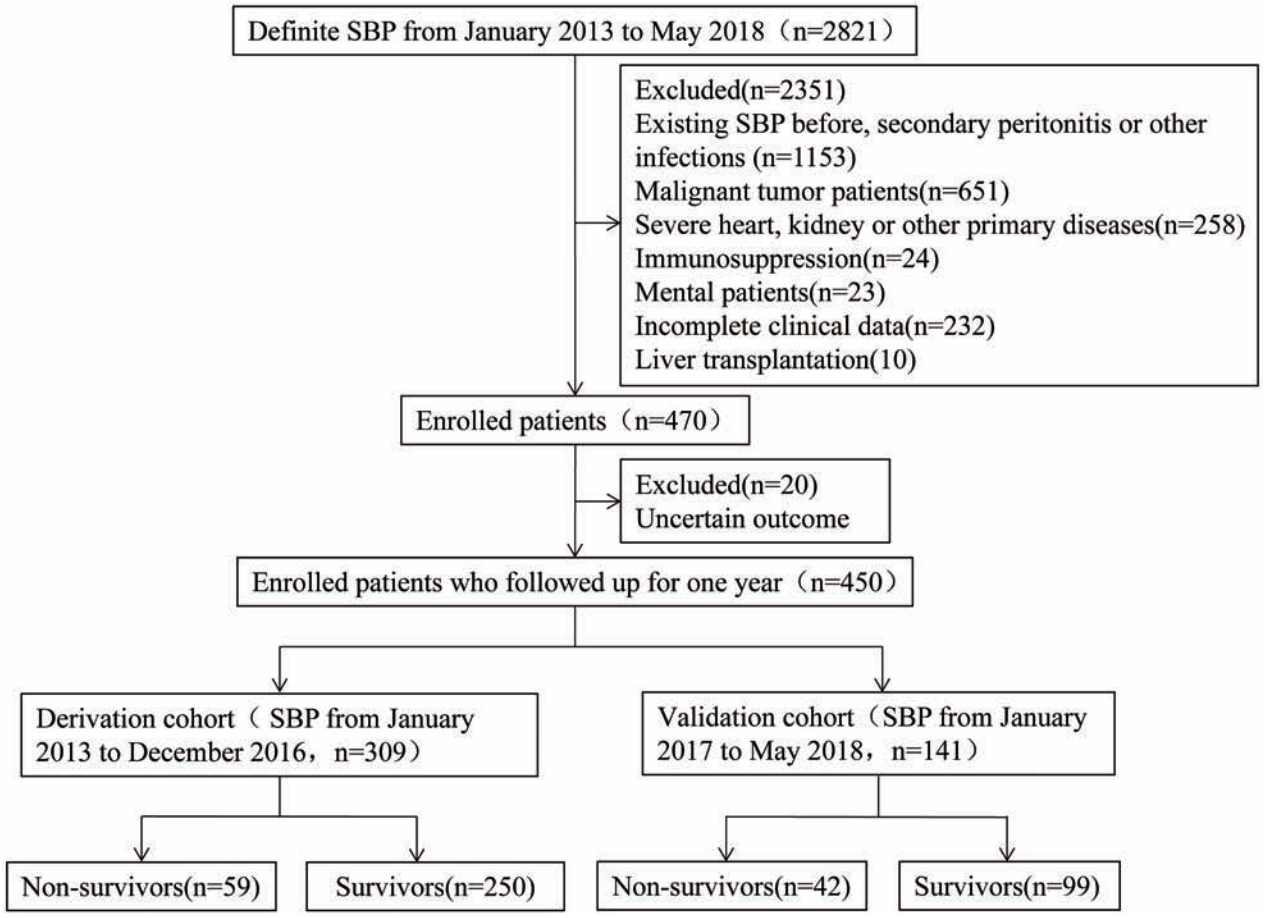

Fig. 1. Study flow chart for derivation and validation cohort. After exclusion, 450 of the 2821 definite SBP cases were identified in our study. Derivation and validation cohort included 309 and 141 cases, respectively.

na, either at hospital admission or during hospitalization due to liver cirrhosis. Each record was reviewed, abstracted and crosschecked by two clinicians (YXH, HML). The exclusion criteria were as follows: (1) previous SBP; (2) secondary peritonitis or human immunodeficiency virus (HIV) infection; (3) concomitant infections at other sites, such as the lung, urinary tract, or soft tissue; (4) tumor, mental illness, or use of immunosuppressants; (5) severe heart, kidney or other primary diseases; (6) incomplete clinical data; (7) previous liver transplantation or liver transplantation within 1 year following the SBP infection. Only the first episode of SBP was included for each patient. This study was approved by the Ethics Committee of Beijing Ditan Hospital at Capital Medical University.

After exclusion criteria were applied, 450 of the 2,821 definite SBP cases were identified and included in our study. We then divided this cohort into the derivation and validation cohorts using the time point of January 1st, 2017. The 309 cases in the derivation cohort were used for variable selection and risk score development. The patient enrolment flowchart is shown in Figure 1.

Definitions

Liver cirrhosis was defined as any two of the following: clinical signs [spider nevi, ascites, hepatic encephalopathy (HE); compatible laboratory data [total bilirubin (TBIL), albumin (ALB), cholinesterase, international normalized ratio (INR)]; imaging findings (nodular liver, varices, splenomegaly]; or corroborative histology.

SBP diagnosis was based on Chinese guidelines on the management of ascites and its related complications in cirrhosis,[12] with two conditions. First, there must be at least one of the following: signs or symptoms of acute peritonitis (abdominal pain, abdominal tenderness or rebound pain, increased abdominal muscle tension, vomiting, diarrhea, or intestinal obstruction), signs or symptoms of systemic inflammatory response syndrome (fever or hypothermia, chills, or tachycardia), deteriorated liver function without obvious inducement, HE, shock, refractory ascites, sudden lack of response to diuretics, renal failure, or acute gastrointestinal bleeding. There must also be at least one of the following test abnormalities: ascitic fluid polymorphonuclear cell count ≥ 250×10^9 cells/mm^3, positive culture of ascites bacteria, or procalcitonin (PCT) > 0.5 ng/mL and excluded infections in other sites.

Model end-stage liver disease (MELD) and Child-Turcotte-Pugh (CTP) scores were calculated according to previously published criteria.[13–15] All definitions and prognostic scores were applied at baseline. The baseline laboratory values were obtained within 3 days when patients were diagnosed as SBP.

Antibiotics and albumin therapy

All patients were treated with antibiotics within 2 weeks.

Due to the retrospective nature of the study, the choice of antibiotics and dosage of ALB were at the discretion of the supervising physician. There were 15 patients in the derivation group whose results in the ascites bacterial culture were positive, and antibiotics were mainly selected according to drug susceptibility. Most of the other patients were recommended cefotaxime, which is an antibiotic similar to the third-generation cephalosporins, or empirical antibiotic therapy according to clinical guidelines.[12,15,16] Additionally, if a patient's serum ALB levels were lower than 30 g/L, they were given an intravenous infusion of human ALB (10 g/day) until serum ALB levels exceeded 30 g/L.

Predictors and outcome

Potential predictive variables that might be associated with the long-term survival in cirrhosis with SBP were collected at hospital admission. These variables include age, sex, etiology of cirrhosis, comorbidities (history of diabetes and hypertension), complications (HE, upper gastrointestinal bleeding, and liver-kidney syndrome), and biochemical parameters including alanine aminotransferase (ALT), aspartate aminotransferase (AST), ALB, TBIL, serum creatinine (Cr), serum sodium, C-reactive protein (CRP), PCT, platelet (PLT) count, neutrophil count, lymphocyte count, and neutrophil-lymphocyte ratio (NLR). The outcome was survival within 1 year of admission. Follow-up and outcome ascertainment were completed over the telephone by trained study coordinators.

Nomogram development and performance assessment

The final predictors in our prediction model were determined based on prior literature, clinical plausibility, data availability, and backward stepwise selection of all covariates in the Cox model. A nomogram was generated using coefficients of independent predictors of 1-year mortality, which were derived from the multivariable Cox regression. The value of each predictor was allocated a score from 0 to 100. We summed all scores to assign a probability of survival for each patient.

To assess the discrimination of our prediction model, we calculated Uno's concordance statistics and integrated time-dependent area under the curve (IAUC),[17] plotted time-dependent receiver operating characteristic (ROC) curves both for the derivation and validation cohorts, and compared them with models using only the MELD or CTP scores.[18] To evaluate the agreement between predicted and observed probabilities, Brier scores and calibration slopes were calculated and calibration curves were generated. We also performed decision curve analyses (DCA) to compare the benefits of our prediction model with the prediction models using only the MELD or CTP scores.

Statistical analysis

Patient characteristics in each cohort were described using frequencies and percentages for categorical variables or means and standard deviations for continuous variables. To assess the association between predictors and 1-year survival, we used univariate and multivariable Cox proportional hazards models to estimate the hazard ratios (HRs) and corresponding 95% confidence intervals.

All statistical analyses were performed with SAS version 9.4 or R version 3.6.0 (SAS Institute, Cary, NC, USA). A *p*-value of less than 0.05 was considered statistically significant.

Results

Patient characteristics

The derivation and validation cohorts included 309 and 141 patients for analysis, respectively. Patients' demographic, clinical, and laboratory characteristics are summarized in Table 1. The mean age was 51 in the derivation cohort and 59 in the validation cohort. There was a total of 239 (77.3%) patients in the derivation cohort, and 95 (67.4%) patients in the validation cohort were male. The etiology of cirrhosis in the two cohorts was primarily hepatitis B, with 169 (57.4%) cases in the derivation cohort and 50 (35.5%) cases in the validation cohort. A total of 59 (19.1%) patients in the derivation cohort and 42 (29.8%) in the validation cohort died within 1 year.

Univariate and multiple Cox regression analyses

Univariate analysis showed that etiology, TBIL, serum sodium, ALB, Cr, history of hypertension, and HE were significantly correlated with 1-year mortality in the derivation cohort. Independent predictors of death identified using the multivariable Cox regression analyses were hepatitis C (HR: 2.94, 95% CI: 1.10–7.89, $p=0.0001$), TBIL (HR: 1.66, 95% CI: 1.28–2.14, $p<0.0001$), serum sodium (HR: 0.94, 95% CI: 0.90–0.98, $p=0.0046$), history of hypertension (HR:2.52, 95% CI: 1.44–4.41, $p=0.0012$), and HE (HR: 2.06,95% CI: 1.13–3.73, $p=0.0178$). Although age (HR: 1.25, 95% CI: 0.92–1.71, $p=0.1557$) was not identified as a statistically significant predictor in the multivariable Cox model, we included it based on clinical knowledge and evidence from prior literature (Table 2).

Nomogram

The nomogram was established based on age, etiology, history of hypertension, HE, TBIL, and serum sodium (Fig. 2). The probability of 1-year survival can be obtained by reading this nomogram. For example, a 50 year-old (20 points) first-time SBP patient with an etiology of alcohol (12.5 points), no history of hypertension (0 points), no previous history of HE (0 points), TBIL of 12.2 μmol/L (27.5 points), and serum sodium of 140 mmol/L (22.5 points) would have a total nomogram score of 82.5 and a <0.1 probability of 1-year death. In comparison, a 70 year-old (36.25 points) first-time SBP patient with an etiology of hepatitis B (0 points), history of hypertension (33.75 points), previous history of HE (26.25 points), TBIL of 33.1 μmol/L (45 points), and serum sodium of 130 mmol/L (44 points) would have a total nomogram score of 185.25 and a 0.55 probability of 1-year death.

Validation of the prognostic nomogram

Uno's concordance of the nomogram was optimal, with 0.77 (95% CI: 0.74–0.80) in the derivation cohort and 0.79 (95% CI: 0.76–0.82) in the validation cohort. The nomogram also had a higher IAUC than the CTP or MELD model in both cohorts (Fig. 3).

Calibration plots showed that the predicted probability was highly consistent with the actual probability in both cohorts (Fig. 4). The Brier score was 0.12±0.22 in the derivation cohort and 0.15±0.21 in the validation cohort. The calibration slope was 1.05 for the derivation cohort and 1.12 for the validation cohort. In the DCA, our nomogram provided

Table 1. Baseline characteristics of enrolled spontaneous bacterial peritonitis patients in the derivation and validation cohorts

Variables	Derivation cohort, *n*=309	Validation cohort, *n*=141
Age in years	51.9±9.5	59.1±11.4
Male sex	239 (77.3)	95 (67.4)
Diagnosis		
Hepatitis B	169 (54.7)	50 (35.5)
Hepatitis C	21 (6.8)	11 (7.8)
Alcohol	84 (27.2)	44 (31.2)
Other	35 (11.3)	36 (25.5)
Complication		
Diabetes mellitus	69 (22.3)	53 (37.6)
History of hypertension	47 (15.2)	34 (24.1)
HE	56 (18.1)	57 (40.4)
Gastrointestinal hemorrhage	97 (31.4)	50 (35.5)
Hepatorenal syndrome	51 (16.5)	12 (8.5)
ALT in U/L	32.7 (19.1–57.9)	33.8 (19.7–60.5)
AST in U/L	42.5 (28.7–85.5)	27.1 (19.2–42.3)
ALB in g/L	28.5 (25.7–31.1)	28.9 (25.5–32.6)
TBIL in μmol/L	44.6 (22.9–105.7)	36.2 (18.5–97.6)
Cr in μmol/L	69.4 (58.0–89.5)	77.7 (61.2–106.2)
Serum sodium in mmol/L	137.3 (132.7–140.3)	136.0 (133.0–140.1)
Neutrophils count as $\times 10^9$/L	3.8 (2.3–5.7)	3.4 (2.1–5.4)
Lymphocyte count as $\times 10^9$/L	0.8 (0.5–1.3)	0.8 (0.5–1.0)
NLR	4.1 (2.6–7.3)	4.7 (2.7–8.3)
PLT as $\times 10^9$/L	64.0 (45.4–99.4)	71.0 (48.4–98.0)
CRP in mg/L	22.5±26.9	2.8±1.5
PCT in μg/L	3.2±15.0	3.3±8.3
INR	1.5 (1.3–1.8)	1.5 (1.3–2.0)
CTP score	9.8±2.1	9.8±2.6
MELD	12.0±7.5	12.6±9.1

Data in the table are the mean ± standard deviation for continuous variables after calculating the log, and *n* (%), frequency with percentage for categorical variables.

superior net benefit in both cohorts compared to the MELD and CTP score models (Fig. 5).

Discussion

In this study, we established a prognostic model for 1-year survival of patients with first-time SBP under real-world conditions. The performance of this model was satisfactory in terms of discrimination, calibration, and clinical benefit indicators in both the development and validation cohorts. The six variables we used to calculate mortality risk (age, etiology, history of hypertension, serum TBIL level, serum sodium level, and complication of HE) are readily available in most clinical datasets, and as such, this nomogram provides clinicians with an accessible tool to estimate individual patients' risk of death. If the patient's estimated risk is low, the clinician may choose to continue the current treatment, whereas patients estimated to have high risk may require more aggressive treatment.

Previous studies of patients with SBP have focused on predictors of acute or short-term outcomes.[8,10,11] A prior study showed that the mortality rate during hospitalization is high (20% to 43%), and remains high at 1 year after discharge.[6] Our study found a similar prevalence of death within 1 year for first-time SBP patients (19.1%).

This study evaluated a number of independent variables that can be used to predict mortality in patients with SBP, including complications such as HE and upper gastrointestinal bleeding, indicators of infection such as total white blood cells and CRP, and other liver and kidney function indicators, such as AST, TBIL, INR, and Cr.[5,7] Previous studies have shown that MELD and CTP can be used as predictors of death in SBP patients during hospitalization.[10,11,19] However, our study indicates that they are not suitable as independent predictors of long-term prognosis of first-time SBP patients. In addition to the aforementioned common independent variables, we also considered the impact of comorbid hypertension and diabetes, which are two common complications. We found that hypertension was an independent risk factor, suggesting that hemodynamic disorders may play an important role in

Table 2. Univariate and multivariable Cox regression analysis in patients with spontaneous bacterial peritonitis patients from the derivation cohort, *n*=309

	Univariate analysis		Multivariable analysis	
	HR (95% CI)	***p***	**HR (95% CI)**	***p***
Age per 10 years	1.28 (0.96–1.71)	0.0892	1.25 (0.92–1.71)	0.1557
Male sex	0.75 (0.42–1.34)	0.3335		
Diagnosis				
Hepatitis B	1.0 (Reference)		1.0 (Reference)	
Hepatitis C	2.13 (0.78–5.83)	0.1389	2.94 (1.10–7.89)	0.0319
Alcoholic fatty liver	2.38 (1.32–4.28)	0.0038	1.40 (0.74–2.64)	0.3000
Other	2.51 (1.19–5.31)	0.0156	1.96 (0.90–4.29)	0.0922
Complication				
Diabetes mellitus	1.32 (0.75–2.32)	0.3421		
History of hypertension	2.50 (1.43–4.34)	0.0012	2.52 (1.44–4.41)	0.0012
HE	2.46 (1.42–4.26)	0.0013	2.06 (1.13–3.73)	0.0178
Gastrointestinal hemorrhage	1.04 (0.61–1.80)	0.8775		
Hepatorenal syndrome	1.74 (0.95–3.18)	0.0729		
Biochemical parameters				
ALT	1.00 (1.00–1.00)	0.7343		
AST	1.00 (1.00–1.00)	0.7381		
ALB	0.93 (0.89–0.98)	0.0061		
TBIL	1.84 (1.44–2.36)	<0.0001	1.66 (1.28–2.14)	0.0001
Cr	1.01 (1.00–1.02)	0.0035		
Serum sodium	0.93 (0.89–0.96)	<0.0001	0.94 (0.90–0.98)	0.00046
CRP	1.00 (1.00–1.01)	0.3754		
PCT	1.00 (0.99–1.02)	0.9817		
PLT	1.00 (1.00–1.00)	0.6835		
NLR	1.01 (0.97–1.05)	0.6284		
INR	1.98 (1.29–3.03)	0.0019		

the long-term prognosis of SBP patients with cirrhosis. This issue has received little attention in prior literature.

Bacterial infections in advanced liver cirrhosis cause profound changes in systemic hemodynamics, with effects such as peripheral vasodilation, reduced systemic vascular resistance, and reduced responsiveness to vasoconstrictors.[20] As a result, the body needs to increase cardiac output to maintain adequate organ perfusion. However, the cardiac compensation reserve of patients with hypertension may be reduced, resulting in impaired adaptive responses to acute circulatory stress, such as in SBP. At the same time, hypertension is often accompanied by a decrease in renal function, leading to an increased risk of hepatorenal syndrome. A study in Austria showed that non-selective beta-blockers increased the length of hospital stay in cirrhosis patients with SBP and increased the risk of hepatorenal syndrome as well as acute kidney injury.[21] This is related to decreased cardiac output in patients with cirrhosis treated with non-selective beta-blockers. One study in Spain found that serum urea nitrogen, white blood cell count, CTP, and mean arterial blood pressure are independent risk factors for in-hospital mortality in patients with SBP.[10] The aforementioned studies as well as the present study show that hemodynamic disorders significantly affect the prognosis of patients with SBP. Furthermore, hypertension is a component of metabolic syndrome. Metabolic syndrome increases the incidence of liver disease-related events by 49%, and those with both metabolic syndrome and hepatitis B infection were more likely to have liver disease-related events.[22] As such, hypertension likely has an important effect on the long-term prognosis of SBP patients with cirrhosis, though the specific mechanism warrants further research and discussion.

Numerous studies have shown that older age is an important factor in the poor prognosis of liver disease.[23–25] This may be related to a decline in immune function, leading to increased sensitivity to infections.[26,27] Several recent studies have found that serum sodium levels are closely related to clinical progress and prognosis, and that the correction of hyponatremia is an integral part of treatment for patients with decompensated liver cirrhosis.[28,29] For example, tolvaptan can effectively improve hyponatremia, thereby improving the prognosis of patients with cirrhosis and ascites.[30] Our study found that hyponatremia is an independent risk factor for death in patients with SBP, which is consistent with findings from previous studies.[31,32] Hyponatremia causes cellular edema, increased intestinal mucosal permeability, bacterial translocation, and also leads to SBP.[33] Moreover, hyponatremia causes cerebral edema, leading to decreased blood volume and induction of hepatorenal syndrome.

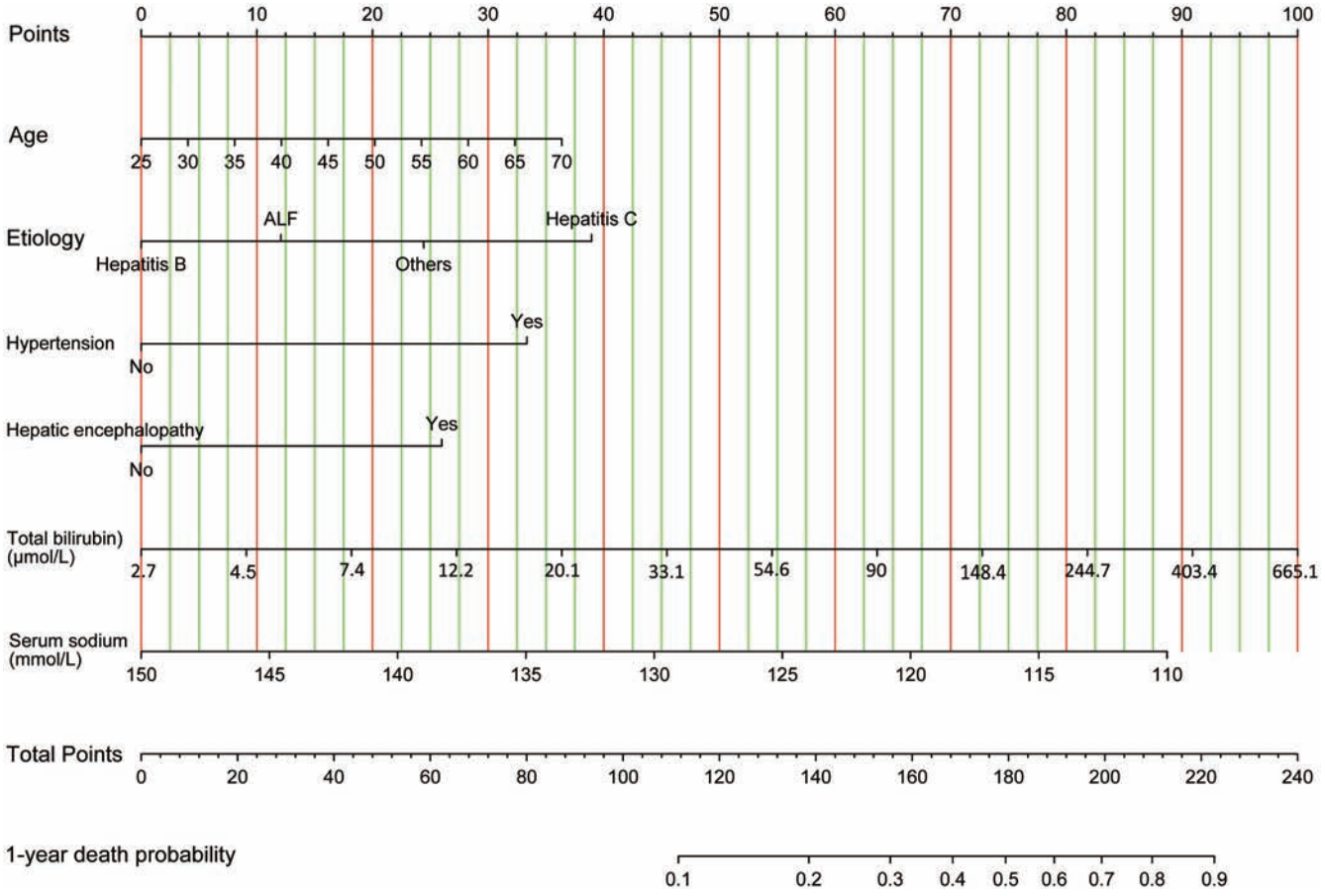

Fig. 2. Nomogram for 1-year survival of cirrhosis patients with first-ever SBP.

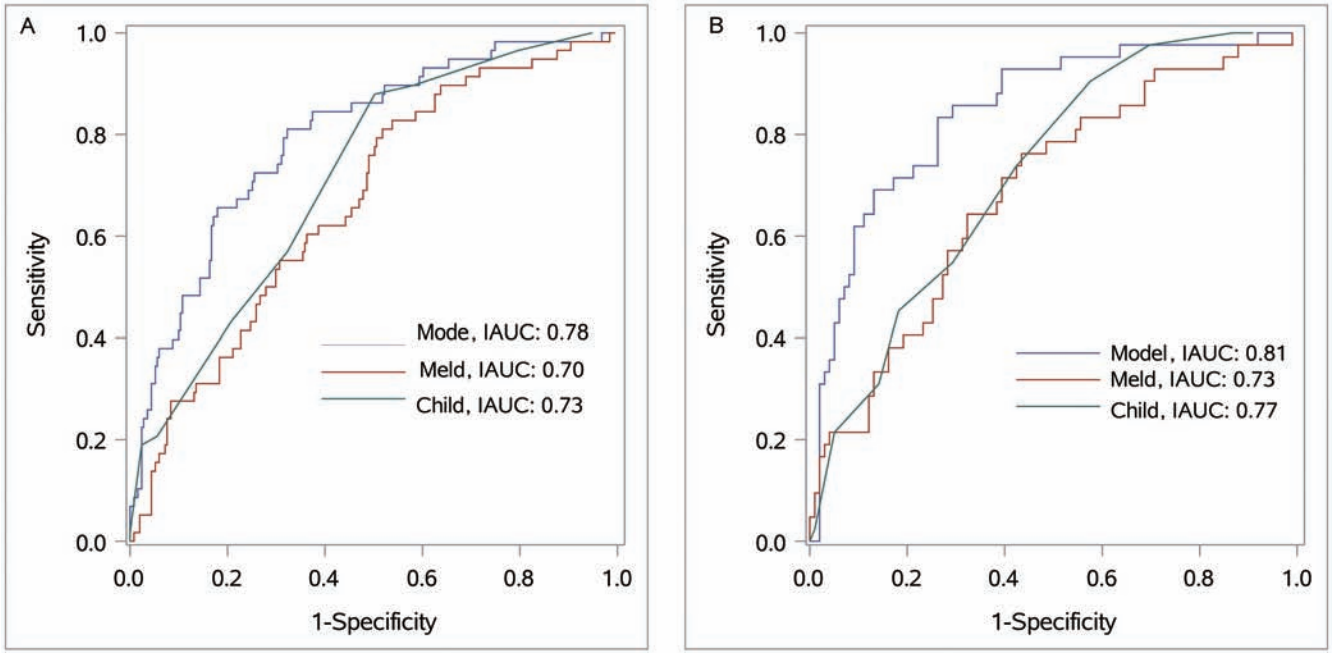

Fig. 3. ROC curves of different models in predicting 1-year prognosis in derivation and validation cohort.

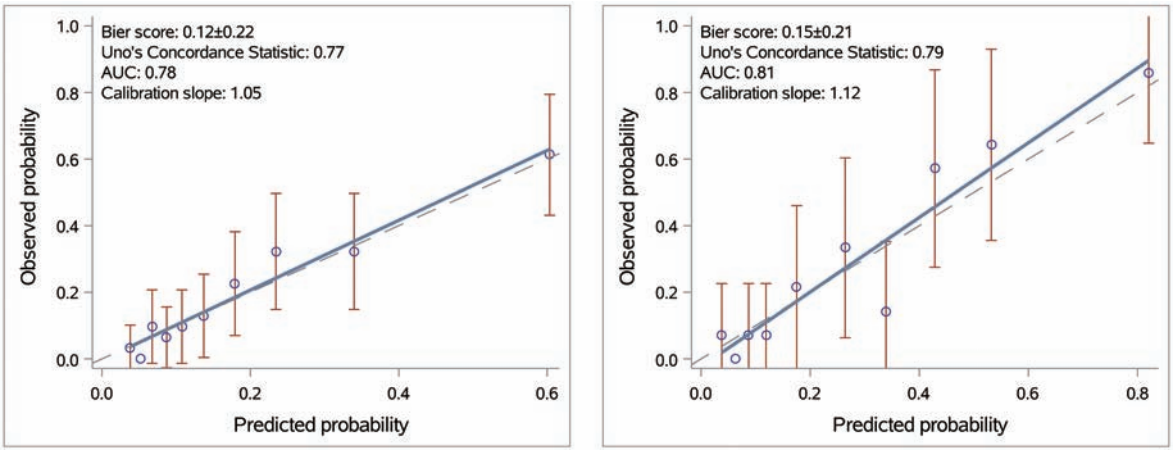

Fig. 4. Calibration curve of overall survival at 1 year for the derivation and validation cohort. Nomogram-predicted probability of survival is plotted on the X-axis, and the actual survival is plotted on the Y-axis. Dashed lines along the 45-degree line through the point of origin represent the perfect calibration models in which the predicted probabilities are identical to the actual probabilities

TBIL is an important indicator for evaluating liver function and is also a major component of the MELD score and the CTP score. HE is another common and serious complication of cirrhosis,[34] reflecting a significant decrease in liver detoxification causing brain dysfunction. Infections such as SBP are an important cause of HE onset,[35,36] which in turn leads to further disease progression.[37] These relationships were corroborated in our study, which identified both TBIL and HE as independent risk factors for cirrhosis and SBP prognosis.

Our study found that compared with hepatitis C, alcohol, and other causes, there was a lower rate of 1-year mortality in cases of SBP caused by the hepatitis B virus (HBV). This may be due to the usage of oral antivirals, such as nucleoside analogs. Previous studies have also supported long-term antiviral therapy to improve CTP scores and prognosis in patients with HBV-related cirrhosis.[38]

Our study included a large number of SBP cases and supports the value of developing similar prediction models in different populations and over different periods of time after the initial SBP episode. However, our study also has several limitations. Generalizability of our findings may be limited since this is a single-center study in China with cohorts of predominantly HBV-related cirrhosis patients. The diagnostic criteria for SBP patients included in this study were defined by the guidelines for diagnosis and treatment of ascites in cirrhosis in China, which is not completely consistent with guidelines issued by the American Association for the Study of Liver Diseases (AASLD) in 2009 and the European Association for the Study of the Liver (EASL) in 2010.[15,16] Therefore, it is unclear whether our nomogram meets the diagnostic criteria of EASL and AASLD and whether it is suitable for the evaluation of SBP prognosis in other settings. Lastly, because the positive rate of ascites culture and blood culture was low, our findings could not explain the correlation between the type of bacterial infection and patient prognoses. Future studies should aim to further explore this relationship with larger sample sizes.

Conclusions

In this study, univariate and multivariable analyses were

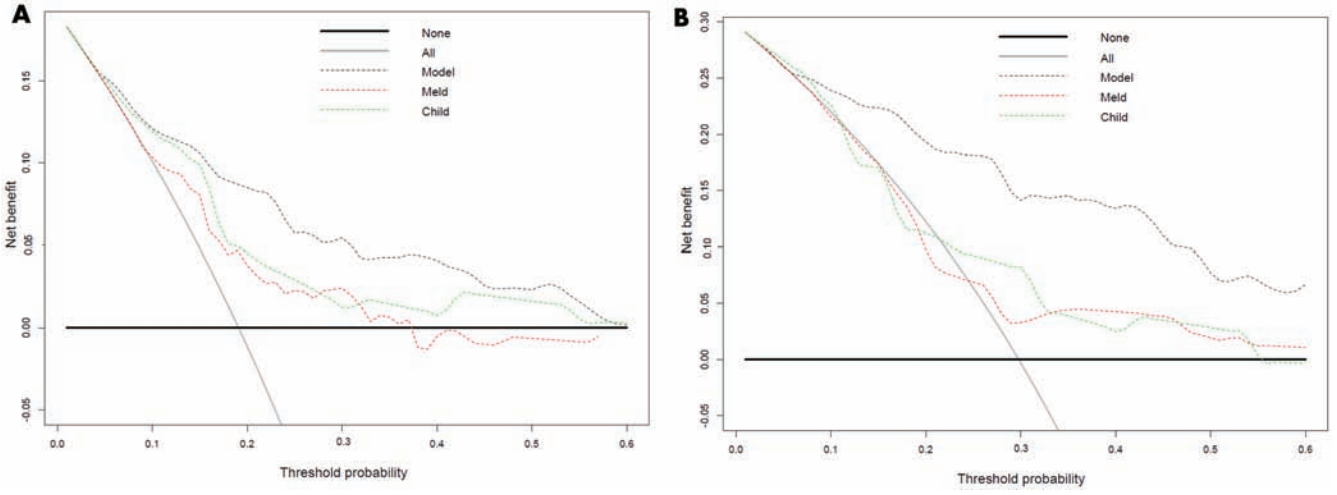

Fig. 5. DCA in the derivation and validation cohort at 1 year. DCA depict the clinical net benefit in pairwise comparisons across the different models. The horizontal solid black line represents the assumption that no patients will experience the event, and the solid gray line represents the assumption that all patients will relapse. In DCA, the nomogram showed superior net benefit compared with other models across a range of threshold probabilities.

performed on SBP patients based on relevant biochemical indices, and a nomogram was established using the multivariable Cox analysis. We found that independent risk factors affecting the prognosis of SBP patients included age, etiology, history of hypertension, serum TBIL level, serum sodium level, and complication of HE. Hypertension was first proposed as an independent factor of SBP but little attention has been paid to it in prior literature. Meanwhile, the long-term prognosis of SBP is closely related to the severity of liver damage. This prediction model performed better than models based on MELD and CTP scores, thus supporting its utility for individualized counseling and clinical treatment.

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Conflict of interest

The authors have no conflict of interests related to this publication.

Author contributions

Conceived and designed the study (YYJ), acquired and analyzed the data. (RRW, HQG, YYW, JXY, YXH, HML, XBW, ZYY), drafted the manuscript (RRW, HQG), interpreted the findings (YYJ, RRW, HQG, YYW, JXY, YXH, HML, XBW, ZYY), and revised the manuscript (YYJ). All authors read and approved the final version of the manuscript.

Data sharing statement

All data and models generated or used during the study appear in the submitted article.

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Original Article

Characteristics and Outcome of Exertional Heatstroke Patients Complicated by Acute Hepatic Injury: A Cohort Study

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Abstract

Background and Aims: Exertional heatstroke (EHS) is associated with strenuous physical activity in hot environments. The present study aimed to investigate dynamic changes of hepatic function indices in EHS patients and determine risk factors for death. ***Methods:*** This single-center retrospective cohort study considered all patients with EHS admitted to the intensive care unit at the General Hospital of Southern Theater Command of PLA from October 2008 to May 2019. Data on general characteristics, organ function parameters, and the 90-day outcome of enrolled patients were collected. Hepatic indices were collected dynamically, and patients with acute hepatic injury (AHI) were identified by plasma total bilirubin (TBIL) ≥34.2 μmol/L and an international normalized ratio ≥1.5, or with any grade of hepatic encephalopathy. ***Results:*** In patients who survived, TBIL, alanine aminotransferase and aspartate aminotransferase were increased at 24 h, peaked at 2–3 days, and began to decrease at 5 days. In non-survivors, TBIL continuously increased post-admission. The area under the receiver operating characteristic curve for the prediction of mortality based on sequential organ failure assessment (SOFA) scores was 89.8%, and the optimal cut-off value was 7.5. Myocardial injury and infection were identified as independent risk factors for death in EHS patients with AHI. ***Conclusions:*** In EHS patients, hepatic dysfunction usually occurred within 24 h. Patients with AHI had more severe clinical conditions, and significantly increased 90-day mortality rates. SOFA scores over 7.5, complicated with myocardial injury or infection, were found to be risk factors for death in EHS patients with AHI.

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Keywords: Exertional heatstroke; Acute hepatic injury; Mortality; TBIL; SOFA.

Abbreviations: AHI, acute hepatic injury; ALT, alanine transaminase; APACHE, acute physiology and chronic health evaluation; APTT, Activated Partial Thromboplastin Time; AST, aspartate transaminase; BUN, Blood Urea Nitrogen; CHS, classical heatstroke; CK, creatine kinase; CK-Mb, creatine kinase-Mb; CNS, central nervous system; CTNI, cardiac troponin I; CRP, C-reactive protein; D.D, D-dimer; EHS, exertional heatstroke; GCS, glasgow coma scale; ICU, intensive care unit; INR, international normalized ratio; IQR, interquartile range; OR, odds ratio; PCT, procalcitonin; PLT, platelets; PT, prothrombin time; SOFA, sequential organ failure assessment; SCR, serum creatinine; TBIL, total bilirubin; WBC, white blood cell.

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Introduction

Heatstroke is a life-threatening condition involving a significant elevation of core body temperature with central nervous system dysfunction, and includes symptoms such as combativeness, delirium, seizures, or even a comatose state.[1,2] It has been reported that at least 3,332 deaths can be attributed to heatstroke between the years of 2006 to 2010 in the USA.[3] As global warming worsens, the morbidity of heatstroke has increased, and it has been predicted that heatstroke-related deaths could increase to nearly 2.5 times the current annual baseline by 2050.[4] Based on the underlying cause, heatstroke can be classified as either classical heatstroke (CHS) or exertional heatstroke (EHS). In both types, the increased core body temperature is attributed to excessive heat accumulation. CHS involves exposure to heat from the environment with poor heat dissipation, whereas EHS is associated with strenuous physical activity in hot environments and excessive production of metabolic heat that overwhelms physiological heat loss.[5] It is still unclear whether the different mechanisms of initiation result in unique pathophysiological disease processes, but organ dysfunction may differ between these two types of heatstroke patients. EHS is usually observed in young, healthy men, most of whom have few underlying diseases. However, the incidence rate of hepatocellular insufficiency in EHS patients is higher compared to that in CHS patients.[1,6] For EHS patients with severe acute liver failure, the hepatic injury due to EHS can result in death that occurs approximately 1 week after the onset of heatstroke, unless a liver transplant is performed.[7,8]

EHS remains a major problem for individuals who regularly meet strenuous physical demands, such as athletes, firefighters, and agricultural workers. The current knowledge regarding EHS patients complicated by acute hepatic injury (AHI) is limited. In the present study, the clinical and prognostic data of heatstroke patients admitted to the intensive care unit (ICU) at a single center in China over a 10-year period were retrospectively collected. The aim was to investigate the dynamic changes of hepatic function indices

in EHS patients and determine the risk factors for death in these patients.

Methods

Study design and participants

Patients diagnosed with EHS in the ICU of the General Hospital of Southern Theater Command in China from October 2008 to May 2019 were considered for inclusion. The inclusion criteria[9] consisted of 1) ≥ 18 years of age, and 2) met the diagnostic criteria of EHS. These diagnostic criteria included a history of strenuous activity (with or without exposure to hot and humid weather), concurrent hyperthermia (central temperature above 40°C), and neurological dysfunction (such as delirium, cognitive disorders, or disturbed consciousness). The exclusion criteria consisted of 1) existing irreversible underlying diseases affecting mortality, and 2) pregnant or breastfeeding women. The study was approved by the Research Ethics Commission of General Hospital of Southern Theater Command of PLA and the requirement for informed consent was waived by the Ethics Commission.

Research procedure

Patient characteristics, organ function parameters, and 90-day outcomes for all enrolled patients were collected. Scores according to the Acute Physiology and Chronic Health Evaluation II (APACHE II), the sequential organ failure assessment (SOFA), and the Glasgow coma scale (GCS) were also collected. The dynamic changes to hepatic indices, including total bilirubin (TBIL), alanine transaminase (ALT), aspartate transaminase (AST), and the international normalized ratio (INR) were evaluated. The time points included admission and 24 h, 2 d, 3 d, 5 d and 7 d after admission. Patients with AHI were identified by plasma levels of TBIL ≥34.2 μmol/L and an INR ≥1.5, or with any grade of hepatic encephalopathy. Myocardial injury was defined by plasma cardiac troponin I >0.2 μg/mL, and kidney injury was defined by serum creatinine >176 μmol/L. Rhabdomyolysis was defined by creatine kinase (CK) >1,000 IU, and patients with procalcitonin (PCT) >2 ng/mL and a white blood cell (WBC) count >10 × 10^9/L were considered to have an infection. Lymphopenia was defined by a lymphocyte count <0.8 × 10^9/L. Patients with a GCS score <8 were considered to have a central nervous system (CNS) disorder.[1] The primary outcome was 90-day mortality, and the secondary outcome was the ICU length of stay.

Statistical analysis

Categorical data were summarized as numbers and percentages, and inter-group comparisons were performed using either Mann-Whitney *U*, χ^2 or Fisher's exact tests. Continuous variables were expressed as the median with interquartile range (IQR) and analyzed using a Wilcoxon rank-sum test, since most continuous variables did not show a Gaussian distribution. Kaplan-Meier survival curves and the log-rank test were used for survival analysis. To determine the independent risk factors of 90-day mortality in severe heatstroke patients with AHI, the Cox proportional hazards model was used. Significant indicators were identified using single-factor analysis, and those with a *p*-value <0.1 were included in the multifactor Cox regression model. The odds ratio (OR) and 95% confidence interval (CI) levels were presented. Statistical analysis was performed using R, version 3.4.0. A two-tailed *p*-value <0.05 was considered statistically significant.

Results

Dynamic changes of hepatic function indices in patients with EHS: Comparison of survivors and non-survivors

Data from a total of 189 patients were collected. Three cases were excluded due to missing data, and 186 cases were included for analysis. All 186 patients were male, with a median age of 21 years (IQR: 19–27) and without any underlying diseases prior to the onset of heatstroke. At admission, the concentration of TBIL in survivors was normal, but TBIL in non-survivors was slightly increased (Table 1). In patients who survived, TBIL was increased at 24 h, reached a peak between 2–3 days, and began to decrease after day 5. The concentrations of ALT and AST also showed similar changes to TBIL. In non-survivors, the levels of TBIL continuously increased following admission. ALT and AST levels were remarkably increased at 24 h until day 3 (Table 1). Though ALT and AST also decreased 5 days after admission, this did not indicate that the liver injury was alleviated; the decreased ALT and AST may have been due to considerable hepatocyte death. In both survivors and non-survivors, INRs increased immediately after the onset of heatstroke. INRs began to decrease after 24 h in the survival group, but remained high in the non-survival group. These results indicate that if hepatic function indices of an EHS patient are slightly increased between 24 h and 3 days following the onset of heatstroke, and the serum levels of TBIL continuously increase, the patient may have a higher risk of mortality.

Characteristics and outcomes of EHS patients: Comparison of patients with and without AHI

To further investigate the characteristics of patients with AHI, all patients were divided into either the AHI group or non-AHI group. Among the 186 cases, 69 showed increased TBIL (≥34.2 μmol/L) and INR (≥1.5) and were placed in the AHI group (Table 2). Compared with the non-AHI group, patients in the AHI group showed increased WBC and neutrophil counts, and decreased lymphocyte and platelet counts. In addition, other organ dysfunction indices were also increased in the AHI group, including kidney (urea nitrogen, serum creatinine), muscle (rhabdomyolysis; creatine kinase (CK), CK-Mb), and cardiac (CTNI) injuries. Patients in the AHI group also showed decreased GCS, and increased APACHE II and SOFA scores compared to the non-AHI group. In addition, patients with AHI had increased ICU length of stays and 90-day mortality rates (Table 3). The 90-day mortality of EHS patients with AHI was 27.5% (19/69) but was only 2.6% (3/117) for patients without AHI. The survival times of EHS patients with AHI were also significantly lower compared to those without AHI (Fig. 1).

Risk factors for EHS patients with AHI

To further determine the risk factors for death in EHS patients with AHI, the organ function indices of the survivors and non-survivors were compared (Table 4). The non-survivors had more severe disease conditions com-

Table 1. Dynamic changes of the hepatic function indexes in the survivor and non-survivor patients with EHS

	Overall (*n*=186)	Survivor (*n*=164)	Non-survivor (*n*=22)	*p*
TBIL in μmol/L				
Ad	15.85 [10.10, 29.40]	14.75 [9.80, 25.47]	29.90 [14.98, 118.08]	0.002
24 h	27.10 [15.45, 55.65]	23.70 [15.07, 47.85]	95.60 [53.53, 176.45]	<0.001
2 d	36.65 [17.20, 87.28]	28.90 [15.70, 55.70]	176.00 [96.05, 198.20]	<0.001
3 d	32.60 [17.60, 97.10]	24.50 [16.40, 59.75]	233.45 [166.45, 373.32]	<0.001
5 d	23.85 [10.20, 83.05]	18.90 [9.80, 36.10]	390.30 [328.10, 422.00]	<0.001
7 d	20.90 [12.20, 73.60]	16.10 [9.70, 28.85]	400.20 [251.33, 424.70]	<0.001
ALT in U/L				
Ad	34.50 [20.00, 222.75]	32.00 [19.00, 149.50]	170.50 [61.50, 1,648.25]	<0.001
24 h	234.50 [56.25, 956.25]	174.50 [47.25, 652.00]	1,530.00 [792.75, 2,649.50]	<0.001
2 d	383.00 [134.00, 1,389.50]	355.00 [134.00, 1,112.50]	1,660.00 [460.00, 3,474.00]	0.017
3 d	468.00 [164.25, 1,431.00]	407.50 [144.75, 1,094.75]	2,573.50 [1,228.50, 4,911.50]	<0.001
5 d	373.50 [129.00, 666.00]	348.00 [110.00, 609.00]	488.00 [370.50, 870.50]	0.077
7 d	192.00 [106.00, 309.00]	205.00 [94.50, 312.00]	148.50 [115.75, 217.50]	0.726
AST in U/L				
Ad	66.50 [34.75, 228.00]	62.00 [33.50, 163.50]	356.00 [110.00, 1,645.00]	<0.001
24 h	214.00 [60.00, 751.75]	166.00 [57.00, 559.00]	2,545.00 [631.00, 5,690.00]	<0.001
2 d	278.00 [91.50, 947.50]	250.00 [89.00, 625.00]	2,635.00 [421.75, 5,159.00]	<0.001
3 d	183.00 [70.50, 568.50]	162.00 [66.50, 462.00]	1,629.50 [359.50, 5,276.75]	0.001
5 d	118.50 [59.00, 219.00]	116.00 [56.00, 190.00]	220.00 [101.00, 355.00]	0.058
7 d	67.50 [39.25, 101.50]	58.50 [34.50, 80.75]	102.50 [75.00, 126.50]	0.003
INR				
Ad	1.29 [1.09, 1.76]	1.24 [1.09, 1.47]	3.22 [1.86, 4.82]	<0.001
24 h	1.29 [1.09, 1.70]	1.19 [1.07, 1.54]	2.76 [2.20, 3.28]	<0.001
2 d	1.14 [1.04, 1.65]	1.10 [1.02, 1.32]	2.82 [2.04, 3.59]	<0.001
3 d	1.05 [0.97, 1.70]	1.02 [0.96, 1.21]	3.10 [2.07, 3.53]	<0.001
5 d	1.01 [0.93, 1.21]	0.98 [0.93, 1.11]	3.02 [2.08, 4.04]	<0.001
7 d	1.07 [0.99, 1.33]	1.03 [0.98, 1.11]	2.38 [1.53, 2.57]	<0.001

d, day.

pared to survivors, as evidenced by higher levels of serum creatinine, CK, CTNI, PCT *etc.* at the time of admission. The non-survivors also had higher SOFA scores. The area under the receiver operating characteristic curve for the prediction of mortality based on the SOFA scores at the time of admission was 89.8%, and the optimal cutoff value was 7.5 (sensitivity of 90.0%, specificity of 80.6%; Fig. 2). Among the patients in the AHI group, 44 cases had myocardial injuries, 32 cases had injury to the kidney, 57 cases were experiencing rhabdomyolysis, 18 cases had a CNS disorder, 45 cases had an infection, and 54 cases showed signs of lymphopenia. Single variable analysis showed that complications due to myocardial injuries, a CNS disorder or infection were risk factors for death in patients with AHI (Table 5). The ORs were 13.10 (95% CI: 1.74, 98.26; p=0.012), 2.86 (95% CI: 1.15, 6.96; p=0.024) and 6.14 (95% CI: 1.42, 26.62; p=0.025), respectively. In the multi-variate analysis, myocardial injury and infection were found to be independent risk factors for death in EHS patients with AHI, with ORs of 12.169 (95% CI: 1.526, 94.806; p=0.023) and 5.637 (95% CI: 1.219, 26.064; p=0.027), respectively.

Discussion

The present retrospective cohort study revealed that hepatic dysfunction usually occurred within 24 h after admission in EHS patients. The patients with AHI had more severe clinical conditions, significantly increased 90-day mortality rates, and shorter survival times. Complications with myocardial injuries or infection were found to be independent risk factors for death in EHS patients with AHI.

The time course of liver damage was found to differ from the damage-associated markers of other organs, since liver damage was often not detected at the onset of heatstroke.[10] These results revealed that TBIL levels were increased at 24 h, peaked at 2–3 days, and began to decrease after day 5 in the survivors, while the non-survivors showed continuously increased levels of TBIL after admission. An animal heatstroke model has indicated that heat stress leads to extensive hepatocyte ballooning degeneration and necro-

Table 2. Comparison of the characteristics between the EHS patients in the non-AHI group and AHI group

	Overall (n=186)	Non-AHI (n=117)	AHI (n=69)	*p*
Age	21.00 [19.00, 27.00]	20.00 [19.00, 27.00]	23.00 [19.00, 27.00]	0.263
WBC, $\times10^9$/L	11.34 [8.72, 14.62]	10.39 [8.61, 14.25]	12.13 [9.04, 15.82]	0.057
Neutrophil, $\times10^9$/L	8.86 [6.54, 12.44]	8.26 [5.80, 11.41]	10.14 [7.42, 13.22]	0.003
Lymphocyte, $\times10^9$/L	1.11 [0.58, 1.89]	1.35 [0.79, 2.12]	0.73 [0.40, 1.63]	<0.001
Monocyte, $\times10^9$/L	0.68 [0.38, 0.99]	0.68 [0.40, 0.97]	0.66 [0.35, 1.00]	0.581
PLT, $\times10^9$/L	165.0 [82.00, 219.0]	185.5 [148.8, 232.25]	80.00 [35.00, 132.0]	<0.001
BUN in mmol/L	5.75 [4.50, 7.60]	5.20 [4.20, 6.50]	6.80 [5.40, 8.70]	<0.001
SCR in μmol/L	127.5 [92.00, 162.3]	107.0 [82.00, 137.0]	159.0 [128.0, 201.0]	<0.001
CK in U/L	904.0 [346.75, 2,537.0]	572.0 [244.0, 1,810.0]	1,452.0 [853.0, 4,573.0]	<0.001
PT in s	15.90 [14.10, 20.58]	14.70 [13.70, 16.10]	23.40 [18.80, 35.00]	<0.001
APTT in s	38.95 [33.52, 49.45]	36.10 [32.60, 40.90]	50.60 [39.20, 91.20]	<0.001
Fib in g/L	2.50 [2.00, 2.80]	2.60 [2.30, 3.10]	2.00 [1.58, 2.60]	<0.001
D.D in mg/L	1.86 [0.51, 7.00]	0.70 [0.36, 2.16]	9.49 [3.51, 14.55]	<0.001
CK-Mb in ng/mL	469.40[128.9, 1,000.0]	239.1 [64.75, 594.5]	1,000.0 [381.5, 1,000.0]	<0.001
CTNI in ng/mL	110.00 [21.40, 432.45]	50.00 [10.00, 143.22]	410.0 [125.90, 1,407.5]	<0.001
CRP in mg/dL	3.30 [1.48, 5.98]	3.18 [0.67, 6.86]	3.38 [3.14, 5.27]	0.3
PCT in ng/mL	1.70 [0.76, 4.14]	1.43 [0.56, 3.97]	2.05 [1.08, 4.69]	0.083
GCS	12.00 [7.00, 14.00]	12.00 [9.00, 14.00]	8.00 [6.00, 13.00]	0.005
SOFA score	3.00 [2.00, 6.00]	3.00 [2.00, 4.00]	6.00 [4.00, 9.00]	<0.001
APACHE II score	10.50 [8.00, 15.75]	9.00 [7.00, 13.00]	15.00 [10.00, 21.00]	<0.001

APACHE, acute physiology and chronic health evaluation; APTT, activated partial thromboplastin time; BUN, blood urea nitrogen; CK, creatine kinase; CK-Mb, creatine kinase-Mb; CTNI, cardiac troponin I; CRP, C-reactive protein; D.D, D-dimer; GCS, glasgow coma scale; PCT, procalcitonin; PLT, platelets; PT, prothrombin time; SOFA, sequential organ failure assessment; SCR, serum creatinine; WBC, white blood cell.

sis.[11] Data from patient autopsies have revealed that EHS-associated liver damage is characterized by centrilobular degeneration and necrosis with parenchymal damage.[1,12] Previous studies have also found that the inhibition of inflammatory mediators, such as high-mobility group box 1 (HMGB1), could alleviate liver injury in a rat model of heatstroke, as evidenced by decreased levels of ALT and AST.[11,13] The modulation of coagulation by thrombomodulin was also shown to improve liver function.[14] Therefore, the hepatocyte damage in heatstroke patients is thought to be caused by a multifactorial damaging effect, including hyperthermia in combination with hypoxia, inflammatory stimuli, ischemia, and disseminated intravascular coagulation.[15,16]

Liver dysfunction is not usually detected at the onset of heatstroke, unlike damages to other organs that can be detected much earlier, such as injury to the myocardia or coagulation dysfunction. Currently, the liver is regarded as one of the first organs that becomes injured during heatstroke. Heat stress and the subsequent inflammatory response and coagulation dysfunction could lead to the damage of hepatocytes as well as liver sinusoidal endothelial cells and intrahepatic biliary epithelial cells, characterized by an increase in aminopherase and bilirubin. However, since liver compensatory mechanisms can maintain liver function, the dysfunction indices do not always directly correlate with damage to the liver. In addition, the secondary factors of heat stress, such as the systemic inflammatory response or liver ischemia, could further aggravate liver damage. These factors might also contribute to the delayed detection of liver damage. In the current study, non-survivor EHS patients had concentrations of TBIL over 2-fold higher at 24 h after admission, and these levels continuously increased, suggesting that a dramatic and continuous increase of TBIL may indicate a poor prognosis. Notably, since the liver plays a significant role in the synthesis of coagulation factors, INR was also regarded as a major index for liver function. However, during the pathological process of heatstroke, coagulation dysfunction occurs at an early stage due to the activation of endothelial cells. Therefore, INR was increased at the onset of heatstroke, which differed from other liver function indices.

During the pathophysiological process of heatstroke, the primary cell damage was due to heat-induced necrotic and apoptotic cell death. In later stages, thermoregulatory failure

Table 3. Comparison of the outcome between the EHS patients in Non-AHI group and AHI group

	Overall (n=186)	Non-AHI (n=117)	AHI (n=69)	*p*
ICU time in days	5.00 [3.00, 9.00]	4.00 [3.00, 7.00]	8.00 [5.00, 14.00]	<0.001
Outcome, %				<0.001
Survive	164 (88.2)	114 (97.4)	50 (72.5)	
Death	22 (11.8)	3 (2.6)	19 (27.5)	

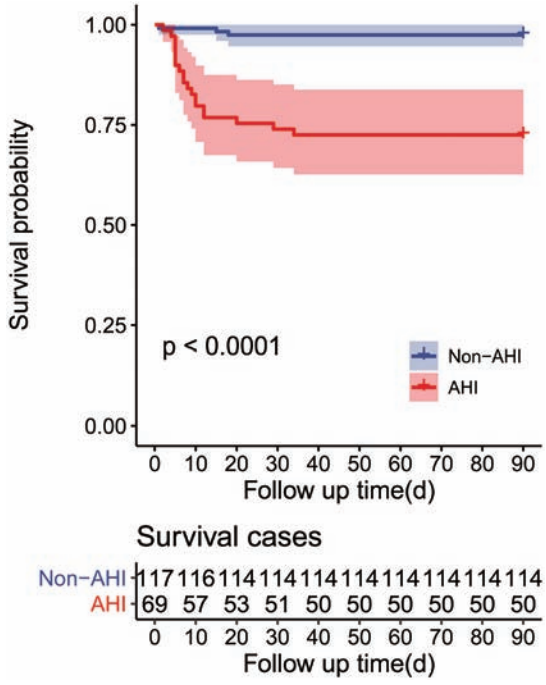

Fig. 1. Survival curves of 90-day mortality rate in the AHI group and non-AHI group.

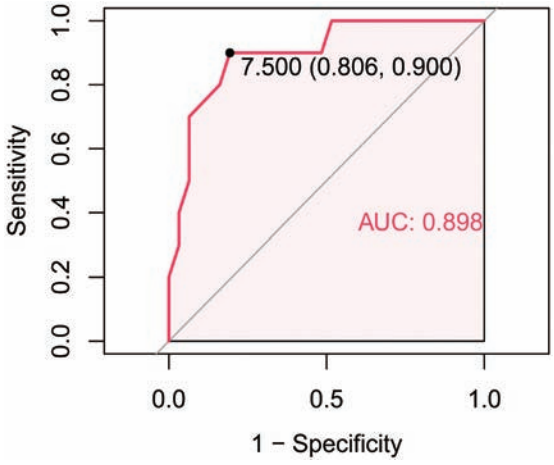

Fig. 2. Receiver operating characteristic curve analysis of SOFA to predict the 90-day mortality of patients with AHI.

combined with an inflammatory reaction results in multiorgan failure that can cause death. Autopsy studies show that end-organ failure following heatstroke is accompanied by widespread microthrombosis, hemorrhage, and inflammatory injury.[5,17,18] In the current study, EHS patients with AHI showed a higher all-cause mortality compared to those without AHI. Hepatocyte damage could result in a weakened detoxification process and decreased protein synthesis, which further increases the risk of infection and coagulation dysfunction. In addition, another explanation could be that EHS patients with AHI were in a more severe condition. Compared to patients in the non-AHI group, the organ dysfunction indices were increased in the AHI group, including those that indicate kidney injury, rhabdomyolysis, and cardiac injury.

To further clarify the risk factors for EHS patients with AHI,

Table 4. Comparison of the characteristics between the survivors and non-survivors in the AHI group

	Overall (n=69)	Survivor (n=50)	Non-survivor (n=19)	*p*
Age	23.00 [19.00, 27.00]	23.00 [20.00, 27.00]	21.00 [18.00, 23.50]	0.103
WBC, ×10^9/L	12.13 [9.04, 15.82]	12.22 [9.37, 15.71]	10.56 [8.46, 15.48]	0.648
Neutrophil, ×10^9/L	10.14 [7.42, 13.22]	10.61 [7.95, 13.71]	8.86 [6.38, 13.18]	0.323
Lymphocyte, ×10^9/L	0.73 [0.40, 1.63]	0.73 [0.51, 1.55]	0.67 [0.33, 2.81]	0.909
Monocyte, ×10^9/L	0.66 [0.35, 1.00]	0.64 [0.36, 1.00]	0.73 [0.26, 0.90]	0.92
PLT, ×10^9/L	80.00 [35.00, 132.00]	85.50 [42.25, 167.00]	72.00 [29.00, 89.00]	0.097
BUN in mmol/L	6.80 [5.40, 8.70]	6.55 [5.40, 8.10]	8.10 [6.30, 9.25]	0.209
SCR, μmol/L	159.00 [128.00, 201.00]	146.00 [125.50, 171.50]	228.00 [189.00, 280.00]	<0.001
CK in U/L	1,452.0 [853.00, 4,573.0]	1,278.0 [803.00, 3,769.0]	3,220.0 [1,014.0, 7,931.5]	0.076
PT in s	23.40 [18.80, 35.00]	21.25 [16.33, 26.90]	37.50 [25.70, 45.00]	<0.001
APTT in s	50.60 [39.20, 91.20]	44.90 [37.68, 68.18]	91.20 [77.35, 122.85]	<0.001
Fib in g/L	2.00 [1.58, 2.60]	2.20 [1.70, 2.60]	1.30 [0.90, 1.90]	0.001
D.D in mg/L	9.49 [3.51, 14.55]	5.32 [1.61, 13.06]	10.27 [10.00, 20.00]	0.001
CK-Mb in ng/mL	1,000.0 [381.48, 1,000.0]	789.00 [264.90, 1,000.0]	1,000.0 [967.55, 1,000.0]	0.088
CTNI in ng/mL	410.00 [125.90, 1,407.50]	230.00 [100.00, 699.90]	1,530.0 [952.80, 2,930.0]	<0.001
CRP in mg/dL	3.38 [3.14, 5.27]	3.37 [2.13, 5.37]	3.38 [3.30, 3.58]	0.668
PCT in ng/mL	2.05 [1.08, 4.69]	2.02 [0.97, 4.08]	2.95 [1.46, 5.81]	0.263
GCS	8.00 [6.00, 13.00]	10.00 [6.00, 14.00]	5.50 [3.00, 7.00]	0.003
SOFA score	6.00 [4.00, 9.00]	5.00 [3.00, 7.00]	11.50 [9.25, 13.75]	<0.001
APACHE II score	15.00 [10.00, 21.00]	14.00 [8.00, 16.50]	22.50 [18.50, 23.75]	0.001

Table 5. Risk factors for the EHS patients with AHI

Variable	Univariate		Multivariate	
	OR (95 % CI)	*p*	OR (95 % CI)	*p*
Myocardial injury	13.10 (1.744, 98.26)	0.012	12.169 (1.562, 94.806)	0.023
Kidney injury	1.156 (0, Inf)	0.997		
Rhabdomyolysis	2.077 (0.476, 9.992)	0.328		
CNS disorder	2.862 (1.147, 6.96)	0.024	1.106 (0.425, 2.879)	0.837
Infection	6.143 (1.418, 26.62)	0.153	5.637 (1.219, 26.064)	0.027
Lymphopenia	28.78 (0, Inf)	0.998		

Inf, infinity.

since most EHS patients in the AHI group were also experiencing dysfunction of other organs, a Cox hazard analysis was used to investigate the risk factors for death. A single variable analysis showed that complications with myocardial injuries, CNS disorders or infection were risk factors for death. In the multi-variate analysis, myocardial injuries and infection were found to be independent risk factors for death in EHS patients with AHI. In healthy individuals, bacteria are rarely cultured from the systemic circulation, and the liver plays an important role in eliminating micro-organisms from the blood.[19] As a result, AHI may contribute to increased circulating endotoxin levels in heatstroke patients due to decreased bacterial clearance function. In EHS patients with AHI and myocardial injury or infection, the circulatory stability was affected. Unstable circulation may aggravate injuries of other organs due to hypoperfusion, leading to an increased risk of death, especially in the acute phase.

Conclusions

In EHS patients, hepatic dysfunction usually occurred 24 h after onset. EHS patients with AHI had more severe clinical conditions, significantly increased 90-day mortality rates, and shorter survival times. Complications of myocardial injuries and infection were found to be independent risk factors for death in EHS patients with AHI.

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Conflict of interest

The authors have no conflict of interests related to this publication.

Author contributions

Study concept and design (ZfL, JJ), data collecting (JG, CW, LO, ZyL), statistical analysis (ZfL, JJ), manuscript drafting (JJ, JG, ZfL).

Data sharing statement

The data sets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Original Article

Potential Role and Clinical Value of PPP2CA in Hepatocellular Carcinoma

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Abstract

Background and Aims: Protein phosphatase 2A (PP2A) is associated with many cancers. This study aimed to clarify whether PPP2CA, which encodes the alpha isoform of the catalytic subunit of PP2A, plays a role in hepatocellular carcinoma (HCC) and to identify the potential underlying molecular pathways. ***Methods:*** Based on bioinformatics, public databases and our in-house RNA-Seq database, we analyzed the clinical value and molecular mechanism of PPP2CA in HCC. ***Results:*** Data were analyzed from 2,545 patients with HCC and 1,993 controls without HCC indexed in The Cancer Genome Atlas database, the Gene Expression Omnibus database and our in-house RNA-Seq database. PPP2CA expression was significantly higher in HCC tissue than in non-cancerous tissues (standardized mean difference: 0.69, 95% confidence interval [CI]: 0.50–0.89). PPP2CA expression was able to differentiate HCC from non-HCC, with an area under the summary receiver operator characteristic curve of 0.79 (95% CI: 0.75–0.83). Immunohistochemistry of tissue sections confirmed that PPP2CA protein was up-regulated in HCC tissues. High PPP2CA expression in HCC patients was associated with shorter overall, progression-free and disease-free survival. Potential molecular pathways through which PPP2CA may be involved in HCC were determined using miRWalk 2.0 as well as analysis of Gene Ontology categories, Kyoto Encyclopedia of Genes and Genomes pathways, and protein-protein interaction networks. ***Conclusions:*** PPP2CA is up-regulated in HCC and higher expression correlates with worse prognosis. PPP2CA shows potential as a diagnostic marker for HCC. Future studies should examine whether PPP2CA contributes to HCC through the candidate microRNAs, pathways and hub genes identified in this study.

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Introduction

Many patients with hepatocellular carcinoma (HCC) are diagnosed when the disease is already advanced, resulting in poor prognosis. HCC involves the actions and interactions of numerous genes,[1,2] and increasingly powerful bioinformatics tools and datasets, such as the Gene Expression Omnibus (GEO) and the Cancer Genome Atlas (TCGA), have facilitated explorations of how specific proteins and pathways contribute to the disease. This may accelerate new discoveries about the disease and its treatment.

Protein phosphatase 2A (PP2A), a serine/threonine phosphatase highly conserved among eukaryotes, is associated with many cancers. The heterotrimeric enzyme comprises a backbone subunit A, regulatory subunit B, and catalytic subunit C.[3] The C subunit occurs as two isoforms, PPP2CA or PPP2CB, and the former is approximately 10 times more abundant than the latter.[4,5] Few studies have examined the potential involvement of either isozyme in HCC.

In the present study, bioinformatics and public databases were used to compare PPP2CA expression between HCC and non-cancerous tissues. The results were verified against our in-house RNA-Seq database and immunohistochemistry of our archived tissue samples. We compared survival between patients showing low or high PPP2CA expression, and we used various bioinformatics programs to explore molecular pathways through which the protein may

Keywords: Hepatocellular carcinoma; PPP2CA; Molecular mechanism; Diagnostic marker.

Abbreviations: DFS, disease-free survival; GEO, Gene Expression Omnibus; GEPIA2, Gene Expression Profiling Interactive Analysis; GO, Gene Ontology; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; KEGG, Kyoto Encyclopedia of Genes and Genomes; OS, overall survival; PFS, progression-free survival; PPI, protein-protein interaction; PP2A, Protein phosphatase 2A; sROC, Summary receiver operating characteristic; TCGA, The Cancer Genome Atlas.

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contribute to HCC.

Methods

Data collection

GEO database searching: We searched the GEO database (www.ncbi.nlm.gov/geo) through 30 September 2019 for microarrays related to HCC.[6] The search string was *(malignan* OR neoplas* OR cancer OR tumour OR tumor OR carcinoma) AND (hepatocellular OR hepatic OR liver)*. The specific search strategy is shown (Supplementary Fig. 1). Array data were filtered according to pre-set inclusion and exclusion criteria. The inclusion conditions were as follows: (1) cancer and adjacent liver tissue samples obtained from HCC patients; (2) each dataset included HCC tissue diagnosed based on pathology as well as adjacent non-cancerous tissue (or healthy liver tissue); and (3) data on PPP2CA mRNA levels available. Data were excluded from: (1) HCC tissues of patients who had not been diagnosed based on pathology; (2) human blood, cell lines or animal models; (3) patients for whom only cancerous tissue or adjacent non-cancerous tissues were available; (4) HCC patients who had received chemotherapy, radiotherapy, interventional treatment or immunotherapy; (5) cholangiocarcinoma or mixed liver cancer; and (6) poor-quality samples.

TCGA data on 361 samples and 50 normal tissues: RNA-Seq data in the liver hepatocellular carcinoma (known as LIHC) database were downloaded on October 3, 2019 from the TCGA database (https://portal.gdc.cancer.gov/), hosted by the University of California Santa Cruz (https://xena.ucsc.edu/). Patients who had been diagnosed with fibrolamellar carcinoma or mixed liver cancer based on pathology were excluded.

The forest plot of subgroup meta-analysis for PPP2CA expression between HCC and control tissues, based on random-effect meta-analysis. The forest plot of PPP2CA expression for alcohol, hepatitis B virus (HBV), hepatitis C virus (HCV) and other subgroups in TCGA, GSE62232 and in-house RNA-Seq datasets.

Data extraction

Two researchers (JYL and XQ) checked and then extracted data on levels of PPP2CA mRNA from microarrays and the TCGA database, together with relevant clinicodemographic information. Considering that the levels of PPP2CA mRNA may be affected by etiology of HCC, all extracted data underwent second extraction. HCC was divided into HBV-related HCC, alcohol-related HCC, HCV-related HCC and other HCC. Datasets with samples greater than 50 and etiological information were extracted for subgroup analysis. Any disagreements were resolved by discussion, mediated by a third researcher (CLY).

Meta-analysis of PPP2CA mRNA levels

Data on PPP2CA mRNA levels were $\log_2$-transformed (X+0.001) and meta-analyzed using Stata/SE 15.1. The standardized mean difference and corresponding 95% confidence interval (CI) between HCC tissues and control tissues were determined. The robustness of the meta-analysis was assessed by performing sensitivity analysis. Heterogeneity was assessed using the chi-squared-based Q test and the I^2 test, and it was considered significant if $p<0.05$ or $I^2>50\%$. Risk of publication bias was assessed using Egger's and Begg's tests. PPP2CA mRNA data of different types of HCC were analyzed in the same way.

Clinical significance of PPP2CA in HCC

The expression of PPP2CA was imported into SPSS software (IBM Corp., Armonk, NY, USA) to calculate the number of true positives, true negatives, false positives, and false negatives. The potential diagnostic value of PPP2CA expression was assessed in terms of the area under a summary ROC curve (AUC) drawn using Stata/SE 15.1 (College Station, TX, USA). An AUC >0.7 was considered to indicate appreciable discriminatory ability.

Potential relationships between PPP2CA expression and clinicopathological parameters of HCC were assessed based on clinical information from the TCGA database. After selecting appropriate cut-off values, we compared the overall survival (OS) and progression-free survival (referred to as PFS) of patients showing high or low PPP2CA expression using the Kaplan-Meier plotter (http://kmplot.com/analysis/index.php?p=service).[7] OS and disease-free survival (DFS) were also examined based on Gene Expression Profiling Interactive Analysis (GEPIA2; http://gepia.cancer-pku.cn/).[8]

Exploration of potential pathways of PPP2CA involvement in HCC

We predicted microRNAs (miRNAs) that may target PPP2CA using miRWalk2.0 (http://zmf.umm.uni-heidelberg.de/apps/zmf/mirwalk2/genepub.html), a comprehensive atlas of predicted and validated miRNA-target interactions. Only miRNAs predicted by more than nine of the twelve algorithms in that software were considered in the present study.

Using GEPIA2, we explored genes whose expression may correlate with that of PPP2CA. Using DAVID (http://david.abcc.ncifcrf.gov/), we examined candidate co-expressed genes for enrichment in Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways and Gene Ontology (GO) biological processes, molecular functions and cellular components. The results of GO and KEGG analyses were depicted in bubble charts, plotted by R version 4.0.2. A protein-protein interaction (PPI) network was constructed using the online STRING database (https://string-db.org/). The expression of hub genes extracted from the PPI network was explored using data from the TCGA database.

Tissue samples and follow-up

To complement our analyses of data from public databases, we performed RNA sequencing and immunohistochemistry of samples of HCC tissues and matched non-tumor tissues from patients treated in the Department of Hepatobiliary Surgery of Guangxi Medical University Cancer Hospital between July 2017 and July 2019. All procedures involving patient samples were approved by the Ethics Committee of Guangxi Medical University Cancer Hospital, and written informed consent was obtained from all patients.

To be included in the study, samples had to be from patients who (1) underwent radical hepatocarcinoma resection, (2) had been definitively diagnosed with HCC based on postoperative pathology, (3) had not received any anticancer treatment before surgery, and (4) did not have any malignancies in addition to HCC.

All patients were followed up in the hospital for the first month after surgery. If there was no recurrence or death,

follow-up was repeated every 3 months during 2 years. Follow-up tests included physical examination, serum alpha-fetoprotein test, serum liver function test, abdominal ultrasonography and computed tomography/magnetic resonance imaging. DFS and OS were defined as the time from the day of surgery to the discovery of tumor recurrence or last follow-up, which was July 2020.

RNA-sequencing

Total RNA was extracted from HCC and matched non-tumor tissues using Trizol reagent (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. RNA purification, reverse transcription, library preparation and sequencing were performed by Wuxi NextCODE (Shanghai, China). RNA-Seq libraries were inspected for quality using FastQC, and the reads were compared against the reference human genome Hg19 using Salmon.[9]

Immunohistochemistry

Specimens of HCC and matched non-tumor tissues were fixed with 10% formalin solution, embedded in paraffin, and cut into sections approximately 4 μm thick. Sections were deparaffinized in xylene at 37°C, rinsed in a graded ethanol series, incubated in 10 mmol/L citrate buffer (pH 6.0) for antigen retrieval, rinsed in phosphate-buffered saline (PBS), incubated in 0.3% H_2O_2 for 10 min at room temperature to inhibit endogenous peroxidases, then rinsed again with PBS. Sections were incubated at 37°C for 1 h with anti-PPP2CA antibody (1:1,000) (ab106262; Abcam, Cambridge, UK), then thoroughly rinsed in PBS. Sections were incubated with MaxVision™/horseradish peroxidase at room temperature for 30 m, washed with PBS, stained with diaminobenzidene and hematoxylin. Sections were dehydrated through a graded ethanol series, allowed to dry, and sealed with neutral gum. In parallel, retinal tissue was processed as a positive control, while HCC tissue was processed with PBS instead of primary antibody to serve as a negative control.

Immunohistochemical staining was semi-quantified using a two-component score. First, staining intensity was assigned 0 points (uncolored), 1 point (light yellow), 2 points (yellow) or 3 points (dark yellow). Second, the percentage of total observed cells that were stained was assigned 1 point (≤25%), 2 points (26–50%), 3 points (51–75%) or 4 points (>75%). The two scores were multiplied together to obtain an overall score of 0–12 points. Overall scores of 0–4 were considered "low PPP2CA expression", while scores of 5–12 were considered "high expression". All sections were assessed independently by two senior pathologists blinded to tissue data. Disagreements were re-assessed.

Statistical analysis

Statistical analyses were carried out using SPSS 25.0 software. Parametric data were presented as mean±standard deviation. Differences between groups were assessed for significance using Student's *t*-test. Relationships between PPP2CA and clinicopathological parameters were analyzed using Student's *t*-test, chi-squared test or Fisher's exact test, as appropriate. The optimal cut-off for binary outcomes was obtained using the pROC package in R version 4.0.2, and patients were stratified by high or low PPP2CA expression based on RNA-Seq data. OS and DFS were analyzed using the Kaplan-Meier method, and curves were compared using the log-rank test in R. Differences associated with $p<0.05$ were considered significant.

Results

PPP2CA mRNA levels in HCC and non-HCC tissues based on GEO and TCGA data

After rigorous screening and evaluation by three researchers, 41 GEO datasets were included (Table 1). Among these, 35 datasets showed higher PPP2CA expression in the HCC group than in the non-HCC group, but only 22 datasets had statistical significance. In another six datasets with lower PPP2CA expression, only one dataset showed a significant difference between the HCC group and non-HCC group.

We collected data on 361 HCC tissues and 50 adjacent non-cancerous tissues from the TCGA database. PPP2CA expression tended to be higher in HCC tissues (11.388±0.360 vs. 11.328±0.306), but the difference was not significant ($p=0.265$).

Meta-analysis of PPP2CA expression based on TCGA, GEO and RNA-Seq databases

We performed a comprehensive meta-analysis based on TCGA, GEO and RNA-Seq data from 2,545 HCC cases and 1,993 non-cancerous cases. PPP2CA expression was higher in HCC tissues than in normal tissues (Fig. 1): the pooled standardized mean difference was 0.69 (95% CI: 0.50 to 0.89, $p<0.001$) based on the random-effects model, with $I^2=86.8\%$ ($p<0.001$). Risk of publication bias was low according to Begg's test ($p=0.530$) and Egger's test ($p=0.305$). Sensitivity analysis did not clearly identify individual studies that contributed to the heterogeneity (Supplementary Fig. 2), suggesting that the results were robust. Subgroup meta-analysis was conducted based on GSE62232, TCGA and RNA-Seq data. The subgroup meta-analysis results showed that PPP2CA expression was higher in HBV-related HCC tissues than normal tissues (Fig. 2A). However, in other subgroups, there were no statistical differences for PPP2CA expression between HCC tissues and normal tissues. The result indicated that PPP2CA may be closely related with HBV-related HCC. However, the heterogeneity of the HBV-related HCC subgroup was still high. After deleting the HBV-related samples of the TCGA dataset, the heterogeneity dropped to 0 (Fig. 2B), indicating that the heterogeneity came from the TCGA dataset.

Clinical usefulness of PPP2CA in HCC

Rates of true positives, false positives, false negatives, and true negatives were calculated for each study in the TCGA, GEO and RNA-Seq datasets (Table 1). PPP2CA showed strong diagnostic potential for HCC (AUC: 0.79, 95% CI: 0.75–0.83; Fig. 3).

In the TCGA dataset, PPP2CA expression was significantly associated with chronic HBV infection ($p=0.041$) but not other clinicopathologic parameters (Supplementary Table 2). Kaplan-Meier analysis indicated that PPP2CA expression above an optimal cut-off was associated with significantly shorter OS ($p<0.001$) and PFS ($p=0.039$). GEPIA2 analysis also indicated that high expression was associated with significantly shorter OS ($p=0.025$), and it tended to be associated with shorter DFS ($p=0.05$) (Fig. 4A–D). These results suggest that extremely high PPP2CA expression may have a

negative impact on HCC survival.

Validation of the clinical significance of PPP2CA using RNA-Seq data

PPP2CA expression was significantly higher in HCC tissues (17.655±6.247) than in adjacent tissues (12.309±2.916, $p<0.001$). Based on a cut-off of 17.664, patients with high PPP2CA expression showed significantly shorter OS ($p=0.0065$) and DFS ($p=0.0045$) than patients with low expression (Fig. 4E, F). Patients with high PPP2CA expression were significantly younger than those with low expression ($p=0.010$) and showed significantly higher prevalence of type 2 diabetes ($p=0.014$), HBV infection ($p=0.046$), gross vascular invasion ($p=0.013$), microvascular invasion ($p=0.019$) and tumor recurrence ($p=0.007$) (Table 1). In addition, patients with high expression showed significantly more advanced Barcelona Clinic Liver Cancer stage ($p=0.039$). Multivariate cox analysis showed that PPP2CA may not be an independent prognostic factor ($p>0.05$).

Validation of the clinical significance of PPP2CA using immunohistochemistry

Samples from 123 HCC patients were included in the immunohistochemistry analysis, comprising 104 men and 19 women with a median age of 51.3±11.2 years. PPP2CA was expressed mainly in the cytoplasm. It was expressed strongly in some HCC tissues but weakly in adjacent tissues. In 107 adjacent tissues, 9 were PPP2CA-negative, 79 weakly positive, 19 moderately positive and 0 strongly positive. We classified 88 cases (82.2%) as showing low PPP2CA expression, and 21 cases (17.8%) as showing high expression. In 123 HCC tissues, 45 were negative, 39 weakly positive, 28 moderately positive and 11 strongly positive (Fig. 5 and Supplementary Table 3). We classified 84 cases (68.3%) as showing low expression and 39 cases (31.7%) as showing high expression. PPP2CA expression was significantly higher in HCC tissues than in adjacent tissues ($\chi^2=5.905$, $p=0.015$; Table 2).

Prediction of miRNAs targeting PPP2CA

We predicted miRNAs that may target the PPP2CA mRNA using miRWalk 2.0. At least nine of the prediction algorithms in the suite identified the following candidate miRNAs: miR-139-5p, miR-141-3p, miR-548o-3p, and miR-200a-3p. We found that miR-548o-3p was expressed at significantly higher levels ($p=0.003$), while miR-139-5p and miR-200a-3p were expressed at significantly lower levels ($p<0.001$) in HCC tissues than in normal liver tissues (Supplementary Fig. 3). In contrast, levels of miR-141-3p did not differ significantly between HCC and normal liver tissues.

GO and KEGG pathway analysis

We analyzed the enrichment of GO and KEGG pathways among 200 genes co-expressed with the PPP2CA gene (Supplementary Fig. 4A–D). The most important GO biological process was vesicle-mediated transport from the endoplasmic reticulum to the Golgi. The most important cellular component was cytoplasm. The most important molecular function was nucleic acid binding. Of the 7 KEGG pathways identified, the most important was the spliceosome, followed by RNA transport.

PPI network of PPP2CA-associated genes

The results of the PPI network generated using the online STRING database showed relationships between PPP2CA and the top 150 selected co-expressed genes (Supplementary Fig. 4E). SKIV2L2, HNRNPK and ABCE1 were extracted as hub genes showing no fewer than 31 edges. The TCGA database showed that SKIV2L2 was significantly up-regulated and ABCE1 significantly down-regulated in HCC tissues compared with normal liver tissues ($p<0.001$). Expression of HNRNPK, in contrast, did not differ significantly between HCC and normal liver tissues ($p=0.079$; Supplementary Fig. 5).

Discussion

PPP2CA encodes the alpha isoform of the catalytic subunit of PP2A, which regulates PP2A activity by selecting PP2A regulatory subunits.[10–12] Down-regulation of PPP2CA leads to lower PP2A activity.[13] Lower levels of PPP2CA have been associated with gastric cancer, such as higher risk of gastric cancer among Chinese,[14] as well as with colorectal cancer and poor prognosis in that disease.[15] Down-regulation of PPP2CA has also been associated with anaplastic thyroid carcinoma (referred to as ATC), and its levels negatively correlate with those of miR-650, which targets PPP2CA in ATC cells.[16] Conversely, PPP2CA overexpression reduces the invasion and metastasis ability of prostate cancer cells.[17]

In other cancers, PPP2CA seems to be overexpressed rather than down-regulated. Up-regulation of PPP2CA in breast cancer is associated with poor prognosis,[18] and up-regulation in malignant osteosarcoma can promote cell proliferation and migration.[19] Thus, PPP2CA expression is altered in different ways in different cancers, suggesting multiple mechanisms of action. This may help explain why previous studies have not clarified whether PP2A promotes or suppresses HCC.[20,21] Another explanation is that many studies of the enzyme in HCC have not taken into account which C subunit isoforms are involved. Our preliminary data suggest that the expression levels of PPP2CA and PPP2CB may be inversely related (data not shown), highlighting the importance of clarifying the specific roles of each C subunit isoform in HCC.

Meta-analysis of 2,545 HCC tissues and 1,993 non-cancerous tissues in the GEO, TCGA and our in-house databases showed that PPP2CA expression was significantly higher in HCC tissues than in non-cancerous tissues. The heterogeneity of this pooled result was high, and sensitivity analysis did not identify any single study that accounted for most of it. In order to further explore the relationship between the expression of HCC with different etiologies and PPP2CA and the source of heterogeneity, we selected datasets with samples greater than 50, which contained specific etiology information of HCC for subgroup analysis. The results showed that the high expression of PPP2CA was related to HBV-related HCC, while it was not related with alcohol- or HCV-related HCC. PPP2CA expression may be related to HBV infection as we found here in our analysis of data and as other studies have reported,[22,23] and it may be related to hepatitis C virus infection,[24] but it may not be associated with alcoholic hepatitis. The subgroup meta-analysis showed that PPP2CA expression was not related to HCV-related HCC. It may also be due to the small sample size. This result needs to be further explored. In terms of heterogeneity, after the HBV-related TCGA dataset was excluded, the heterogeneity of the four subgroups was reduced to 0, while the overall heterogeneity was still high. Thus, we considered that the heterogeneity came from different etiology of HCC and HBV-related TCGA data.

Table 1. Relationships between PPP2CA expression based on RNA-Seq and clinicopathologic parameters of our in-house patient sample

Clinicopathologic parameter	n	PPP2CA expression		*p*
		Low (*n*=52)	High (*n*=64)	
PPP2CA expression	116	12.633.41	22.264.21	<0.001*
Age in years				0.010*
<60	85	32	53	
≥60	31	20	11	
Sex				0.337
Male	101	5	10	
Female	15	47	54	
Hypertension				0.319
Yes	18	10	8	
No	98	42	56	
Type 2 diabetes				0.014*
Yes	7	0	7	
No	109	52	57	
Barcelona Clinic Liver Cancer stage				0.039*
A	58	29	29	
B	32	17	15	
C	26	6	20	
Hepatitis B virus infection				0.046*
Yes	96	39	57	
No	20	13	7	
Hepatitis C virus infection				0.114
Yes	3	0	3	
No	113	52	61	
Alpha-fetoprotein, ng/mL				0.484
<400	71	30	41	
≥400	45	22	23	
Gross vascular invasion				0.013*
Yes	23	5	18	
No	93	47	46	
Cirrhosis				0.230
Yes	54	21	33	
No	62	31	31	
Pathology grade				0.191
I–II	59	30	29	
III–IV	56	21	35	
Microvascular invasion				0.019*
Yes	71	26	45	
No	44	24	18	
Recurrence				0.007*
yes	63	21	42	
no	53	31	22	

*$p<0.05$.

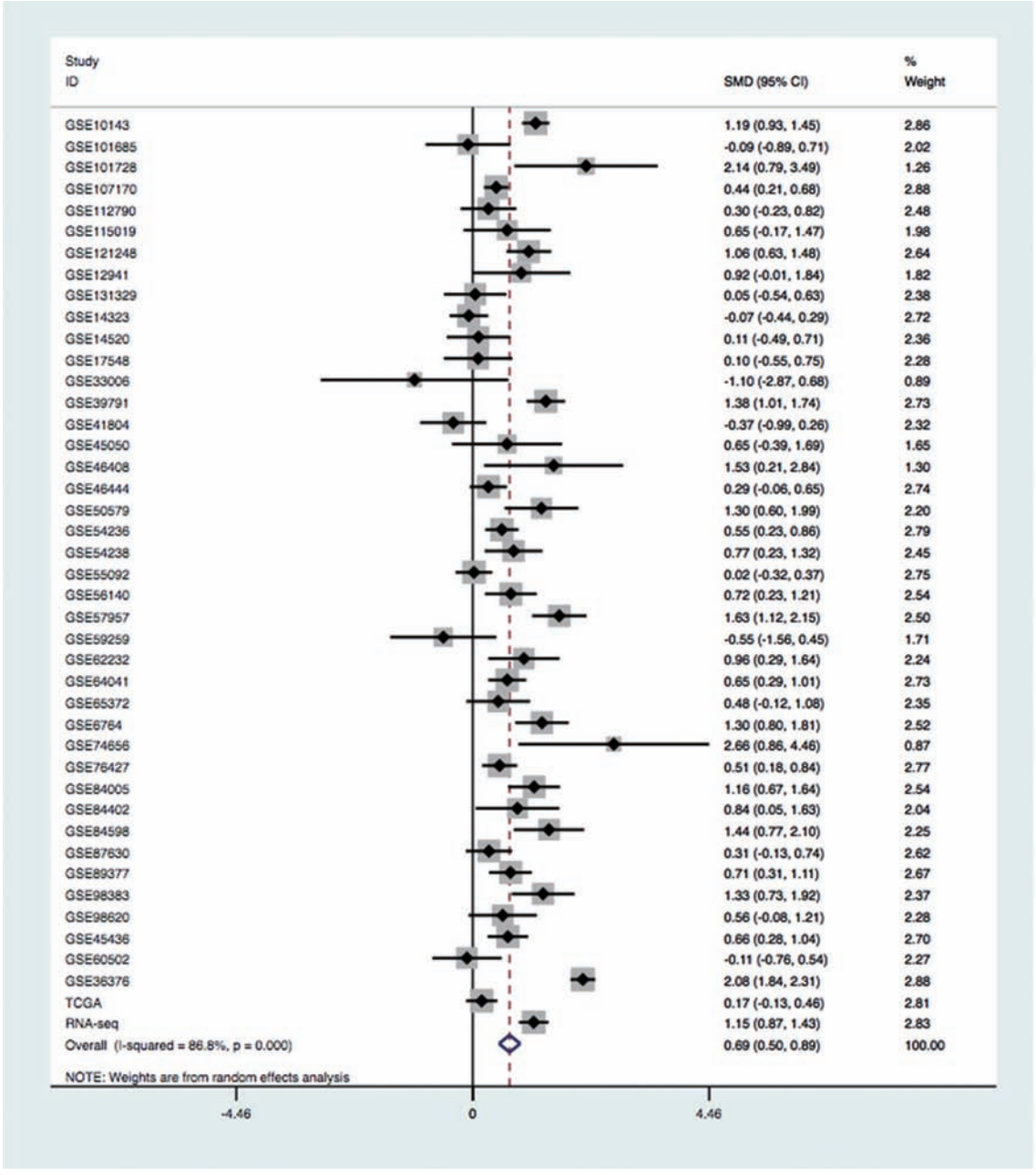

Fig. 1. Forest plot of the pooled standardized mean difference (SMD) for PPP2CA expression between HCC and control tissues, based on random-effect meta-analysis. HCC, hepatocellular carcinoma.

We found evidence that PPP2CA expression shows diagnostic potential in HCC. In addition, high PPP2CA expression in HCC was associated with poor OS, PFS and DFS. Nevertheless, our results should be interpreted cautiously because our follow-up was shorter than that in the TCGA database. We performed a multivariate analysis, which showed that the PPP2CA gene may not be an independent risk factor for the poor prognosis of HCC. Gong *et al*.[23] reported that PP2Ac expression plays a role in HCC tumorigenesis induced by HBV X protein, and PP2Ac protein overexpression is an independent predictor of poor OS of HCC patients. It can be seen that there are obvious differences between the two, which further proves the necessity of studying different subtypes of PP2Ac protein. We confirmed here with immunohistochemistry that PPP2CA protein is overexpressed in HCC tissues. These results may improve the diagnosis of HCC and prediction of prognosis. However, the most ideal biomarkers would be non-invasive. We look forward to seeing more ideal reports of PPP2CA in the peripheral blood of HCC patients in the future.

We identified miR-139-5p, miR-200a-3p, miR-548o-3p, and miR-141-3p as potential miRNAs targeting PPP2CA. The first two were expressed at lower levels in HCC tissues than in normal liver tissues, while the third showed the inverse pattern. Consistent with our results, other studies showed down-regulation of miR-139-5p in HCC samples.[25,26] Its low

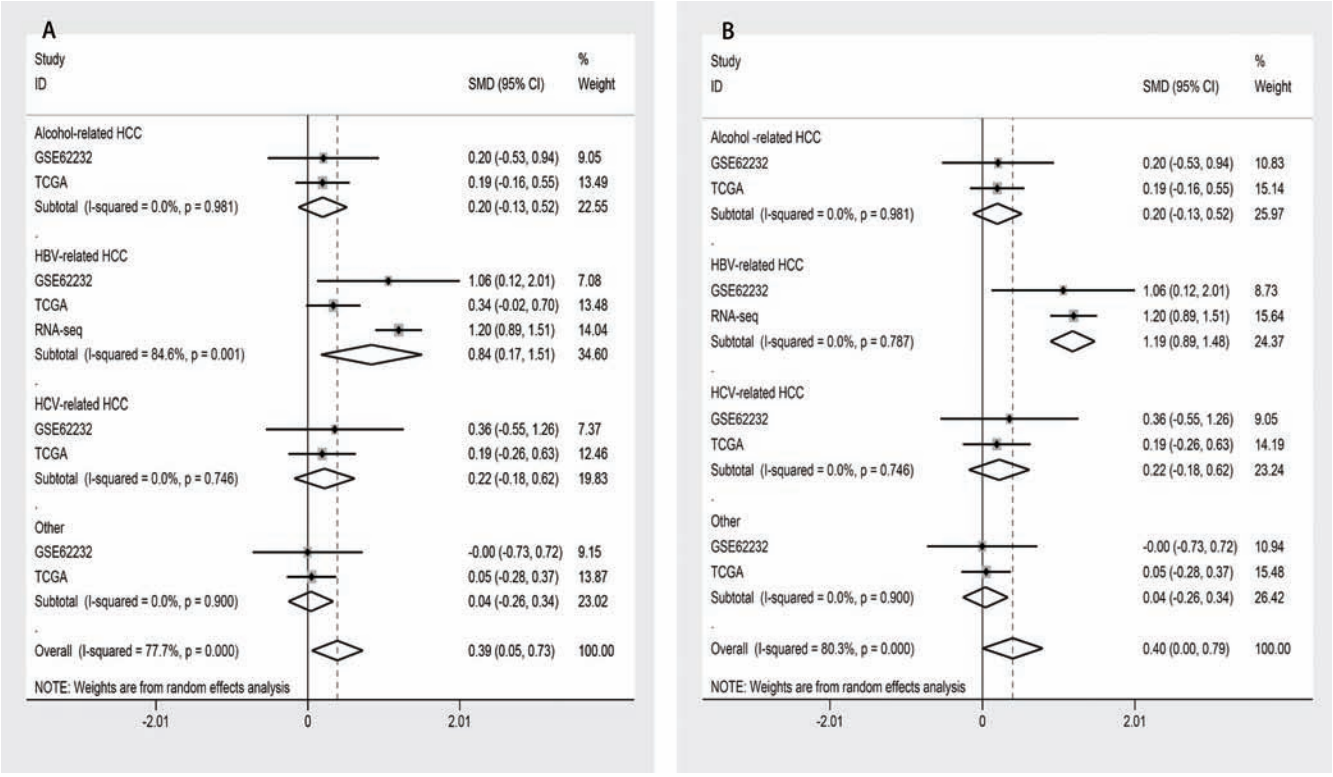

Fig. 2. The subgroup meta-analysis of PPP2CA expression levels between cancerous tissues and non-cancerous tissues in HCC. (A) Forest plot of the PPP2CA expression levels based on TCGA, GSE62232 and in-house RNA-Seq datasets. (B) Forest plot of the PPP2CA expression levels after eliminating HBV-related HCC considered to be sources of heterogeneity. HCC, hepatocellular carcinoma; TCGA, The Cancer Genome Atlas; HBV, hepatitis B virus.

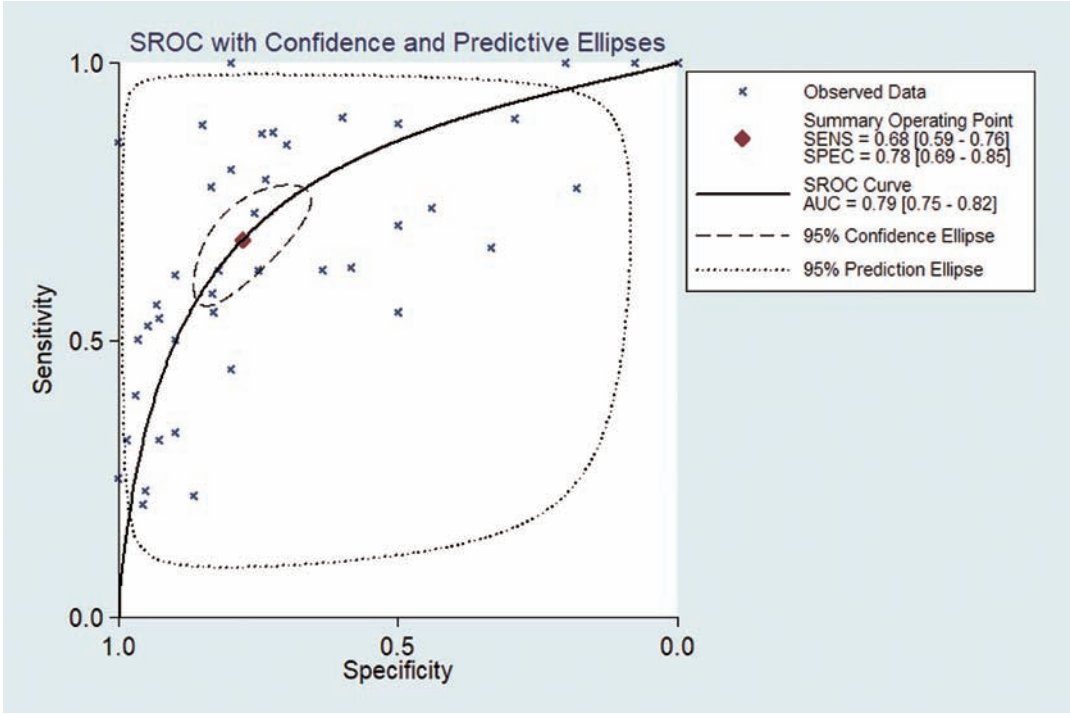

Fig. 3. Summary receiver operating characteristic (sROC) curve assessing the ability of PPP2CA expression to diagnose HCC, based on data from GEO microarrays and the TCGA database. The y-axis represents specificity, and the x-axis represents sensitivity. HCC, hepatocellular carcinoma; GEO, Gene Expression Omnibus; TCGA, The Cancer Genome Atlas.

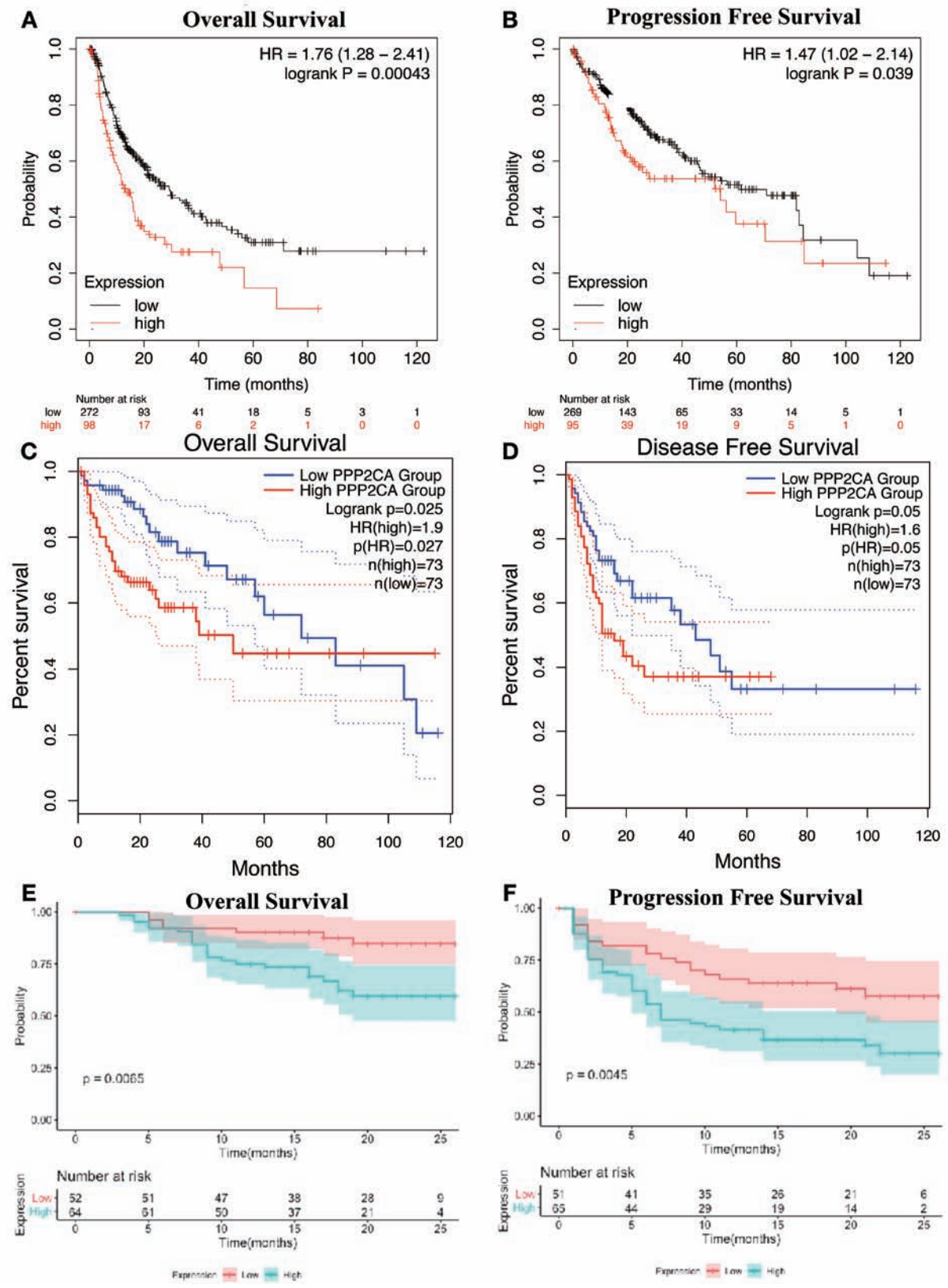

Fig. 4. Overall survival and progression-free survival of hepatocellular patients, stratified based on PPP2CA expression, according to the Kaplan-Meier plotter (A–B), GEPIA (C–D) and our in-house RNA-Seq database (E–F). GEPIA2, Gene Expression Profiling Interactive Analysis.

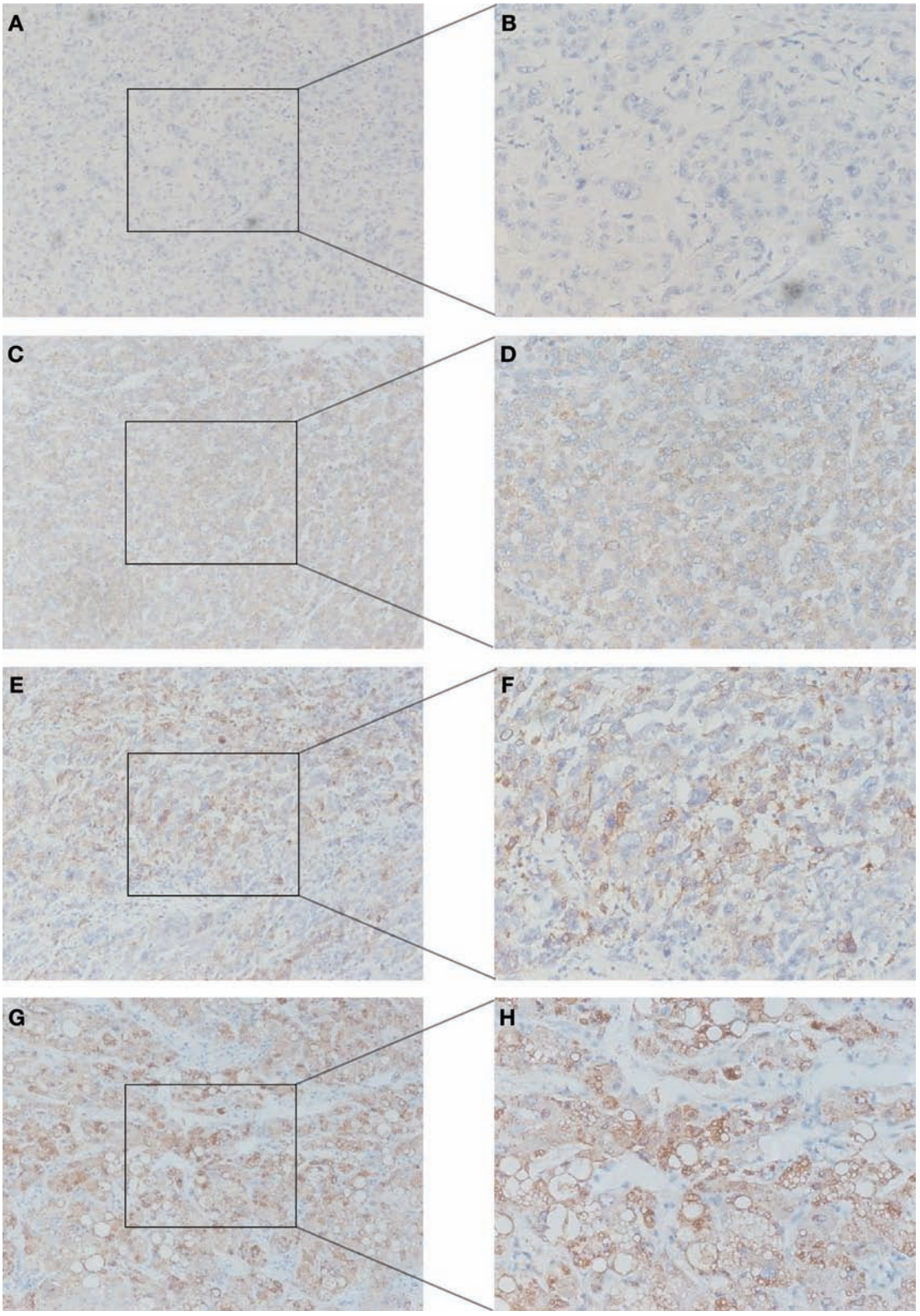

Fig. 5. Expression of PPP2CA in HCC tissues, showing primarily cytoplasmic localization. (A–B) A representative tissue slice classified as showing negative expression (0 points in overall score). Magnification, ×100 (A), ×200 (B). (C–D) A representative tissue slice classified as showing weakly positive expression (1–4 points in overall score). Magnification, ×100 (C), ×200 (D). (E–F) A representative tissue slice classified as showing moderately positive expression (5–8 points in overall score). Magnification, ×100 (E), ×200 (F). (G–H) A representative tissue slice classified as showing strongly positive expression (9–12 points in overall score). Magnification, ×100 (G), ×200 (H). HCC, hepatocellular carcinoma.

Table 2. PPP2CA expression in HCC and adjacent tissues

Group	Low expression, *n* (%)	High expression, *n* (%)	Total	χ^2	*p*
HCC tissue	84 (68.3%)	39 (31.7%)	123	5.905	0.015*
Adjacent tissue	88 (82.2%)	19 (17.8%)	107		
Total	172 (100%)	58 (100%)	230		

*$p<0.05$. HCC, hepatocellular carcinoma.

expression has been associated with poor prognosis,[25] and its anti-tumor effects in HCC have been attributed to an ability to target SPOCK1.[26] Also consistent with our results, another study found that miR-200a-3p was down-regulated in HCC tissues, and that circ-ZEB1.33 promoted HCC proliferation by sponging this miRNA.[27] Therefore, we speculate that low levels of miR-139-5p and miR-200a-3p allow higher PPP2CA expression, thereby promoting HCC. Future studies should test this hypothesis and examine whether and how miR-548o-3p is involved in HCC or other tumors. We also expect that the roles of these miRNAs can be deeply studied in the peripherals of HCC patients to prove whether they may serve as surrogate soluble biomarkers.

KEGG pathway analysis of the 200 genes most closely related to PPP2CA showed the most enriched pathways to be spliceosome and RNA transport. HCC involves up-regulation of the spliceosome pathway and its related genes,[28] and mutations in exportins that affect RNA transport pathways are associated with worse OS among patients.[29] We speculate that PPP2CA may affect HCC through the spliceosome and RNA transport. Our PPI network further identified SKIV2L2 as a hub gene up-regulated in HCC, consistent with a previous report that it is overexpressed in the disease and is a predictor of poor prognosis.[30] Future studies should clarify interactions among SKIV2L2 and the other two hub genes, ABCE1 and HNRNPK, in HCC.

In conclusion, our study shows that PPP2CA expression is significantly higher in HCC tissue than in adjacent tissue, and that it may work as a tumor promoter in HCC. Its high expression correlates with poor prognosis. It may also be a reliable diagnostic marker for HCC. Our study also identifies several miRNAs, key pathways and hub genes that may be related to HCC and therefore merit further study.

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Conflict of interest

The authors have no conflict of interests related to this publication.

Author contributions

Designed the research, analyzed the data, and wrote the manuscript (ZMZ, BDX, YMZ), participated in data preparation, experiments, analysis of data, and figure preparation (CLY, XQ, JYL, XYC, XYH, JHZ, ST, XYL). All authors read and approved the manuscript for publication.

Data sharing statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Original Article

Hepatic Resection Versus Stereotactic Body Radiation Therapy Plus Transhepatic Arterial Chemoembolization for Large Hepatocellular Carcinoma: A Propensity Score Analysis

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Abstract

Background and Aims: There are no comparative studies on the efficacy of hepatic resection (HR) and CyberKnife stereotactic body radiation therapy (CK-SBRT) plus transhepatic arterial chemotherapy embolization (TACE) in the treatment of large hepatocellular carcinoma (HCC). Therefore, this study aimed to compare the efficacy of HR and CK-SBRT+TACE in large HCC. ***Methods:*** A total of one hundred and sixteen patients were selected from November 2011 to December 2016. Among them, 50 were allocated to the CK-SBRT+TACE group and 66 were allocated to the HR group. The Kaplan-Meier method was applied to calculate overall survival (OS) and progression-free survival (PFS) rates. Propensity score matching was performed to control for baseline differences between the groups. ***Results:*** Thirty-six paired patients were selected from the CK-SBRT+TACE and HR groups. After propensity score matching, the 1-, 2- and 3-year OS rates were 83.3%, 77.8% and 66.7% in the HR group and 80.6%, 72.2% and 52.8% in the CK-SBRT+TACE group, respectively. The 1-, 2- and 3-year PFS rates were 71.6%, 57.3% and 42.3% in the HR group and 66.1%, 45.8% and 39.3% in the CK-SBRT+TACE group, respectively (OS: $p=0.143$; PFS: $p=0.445$). Both a high platelet count and low alpha-fetoprotein value were revealed as influencing factors in improving OS and PFS. ***Conclusions:*** CK-SBRT+TACE brought local effects that were similar to those of HR in HCC patients with a large and single lesion. Moreover, the liver injury occurrence rate was acceptable in both groups.

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Keywords: CyberKnife; Radiation-induced liver disease; Survival rates; Large hepatocellular carcinoma.

Abbreviations: AFP, alpha-fetoprotein; ALB, albumin; ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; CK-SBRT, CyberKnife stereotactic body radiation therapy; CT, computed tomography; ECOG PS, Eastern Cooperative Oncology Group performance score; HCC, hepatocellular carcinoma; HR, hepatic resection; LC, Local control; LT, liver transplantation; MRI, Magnetic resonance imaging; OS, overall survival; PFS, progression-free survival; PLT, platelet; PSM, propensity score matched analysis; RFA, radiofrequency ablation; RILD, radiation-induced liver disease; TACE, transhepatic arterial chemoembolization; TAE, transcatheter arterial embolization.

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Introduction

Hepatocellular carcinoma (HCC) is the sixth most common cancer worldwide and the fourth most common cause of cancer-related death.[1] Hepatic resection (HR), radiofrequency ablation (RFA) and liver transplantation (LT) are the main curative methods for HCC, especially for early-stage HCC.[2] However, without any related syndrome in the early stages of the disease, some HCC patients are at an advanced stage at the time of diagnosis and lose the opportunity for radical treatment.

HR and transhepatic arterial chemotherapy embolization (TACE) are widely used for patients with a tumor diameter of 5–10 cm. With the advancement of radiotherapy technologies, CyberKnife stereotactic body radiation therapy (CK-SBRT) has also been applied to patients with large HCC and prolonged their survival, especially among those who were not suitable for or refused other treatments.[3,4] Previous studies have reported improved outcomes using radiotherapy+TACE combination therapy compared with TACE or radiotherapy alone.[5–7] However, there are no comparative studies on the efficacy of HR and CK-SBRT+TACE in the treatment of large HCC. Therefore, we conducted a retrospective analysis to compare long-term survival following CK-SBRT+TACE versus HR for patients with large HCCs (5–10 cm) that were treated in our medical center.

Methods

The study profile is shown in Figure 1. One hundred and sixteen patients were enrolled in this study from November 2011 to December 2016. Among them, 50 were in the CK-

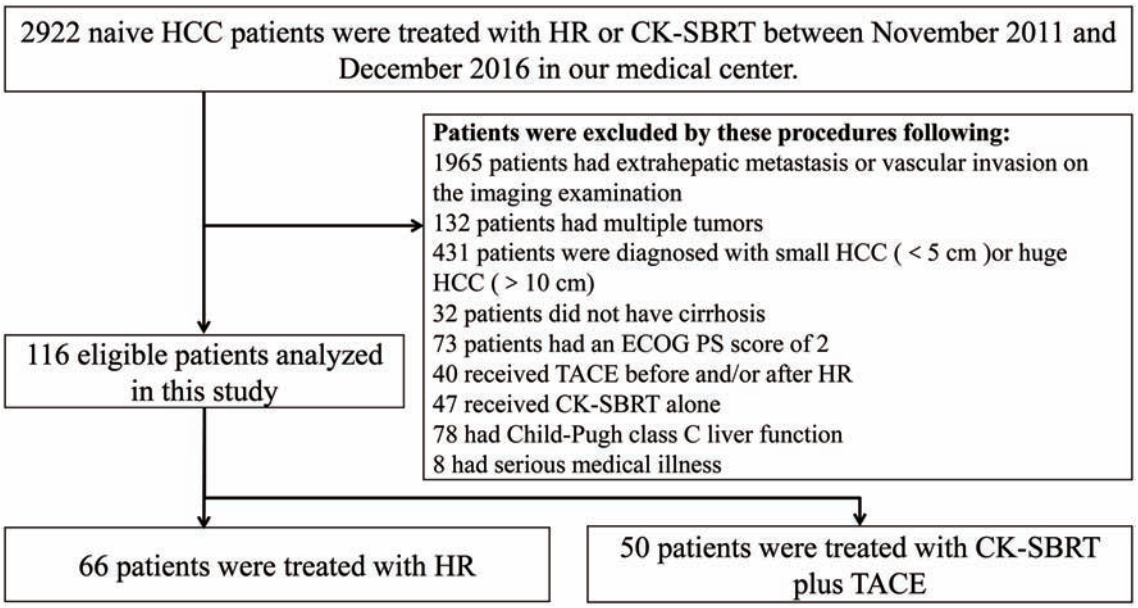

Fig. 1. Flow diagram showing the screening, enrollment, and treatment allocation of patients.

SBRT+TACE group and 66 were in the HR group.

Eligibility criteria were as follows: 1) HCC patients diagnosed according to an imaging examination, laboratory tests or pathology; 2) a single lesion with a diameter of 5–10 cm; 3) no prior treatment; 4) Child-Pugh classification A or B; 5) no portal vein tumor thrombus on imaging examination; 6) an Eastern Cooperative Oncology Group performance score (commonly referred to as ECOG PS) of 0 or 1; 7) (for patients in the HR group) an indocyanine green retention rate at 15 minutes less than 10%; and 8) (for patients in the CK-SBRT group) normal residual liver volume ≥700 cc and having undergone gastroscopy before treatment.

CK-SBRT procedure

All patients in the CK-SBRT group received fiducial marker implantation before TACE and underwent computed tomography (CT) localization imaging after their liver function recovered. Plain CT scan images were benchmark images, and contrast-enhanced CT or contrast-enhanced magnetic resonance images were used as auxiliary images for fusion. An oncologist contoured the gross tumor volume, planning target volume and organs-at-risk. Planning target volume was defined as 3–5 millimeter expansion of gross tumor volume and avoided organs-at-risk. Prescribed doses were 50–54 Gy/5–6 fx. All plans were calculated by G4 CyberKnife MultiPlan (version 4.0.2) and VSI CyberKnife MultiPlan (version 4.6.1) (Accuracy, USA). Normal tissue tolerance doses were determined according to the AAPM TG-101 report.[3]

TACE procedure

The patients underwent TACE between fiducial marker implantation and CK-SBRT execution. The femoral artery was accessed via catheterization. Hepatic angiography was performed to observe the common hepatic artery, left and right hepatic arteries, and splenic artery. After localized tumor staining, intervention radiologists inserted a microcatheter into the blood supply vessel and infused it with a mixture of 5–20 mL iodinated oil injection (Lipiodol; Guerbet, Aulnay-sous-Bois, France) plus epirubicin (10 mg). If a patient's tumor had an arteriovenous fistula, gelatin sponge particles (Cutanplast; Mascia Bruneili S.p.A., Milano, Italy) were applied for embolization. After CK-SBRT, the patients received TACE once a month.

HR procedure

Segmental hepatectomy, left hepatectomy and right hepatectomy were applied to remove the tumor. The residual liver volume was estimated from a preoperative volumetric CT scan. Hepatectomy was not executed in patients with a remnant volume less than 30% of the total liver volume, excluding the lesion.[4] Intraoperative ultrasound was routinely applied to evaluate the extent of parenchymal resection that could be safely performed. HR could receive R0 resection.

Toxicity reactions and follow-up

Toxicity reactions were evaluated according to the Common Terminology Criteria for Adverse Events version 4.0.[5] Radiation-induced liver disease (referred to as RILD) was observed among the patients in the CK-SBRT+TACE group.

All patients underwent laboratory tests at least every 3 days during CK-SBRT+TACE/HR treatment. After treatment, the patients were reviewed every 3 months for 1 year and every 6 months thereafter until March 2020 or death.

Statistical analysis

Overall survival (OS) was defined as the period between the beginning of treatment and the final follow-up or death. Progression-free survival (PFS) was defined as the period between the beginning of treatment and the final follow-up or tumor progression. Local control was defined as the pe-

riod between the beginning of treatment to the progression of the previously treated lesion or the final follow-up. Propensity score matching (PSM) at a 1:1 ratio was performed to balance the CK-SBRT+TACE and HR patient cohorts. The Kaplan-Meier method was applied to calculate survival rates. The log-rank test was used to compare survival outcomes of the two groups. The $\chi 2$ test or Fisher's exact test was used to compare baseline variables of the two groups. All statistical analyses were performed using STATA (version 15.0; STATA Corp., College Station, TX, USA) and SPSS (version 23.0; IBM Corp., NY, Armonk, USA). A *p*-values of < 0.05 was considered statistically significant.

Results

The patients' characteristics are shown in Table 1. The proportions of patients with an alpha-fetoprotein (AFP) value ≥200 ng/mL or a low platelet (PLT) count were higher in the CK-SBRT+TACE group than in the HR group. After PSM, 36 paired patients were selected from the two groups, and no significant differences in variables were observed.

Recurrence / metastasis, subsequent treatment and cause of death

By March 2020, 75 patients had developed relapse or metastasis (44 patients in the HR group and 31 patients in the CK-SBRT+TACE group), and 60 patients had died (31 patients in the HR group and 29 patients in the CK-SBRT+TACE group). After PSM, 48 patients had developed recurrence or metastasis (24 patients in the HR group and 24 patients in the CK-SBRT+TACE group), and 38 patients had died (16 patients in the HR group and 22 patients in the CK-SBRT+TACE group). The details are shown in Table 2.

Survival analyses

Before PSM, the 1-, 2- and 3-year OS rates were 80.0%, 70.0% and 54.0% in the CK-SBRT+TACE group and 83.3%, 71.2% and 63.6% in the HR group, respectively ($p=0.213$; Fig. 2A). The 1-, 2- and 3-year PFS rates were 65.4%, 46.2% and 41.6% in the CK-SBRT+TACE group and 63.1%, 49.3% and 41.4% in the HR group, respectively ($p=0.923$; Fig. 2B). After PSM, the 1-, 2- and 3-year OS rates were 83.3%, 77.8% and 66.7% in the HR group and 80.6%, 72.2% and 52.8% in the CK-SBRT+TACE group, respectively ($p=0.143$; Fig. 2C). The 1-, 2- and 3-year PFS rates were 71.6%, 57.3% and 42.3% in the HR group and 66.1%, 45.8% and 39.3% in the CK-SBRT+TACE group, respectively ($p=0.445$; Fig. 2D). There was no significant difference in OS and PFS. The influencing factors of OS and PFS are shown in Table 3, and we found that a low AFP value and high PLT count were influencing factors in improving OS and PFS for all patients with large HCC.

Two patients are shown in the figures, including one who received CK-SBRT+TACE (Fig. 3A–C) and one who received HR (Fig. 3D–F).

Toxicity reactions and complications

The main common adverse reactions in the two groups were grade 1–2 gastrointestinal reactions, including nausea, vomiting and anorexia. No grade ≥3 gastrointestinal toxicities were observed. The proportion of patients with abdominal pain in the HR group was higher than that in the CK-SBRT+TACE group, as was the proportion of patients with ascites or hydrothorax. Transient liver dysfunction occurred mainly in the HR group and showed mainly a decrease in albumin and an elevation in transaminase. Six patients in the CK-SBRT+TACE group were diagnosed with RILD before PSM, and the details of their liver function before and after CK-SBRT are shown in Table 4.

None of these patients died from the toxicity outcomes and complications of HR or CK-SBRT+TACE. The toxicity reactions and complications in the two groups are shown in Table 5.

Discussion

The treatment of patients with large HCC is a considerable challenge. Some studies reported that ablation or HR therapy could achieve certain efficacy. Xu *et al*.[6] reported the outcomes of patients with 5–6 cm unresectable HCCs who received microwave ablation. They found that the 1-, 3- and 5-year OS rates were 92.7%, 63.4% and 41.1%, respectively, and the corresponding recurrence-free survival rates were 65.9%, 31.7% and 23.0%, respectively. Although the tumor size was smaller in their study than in ours, their 3-year OS rate was similar to ours, and the 3-year recurrence-free survival rates were lower than our PFS rates. Zhao *et al*.[7] conducted a retrospective analysis of patients with large HCC undergoing HR. Ninety-nine patients were enrolled in their study. Two patients died of hepatic failure within 30 days after surgery. The 1-, 3- and 5-year disease-free survival and OS rates following HR were 67% and 49% and 37% and 77%, 56%, and 43%, respectively. Hsu *et al*.[8] evaluated the long-term outcomes after HR in elderly patients with resectable large HCC compared with those in younger patients. The 1-, 3-, 5- and 7-year OS rates in the elderly/younger groups were 76%/79%, 55%/57%, 48%/51% and 42%/49%, respectively. The 1-, 3-, 5- and 7-year disease-free survival rates in the elderly/younger groups were 60%/54%, 40%/36%, 38%/32%, and 27%/32%, respectively. The OS rate in our study was higher than that in theirs. We believe that this finding may be related to the fact that all patients in the HR group of our study were of Child-Pugh A classification and with single lesion, both of which are influencing factors for improving prognosis.

SBRT has been applied in the HCC treatment field for over 20 years[9] and has achieved a satisfactory effect on HCC patients, and the number of related studies published in recent years has increased. To date, there have been more studies on patients with small HCC[10–12] than on patients with large HCC. Shibata *et al*.[13] applied proton beam therapy to patients with large HCC, in which the tumor size ranged from 5.0 to 13.9 cm. Twenty-four patients were classified as Child-Pugh A, and five patients were classified as Child-Pugh B. The 2-year Local control (LC), PFS and OS rates were 95%, 22% and 61%, respectively. Beaton *et al*.[14] described 13 patients with large HCC whose median tumor size ranged from 5.1 to 9.7 cm and were treated with SBRT. The prescribed doses were 40–45 Gy in five fractions. They reported a median OS of 17.7 months and a 1-year OS rate of 62%. SBRT provides an effective treatment for patients with large HCC, especially for patients who are not suitable for or unwilling to receive other treatments.

The AFP value and PLT count were significant factors of OS and PFS in our study. A similar AFP value was reported in previous studies.[15,16] The PLT count could serve as an indicator for the degree of cirrhosis by indicating the degree of hypersplenism and portal hypertension,[17] and complications of cirrhosis were the main cause of death. Moreover,

Table 1. Characteristics of patients before and after PSM in this study

Patients details	Total enrolled patients	Before PSM				After PSM			
		HR group	CK-SBRT+ TACE group	*p*	Std. mean diff	HR group	CK-SBRT+ TACE group	*p*	Std. mean diff
Number of patients	116	66	50			36	36		
Sex									
Male	97 (83.6)	56 (84.8)	41 (82.0)	0.681	0.079	30 (83.3)	29 (80.6)	0.759	−0.077
Female	19 (16.4)	10 (15.2)	9 (18.0)	0.681	0.079	6 (16.7)	7 (19.4)	0.759	−0.077
Age in years	54.1±10.27	52.52±8.98	56.2±11.51	0.055	−0.410	53.72±8.66	53.5±10.92	0.943	0.019
Diameter of tumor in cm	6.74±1.34	6.93±1.27	6.49±1.41	0.079	0.349	6.73±1.19	6.74±1.51	0.978	−0.007
Type of chronic hepatitis									
Hepatitis B virus infection	101 (87.1)	58 (87.9)	43 (86.0)	0.174	0.109	32 (88.9)	32 (88.9)	1.000	0.000
Hepatitis C virus infection	5 (4.3)	1 (1.5)	4 (8.0)	0.174	−0.527	1 (2.8)	1 (2.8)	1.000	0.000
Without hepatitis virus infection	10 (8.6)	7 (10.6)	3 (6.0)	0.174	–	3 (8.3)	3 (8.3)	1.000	–
Child-Pugh classification									
CP-A	112 (96.6)	66 (100.0)	46 (92.0)	0.009	–	36 (100.0)	36 (100.0)	1.000	–
CP-B	4 (3.4)	0 (0.0)	4 (8.0)	0.009	–	0 (0.0)	0 (0.0)	1.000	–
Alpha fetoprotein value in ng/mL									
<200	84 (72.4)	53 (80.3)	31 (62.0)	0.029	−0.132	23 (63.9)	24 (66.7)	0.804	−0.059
≥200	32 (27.6)	13 (19.7)	19 (38.0)	0.029	−0.132	13 (36.1)	12 (33.3)	0.804	−0.059
ECOG PS score									
0	77 (66.4)	45 (68.2)	32 (64.0)	0.637	0.001	22 (61.1)	21 (58.3)	0.810	−0.056
1	39 (33.6)	21 (31.8)	18 (36.0)	0.637	−0.001	14 (38.9)	15 (41.7)	0.810	0.056
White blood count as $\times 10^9$/L	5.46±1.80	5.26±1.85	5.61±1.75	0.307	0.197	5.59±2.01	5.41±1.80	0.688	0.103
Platelet count as $\times 10^9$/L	159.60±68.74	176.47±66.78	137.34±65.46	0.002	0.586	154.64±58.79	150.78±66.15	0.794	0.058

CK-SBRT, CyberKnife stereotactic body radiation therapy; ECOG PS: Eastern Cooperative Oncology Group performance score; HR, hepatic resection; PSM, propensity score matched analysis; TACE, transhepatic arterial chemoembolization.

Table 2. Recurrence, metastases, treatment and cause of death of patients before and after PSM in this study

Patients details	Before PSM			After PSM		
	HR group	CK-SBRT+TACE group	*p*	HR group	CK-SBRT+TACE group	*p*
Number of patients with metastases	44	31	0.603	24	24	1.000
Single organ metastasis	40 (90.9)	29 (93.5)		21 (87.5)	21 (87.5)	
Liver	35 (79.5)	22 (71.0)		19 (79.2)	19 (79.2)	
Lung	3 (6.8)	3 ((9.7)		2 (8.3)	1 (4.2)	
Lymph node	0 (0.0)	2 (6.5)		0 (0.0)	0 (0.0)	
Bone	2 (4.6)	2 (6.5)		0 (0.0)	1 (4.2)	
Adrenal gland	1 (2.3)	0 (0.0)		0 (0.0)	0 (0.0)	
Multiple organ metastasis	4 (9.1)	2 (6.5)		3 (12.5)	3 (12.5)	
Subsequent therapy						
Single treatment	29 (65.9)	15 (48.4)		15 (62.5)	12 (50.0)	
Hepatic resection	2 (4.6)	0 (0.0)		1 (4.2)	0 (0.0)	
Trans-arterial chemoembolization	14 (31.8)	0 (0.0)		4 (16.7)	0 (0.0)	
Radio-frequency ablation	7 (15.9)	0 (0.0)		5 (20.8)	0 (0.0)	
CK-SBRT	5 (11.4)	14 (45.2)		4 (16.7)	11 (45.8)	
Target therapy or immunotherapy	1 (2.3) (Target therapy)	1(3.2) (Immunotherapy)		1 (4.2) (Target therapy)	1(4.2) (Immunotherapy)	
Multiple treatments	4 (9.1)	1 (3.2) (SBRT+RFA)		1 (4.2)	1 (4.2)	
Conservative treatment	11 (25.0)	15 (48.4)		8 (33.3)	11 (45.8)	
Number of dead patients	31	29	0.239	16	22	0.157
Cause of death						
Liver failure	12 (38.7)	10 (34.5)		4 (25.0)	8 (36.4)	
Upper gastrointestinal hemorrhage	5 (16.1)	6 (20.7)		3 (18.8)	5 (22.7)	
Infectious shock	8 (25.8)	1 (3.4)		6 (37.5)	0 (0.0)	
Other causes	1 (3.2)	5 (17.2)		1 (6.2)	3 (13.6)	
Unknown	5 (16.1)	7 (24.1)		2 (12.5)	6 (27.3)	

CK-SBRT, CyberKnife stereotactic body radiation therapy; HR, hepatic resection; PSM, propensity score matched analysis; TACE, transhepatic arterial chemoembolization.

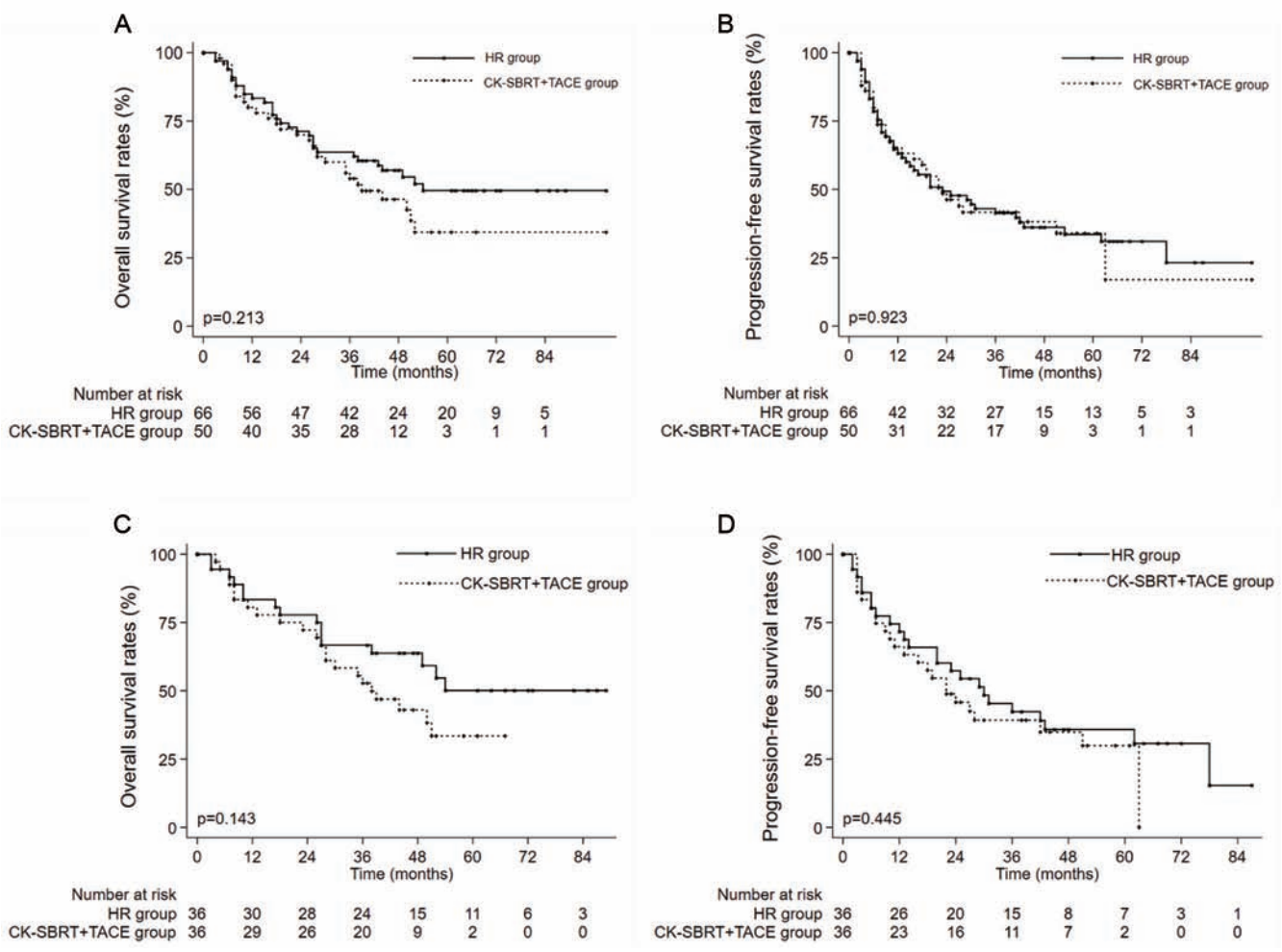

Fig. 2. Comparison of the groups who received CK-SBRT+TACE and HR group. (A) OS rates (p=0.213). (B) PFS rates (p=0.923). (C–D) Following PSM, OS rates (C) (p=0.143) and PFS rates (D) (p=0.445). CK-SBRT, CyberKnife stereotactic body radiation therapy; HR, hepatic resection; TACE, transhepatic arterial chemoembolization.

an adequate PLT count was one of the necessary conditions for treatment of relapse.

This study is the first to compare the efficacy and complications of SBRT+TACE and HR in patients with large HCC. The main adverse reactions in the SBRT group were nausea and vomiting, which were mainly related to exposure of the gastrointestinal tract to radiation during therapy. We found that RILD occurred only in six patients, and there were no deaths. Compared to conventional radiation therapy, CK-SBRT improved accuracy through noncoplanar irradiation and better protected normal residual liver function by adopting fiducial marker tracking combined with dynamic respiration tracking. Fatigue and abdominal pain were the main syndromes in the HR group, and some patients had ascites or hydrothorax, which was mainly related to surgical trauma, and most patients recovered within 3 weeks. Although the OS curve of HR seems to be higher than that of CK, the difference in OS between the two groups was not statistically significant before and after PSM. After relapse or metastases, patients in the HR group received more types of follow-up treatment, and the proportion of patients who received multiple treatments was higher than that of those who received CK-SBRT+TACE. However, the majority of patients in the CK-SBRT+TACE group received repeated CK-SBRT only. Moreover, the proportion of patients in the CK-SBRT+TACE group who received conservative therapy was higher than that in the HR group. Although the choice of treatment was related to the patients, it may have affected the prognosis.

TACE has been widely applied to patients with large HCC in clinical practice. Jin *et al*.[18] compared the OS outcomes of patients with large HCC and a single tumor treated with HR and TACE. In their study, 206 patients were in the HR group, and 489 patients were in the TACE group. The cumulative OS rates at 1, 3 and 5 years in the HR group were significantly higher than those in the TACE group. Previous studies showed that CK-SBRT combined with TACE could prolong survival in patients with nonresectable HCC. Wong *et al*.[19] conducted a retrospective study of two centers in Hong Kong. After PSM, 49 patients were in the TACE+SBRT group, and 98 patients were in the TACE alone group. The 1- and 3-year OS rates in the TACE+SBRT group and TACE alone group were 67.2% versus 43.9% and 36.5% versus 13.3%, respectively. The 1- and 3-year PFS rates in the TACE + SBRT group and TACE alone group were 32.5% versus 21.4% and 15.1% versus 5.1%, respectively. Su *et al*.[20] described 77 patients who received SBRT followed by transcatheter arterial embolization (commonly known as TAE)

Table 3. Univariate and multivariate Cox hazard analyses of risk factors for OS and PFS in all patients enrolled in this study

Patients details	OS				PFS			
	Univariate Cox regression		Multivariate Cox regression		Univariate Cox regression		Multivariate Cox regression	
	p value	Hazard ratio (95% CI)	*p* value	Hazard ratio (95% CI)	*p* value	Hazard ratio (95% CI)	*p* value	Hazard ratio (95% CI)
Sex								
Male versus female	0.983	1.007 (0.510–1.988)	0.739	1.134 (0.541–2.377)	0.450	1.293 (0.664–2.520)	0.361	1.409 (0.676–2.939)
Age in years	0.613	1.007 (0.510–1.988)	0.162	0.981 (0.955–1.008)	0.445	0.992 (0.971–1.013)	0.217	0.985 (0.962–1.009)
Diameter of tumor in cm	0.506	1.067 (0.881–1.293)	0.235	1.134 (0.921–1.395)	0.345	1.091 (0.911–1.307)	0.268	1.114 (0.920–1.347)
Type of chronic hepatitis								
B versus C versus none	0.614	0.907 (0.621–1.326)	0.078	0.650 (0.402–1.050)	0.693	0.932 (0.657–1.323)	0.245	0.778 (0.509–1.189)
Child-Pugh classification								
CP-A versus CP-B	0.048	2.354 (1.006–5.505)	0.066	2.353 (0.947–5.851)	0.581	1.327 (0.486–3.625)	0.518	1.424 (0.488–4.155)
Alpha fetoprotein value in ng/mL								
<200 versus ≥200	0.093	1.559 (0.928–2.618)	0.025	1.842 (1.079–3.147)	0.086	1.522 (0.942–2.458)	0.025	1.765 (1.073–2.902)
ECOG PS								
0 versus 1	0.797	0.935 (0.560–1.560)	0.832	1.062 (0.611–1.846)	0.755	1.076 (0.680–1.702)	0.579	1.154 (0.696–1.911)
White blood count as $\times 10^9$/L	0.671	0.967 (0.830–1.127)	0.504	1.069 (0.879–1.299)	0.395	1.058 (0.929–1.204)	0.259	1.103 (0.930–1.307)
Platelet count as $\times 10^9$/L	0.080	0.997 (0.993–1.000)	0.008	0.992 (0.987–0.998)	0.569	1.327 (0.486–3.625)	0.045	0.995 (0.990–1.000)

ECOG PS, Eastern Cooperative Oncology Group performance score; OS, overall survival rates; PFS, progression-free survival rates.

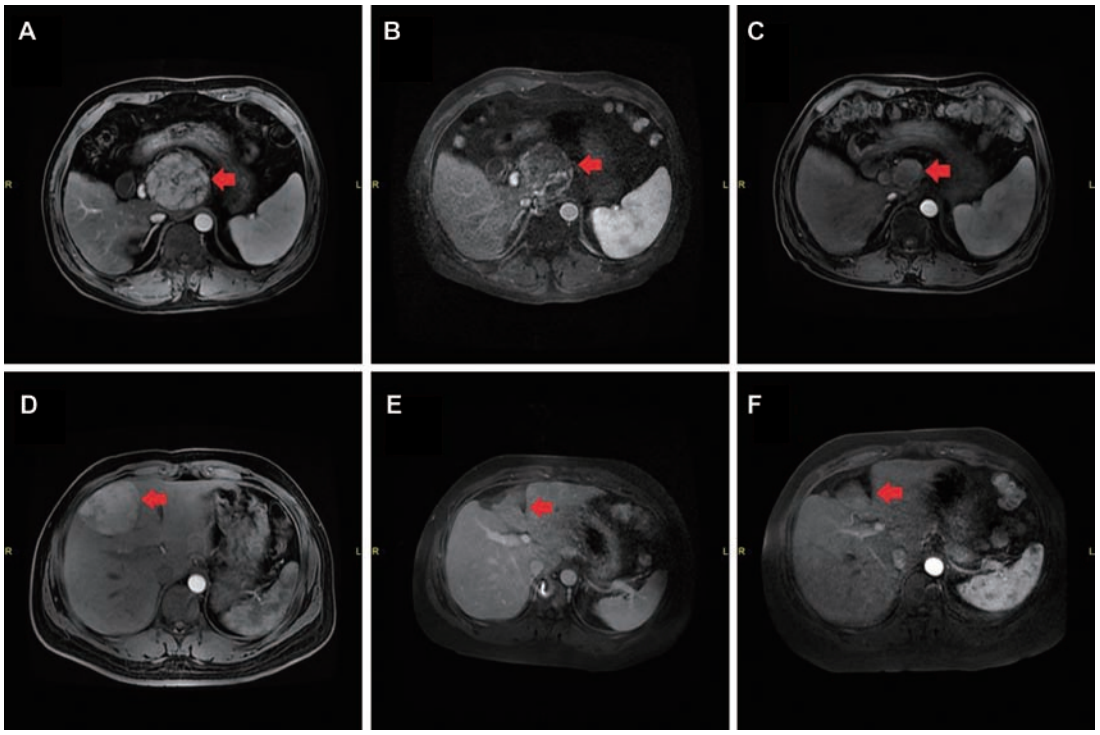

Fig. 3. Magnetic resonance imaging (MRI) of patients with large HCC who were treated with CK-SBRT+TACE and HR. (A–C) are for a patient treated with CK-SBRT+TACE, primary abdominal scan showing the HCC lesion (A), at 6 months after CK-SBRT+TACE (B), and at 2 years after CK-SBRT+TACE (C). (D–F) are for a patient treated with HR, primary abdominal MRI scan showing the HCC lesion (D), at 6 months after HR (E), and at 2 years after HR (F). CK-SBRT, CyberKnife stereotactic body radiation therapy; HCC, hepatocellular carcinoma; HR, hepatic resection; MRI, Magnetic resonance imaging; TACE, transhepatic arterial chemoembolization.

or TACE and 50 patients who received SBRT alone. The 1-, 3- and 5-year OS rates were 75.5%, 50.8% and 46.9% in the TAE/TACE+SBRT group and 62.4%, 32.9% and 32.9% in the SBRT group, respectively. All their results showed that SBRT combined with TACE could better improve survival than SBRT or TACE alone. We conjectured that SBRT may normalize the tumor vasculature and increase embolization rates and that TACE/TAE could eliminate subclinical lesions, which prolonged PFS and OS.

It is difficult to carry out a prospective randomized controlled cohort study to compare the efficacy of CK-SBRT+TACE and HR in treating patients with large HCC. However, based on our results, we believe that CK-SBRT combined with TACE could offer a strategy for improving the survival of patients with large HCC, especially those who were not suitable for HR.

Conclusions

Our results showed that CK-SBRT+TACE and HR provide similar OS and PFS benefits for patients with large HCC. CK-SBRT+TACE could offer a treatment option for patients with large HCC who are not suitable for or refuse other treatments. The toxicity reactions and complications in the two groups were acceptable.

Table 4. Details of patients who were diagnosed with RILD before and after CK-SBRT

ALP		Bilirubin		ALT		AST		ALB		Ascites	
Pre-	Post-	Pre-	Post-	Pre-	Post-	Pre-	Post-	Pre-	Post-	Pre-	Post-
Classic RILD											
80	245	17.1	20.1	34	33	36	36	43	35	–	+
Non-classic RILD											
98	112	12.4	32.1	33	424	32	286	38	32	–	+
98	121	20.1	51.7	40	34	42	114	35	33	–	+
67	136	22.7	55.6	30	608	28	363	36	40	–	–
165	144	26.9	43.8	19	68	40	75	31	28	–	+
79	81	18.5	35.6	28	17	37	42	39	34	–	–

ALB, albumin; ALT, alanine aminotransferase; ALP, alkaline phosphatase; AST, aspartate aminotransferase; CK-SBRT, CyberKnife Stereotactic body radiation therapy; RILD, radiation induced liver injury.

Table 5. Toxicity reaction and complications of patients before and after PSM in this study

Adverse reaction	Before PSM			After PSM		
	HR group	CK-SBRT+TACE group	*p*	HR group	CK-SBRT+TACE group	*p*
Number of patients	66	50		36	36	
Nausea/vomiting						
Grade 1–2	0 (0.0)	28 (56.0)	0.000	0 (0.0)	5 (13.9)	0.064
Anorexia						
Grade 1–2	22 (33.3)	32 (64.0)	0.001	8 (22.2)	15 (41.7)	0.077
Fatigue						
Grade 1–2	17 (25.8)	16 (32.0)	0.461	11 (30.6)	7 (19.4)	0.276
Grade ≥3	6 (9.1)	0 (0.0)	0.077	2 (5.7)	0 (0.0)	0.473
Abdominal pain						
Grade 1–2	32 (48.5)	5 (10.0)	0.000	15 (41.7)	4 (11.1)	0.007
Grade ≥3	7 (10.6)	0 (0.0)	0.047	2 (5.7)	0 (0.0)	0.473
Anemia						
Grade 1–2	15 (22.7)	3 (6.0)	0.027	6 (16.7)	0 (0.0)	0.033
Grade ≥3	2 (3.0)	0 (0.0)	0.602	0 (0.0)	0 (0.0)	1.000
Ascites	15 (22.7)	4 (8.0)	0.062	8 (22.2)	2 (5.6)	0.088
Hydrothorax	5 (75.8)	0 (0.0)	0.127	1 (2.8)	0 (0.0)	1.000
ALT increase						
Grade 1–2	33 (50.0)	10 (20.0)	0.001	15 (41.7)	7 (19.4)	0.041
Grade ≥3	18 (27.3)	2 (4.0)	0.002	5 (13.9)	0 (0.0)	0.064
Child-Pugh score increasing by two points (one of the RILD criteria)	19 (28.8)	5 (10.0)	0.013	10 (27.8)	3 (8.3)	0.066
Patients with RILD	–	6 (12.0)		–	3(8.3)	

ALT, alanine aminotransferase; CK-SBRT, CyberKnife stereotactic body radiation therapy; HR, hepatic resection; PSM, propensity score matched analysis; RILD, radiation-induced liver disease; TACE, transhepatic arterial chemoembolization.

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Conflict of interest

The authors have no conflict of interests related to this publication.

Author contributions

Data analysis and interpretation, and drafting and revision of the manuscript for critically important intellectual content (JS, WGL, QW), data acquisition (HBW, TZ, YZF), manuscript preparation (WPH, AMZ, PH, YZS), and provision of final approval of the version to be published (XZD). All authors have read and approved the final version.

Ethics statement

This study was approved by the Institutional Review Board of Beijing 302 Hospital and was conducted in accordance with the Declaration of Helsinki and internationally accepted ethical guidelines. All patients signed written informed consent for their information to be stored in the hospital databases and used for research.

Data sharing statement

All data are available upon request.

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Original Article

A Simple and Quick Screening Method for Intrapulmonary Vascular Dilation in Cirrhotic Patients Based on Machine Learning

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Abstract

Background and Aims: Screening for hepatopulmonary syndrome in cirrhotic patients is limited due to the need to perform contrast enhanced echocardiography (CEE) and arterial blood gas (ABG) analysis. We aimed to develop a simple and quick method to screen for the presence of intrapulmonary vascular dilation (IPVD) using noninvasive and easily available variables with machine learning (ML) algorithms. ***Methods:*** Cirrhotic patients were enrolled from our hospital. All eligible patients underwent CEE, ABG analysis and physical examination. We developed a two-step model based on three ML algorithms, namely, adaptive boosting (termed AdaBoost), gradient boosting decision tree (termed GBDT) and eXtreme gradient boosting (termed Xgboost). Noninvasive variables were input in the first step (the NI model), and for the second step (the NIBG model), a combination of noninvasive variables and ABG results were used. Model performance was determined by the area under the curve of receiver operating characteristics (AUCROCs), precision, recall, F1-score and accuracy. ***Results:*** A total of 193 cirrhotic patients were ultimately analyzed. The AUCROCs of the NI and NIBG models were 0.850 (0.738–0.962) and 0.867 (0.760–0.973), respectively, and both had an accuracy of 87.2%. For both negative and positive cases, the recall values of the NI and NIBG models were both 0.867 (0.760–0.973) and 0.875 (0.771–0.979), respectively, and the precisions were 0.813 (0.690–0.935) and 0.913 (0.825–1.000), respectively. ***Conclusions:*** We developed a two-step model based on ML using noninvasive variables and ABG results to screen for the presence of IPVD in cirrhotic patients. This model may partly solve the problem of limited access to CEE and ABG by a large numbers of cirrhotic patients.

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Introduction

Hepatopulmonary syndrome (HPS) is characterized by intrapulmonary vascular dilation (IPVD) and remodeling in the alveolar microcirculation resulting in impaired gas exchange.[1] The presence of HPS increases the mortality risk and decreases the patients' quality of life,[2] and it can also award liver transplantation (LT) candidates exceptional points while awaiting a donor liver.[3] The diagnosis of HPS is based on the presence of chronic liver disease, IPVD as evidenced by contrast-enhanced echocardiography (CEE) and abnormal arterial oxygenation.[4] However, the screening and diagnosis of HPS are limited; only 0.45% of cirrhotic patients in a large cohort were diagnosed with HPS according to the International Classification of Diseases code, with an accuracy of only 22.5%.[1] More importantly, only 143 out of 194 diagnosed HPS patients underwent CEE, and 61 out of 98 IPVD patients had arterial blood gas (ABG) analysis results,[1] which indicated that although CEE and ABG are common, screening for IPVD or HPS is limited because most of the patients are asymptomatic. A simpler, CEE- or ABG-free method for screening IPVD or HPS would improve the

Keywords: Hepatopulmonary syndrome; Intrapulmonary vascular dilation; Cirrhosis; Screening; Machine learning.

Abbreviations: A-a gradient, alveolar-arterial gradient; ABG, arterial blood gas; AdaBoost, adaptive boosting; AUCROC, area under the receiver operating characteristic curve; CEE, contrast enhanced echocardiography; CI, confidence interval; FNI, full noninvasive; GBDT, gradient boosting decision tree; HPS, hepatopulmonary syndrome; INI, important noninvasive; INR, international normalized ratio; IPVD, intrapulmonary vascular dilation; MELD, model for end-stage liver disease; ML, machine-learning; NI, noninvasive; NIBG, noninvasive variables and the results of ABG analysis; NPV, negative predictive value; $PaCO_2$, arterial CO_2; PaO_2, arterial O_2; PPV, positive predictive value; SpO2, pulse oxygen saturation; Xgboost, eXtreme gradient boosting.

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present situation of HPS patient management.

In the past, researchers attempted to use single variables, such as spider angioma, acropachy and cyanosis, Child-Pugh score and bilirubin, prothrombin time, creatinine, the difference between the SpO_2 (supine) and SpO_2 (upright), to predict the presence of IPVD without CEE, but with unsatisfactory results.[4–7] Since the underlying mechanism of IPVD is largely unknown, it would be helpful to use multiple variables to predict the presence of IPVD. This could be further improved by applying new algorithms for model construction to overcome the limitations of conventional statistical methods.

Machine learning (ML) methods, a subset of artificial intelligence, may offer an alternative approach for predicting IPVD to overcome existing limitations. Due to the complexity and heterogenicity of liver diseases, artificial intelligence- or ML-based methods would be quite promising in for identification, prediction, and assessment of multiple liver diseases, comparing over conventional methods.[8] Recently, Kanwal *et al*.[9] developed and validated a model based on gradient descent boosting algorithm to predict the 1-year mortality risk with better discrimination than the conventional scoring method. To date, there has been no investigation applying ML methods to predict the presence of IPVD in cirrhotic patients, largely based on routine clinical data. Therefore, we aimed to develop a simple and quick model based on routine clinical data to screen IPVD patients (with both normal and abnormal arterial oxygenation) without CEE from cirrhotic patients, especially hospitalized patients, with the goal of achieving better patient management.

Methods

Patients in the current study were retrospectively analysed from a prospective study approved by the institutional ethics committee of the First Affiliated Hospital of Third Military Medical University (No. 2017(35) on July 10, 2017), and written informed consent was obtained from each patient. We enrolled cirrhotic patients scheduled for elective surgery at our hospital between July 27, 2017, and March 14, 2018.

The inclusion criteria were as follows: a diagnosis of liver cirrhosis based on biopsy, typical clinical findings, imaging studies and characteristic laboratory results; age ranging from 18 to 80 years-old; American Society of Anaesthesiologists score of II–III; ability to comply with research programmes; and no primary cardiopulmonary disease (heart disease, emphysema, pneumonia, asthma).

Study procedure

Patients were screened for eligibility 1 day before elective surgery and were assessed by the preoperative interview, CEE, physical examination, and ABG sampling.

CEE

All patients underwent CEE to detect the presence of IPVD using Fujifilm sonosite Edge (Sonosite, Bothell, WA, USA) equipped with a 2.5 MHz transducer. A baseline two-dimensional apical four-chamber view was acquired and 20 mL of agitated 0.9% saline solution was injected via the peripheral vein. The presence of microbubbles in the left cardiac chambers, identified between 3 and 6 heartbeats after visualization in the right cardiac chambers, was considered a positive result for IPVD.[10]

Oxygen saturation

Pulse oxygen saturation (SpO_2) was performed using a digital pulse oximeter after the participant maintained an upright posture (seated) for 5 minutes after, which he or she was repositioned supine for 5 minutes. One sample of arterial blood was obtained by percutaneous radial artery puncture while the patient was seated and breathed room air. The blood sample was analyzed by a standard blood gas analyzer (ABL800 FLEX; Radiometer, Copenhagen, Denmark). Arterial pH, O_2 (PaO_2) and CO_2 ($PaCO_2$) were documented and the alveolar-arterial gradient (A-a gradient) was determined according to the alveolar gas equation.[11] Age-related threshold[12] was defined as [10 + 0.43 × (age in years − 20)]. Abnormal arterial oxygenation was determined as A-a gradient greater than the age-related threshold.[13]

Clinical data collection

Clinical data collected from the electronic medical record included demographics, causes of liver cirrhosis, smoking and drinking history and morbidities such as hypertension and diabetes, as shown in Table 1. All the participants received a thorough physical examination and consultation to obtain details of spider angioma, acropachy, liver palm, ascites, encephalopathy and dyspnea. Serum albumin, total bilirubin, direct bilirubin, aspartate transaminase, alanine transaminase, creatinine, hemoglobin, prothrombin time, international normalized ratio (commonly known as INR) and total bile acid concentrations were measured for each patient using standard laboratory methods and via the instruments of Sysmex XE 2100, Sysmex CS 5100 (Wakinohama-kaigandori, Chuo-ku, Kobe, Japan) and Beckman-Coulter AU5800 (Brea, CA, USA). Laboratory results closest to the date of the ABG analysis were recorded. Model for end-stage liver disease (commonly referred to as MELD) scores were calculated using the following formula:[14] 10×[(0.378×ln [bilirubin])+(0.957×ln[creatinine])+(1.12 ×ln[INR])+6.43. The Child-Pugh score was calculated by hepatic encephalopathy grade, ascites, INR, albumin, and total bilirubin.[14]

ML methodology

The whole process of our model establishment was shown in Figure 1. First, the whole dataset was randomly divided into training (154) and testing (39) datasets. Data pre-processing was conducted separately on the training and testing datasets using binarization, categorization and Z-score normalization. For the features with only two candidate values, such as hypertension, diabetes, drinking and certain clinical symptoms, binarization was used. Age, body mass index, number of spider angiomas and SpO_2 difference between seated and supine positions were categorized. For instance, the number of spider angiomas was classified into two categories, namely, less than two and more than three. For PCO_2, PaO_2, A-a gradient and pH, we performed Z-score normalization, which produces a mean and standard variance of 0 and 1, respectively. The formula of Z-score normalization is:

$$x^* = \frac{x - x_M}{\sigma},$$

where, x_M and σ are the mean and standard variance of the original data, respectively. The detailed valuations of noninvasive variables and ABG analysis results are shown in Supplementary Table 1.

Table 1. Comparison of patient characteristics according to the presence of IPVD

Variable	IPVD, *n*=117	non-IPVD, *n*=76	$t/\chi^2/U$	*p*
Age in years, mean (SD)	50.3 (12.3)	47.1 (13.4)	−1.70	0.090
Male, *n* (%)	90 (76.9%)	53 (69.7%)	1.24	0.266
BMI in kg/m^2, mean (SD)	23.6 (3.6)	23.0 (3.6)	−1.07	0.287
Child-Pugh score, median (IQR)	9 (7–10)	8 (7–9)	4.21	<0.001
MELD score, median (IQR)	11.9 (5.8–16.3)	10.1 (5.9–14.3)	1.37	0.172
Cause of liver cirrhosis, *n* (%)			16.72	0.005
Hepatitis B	92 (78.6%)	59 (77.6%)		
Alcohol	13 (11.1%)	2[a] (2.6%)		
Hepatitis C	2 (1.7%)	1 (1.3%)		
Primary biliary cholangitis	3 (2.6%)	0 (0%)		
Drug-induced hepatitis	7 (6.0%)	8 (10.5%)		
Autoimmune hepatitis	0 (0%)	4[a] (5.3%)		
Nonalcoholic fatty liver disease	0 (0%)	2 (2.6%)		
Hypertension, *n* (%)	15 (12.8%)	7 (9.2%)	0.59	0.441
Diabetes, *n* (%)	15 (12.8%)	10 (13.2%)	0.01	0.946
Drinking, *n* (%)	45 (38.5%)	21 (27.6%)	2.40	0.121
Smoking index, median (IQR)	0 (0–400)	0 (0–200)	1.56	0.119
Acropachy, *n* (%)	99 (84.6%)	38 (50.0%)	26.80	<0.001
Liver palm, *n* (%)	106 (90.6%)	44 (58.7%)	27.27	<0.001
Spider angioma, median (IQR)	2 (0–4)	0 (0–1.75)	5.96	<0.001
Dyspnea, *n* (%)	72 (61.5%)	17 (22.4%)	28.45	<0.001
Ascites, *n* (%)	87 (74.4%)	31 (40.8%)	21.85	<0.001
Encephalopathy, *n* (%)	16 (13.7%)	0 (0)	11.33	0.001
SpO_2 seated, %, median (IQR)	97 (96–98)	98 (97–98)	−4.45	<0.001
SpO_2 supine, %, median (IQR)	98 (97–98)	98 (98–98)	−2.89	0.004
pH median (IQR)	7.45 (7.42–7.48)	7.43 (7.41–7.45)	2.38	0.017
$PaCO_2$ mmHg, mean (SD)	36.2 (4.5)	37.4 (3.9)	1.85	0.065
PaO_2 mmHg, median (IQR)	79.4 (70.8–85.0)	94.6 (83.8–107.0)	−6.76	<0.001
A-a gradient mmHg, median (IQR)	25.8 (19.9–32.1)	8.3 (−3.6–17.5)	7.50	<0.001
Elevated A-a gradient, *n* (%)	71 (59.6%)	12 (15.8%)	37.89	<0.001
TBA μmol/L, median (IQR)	97.4 (31.0–210.6)	40.6 (15.6–189.6)	1.97	0.049
Hemoglobin g/L, mean (SD)	108.5 (22.1)	114.1 (23.8)	1.66	0.099
ALT U/L, median (IQR)	50. 6 (28.8–103.0)	65.1 (34.1–146.8)	−1.55	0.121
AST U/L, median (IQR)	67.1 (43.3–119.3)	57.2 (38.8–126.4)	0.30	0.767
Albumin g/L, median (IQR)	31.4 (28.4–35.3)	33.3 (29.9–37.6)	−2.40	0.017
Globin g/L, median (IQR)	30.5 (26.7–35.9)	29.6 (23.5–36.4)	0.72	0.469
TBIL μmol/L, median (IQR)	68.0 (25.6–177.8)	55.6 (19.8–156.5)	1.20	0.230
DBIL μmol/L, median (IQR)	45.6 (14.1–135.1)	32.8 (8.4–109.2)	1.22	0.222
IBIL μmol/L, median (IQR)	28.6 (13.1–54.3)	21.0 (11.1–38.2)	2.00	0.046
PT second, median (IQR)	16.5 (13.5–19.6)	13.7 (11.9–16.4)	4.19	<0.001
INR median (IQR)	1.4 (1.1–1.7)	1.2 (1.0–1.4)	4.40	<0.001
Creatinine μmol/L, median (IQR)	65.1 (52.3–74.8)	69.0 (56.6–81.0)	−1.94	0.052

Mean (standard deviation, SD) presented for normally distributed continuous variables, while median (interquartile range, IQR) was given to those with non-normally distributed continuous variable. Unless otherwise stated, *n* is as indicated in the column headings. Prevalence of liver disease etiology was statistically compared between IPVD and non-IPVD patients ([a]$p<0.05$). BMI, body mass index; DBIL, direct bilirubin; IBIL, indirect bilirubin; PT, prothrombin time; TBA, total bile acid; TBIL, total bilirubin.

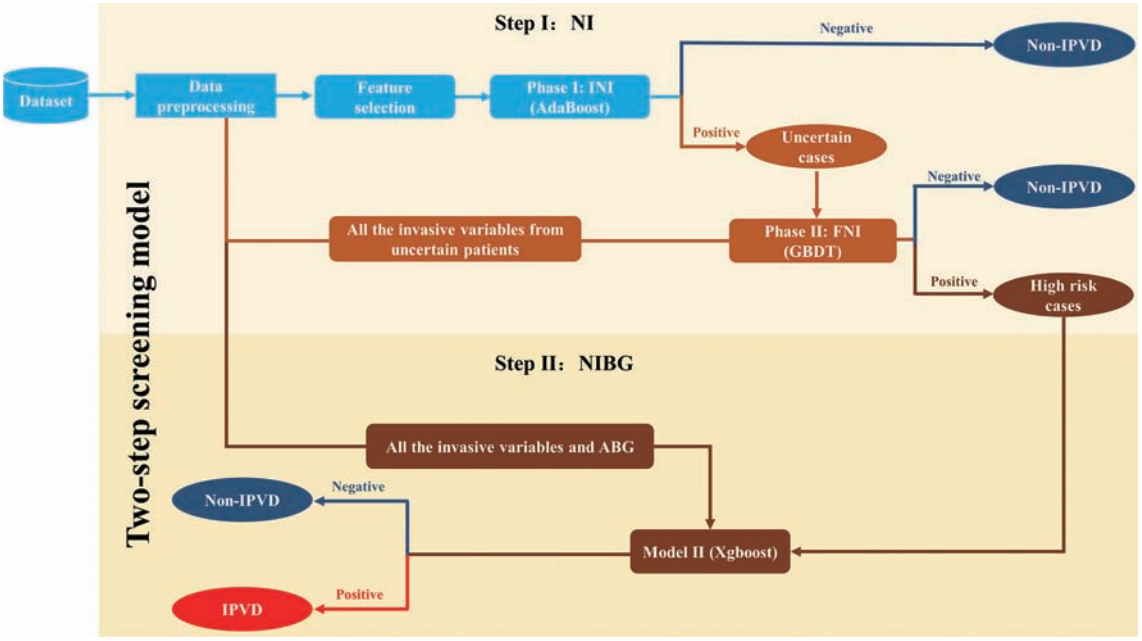

Fig. 1. The whole process for establishing our two-step screening model with the training dataset. When the predicted value of the INI model was less than 0.5, the result of the NI model was determined according to the results of the INI model; if the predicted value of the INI model was more than 0.5, the result of the NI model was determined by the results of the FNI model. When the result of the NI model was positive, we used the results of the NIBG model. The model fitting method for the INI, FNI, and NIBG model were AdaBoost, GBDT, and Xgboost, respectively.

Based on the Python packages, including "numpy" and "scikit-learn (sk learn)", we analyzed the patients' data with three algorithms: adaptive boosting (AdaBoost), gradient boosting decision tree (GBDT), and eXtreme gradient boosting (Xgboost). Based on these algorithms, we aimed to screen IPVD from cirrhotic patients by establishing a two-step model that could be used by different hospitals. This two-step model consists of a noninvasive (NI) model and a model that incorporates both noninvasive variables and the results of ABG analysis (the NIBG model). The model was constructed on the training dataset. The NI model was conducted by a two-phase method based on the noninvasive variables. The first phase of the NI model is called the important noninvasive (INI) model, and the second phase is the full noninvasive (FNI) model. In the INI model, the input of variables were selected by statistical methods (shown in Supplementary Table 2). When a single patient was predicted as positive, we called him or her an uncertain case. To further determine whether the uncertain cases had IPVD, they were re-evaluated by the FNI model. The input variables of the FNI model were all the noninvasive variables, and the trained cases were only of the uncertain cases. AdaBoost was used for model fitting for the INI model and GBDT for the FNI model. The parameter of the NI model is shown in Supplementary Table 7. When the predicted value of the INI model was less than 0.5, the result of the NI model was determined according to the results of INI model; on the other hand, if the predicted value of INI was more than 0.5, the result of the NI model was determined by the FNI model. As shown in Figure 1, when the result of the NI model was positive, we implemented the second-step model, the NIBG model. The input variables included all the noninvasive variables and the ABG analysis results. The training data for construction of the NIBG model were the patient data that yielded positive results in the NI model. The model fitting method for the NIBG model was Xgboost. The parameters of the Xgboost algorithm are shown in Supplementary Table 7.

Model performance was evaluated by precision, recall, F1 score and the area under curve of receiver operating characteristics (AUCROC). Precision, recall and F1 score were calculated by a confusion matrix as follows:

$$\text{Precision} = \frac{\text{TP}}{\text{TP}+\text{FP}},\ \text{Recall} = \frac{\text{TP}}{\text{TP}+\text{FN}},$$

$$\text{F1 score} = \frac{2\times\text{Precision}\times\text{Recall}}{\text{Precision}+\text{Recall}},$$

where TP means true positive, FP means false positive, and FN means false negative. In our current study, we calculated the recall, precision, and F1-score for positive cases (recall (1), precision (1), F1 score (1)) and negative cases (recall (0), precision (0), F1 score (0)). Recall (1), recall (0), precision (1) and precision (0) are also called sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV), respectively. The AUCROC is an important indicator for the model discrimination.

Statistical analysis

Descriptive statistics are summarized as the mean±standard deviation or median (interquartile range). Comparisons between groups or datasets were made with the Student's *t*-test or Mann-Whitney *U* test for quantitative variables, and with the χ^2 test or Fisher's test for category variables. All statistical tests were two-sided, and *p* values less than 0.05 indicated statistical significance. The statistical analyses were performed using SPSS software for Windows, V.19.0 (IBM Corp., Armonk, NY, USA).

Ethics approval

The study protocol was approved by the institutional ethics

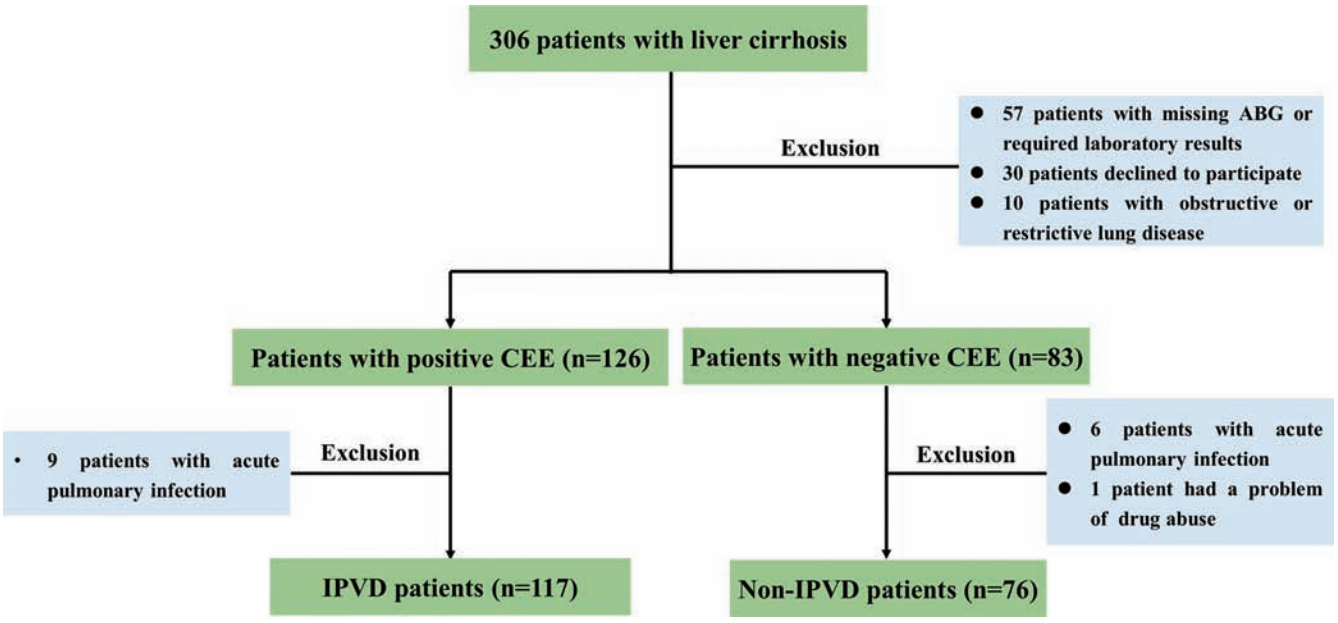

Fig. 2. Flow chart of the study population.

committee of the First Affiliated Hospital of Third Military Medical University (No. 2017(35), approved as of July 10, 2017 for the study titled "Perioperative application of specific COX-2 receptor inhibitor in lung protection in cirrhotic patients", of which this is the first part), and written informed consent was obtained from each patient.

Results

Baseline characteristics

As shown in Figure 2, 193 patients were finally included in the analysis. Between the training and testing datasets, except for smoking, there were no significant differences in all the other baseline characteristics, nor were there significant differences in the distribution of patients in the IPVD and non-IPVD patients (shown in Supplementary Table 2).

Patient characteristics according to findings on CEE

As shown in Table 1, 117 of the 193 patients (60.6%) had IPVD, as evidenced by CEE. There were no statistically significant differences in demographics, comorbidities, MELD score, drinking and smoking index between the IPVD and non-IPVD patients. Cirrhotic patients induced by various of causes would occur with IPVD, and in our study most of the cirrhotic patients were caused by hepatitis B. Patients with IPVD had significantly higher Child-Pugh scores ($p<0.001$), prothrombin time ($p<0.001$) and indirect bilirubin ($p=0.046$) than those without IPVD. Clinical features were significantly different between IPVD and non-IPVD patients, namely, the presence of acropachy (84.6% vs. 50.0%, $p<0.001$), liver palm (90.6% vs. 58.7%, $p<0.001$), spider angioma ($p<0.001$), dyspnea (61.5% vs. 22.4%, $p<0.001$), ascites (74.4% vs. 40.8%, $p<0.001$) and encephalopathy (13.7% vs 0.0%, $p<0.001$). Notably, 16 of the 193 patients suffered from mild encephalopathy. IPVD patients had significantly lower SpO_2 at both positions compared to non-IPVD patients. IPVD patients had a lower level of PaO_2 (79.4 mmHg vs.94.6 mmHg, $p<0.001$) and a higher A-a gradient (25.8 mmHg vs. 8.3 mmHg, $p<0.001$) than non-IPVD patients. Furthermore, according to the age-related threshold, the prevalence of elevated A-a gradient in IPVD and non-IPVD patients was 61% and 16%, respectively ($p<0.001$).

Model performance of NI model on training and testing dataset

The NI model was composed of the two-phase model, namely, the INI and FNI models. After data pre-processing, dyspnea, liver palm, number of spider angiomas, ascites, acropachy, encephalopathy, and SpO_2 differences between seated and supine positions were found to be statistically significantly different between IPVD and non-IPVD patients in the training dataset (shown in Supplementary Table 3). Therefore, these seven features were used as the input noninvasive variables for the INI model. If the patient's predictive value calculated by the INI model was higher than 0.5, the patient was called an uncertain case. Uncertain cases were re-evaluated by the FNI model, of which the input variables were all the noninvasive variables. The final model performance of the NI model is presented in Table 2. For the training dataset, the AUCROC, F1-score (0), F1-score (1) and accuracy of the NI model were 0.952 [95% confidence interval (CI): 0.918–0.986], 0.857 (95% CI: 0.802–0.912), 0.901 (95% CI:0.854–0.948) and 88.3%, respectively. The NPV, PPV, specificity and sensitivity of the NI model for the training dataset were 0.831 (95% CI: 0.772–0.890), 0.921 (95% CI: 0.879–0.964), 0.885 (95% CI: 0.835–0.936) and 0.882 (95% CI: 0.831–0.933), respectively. For the testing dataset, the AUCROC, F1-score (0), F1-score (1) and accuracy of the NI model were 0.850 (95% CI: 0.738–0.962), 0.839 (95% CI:0.723–0.954), 0.894 (95% CI: 0.797–0.990) and 87.2%, respectively. The NPV, PPV, specificity and sensitivity of the NI model for the testing dataset were 0.813 (95% CI: 0.690–0.935), 0.913 (95% CI: 0.825–1.000), 0.867 (95% CI: 0.760–0.973) and 0.875 (95% CI: 0.771–0.979), respectively.

Table 2. Model performances of the NI and NIBG model

	AUROC	Precision (0)	Precision (1)	Recall (0)	Recall (1)	F1-score (0)	F1-score (1)	Accuracy
NI model								
training dataset	0.952 (0.918–0.986)	0.831 (0.772–0.890)	0.921 (0.879–0.964)	0.885 (0.835–0.936)	0.882 (0.831–0.933)	0.857 (0.802–0.912)	0.901 (0.854–0.948)	0.883
testing dataset	0.850 (0.738–0.962)	0.813 (0.690–0.935)	0.913 (0.825–1.000)	0.867 (0.760–0.973)	0.875 (0.771–0.979)	0.839 (0.723–0.954)	0.894 (0.797–0.990)	0.872
NIBG model								
training dataset	0.966 (0.937– 0.995)	0.845 (0.788–0.902)	0.988 (0.971–1.005)	0.984 (0.964–1.004)	0.882 (0.831–0.933)	0.909 (0.864–0.954)	0.932 (0.892–0.972)	0.922
testing dataset	0.867 (0.760–0.973)	0.813 (0.690–0.935)	0.913 (0.825–1.001)	0.867 (0.760–0.973)	0.875 (0.771–0.979)	0.839 (0.723–0.954)	0.894 (0.797–0.990)	0.872

Statistical quantifications were demonstrated with 95% CI, when applicable.

Model performance of NIBG model on training and testing dataset

Patients with predicted values higher than 0.5 as calculated by the NI model were considered high-risk cases. When the hospital had access to CEE, high-risk patients underwent CEE and ABG analysis for the diagnosis of IPVD or HPS. However, if the hospital had limited access to CEE, high-risk patients only underwent ABG analysis and were re-evaluated by the NIBG model. The input variables of the NIBG model were the noninvasive variables and the ABG results. For the training dataset, the AUCROC, F1-score (0), F1-score (1) and accuracy of the NI model were 0.966 (95% CI: 0.937–0.995), 0.909 (95% CI: 0.864–0.954), 0.932 (95% CI: 0.892–0.972) and 92.2%, respectively. The NPV, PPV, specificity and sensitivity of the NI model for the training dataset were 0.845 (95% CI: 0.788–0.902), 0.988 (95% CI: 0.971–1.005), 0.984 (95% CI: 0.964–1.004) and 0.882 (95% CI: 0.831–0.933), respectively. For the testing dataset, the AUCROC, F1-score (0), F1-score (1) and accuracy of the NIBG model were 0.867 (95% CI: 0.760–0.973), 0.839 (95% CI: 0.723–0.954), 0.894 (95% CI: 0.797–0.990) and 87.2%, respectively. The NPV, PPV, specificity and sensitivity of the NI model for the testing dataset were 0.813 (95% CI: 0.690–0.935), 0.913 (95% CI: 0.825–1.001), 0.867 (95% CI: 0.760–0.973) and 0.875 (95% CI: 0.771–0.979), respectively.

Discussion

IPVD is one form of the extrahepatic vasculature changes induced by various chronic liver diseases, such as alcoholic cirrhosis, hepatitis B or C infection, and nonalcoholic fatty liver disease.[7,15] IPVD is also deemed to be an essential criterion for the diagnosis of HPS, while most of the researchers considered that there was no need to screen IPVD patients with normal oxygenation from cirrhotic patients in the past. However, Manual *et al*.[16] found that approximately 35% of IPVD patients with normal gas exchange developed HPS by serial ABG measurement. IPVD-only patients were found to be in a hyperdynamic state, presenting as a higher cardiac output, cardiac index, and left ventricular stroke volume, leading to higher risk of dyspnea.[7] IPVD was also found to be associated with a higher prevalence of obstructed intrahepatic portal branches, of slowed or hepatofugal portal blood flow, and of large abdominal portosystemic shunts,[15] which was in accordance with the intra- and extrahepatic vasculature changes in cirrhosis.[17] Furthermore, Jin *et al*.[18] observed that HPS reversed in 95.8% of the liver transplant patients at 6 months; however, the prevalence of IPVD was 69.2% at 6 months, suggesting a difference between HPS and IPVD reversibility. It was also reported that cirrhosis regression induced a significant reduction in portal pressure accompanied by a normalization of systemic hemodynamics; however, there was no change in extrahepatic vascular structures.[19] These findings show that IPVD screening is important not only for indicating HPS but also for evaluating the changes in extrahepatic vasculature. Thus, IPVD patients should be treated with cirrhosis-related routine measures and measures for liver-derived hyperdynamic states, which may help to prevent IPVD patients with normal gas exchange from developing HPS.

As shown in Supplementary Table 4, many researchers have attempted to use single variables to predict the presence of IPVD but most of the results have been unsatisfactory. Spider angioma was thought to be a skin marker of HPS,[20,21] while in other studies, spider angioma and liver palm were found to be ineffective for detecting HPS or IPVD.[7,10,13] Dyspnea and acropachy were found to be good

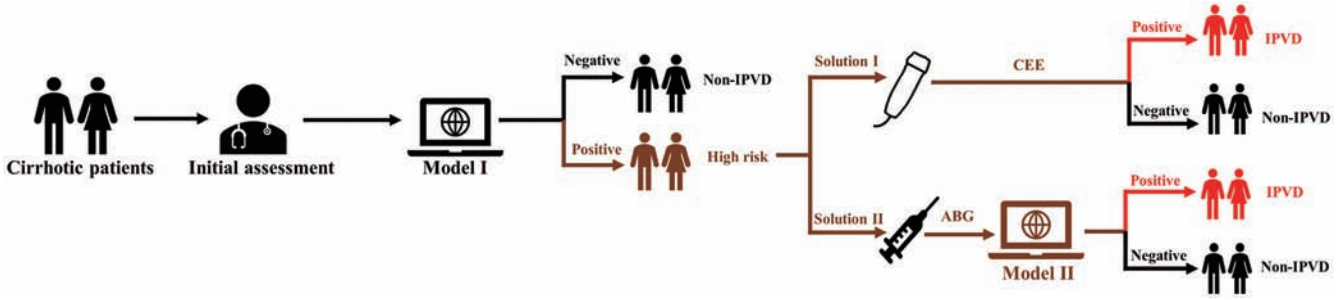

Fig. 3. Flow chart of the screening method for clinical use. For an individual cirrhotic patient, we initially evaluated him/her with model I (the NI model) to determine if he/she was a high-risk patient for IPVD; if the patient was determined to be high-risk, according to the reality of clinics, he/she can undergo CEE and ABG for final confirmation or ABG and prediction by model II (the NIBG model).

clinical features in HPS patients,[5] while in IPVD patients with normal gas exchange, the two features lacked significance.[7] As shown in Supplementary Table 5, spider angioma was a good indicator for IPVD, with an AUCROC, sensitivity and specificity of 0.74, 66.7% and 75%, respectively. When the cut-off value of the A-a gradient was 19.83 mmHg, the AUCROC, sensitivity and specificity were 0.82, 76.9% and 81.6%, respectively. However, approximately 40% of the IPVD patients in our study had a normal A-a gradient, so using the A-a gradient as a single predictor would cause loss of predictive information. Although commonly used, SpO_2 <96% had low AUCROC (0.68) and sensitivity (36.8%), which is constant with Forde's study (AUCROC: 0.59, sensitivity: 28%).[22] Although some laboratory indicators showed significant differences between IPVD and non-IPVD patients, we found that routine laboratory indicators alone were not good indicators for IPVD, which is constant with previously reported findings.[7,18] We also attempted to use noninvasive variables and ABG results to develop one-step models for screening for the presence of IPVD in cirrhotic patients, with unsatisfactory results (shown in Supplementary Table 6). Previous studies and our results suggest that it would be more practical to combine variables rather than using single variables for model construction. Meanwhile, two-step models should be considered and applied to patients with lower and higher risks.

The AUCROCs of the NI and NIBG model for the testing dataset were all larger than 0.85 and the accuracy of the two models were higher than 85%, which indicated that our two-step model had satisfactory discrimination. Considering that our aim was to screen IPVD patients from large numbers of asymptomatic cirrhotic patients, the ideal model should screen out as many patients as possible and miss as few IPVD patients as possible. Therefore, except for the discriminability, the PPV and NPV are of equal importance. If we assume that the prevalence of IPVD is similar to that observed in our current study, the PPV and NPV were 81.3% and 91.3%, respectively. For clinical use, the PPV and NPV should usually be adjusted to the prevalence of the positive cases. The prevalence of IPVD varies from 20.7% to 84.0% due to differences in the study populations.[7,10,18,23,24] However, IPVD patients with abnormal gas exchange, with a prevalence of approximately 30%,[25] should not be overlooked and need intervention. Thus, the adjusted PPV and NPV were 73.8% and 94.2% for both the NI and NIBG model, respectively, for the whole model, when the prevalence of positive cases was 30%. Our results suggest that regardless of whether the PPV and NPV are adjusted, the NPV was higher than 90%, indicating that missed diagnosis rate was less than 10%. As for the PPV of the NI model being higher than 70%, given that the prevalence of positive cases in the second step was higher, the PPV of the whole model would be higher than the calculated value. Moreover, the first step of our model used only noninvasive and economic variables to screen out high risk patients; then, according to the reality, patients could undergo CEE and ABG for final confirmation or undergo ABG and then be predicted by our second-step evaluation (shown in Fig. 3).

Limitations

Our study had several limitations. This was a single-centre and small sample size study, for which bias is difficult to avoid. Four different researchers performed the electronic medical records data collection and physical examination, ABG analysis, CEE and final diagnosis to decrease the bias. In the near future, multicenter research should be performed to validate our model.

Conclusions

We developed a two-step model based on ML methods using noninvasive variables and ABG analysis to screen for the presence of IPVD in cirrhotic patients. This model may prove to be promising for improving the quality of management for cirrhotic patients with intra- and extrahepatic vascular complications.

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Conflict of interest

The authors have no conflict of interests related to this publication.

Author contributions

Study conception (BY, YWC, ZYX, JZ), data collection (XT, ZYY, XHB, PL, HYZ, XJL, YC, PD), data analysis (YJL, KHZ,

YC, XLQ), administrative support (LZK, JTG, JLN), manuscript drafting (YJL, XT, KHZ). All authors read and approved the final version of the manuscript.

Data sharing statement

The data analyzed during the current study are available from the corresponding author upon reasonable request.

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Original Article

UMSCs Attenuate LPS/D-GalN-induced Acute Liver Failure in Mice by Down-regulating the MyD88/NF-κB Pathway

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Abstract

Background and Aims: Acute liver failure (ALF) is an inflammatory process of acute liver cell injury. Mesenchymal stem cells (MSCs) are undifferentiated, primitive cells with anti-inflammatory, anti-apoptotic, and multi-directional differentiation abilities. This study aimed to explore the therapeutic mechanism of umbilical cord (U)MSCs in ALF. ***Methods:*** D-galactosamine (D-GalN) combined with lipopolysaccharide (LPS) was used to establish an ALF model. After model establishment, UMSCs were injected via the tail vein. After UMSC transplantation, the number of mouse deaths was monitored every 12 h. A fully automatic biochemical analyzer was used to detect changes in biochemical analysis. Pathological changes was observed by stained with hematoxylin and eosin.The expression of My D88 was detected by immunohistochemical analysis, quantitative reverse transcription, and western blotting. The expression of NF-κB was detected by quantitative reverse transcription, western blotting.The expression of Bcl-2,Bax were detected by quantitative reverse transcription, western blotting.The expression of TNF-α, IL-1β, IL-6 were detected by enzyme-linked immunosorbent assay. ***Results:*** The 48-h survival rate of the UMSC-treated group was significantly higher than that of the LPS/D-GalN-exposed group. After 24 h of LPS/D-GalN exposure, UMSCs reduced serum alanine aminotransferase and aspartate aminotransferase levels and improved the liver structure. Western blot and real-time fluorescence quantitative nucleic acid amplification analyses showed that UMSCs decreased MyD88 expression, thereby inhibiting LPS/GalN-induced phosphorylation and degradation of inhibitor of nuclear factor (NF)-κB (IκB). Additionally, NF-κB p65 underwent nuclear translocation, inhibiting the production of the inflammatory factors interleukin (IL)-1β, IL-6 and tumor necrosis factor (TNF)-α and played a protective role in ALF by down-regulating the pro-apoptotic gene Bax and up-regulating the anti-apoptotic gene Bcl-2. In summary, these findings indicate that UMSCs play a protective role in LPS/GalN-induced acute liver injury via inhibition of the MyD88 pathway and subsequent inhibition of NF-κB-mediated cytokine production. ***Conclusions:*** Through the above mechanisms, UMSCs can effectively reduce LPS/D-GalN-induced ALF, reduce mouse mortality, and restore damaged liver function and damaged liver tissue.

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Introduction

Acute liver failure (ALF) refers mainly to interactions among multiple factors that lead to the acute necrosis of liver cells and rapid loss of liver function; in such, liver function is decompensated, and severe liver disease may eventually lead to functional failure. This syndrome[1–2] is clinically characterized by acute onset, rapid progression, and high mortality. Since 1983, liver transplantation has been recognized as the most effective treatment for ALF.[3] However, as living standards have continuously improved, the incidence of ALF has also increased annually. Thus, the gap between the number of patients awaiting transplant and the supply of organs is widening, and the development of a treatment to replace liver transplantation is urgently needed. As early as the 1980s, Arnold Caplan proposed mesenchymal stem cells (MSCs) and MSC-based treatments.[4] Studies have reported that transplanted MSCs can secrete many cytokines, promote liver tissue repair, inhibit immune cell proliferation and migration to the liver, and regulate liver function and the systemic immune inflammatory response.[5] Knowledge on stem cell (SC) biology has increased rapidly, opening new avenues for SC-based therapies and the use of SCs as a cell therapy platform for ALF.[6]

SC transplantation therapy has become another important option to improve ALF treatment. SCs are undifferentiated, primitive progenitor cells with the characteristics of self-renewal, proliferation potential, and differentiation potential,[7] and they can differentiate into multifunctional cells under certain conditions. In addition, studies have shown that SCs have other characteristics, including anti-inflammatory activity, apoptosis resistance, antioxidant activity, immunosuppressive activity, tissue repair capability, and growth factor expression.[8] In humans, umbilical cord (U)MSCs can be derived from different compartments of the organ, including the amniotic membrane region, Wharton's jelly, perivascular zone[9]

Keywords: Acute liver failure; Stem cells; Inflammation; Signaling pathway.
Abbreviations: ALF, acute liver failure; ALT, alanine aminotransferase; AST, aspartate aminotransferase; Bax, Bcl 2-Associated X Protein; Bcl-2, B-cell lymphoma-2; D-GalN, D-galactosamine; IL-1β, interleukin-1β; IL-6, interleukin-6; IkB, inhibitor of nuclear factor-κB; LPS, lipopolysaccharide; MSCs, mesenchymal stem cells; My D88, Myeloid differentiation factor 88; NF-κB, nuclear factor-κB; P-IkB, phosphorylated-IκB; TBil, total bilirubin; TNF-α, tumor necrosis factor-α; UMSCs, umbilical cord mesenchymal stem cells.

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of the vascular wall, and endothelial region in the middle and outer membrane region. Among all MSC groups, UMSCs have a strong proliferative ability. Research shows that UMSCs can maintain a stable doubling time in multiple passages, as the doubling time of the bone mesenchymal stem cells increased significantly after only six passages.[10] Compared to other MSCs, UMSCs are more primitive, and because of their unique gene expression profile, UMSCs produce few teratomas.[11] In addition, because the placenta has a barrier, there is a low risk of infection from UMSC transplants, and hence, UMSCs are more suitable for clinical research.

Lipopolysaccharide (LPS) combined with D-galactosamine (D-GalN) is often used to establish animal models of liver failure, which is characterized by the activation of nuclear factor (NF)-κB and the excessive secretion of inflammatory cytokines/mediating factors, leading to a systemic inflammatory response.[12] Accumulating evidence indicates that various proinflammatory cytokines/mediators, such as tumor necrosis factor (TNF)-α, leukocyte-derived interleukin (IL)-1β, IL-6, inducible nitric oxide synthase and cyclooxygenase-2, are involved in LPS/GalN-induced liver toxicity.[13] Therefore, inhibiting the inflammatory response may be an approach for treating liver failure.

Myeloid differentiation factor (MyD88) is an important Toll-like receptor (TLR)/IL-1 receptor superfamily member, and transduction of all TLRs into the cytoplasm by MyD88, in whole or in part, makes it a necessary adaptor protein to activate NF-κB and the mitogen-activated protein kinase signaling pathway.[14] LPS is recognized by TLR4 expressed by the cell and activates innate immunity through a MyD88-dependent pathway. Studies have shown that after genetic knockout of MyD88 in mice, LPS-induced activity almost completely disappeared. After intraperitoneal injection of high concentrations of LPS, mice can survive for more than 96 h, but all mice without MyD88 gene knockout die within 96 h, indicating that MyD88 plays an important role in LPS activation. NF-κB is comprised of the p50 and p65 subunits; in addition, the inhibitor of NF-κB (IκB) is essential for host defense and mediates expression of the above-mentioned pro-inflammatory mediators and cytokines. In addition, NF-κB controls apoptosis. Therefore, the inhibition of pro-inflammatory mediators and apoptosis is considered a potential strategy for ALF prevention and treatment. Studies have shown that overactivated MyD88 signaling is a key factor in the development of many immune-mediated diseases, which provides us with new therapeutic areas targeting MyD88 signaling pathways to reduce the intensity of immune responses.[15]

On the basis of the characteristics of the LPS/D-GalN model, we suspect that UMSCs may protect liver injury by inhibiting the inflammatory pathway and apoptosis pathway. Therefore, in this study, a mouse model of ALF was established via the administration of D-GalN combined with LPS. The therapeutic effect of UMSCs on ALF was evaluated, and the potential mechanism was revealed.

Methods

Mouse model establishment and cell transplantation

Healthy female BALB/c mice (weight, 20–22 g; age, 6–8 weeks; no specific pathogen grade) were purchased from the Animal Experiment Center of Kunming Medical University (China). Mice were housed in an environmentally controlled room (temperature, 24°C; humidity, 40–80%) under a 12-h dark/12-h light cycle and had free access to food and water. All experiments were approved by the Medical Ethics Committee of Kunming Medical University and were conducted in accordance with the experimental animal care principles of the University. Sixty-eight mice were randomly divided into three groups, namely, the control group (n=10), the LPS/D-GalN group (n=28), and the LPS/D-GalN+UMSCs group (n=28). D-GalN (900 mg/kg; Sigma-Aldrich, St. Louis, MO, USA) was administered via intraperitoneal injection at 12-h intervals for a total of two times. After the second intraperitoneal injection of D-GalN, LPS (10 μg/kg; Sigma-Aldrich) was also administered to establish the ALF model. At 24 h after the LPS/D-GalN injection, mice in the LPS/D-GalN + UMSCs group were injected with UMSCs (5×10^6 cells/mouse; Beike Bio, Shenzhen, China) via the tail vein. Mice in the LPS/D-GalN group were not given any treatment. At 12 h, 1 day, 2 days, 5 days, 7 days, 14 days, 21 days, and 28 days after cell transplantation, mice were anaesthetized with 40 mg/kg pentobarbital sodium (Sigma-Aldrich) via intraperitoneal injection, and blood samples and liver tissue samples were collected for histopathological studies and protein detection.

Long-term survival analysis and biochemical analysis

After UMSC transplantation, the number of mouse deaths was monitored every 12 h. At 12 h, 24 h, and 48 h after UMSC transplantation, mice were anaesthetized by the intraperitoneal injection of pentobarbital sodium. Mouse eyeballs were then removed for blood collection. Each blood sample was incubated at room temperature for 30 m and was then centrifuged at 3,000 rpm for 15 m at 4°C. The upper layer of serum was stored at −20°C. A fully automatic biochemical analyzer (Beckman AU-5421; Beckman-Coulter, Brea, CA, USA) was used to detect changes in serum alanine aminotransferase (ALT), aspartate aminotransferase (AST) and total bilirubin (TBil) levels.

Pathological and immunological analyses

Mice were sacrificed at the designated time points (12 h, 1 day, 2 days, 5 days, 7 days, 14 days, 21 days, and 28 days) after UMSC transplantation. Liver lobes were excised from the same site and fixed with 4% paraformaldehyde (Solarbio, Beijing, China) for 24 h for histological and immunological analyses. Paraffin-embedded liver tissue was sliced into 5-μm sections and stained with hematoxylin and eosin (HE; Solarbio). Pathological changes in liver tissue were assessed under an optical microscope. For immunohistochemical analysis, the tissue sections were heated in citric acid buffer (0.02 mol/L, pH=5.8) (Solarbio) for antigen retrieval. Bovine serum albumin (5%; Sigma-Aldrich) in phosphate-buffered saline (Solarbio) was used to block non-specific binding. Then, according to the instructions of the reagent manufacturer, the sections were incubated with an anti-MyD88 antibody (Abcam, Cambridge, UK) overnight at 4°C. The sections were then incubated with a horseradish peroxidase-conjugated secondary antibody (Abcam) at 37°C for 1 h and evaluated under an optical microscope. The optical density value was calculated by ImagePro Plus software (Media Cybernetics, Inc., Rockville, MD, USA).

Enzyme-linked immunosorbent assay (referred to as ELISA)

Blood was collected from mouse eyeballs, incubated at room temperature for 30 m and centrifuged at 3,000 rpm for 15 m at 4°C. The supernatant was collected, and ELISA kits (Jiang Lai, Shanghai, China) were used to determine serum TNF-α, IL-1β, and IL-6 cytokine levels. The absorbance was measured at 450 nm in a microplate reader (ELx800; Bio-Tek, Winooski, VT, USA).

Quantitative reverse transcription (qRT)-PCR

TRIzol Reagent (Solarbio) was used to extract the total RNA from mouse liver tissue, according to the manufacturer's instructions. RNA was reverse transcribed to prepare the cDNA templates, and qRT-PCR was performed in a Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) under the following cycling conditions: initial denaturation at 95°C for 10 s, 40 cycles of a two-step PCR (95°C for 5 s, 60°C for 30 s), denaturation at 95°C for 15 s, annealing at 60°C for 1 m, and denaturation at 95°C for 15 s. β-actin was used as the housekeeping gene to normalize expression data. All expression levels were analyzed by the ΔCT method. Primers were designed by the Shanghai Jierui Biological Engineering Co., Ltd (Shanghai, China). The primer sequences for IκB were 5′ GGTGCAGGAGTGTTGGTGG 3′, 5′ CTGAGTGAGGTAGGTATCTGAGGC 3′; those for NF-κB were 5′ GATGTGCATCGGCAAGTGG 3′, 5′ AGAAGTTGAGTTTCGGGTAGGC 3′; those for MyD88 were 5′ CCCACTCGCAGTTTGTTG 3′, 5′ CCACCTGTAAAGGCTTCTCG 3′; those for BAX were 5′ CAGGATGCGTCCACCAAGAA 3′, 5′ CAGGATGCGTCCACCAAGAA 3′; those for BcL-2 were 5′ GCTACCGTCGTGACTTCGC 3′, 5′ ATCCCAGCCTCCGTTATCC 3′; and those for M-actin were 5′ TGCTGTCCCTGTATGCCTCT 3′, 5′ TTTGATGTCACGCACGATTT 3′.

Western blotting

RIPA lysis buffer (Solarbio) was used to extract protein from liver tissue. A bicinchoninic acid kit (Sigma-Aldrich) was used to determine protein concentrations. Samples containing equal amounts of protein (50 μg) were subjected to electrophoresis. Proteins were separated on a 10% sodium dodecyl sulfate-polyacrylamide gel and transferred to a polyvinylidene fluoride membrane (Sigma-Aldrich). The membrane was blocked with skim milk (Sigma-Aldrich) and incubated with primary antibodies (Abcam) overnight at 4°C. The next day, the membrane was incubated with Tris-buffered saline containing Tween. After washing, the membrane was incubated with the secondary antibody (Abcam) for 1.5 h at room temperature. Immunoreactions were visualized according to the instructions of the instrument manufacturer (ChemiDoc™ XRS+; Bio-Rad, Hercules, CA, USA), and the intensities of the immunoreactive bands were measured using ImageJ analysis software (National Institutes of Health, Bethesda, MD, USA).

Data analysis

GraphPad Prism version 7.0 (GraphPad Software, San Diego, CA, USA) statistical software was used for statistical analysis. The Kaplan-Meier method with the log rank test was used to analyze survival. Measurement data are expressed as the means±standard deviations, and analysis of variance in a random block design was used for comparisons among multiple groups. A p-value <0.05 was considered to indicate a statistically significant difference.

Results

UMSC transplantation improves survival

Survival analysis is the most straightforward approach to evaluate the effect of UMSCs on ALF. As shown in Figure 1 (panels C) and Table 1, the survival rate of mice was determined every 12 h after UMSC injection. The effective transplantation of UMSCs increased the survival rate by 90% at 48 h, indicating that UMSC transplantation can increase the survival rate of mice with LPS/D-GalN-induced ALF.

UMSC transplantation restores liver function

The serum ALT, AST and TBil levels in mice in the LPS/D-GalN group gradually increased with the time and duration of disease. The serum ALT level in mice in the LPS/D-GalN+UMSCs group peaked 24 h after transplantation (248±37.3 U/L); the AST and TBil levels peaked 12 h after transplantation (379.3±7.2 U/L; 53.9±6.1 mg/dL). Subsequently, the levels of ALT, AST, and TBil in mice in the LPS/D-GalN+UMSCs group gradually decreased with time to 63.3±2.1 U/L, 214.3±7.5 U/L, and 29.9±0.8 mg/dL, respectively, 48 h after transplantation. The ALT, AST, and TBil levels in the LPS/D-GalN+UMSCs group of mice at 12 h, 24 h, and 48 h after UMSC transplantation differed significantly from those in the LPS/D-GalN group of mice (p<0.05; Table 2). This result shows that UMSCs can significantly improve liver function.

UMSC transplantation reduces liver histopathological changes induced by LPS/D-GalN

Liver histopathological analysis revealed the protective effect of UMSCs on LPS/GalN-induced ALF. As shown in Figure 1 (panels A–B), the normal rat liver lobular structure is typical. At 24 h after the injection of D-GalN, the control group lost its normal lobular structure, showing hepatocyte cytoplasmic edema, local regional expansion, and hepatocyte degeneration. The patchy necrosis of hepatocytes was observed, in addition to inflammatory cell infiltration in the necrotic area. At 7 days after transplantation, the LPS/D-GalN+UMSCs group showed a significant recovery in liver structure, resolution of the large areas of degeneration and necrosis, significantly decreased inflammatory cell infiltration, and gradual recovery of the liver lobular structure. Furthermore, we observed significant bile duct hyperplasia in the portal area and normal liver cells around the bile duct. After 28 d, the liver structure basically returned to normal. Hematoxylin-eosin staining showed significant morphological changes in the transplant group compared to the control group (p<0.05).

UMSCs inhibit MyD88 expression

To explore the effect of UMSCs on MyD88, we obtained liver tissues at various time points after LPS/D-GalN induction and UMSC treatment and analyzed them by immunohistochemistry (Fig. 2), Western blotting (Fig. 3 C–E), and real-time fluorescence quantitative nucleic acid amplification and detection (Fig. 3 A–B). The MyD88 expression level increased after LPS/D-GalN induction. However, UMSC treatment significantly inhibited MyD88 expression in a time-dependent manner. Therefore, UMSCs may play a therapeutic role by inhibiting MyD88 expression.

UMSCs inhibit LPS/GalN-induced NF-κB signaling pathway activation

NF-κB is the main regulator of LPS/D-GalN-induced liver inflammation. LPS/D-GalN promoted IκB phosphorylation and degradation (Fig. 4) as well as the nuclear translocation of NF-κB p65 (Fig. 5), and these effects were significantly inhibited after UMSC transplantation. This pattern shows that the UMSC-mediated inhibition of inflammation effectively

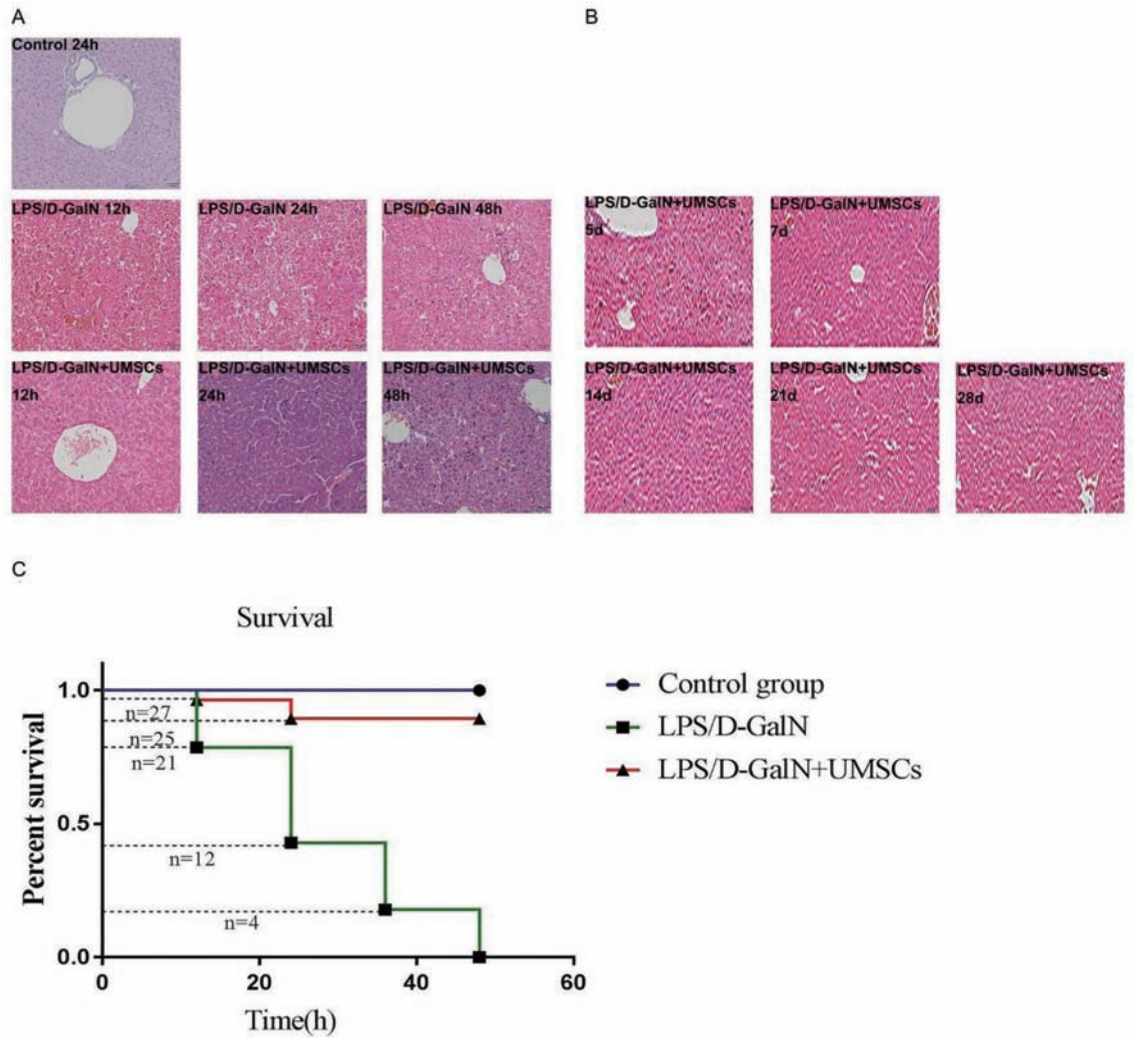

Fig. 1. Liver injury gradually repaired completely after UMSC transplantation. (A–B) Representative histological changes are shown in images of liver tissue from mice in each group (hematoxylin-eosin ×100). (C) Survival curves of the LPS/D-GalN-induced ALF group, control group, and the UMSC treatment group. The survival rate of the LPS/D-GalN+UMSCs group was significantly higher than that of the LPS/D-GalN group at each time point (n=3/group, p<0.01). D-GalN, D-galactosamine; LPS, lipopolysaccharide. My D88, Myeloid differentiation factor 88.

blocked NF-κB signaling pathway activation.

UMSCs inhibit the release of inflammatory factors

The inflammatory cytokines TNF-α, IL-6 and IL-1β play an important role in liver injury. To further study the anti-inflammatory effect of UMSCs, their effect on serum TNF-α, IL-6 and IL-1β secretion was detected by ELISA (Fig. 6). After LPS/D-GalN treatment, the TNF-α, IL-6 and IL-1β levels in serum samples increased significantly, indicating that LPS/D-GalN can stimulate the release of these inflammatory mediators. After UMSC transplantation, the levels of these inflammatory cytokines were significantly decreased and gradually returned to normal as the survival time increased. This pattern shows that UMSCs inhibit the LPS/D-

Table 1. The survival rate of mice at each time point

Time	Number of death		Survival rate	
	LPS/D-GalN	LPS/D-GalN+UMSCs	LPS/D-GalN	LPS/D-GalN+UMSCs
12h	6	1	78.6%	96.4%
24h	10	2	42.9%	89.3%
36h	7	0	17.9%	89.3%
48h	5	0	0%	89.3%

Number of deaths and survival rates at each time point in each group (n=3/group, p<0.01). D-GalN, D-galactosamine; LPS, lipopolysaccharide.

Table 2. Comparison of serum ALT, AST and TBil levels at different time points.

Group (time point)	AST	ALT	TBil
Control	38.6±2.3	41.1±3.5	9.4±1.9
LPS/D-GalN (12 h)	1,927±16.9*	2,120±35.3*	79.6±1.0*
LPS/D-GalN (24 h)	2,303.7±47.3*	3,143±121.6*	95.3±2.0*
LPS/D-GalN (48 h)	1,369±12.8*	3,653±74.9*	76±4.7*
LPS/D-GalN+UMSCs (12 h)	233.3±13#	379.3±7.2#	53.9±6.1#
LPS/D-GalN+UMSCs (24 h)	248±37.3#	268±23.4#	51.1±4.5#
LPS/D-GalN+UMSCs (48 h)	63.3±2.1#	214.3±7.5#	29.9±0.8#

Data are expressed as means±standard deviations (n=3/group): *p<0.05 vs. control; #p<0.05 vs. LPS/D-GalN. ALT, alanine aminotransferase; AST, aspartate aminotransferase; D-GalN, D-galactosamine; LPS, lipopolysaccharide; TBil, total bilirubin.

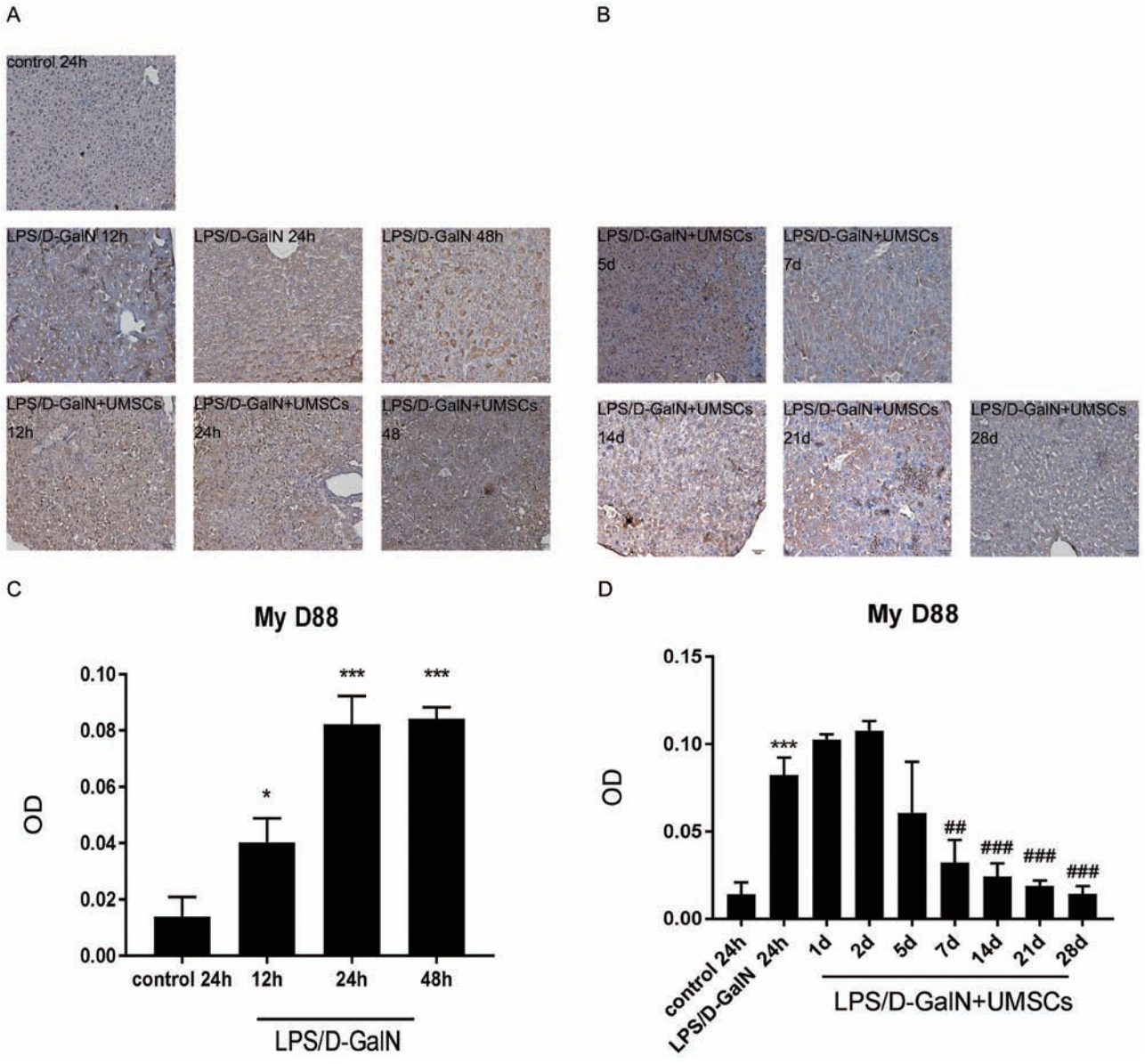

Fig. 2. Protein expression in the liver (immunohistochemistry ×100). The MyD88 expression level increased after LPS/D-GalN induction. At 7 d after transplantation, MyD88 protein expression was significantly inhibited in the LPS/D-GalN+UMSCs group. Data are presented as means±standard deviations (n=3/group). *p<0.05 vs. control; ***p<0.001 vs. control; ###p<0.001 vs. LPS/D-GalN. D-GalN, D-galactosamine; LPS, lipopolysaccharide; My D88, Myeloid differentiation factor 88.

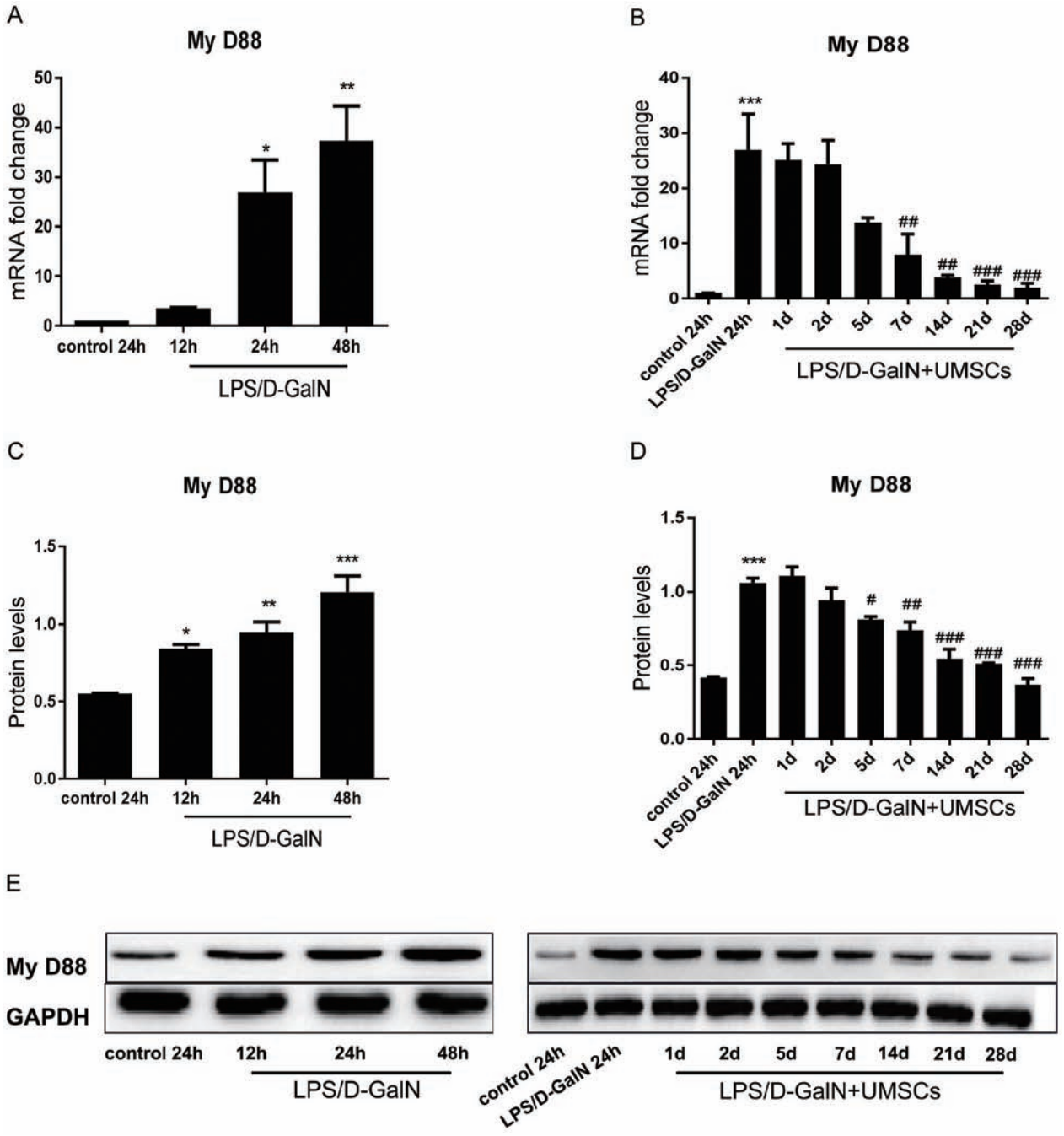

Fig. 3. mRNA (A–B) and DNA (C–E) expression of MyD88 in different groups. Data are presented as means±standard deviations (n=3/group). $^{*}p<0.05$ vs. control; $^{**}p<0.01$ vs. control; $^{***}p<0.001$ vs. control; $^{\#}p<0.05$ vs. LPS/D-GalN; $^{\#\#}p<0.01$ vs. LPS/D-GalN; $^{\#\#\#}p<0.001$ vs. LPS/D-GalN. D-GalN, D-galactosamine; LPS, lipopolysaccharide; My D88, Myeloid differentiation factor 88.

GalN-induced production of inflammatory cytokines.

UMSCs inhibit the expression of apoptosis-related proteins

To explore the inhibitory effect of UMSCs on LPS/D-GalN-induced hepatocyte apoptosis, we evaluated the expression of apoptosis-related signaling proteins by western blot analysis and qRT-PCR. In the LPS/D-GalN group, the expression of Bax increased, while that of Bcl-2 decreased (Fig. 7). In contrast, in the UMSC-treated group, Bax was down-regulated, and Bcl-2 was up-regulated.

Discussion

The liver is an important metabolic tissue and plays an im-

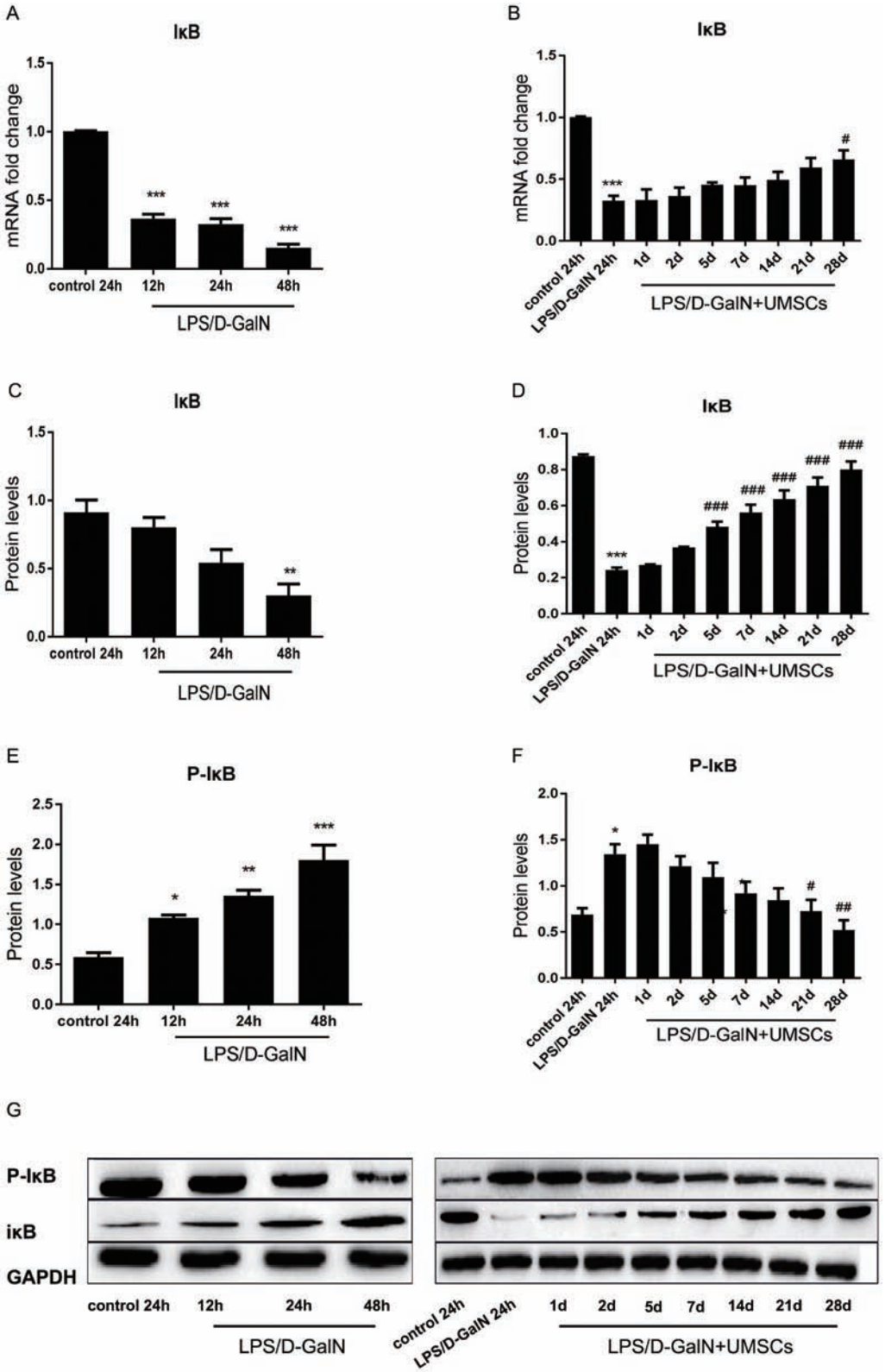

Fig. 4. UMSC therapy inhibited IκB phosphorylation and degradation, mRNA (A–B) and DNA (C–D) expression of IκB in different groups, and DNA expression (E–F, G) of P-IκB in different groups. Data are presented as means±standard deviations (n=3/group). $^{*}p<0.05$ vs. control; $^{**}p<0.01$ vs. control; $^{***}p<0.001$ vs. control; $^{\#}p<0.05$ vs. LPS/D-GalN; $^{\#\#}p<0.01$ vs. LPS/D-GalN; $^{\#\#\#}p<0.001$ vs. LPS/D-GalN.D-GalN, D-galactosamine; IkB, inhibitor of nuclear factor-κB; LPS, lipopolysaccharide; P-IkB, phosphorylated-IκB; UMSCs, umbilical cord mesenchymal stem cells.

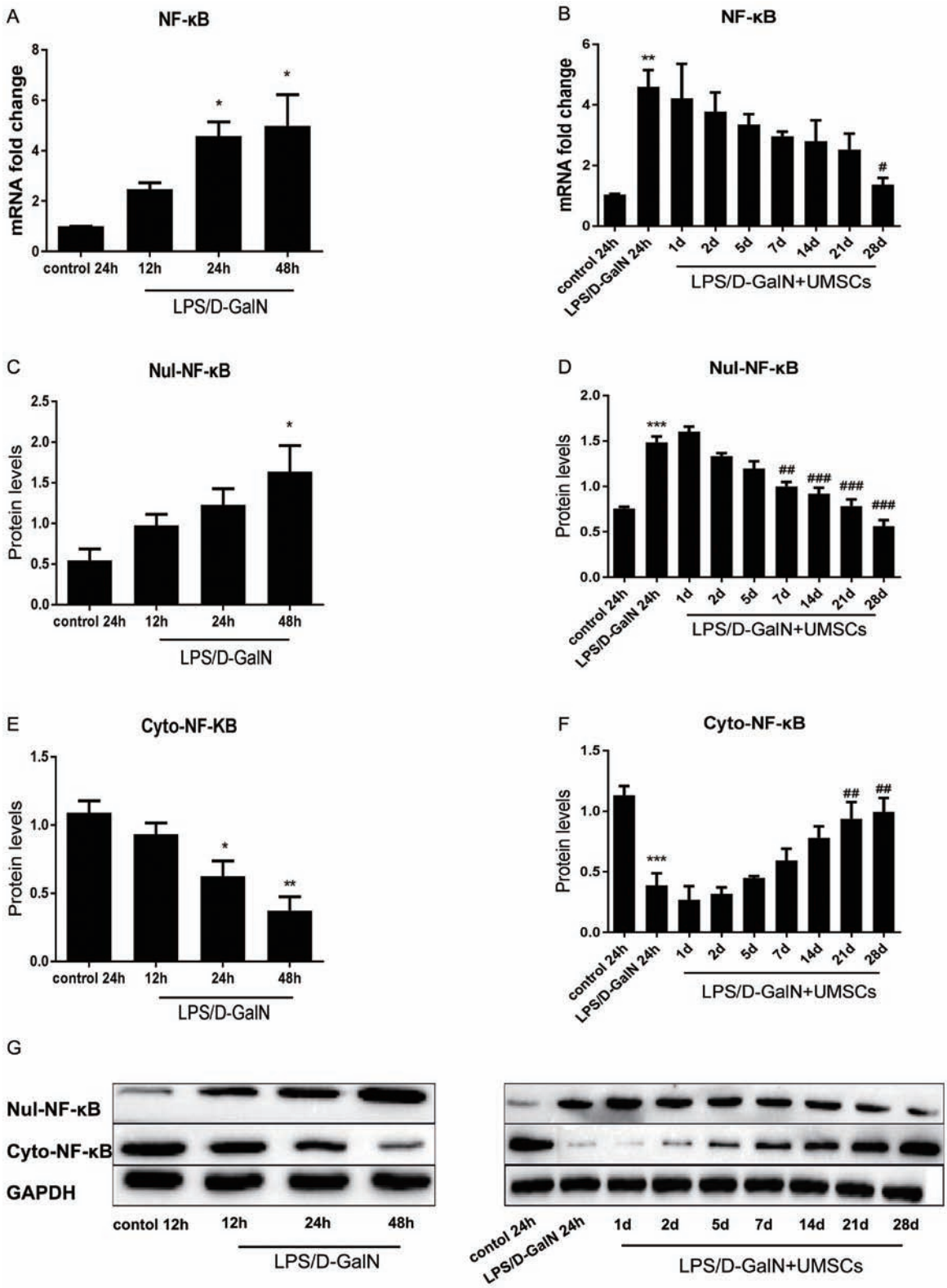

Fig. 5. UMSC therapy inhibited nuclear translocation of NF-κB p65. mRNA (A–B) of NF-κB in different groups, DNA expression (C–D, G) of Nul-NF-κB in different groups, and DNA expression (E–F, G) of Cyto-NF-κB in different groups. Data are presented as means±standard deviations (n=3/group). $^{*}p<0.05$ vs. control; $^{**}p<0.01$ vs. control; $^{***}p<0.001$ vs. control; $^{\#}p<0.05$ vs. LPS/D-GalN; $^{\#\#}p<0.01$ vs. LPS/D-GalN; $^{\#\#\#}p<0.001$ vs. LPS/D-GalN.D-GalN, Cyto-NF-κB, cytoplasmic levels of NF-κB; D-galactosamine; LPS, lipopolysaccharide; NF-κB, nuclear factor-κB; Nul-NF-κB, nuclear levels of NF-κB; UMSCs, umbilical cord mesenchymal stem cells.

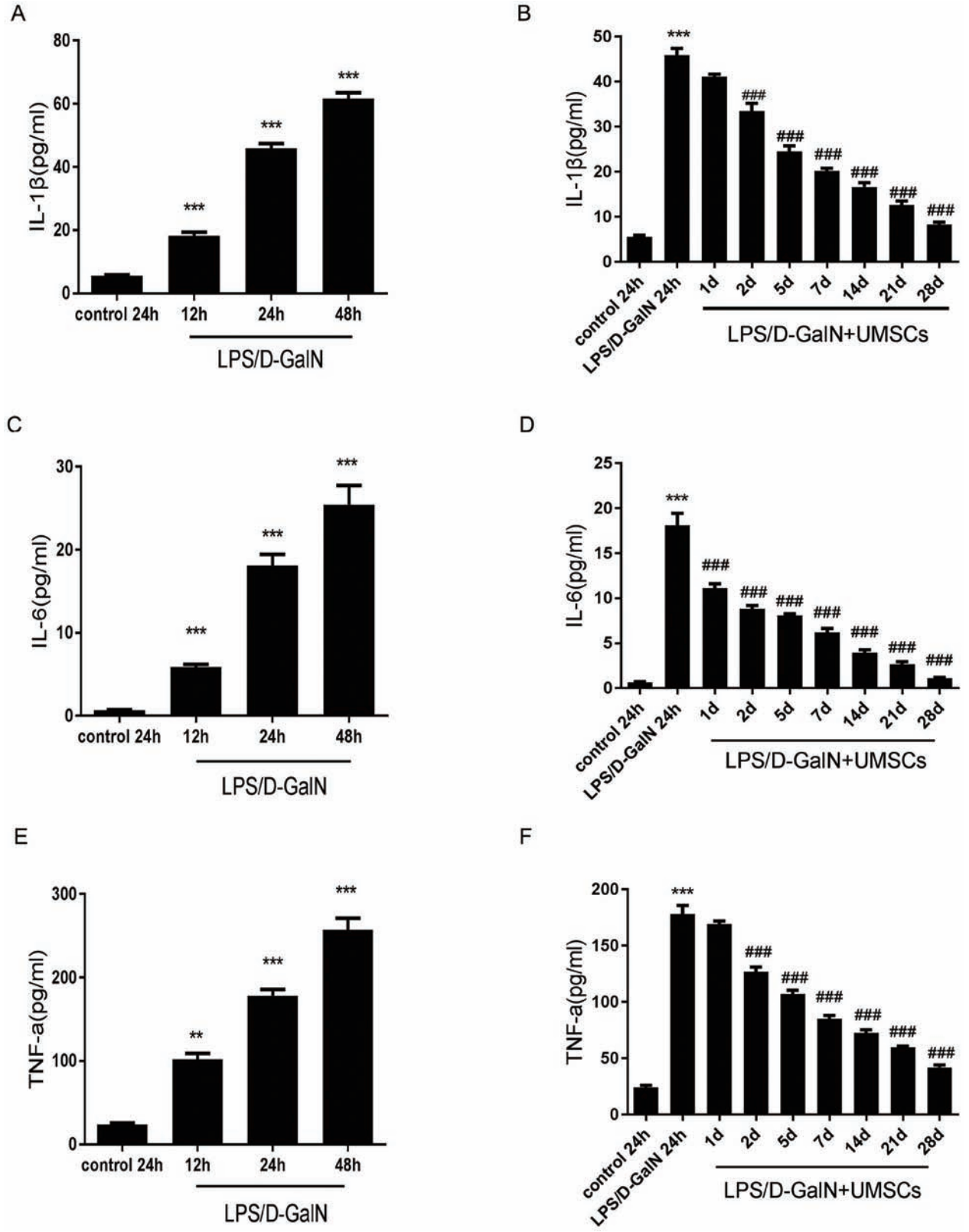

Fig. 6. Effects of UMSCs on inflammatory mediators IL-1β (A–B), IL-6 (C–D), and TNF-α (E–F) in LPS/D-GalN-induced ALF. Data are expressed as means±standard deviations (n=3/group). $^{**}p<0.01$ vs. control; $^{***}p<0.001$ vs. control; $^{\#\#\#}p<0.001$ vs. LPS/D-GalN. D-GalN, D-galactosamine; IL-1β, interleukin-1β; IL-6, interleukin-6; LPS, lipopolysaccharide; TNF-α, tumor necrosis factor-α; UMSCs, umbilical cord mesenchymal stem cells.

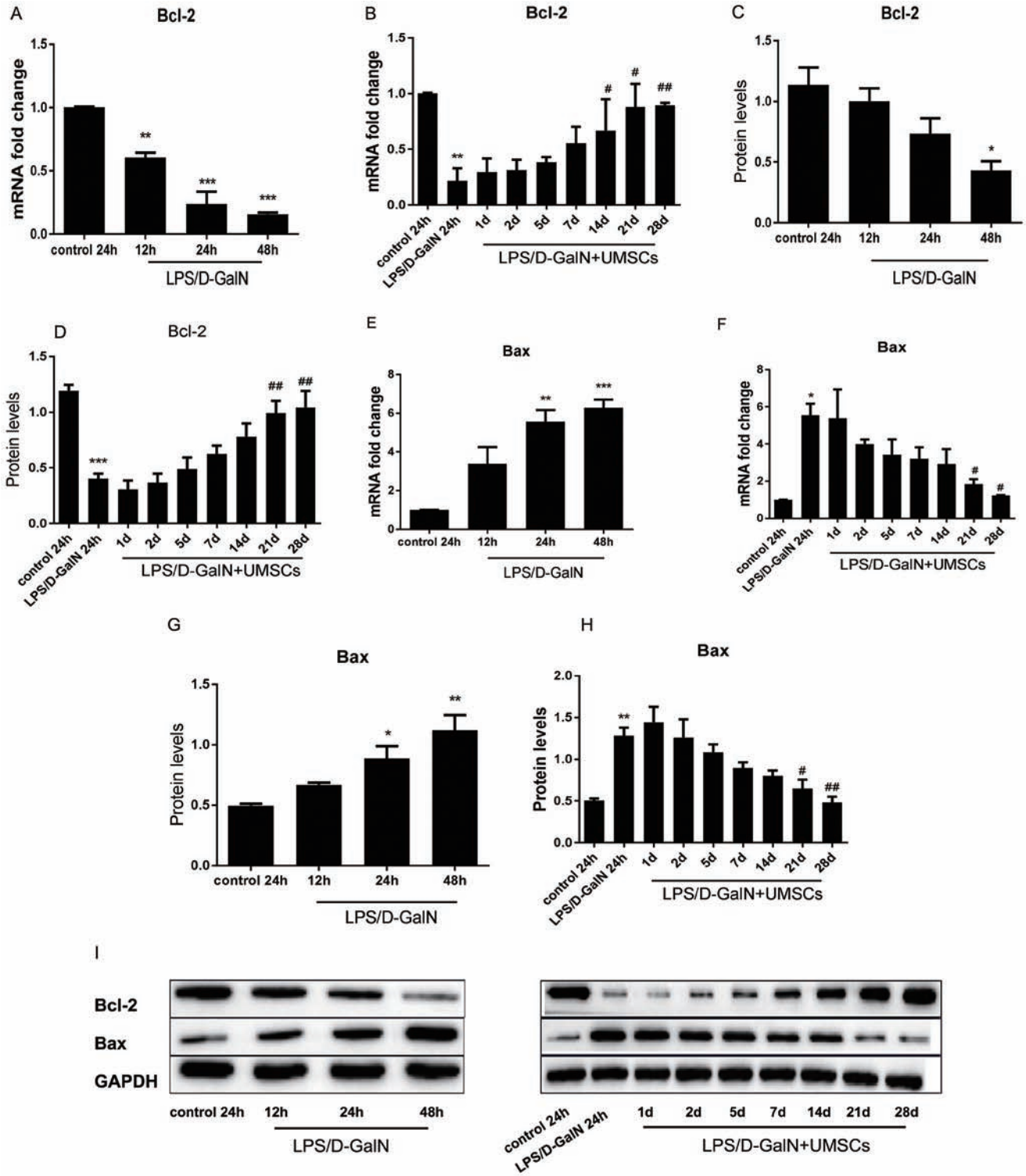

Fig. 7. Effect of UMSCs on apoptosis-related proteins in LPS/D-GalN-induced ALF. mRNA and DNA expressions of Bax in different groups (A–D, I), and mRNA and DNA expressions of Bcl-2 in different groups (E–H, I). These data are expressed as means±standard deviations (n=3/group). $^{*}p<0.05$ vs. control; $^{**}p<0.01$ vs. control; $^{***}p<0.001$ vs. control; $^{\#}p<0.05$ vs. LPS/D-GalN; $^{\#\#}p<0.01$ vs. LPS/D-GalN; $^{\#\#\#}p<0.001$ vs. LPS/D-GalN. Bax, Bcl 2-Associated X Protein; Bcl-2, B-cell lymphoma-2; D-GalN, D-galactosamine; LPS, lipopolysaccharide; UMSCs, umbilical cord mesenchymal stem cells.

portant role in maintaining balance and health. ALF is a life-threatening clinical syndrome characterized by rapid development and high mortality. Finding a clinical method that can effectively treat ALF is an important challenge.

LPS/D-GalN-induced ALF is a well-established experimental model, and inflammation is an important pathogenic mechanism of LPS/D-GalN-induced ALF.[12] A number of studies have shown that UMSCs have the potential for self-renewal and multi-lineage differentiation into terminal cells of many tissues and organs and can participate in immunomodulation.[16] Therefore, it was an important objective of our study to explore whether UMSCs can exhibit anti-inflammatory activity and anti-apoptosis characteristics in ALF. In this study, we investigated the protective effect and the underlying mechanism of UMSCs against ALF in LPS/D-GalN-induced mice with an inflammatory response and apoptosis. The ALF model was successfully established by the intraperitoneal injection of LPS/D-GalN. This model presented a substantial liver injury with obvious changes in histopathological and biochemical parameters. Our results showed the histopathological changes after LPS treatment, including loss of normal lobular structure and showing hepatocyte cytoplasmic edema, local regional expansion, hepatocyte degeneration, necrotic areas filled with inflammatory cells and red blood cells, and inflammatory cell infiltration. Moreover, the biochemical markers of ALT, AST and TBil increased significantly in the LPS/D-GalN group. Liver injury was gradually repaired completely after UMSC transplantation, and there was a marked decrease in the levels of ALT, AST and TBil.

MyD88 is an important TLR/IL-1 receptor superfamily member. LPS is recognized by TLR4 expressed by the cell and activates innate immunity through a MyD88-dependent pathway. Recent studies have shown that the MyD88/NF-κB signaling pathway plays a key role in inflammation. During this process, MyD88 is activated and induces a cytoplasmic signaling cascade, which leads to the activation of NF-κB signaling molecules, in turn leading to excessive Kupffer cell activation.[17] Excessive MyD88 signaling pathway activation can lead to various inflammation-related diseases. Studies have shown that treatment with MyD88 inhibitors or knockdown of MyD88 can reduce inflammatory cell infiltration and protect liver cells against apoptosis, improving the survival rate of mice with acute liver injury.[18] NF-κB is a downstream signaling molecule of MyD88 and an upstream regulator of various inflammation-related genes.[19] NF-κB is retained in an inactive form in the cytoplasm of hepatocytes that interact with IκB inhibitors. However, some stimulants, such as LPS, proinflammatory cytokines, viruses and other substances, can trigger NF-κB activation. The IKK complex is phosphorylated and catalyzes the phosphorylation of IκB, which is followed by its ubiquitination, resulting in its proteasomal degradation. Then, the IκB/NF-κB complex dissociates, resulting in the nuclear translocation of active NF-κB. Through immunohistochemical staining, qRT-PCR and western blot assay, our present study determined that the MyD88/NF-κB signaling pathway was successfully activated by LPS. LPS/D-GalN-induced IκB phosphorylation and degradation and increased NF-κB p65 nuclear translocation, while UMSCs attenuated these effects. These results indicate that UMSCs partially suppress the MyD88/NF-κB signaling pathway activity by inhibiting IκB phosphorylation.

Inflammatory mediators play an important role in LPS/D-GalN-induced ALF. In the liver, LPS first binds to LPS-binding protein, is then transferred to TLR4, and is finally expressed on the surface of Kupffer cells. Activated Kupffer cells can mediate hepatitis progression by secreting TNF-α and other proinflammatory cytokines.[20] TNF-α is an important inflammatory mediator associated with LPS/GalN-induced liver injury and may induce hepatocyte apoptosis, which in turn leads to organ failure.[21] In addition, TNF-α may trigger an inflammatory cascade and induce the production of other cytokines, including IL-1β and IL-6.[22] Previous studies have reported that inhibiting TNF-α synthesis inhibits cytokine production and reduces liver damage.[23] Our results suggest that UMSCs can significantly reduce the production of the inflammatory factors TNF-α, IL-1β and IL-6 by down-regulating the expression of upstream regulators of inflammatory factors after transplantation.

Moreover, previous studies have shown that MyD88 overexpression does not immediately induce a strong apoptotic response.[24] However, after 2–3 days, in cells with high expression of MyD88, the apoptotic response becomes apparent. Therefore, we suspect that after LPS stimulation, the MyD88-induced apoptotic pathway can become activated. Through qRT-PCR and western blot assay, our findings indicated that after LPS/D-GalN stimulation, the expression of the antiapoptotic protein Bcl-2 was significantly down-regulated and that of the proapoptotic protein Bax was up-regulated. However, after UMSC transplantation, this effect was significantly reversed.

In summary, this study suggests that in the mouse model of LPS/D-GalN-induced ALF, UMSCs can reduce liver damage by suppressing inflammatory mediator release and apoptosis. Mechanistically, this effect is achieved via MyD88/NF-κB signaling inhibition, which finally exerts a therapeutic effect on ALF. Therefore, we believe that UMSCs are a potential and valuable therapeutic alternative for ALF.

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Conflict of interest

The authors have no conflict of interests related to this publication.

Author contributions

Study design (HL, JY), performance of experiments (HL, SD), analysis and interpretation of data (HL, SD, TJ, MZ, ZX), manuscript writing (SD, TJ, MZ), critical revision of the manuscript (SD, TJ, MZ, ZX), technical or material support (JY).

Data sharing statement

No additional data are available.

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Original Article

Serum N-terminal DDR1: A Novel Diagnostic Marker of Liver Fibrosis Severity

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Abstract

Background and Aims: The expression of discoidin domain receptor 1 (DDR1) is commonly up-regulated and undergoes collagen-induced ectodomain (N-terminal) shedding during the progression of liver fibrosis. This study aimed to evaluate the clinical utility of N-terminal DDR1 as a diagnostic biomarker for liver fibrosis. ***Methods:*** N-terminal DDR1 shedding was evaluated using cell lines, liver fibrosis mouse models, clinical data of 298 patients collected from February 2019 to June 2020. The clinical data were divided into test and validation cohorts to evaluate the diagnostic performance of serum N-terminal DDR1. ***Results:*** Time- and dosage-dependent N-terminal DDR1 shedding stimulated by collagen I was observed in a hepatocyte cell line model. The type I collagen deposition and serum N-terminal DDR1 levels concurrently increased in the development of liver fibrosis in mouse models. Clinical data demonstrated a significant diagnostic power of serum N-terminal DDR1 levels as an accurate biomarker of liver fibrosis and cirrhosis. The diagnostic performance was further increased when applying N-DDR1/albumin ratio, achieving area under the curve of 0.790, 0.802, 0.879, and 0.865 for detecting histological fibrosis stages F ≥2, F ≥3, F 4 with liver biopsy as a reference method, and cirrhosis according to imaging techniques, respectively. With a cut-off of 55.6, a sensitivity, specificity, positive predictive value, and negative predictive value of 82.7%,76.6%, 67.4%, and 88.3% were achieved for the detection of cirrhosis. ***Conclusions:*** Serum N-terminal DDR1 appears to be a novel diagnostic marker for liver fibrosis.

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Introduction

Liver fibrosis (LF) denotes a series of dynamic pathophysiological changes characterized by hepatocyte degeneration and necrosis due to chronic liver injury, including hepatotoxic drug injury, cholestasis, etc. LF has been the most crucial predictor of liver-related morbidity and mortality in liver diseases.[1] However, studies have revealed that early-stage LF and even liver cirrhosis can be reversed.[2] Moreover, detecting advanced LF and cirrhosis is crucial for choosing adequate support treatment, determining surveillance intervals, and predicting clinical outcomes.[3,4] Although liver biopsy is still the gold standard for clinical diagnosis, its inherent shortcomings, such as sampling error of biopsy specimen caused by different operators,[5] expensive operation cost,[6] and complications such as bleeding and bile fistula caused by the invasive operation, all contribute to its poor tolerance and low repeatability.[7] These problems have led to the question of its applicability as a reference standard for LF.[8,9] Different serological biomarkers combined with clinical parameters, such as body mass index (BMI) and age, were gradually developed into a non-invasive, low-cost, and repeatable alternative method compared with liver biopsy. These markers have been widely studied in nonalcoholic fatty liver disease and viral hepatitis, in which they are mainly used to detect significant fibrosis (F ≥2) or advanced fibrosis (F ≥3). At present, a variety of widely reported markers, including the aspartate to platelet ratio index (commonly referred to as APRI),[10] enhanced liver fibrosis (commonly referred to as ELF) test,[11] Fibrosis 4 (commonly referred to as FIB4)-index[12] or FibroTest[13] are receiving much attention.

The discoidin domain receptors (DDRs), including DDR1 and DDR2, are a unique receptor tyrosine kinase (RTK) family containing a discoidin homology domain in their extracellular region. DDRs are the only collagen-activated RTKs,

Keywords: Liver fibrosis; Serum biomarker; DDR1; FIB-4.
Abbreviations: DDR1, discoidin domain receptor 1; LF, liver fibrosis; ECM, extracellular matrix.
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showing different structural and functional homologies. DDR1 is mainly expressed in epithelial cells. Studies have confirmed that DDR1 plays an essential role in tissue fibrosis, and it is considered an attractive antifibrotic target.[14,15] Several studies have demonstrated that DDR1 expression and function are associated with fibrotic diseases, such as atherosclerosis, arthritis, and many types of cancers.[16–19] In pulmonary fibrosis and chronic kidney disease, activation of DDR1 stimulates inflammatory pathways, including cytokine synthesis.[16,17] Proinflammatory cytokines further enhance inflammatory cell infiltration, extracellular matrix (ECM) synthesis and DDR1 expression, to form a positive feedback loop. The combination of collagen and DDR1 leads to the activation and phosphorylation of DDR1, which transmits various collagen signals from epithelial cells.[20,21] The clustering and activation of DDR1 mediate tractional collagen remodeling, which is vital for mechanical compaction and reorganization of collagen in the ECM during fibrosis.[22] Collagen has been reported to activate DDR1 aggregation, enhancing its binding to collagen, and DDR1 allows collagen remodeling to promote fibrosis.[22,23] Upon collagen binding, shedding of N-terminal DDR1 can be induced by membrane-anchored collagenase, membrane type (MT) 1-, MT2-, and MT3 matrix metalloproteinases (commonly known as MMPs).[14,15,24,25] Collagen specifically stimulates the N-terminal DDR1 shedding but does not affect other transmembrane proteins.[24] Collagen-stimulated shedding of the N-terminal DDR1 has been confirmed in many studies;[14,15,24–26] however, the expression and diagnostic value of serum N-terminal DDR1 in LF remain unclear. This study aims to explore the phenomenological relationship between LF and N-terminal DDR1 shedding.

Methods

Cell culture

Human embryonic kidney (HEK) 293 cells and HL-7702 cells were cultured in Dulbecco's modified Eagle's medium/F12 nutrient mixture (commonly known as DMEM/F12; Invitrogen, Carlsbad, CA, USA) with 10% fetal bovine serum. AML12 medium was also used and supplemented with 2 mM L-glutamine, and 100 U/mL penicillin, and 100 mg/mL streptomycin. All cells were cultured at 37°C and in an atmosphere of 5% CO_2.

Western blot analysis

The serum-free medium's supernatant was collected and added with an equal amount of methanol and 1/4 volume of chloroform. The supernatant protein was obtained by centrifugation of 17,000×*g* for 15 m. After removing the transparent aqueous phase, the white layer on top of the chloroform phase was left (composed of protein). Methanol was added into the remaining liquid, which was centrifuged at 17,000×*g* for 15 m. After decanting the liquid phase, the protein remained at the bottom.

Equal amounts of protein per sample were separated by polyacrylamide gel electrophoresis and then transferred to a polyvinylidene difluoride membrane (Millipore, Billerica, MA, USA). The membranes were blocked in 5% skim milk, followed by incubation with the primary antibodies overnight at 4°C and secondary antibodies 1 h at 25°C. The immunoreactive bands were visualized using an enhanced chemiluminescence detection system and standard autoradiography.

The following antibodies were used: anti-DDR1 (C-20) (sc-532; Santa Cruz Biotechnology, Dallas, TX, USA); DDR1 ECD antibody (AF2396; R&D Systems, Minneapolis, MN, USA); type I collagen immunohistochemistry antibody (ab34710; Abcam, Cambridge, UK); and DDR1 immunohistochemistry antibody (cst5583; Cell Signaling Technology, Danvers, MA, USA).

LF mouse models

The male C57Bl/6 mice were used to construct an LF model via bile duct ligation (BDL) (6–8 weeks). Mice were anesthetized with 4% chloral hydrate. Following abdominal midline skin incision through the musculature, laparotomy ligation was performed with two ligatures close to the liver hilum immediately below the bifurcation and one ligature around the cystic duct. The abdominal incision was closed with absorbable suture, and the skin incision with normal suture. The entire surgical procedures were performed under sterile conditions. The animals were sacrificed at 0, 5, 10, and 15 days after surgery. Liver tissue and serum were harvested. Six control animals were included at each time point.

Carbon tetrachloride (CCl4) was diluted with peanut oil to a concentration of 0.1% and injected intraperitoneally at a dose of 1 mL/100 g twice a week. The mice were sacrificed at the 6th, 9th, and 12th weeks to collect liver tissue and serum. All experimental animals were sacrificed by the carbon dioxide method.

Enzyme-linked immunosorbent assay (ELISA)

According to the manufacturer's protocol, the serum levels of DDR1 were measured using a commercial ELISA kit (CHE0283; Beijing 4A Biotech Co., Ltd., Beijing, China). The OD_{450} value of the detection solution was measured immediately after mixing.

Statistical analyses

All statistical analyses were performed using SPSS v.23.0 (IBM Corp., Armonk, NY, USA). Summary statistics for normally distributed continuous variables were expressed as means and standard deviation, whereas quantitative variables without a normal distribution were expressed as the median and interquartile range (IQR). The means of continuous variables were compared using the Mann-Whitney *U*-test. Categorical data were presented as numbers and frequencies (%) and were compared using the chi-squared test or Fisher's exact test. The Pearson and Spearman correlation tests were used to analyze the relationship between the serum DDR1 levels and various clinical characteristics or categorical data. The receiver operating characteristic (commonly referred to as ROC) curve analysis was performed to assess the diagnostic accuracy. Area under the curve (AUC) was used to identify optimal sensitivity and specificity levels. All *p*-values were two-sided, and differences with *p*-values <0.05 were considered statistically significant.

Ethics approval

All animal experiments were approved by the Ethics Committee of Tongji Hospital, Huazhong University of Science and Technology (HUST). The study was conducted according to the Declaration of Helsinki, and reviewed by the Ethics Committee of the Tongji Hospital (TJ-IRB20190706).

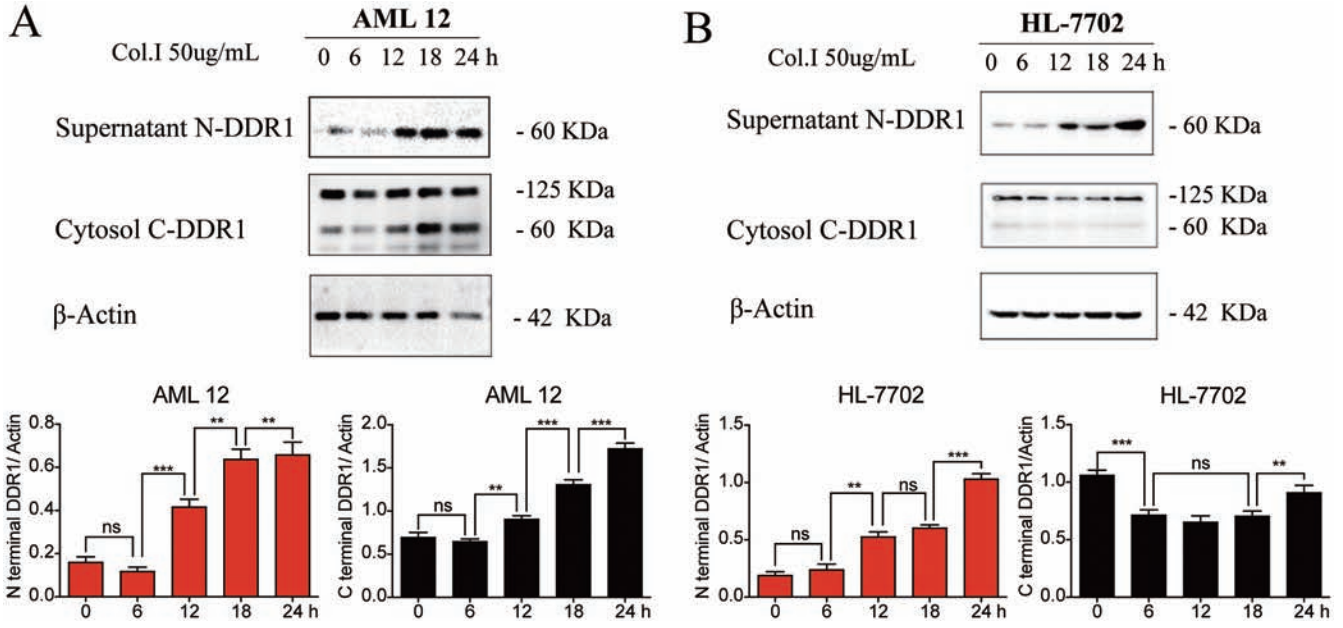

Fig. 1. Type I collagen promotes the N-terminal DDR1 shedding in hepatocytes. (A–F) Western blot analysis of DDR1 shedding at different times in AML12 (A, B) and HL-7702 liver cell line (C–F). Student's *t*-test was applied for statistical analyses, comparing each variant group for multiple comparisons. **p*<0.05, ***p*<0.01, *** *p*<0.001, **** *p*<0.0001.

Results

Type I collagen promotes N-terminal DDR1 shedding in a time- and concentration-dependent manner

Collagen is known to promote the shedding of N-terminal DDR1.[25] To quantify type I collagen-induced DDR1 shedding at different times and concentrations, we transiently transfected HEK293 cells with a DDR1 expression vector and then incubated with serum-free DMEM containing 10 μg/mL type I collagen, and ectodomain shedding was analyzed at 0, 2, 4, 6, and 8 h. The level of N terminal-DDR1 with a molecular size of 60 kDa in the culture supernatant was assayed by immunoblotting (Supplementary Fig. 1A). Upon treatment, the concentration of the N-terminal DDR1 in the culture supernatant increased, which was consistent with previous studies.[14,15,24,26]

Further, we tested N-terminal DDR1 shedding at different collagen concentrations (0, 25, 50, and 75 μg/mL) (Supplementary Fig. 1B). N-terminal DDR1 shedding increased significantly as the collagen concentration increased. This trend, however, was reversed at a collagen concentration of 100 μg/mL. This phenomenon may be explained by the saturation of stimulus that has been reported in A431 cells.[24] Nevertheless, the results clearly showed that N-terminal DDR1 shedding due to type I collagen stimulation is time- and concentration-dependent.

Type I collagen stimulated the shedding of N-terminal DDR1 in hepatocytes

To further investigate if the collagen-induced DDR1 shedding is consistent in hepatocytes expressing endogenous DDR1, AML12 and HL-7702 cells were cultured. We then used 50 μg/mL collagen I as the optimal concentration to treat normal hepatocytes for 0, 6, 12, 18, and 24 h, respectively. Similar findings of DDR1 shedding were found in the hepatocyte cell lines. Notably, HL-7702 cells quantitatively exhibited small amounts of shedding, even in the absence of collagen. Furthermore, the shedding level progressively increased over time. This phenomenon may indicate that N-terminal DDR1 undergoes minor spontaneous shedding at a basal level, while the addition of collagen significantly increases shedding. After collagen stimulation for 12 h, AML12 cells also exhibited DDR1 shedding (Fig. 1A, B). These results thus confirmed collagen-induced endogenous DDR1 shedding in normal liver cell lines.

Serum levels of N-terminal DDR1 were associated with the severity of LF

To further confirm the relationship between type I collagen deposition and LF, the LF models were generated using CCl4 treatment and BDL in C57BL/6 mice. To confirm the successful induction of LF, hematoxylin and eosin, Sirius red, and collagen staining was performed in liver tissues (Fig. 2A, B). In the CCL4-induced mouse model, a small number of fibrous septa and fibrous tissue hyperplasia were observed at the 6th and 9th weeks after model induction. At the 12th week, many fibrous septa and fiber deposition caused structural disorder of the liver tissue (Fig. 2A). In the BDL model mice, on the 5th day after treatment, the fibrous tissue around the vessel proliferated. On the 10th day, the hyperplasia increased with small areas of necrosis. On the 15th day, the liver tissue showed widespread necrosis and apparent fibrous tissue hyperplasia (Fig. 2B). Taken together, type I collagen deposition was increased concurrently with the increased degree of fibrosis *in vivo*.

To verify the correlation between the degree of LF in the mouse model and the N-terminal DDR1 levels in the serum, a commercial ELISA kit was used to measure the serum levels of N-terminal DDR1. The results suggested that the mean serum level was significantly higher than in the control group and increased with the degree of fibrosis (Fig. 2C,

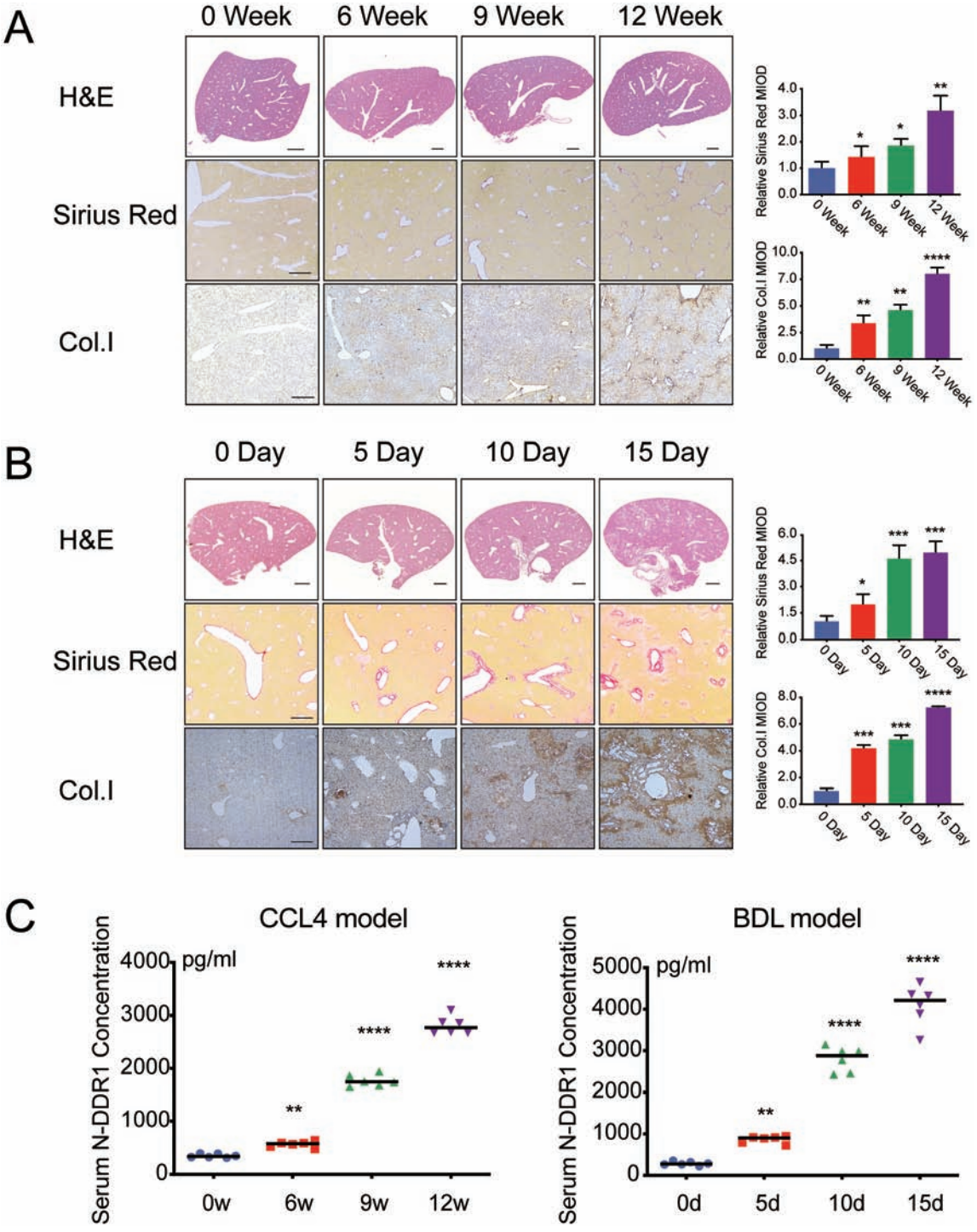

Fig. 2. Type I collagen deposition and serum N-terminal DDR1 in the CCl4-induced and BDL mouse model. (A. B) Representative images of hematoxylin and eosin (HE), Sirius red, and collagen I staining performed the mouse model liver tissue. (C) ELISA was used to measure the serum levels of N-terminal DDR1 in CCL4 and BDL models at 0, 6, 9, and 12 weeks, or 0, 5, 10, 15 days and compared with healthy controls, respectively. (HE, 4×, scale bar: 500 μm; Sirius red and collagen I staining, 40×, scale bar: 500 μm)

Supplementary Fig. 1C, D).

To further investigate the association of serum levels of N-terminal DDR1 and the severity of LF in patients, a total of 298 patients (median age: 53 years; male sex: 54.3%) were included from February 2019 to June 2020, and liver biopsy was performed. We strictly screened patient data, excluded tumor patients, patients with common kidney and lung diseases, osteoarthritis and atherosclerosis, or patients with a history of the above diseases. The serum N-terminal DDR1 levels were measured by ELISA in

Table 1. Patient characteristics

Characteristics	All patients (*n*=298)	Test cohort (*n*=230)	Validation cohort (*n*=68)	*p*-value
Age in years, median (IQR)	53.0 (46–61)	54.0 (46–62)	50.0 (45–57.5)	0.053
Male sex, % (*n*)	54.3 (162)	54.3 (125)	54.4 (37)	0.993
BMI, median (IQR)	23.5 (20.5–26.6)	23.4 (20.4–26.5)	24.0 (21.1–26.7)	0.206
Laboratory parameter				
AST in U/mL, median (IQR)	26.0 (19.0–39.3)	25.0 (19.0–38.0)	31.5 (21.5–47.5)	0.045
ALT in U/mL, median (IQR)	22 (14–37.25)	20 (14–36)	25.5 (18.0–44.5)	0.028
GGT in U/mL, median (IQR)	48.5 (26.0–104.0)	45.5 (24.0–89.0)	75.0 (30.5–137.0)	0.014
Albumin in mg/dL, median (IQR)	41.1 (38.0–43.8)	41.4 (38.3–44.2)	39.8 (36.8–42.9)	0.014
Total bilirubin in mol/L, median (IQR)	12.7 (9.0–17.5)	12.4 (8.9–17.0)	13.2 (9.5–19.7)	0.259
Platelet count in G/L, median (IQR)	173.5 (122.0–237.0)	171.5 (124.0–233.0)	181.0 (118.5–241.5)	0.690
MELD score, median (IQR)	5.7 (5.5–5.9)	5.7 (5.4–5.9)	5.7 (5.5–5.9)	0.712
HBeAg positivity, % (*n*)	60.1 (179)	58.7 (135)	64.7 (44)	0.374
Healthy controls, % (*n*)	6.7 (20)	8.7 (20)	0.0 (0)	n.d
Cirrhosis according to imaging, % (*n*)	36.9 (110)	36.1 (83)	39.7 (27)	0.587
US only	60.0 (66)	56.6 (47)	70.4 (19)	n.d
US+CT	16.4 (18)	18.1 (15)	11.1 (3)	n.d
US+MRI	12.7 (14)	13.3 (11)	11.1 (3)	n.d
US+CT+MRI	10.9 (12)	12.0 (10)	7.4 (2)	n.d
N-DDR1, % (*n*)	100 (298)	100 (230)	100 (68)	n.d
FIB4, % (*n*)	100 (298)	100 (230)	100 (68)	n.d
FibroScan, % (*n*)	93.3 (278)	91.3 (210)	100 (68)	n.d

ALT, alanine transaminase; AST, aspartate transaminase; CT, computed tomography; GGT, γ-glutamyl transpeptidase; HBeAg, hepatitis B e antigen; MRI, magnetic resonance imaging; n.d., not determined; US, ultrasound.

all 110 patients with liver cirrhosis and 188 patients without liver cirrhosis (according to imaging). A total of 210 cases (Department of Liver Surgery, Tongji Hospital) and 20 health personnel (physical examination centers, Tongji Hospital) were investigated in the test cohort. Then, 68 LF patients in the validation cohort (Infectious Diseases Department of Tongji Hospital) were investigated to confirm our research results. The basic data of the cases are shown in Table 1. Among the total 298 samples, according to the METAVIR classification system, there were 20 F 0, 64 F 1, 52 F 2, 89 F 3 and 73 F 4 cases. The mean serum N-terminal DDR1 levels in patients with significant LF (F ≥2) and advanced fibrosis (F ≥3) were 2,386.4 pg/mL (IQR: 1,595.6; 2,750.6, *n*=214) and 2,501.9 pg/mL (IQR: 1,919.7; 2,891.5, *n*=162). Additionally, histopathological examination and staining on F 1-F 4 fibrosis cases were performed, revealing that collagen deposition increased with the degree of LF (Supplementary Fig. 2). Spearman's correlation coefficients of the N-terminal DDR1 levels with degree of LF was 0.574 ($p \leq 0.001$).

The N-terminal DDR1 levels were increased according to the stage of fibrosis. For the diagnosis of LF, in addition to the gold-standard liver biopsy pathological examination, preoperative imaging is also necessary. Because imaging detection of less advanced fibrosis is challenging, imaging is often used to assess liver cirrhosis and its complications.[27] To distinguish, the present study used F 4 to represent pathological end-stage cirrhosis, while cirrhosis was used to describe the end-stage diagnosis of imaging. According to liver biopsy and imaging results, the serum N-terminal DDR1 levels, N-terminal DDR1/albumin ratio, FIB4 test results, and transient elastography (TE) results of all patients are shown in Figures 3 and Supplementary Figure 3. Overall, the N-terminal DDR1 levels increased according to the stage of fibrosis. N-terminal DDR1 levels were significantly higher in patients with F 4 compared to those with F 0 to F 3 (median N-DDR1: 2,853.8 pg/mL, IQR: 2,497.4; 2,947.2 versus 1,951.5 pg/mL, IQR: 1,287.4; 2,570.8, $p<0.0001$), as well as in patients with cirrhosis according to imaging compared to patients without cirrhosis or healthy controls (median N terminal-DDR1: 2,515.4 pg/mL, IQR 2,312.6; 3,060.0 versus 1,566.7 pg/mL, IQR: 1,155.8; 2,227.9 vs. 1,343.4, IQR: 870.6; 1,778.1). Taken together, these data revealed that N terminal-DDR1 levels do not significantly change during the early stages of hepatic fibrosis (F 0–F 3) and were highly elevated in liver cirrhosis (F 4) compared to advanced fibrosis (F 3).

Comparison of the diagnostic accuracy of serum N terminal-DDR1/albumin ratio, FIB-4, and TE for patients with LF

The AUC values of the N-terminal DDR1 ROC curve for detecting F ≥2, F ≥3, and F 4 compared to healthy control and cirrhosis were 0.764, 0.776, 0.845, and 0.827, respectively. Calculating the N-DDR1/albumin ratio increased the AUC to 0.790, 0.802, 0.879, and 0.865, respectively (Supplementary Fig. 4). Spearman's correlation coefficients of the N-DDR1/albumin ratio with degree of LF was 0.627

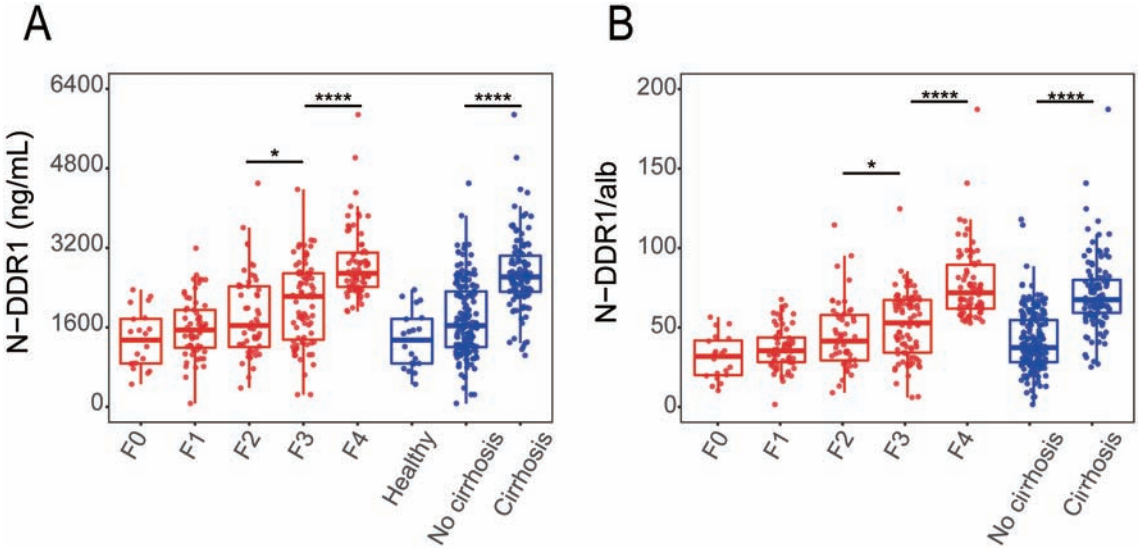

Fig. 3. Serum levels of N-DDR1 (A) and N-DDR1/albumin ratio (B) results stratified according to liver biopsy and the presence of imaging test results.

($p \leq 0.001$). The N-DDR1/albumin ratio, stratified by fibrosis grade and cirrhosis, and the detailed statistical results, are listed in Table 2 and Supplementary Table 1.

The FIB4 test and TE had excellent diagnostic accuracy for the detection of LF and cirrhosis. Data on their diagnostic performance is shown in Supplementary Table 1. In the present study, the N-DDR1/albumin ratio demonstrated acceptable accuracy for detecting F ≥2, F ≥3, F 4, and cirrhosis compared to the FIB4 test (Fig. 4) as well as TE. Spearman's correlation coefficients of the N-terminal

Table 2. Results of non-invasive fibrosis assessment

Fibrosis/Cirrhosis	AUC, 95% CI	Sensitivity, %	Specificity, %	PPV	NPV	Youden's index	Cut-off
N-DDR1							
All patients							
F ≥2	0.764	62.62	85.71	91.8	47.4	0.4833	2,202.44
F ≥3	0.776	70.99	77.21	78.8	69.1	0.4819	2,202.44
F 4	0.845	93.15	66.22	47.2	96.8	0.5937	2,215.5
Cirrhosis	0.827	90.91	65.43	60.6	92.5	0.5633	1,894.492
Hepatitis B virus							
F ≥2	0.788	66.21	91.18	97.0	38.8	0.5738	2,202.44
F ≥3	0.792	73.50	79.03	86.9	61.3	0.5254	2,202.44
F 4	0.830	88.68	69.05	54.7	93.5	0.5773	2,264.6
Cirrhosis	0.827	91.67	66.32	70.6	90.0	0.5798	1,894.492
N-DDR1/albumin							
All patients							
F ≥2	0.790	64.49	84.52	91.4	48.3	0.4901	51.6171
F ≥3	0.802	72.84	78.68	80.3	70.9	0.5152	52.3512
F 4	0.879	98.63	68.00	50.0	99.4	0.6663	52.9793
Cirrhosis	0.865	82.73	76.6	67.4	88.3	0.5932	55.6054
Hepatitis B virus							
F ≥2	0.816	73.79	85.29	95.5	43.3	0.5909	44.2517
F ≥3	0.820	81.2	74.19	85.6	67.6	0.5539	44.3576
F 4	0.860	98.11	64.29	53.6	98.8	0.6340	52.9193
Cirrhosis	0.866	82.14	76.84	75.8	83.0	0.5898	55.6054

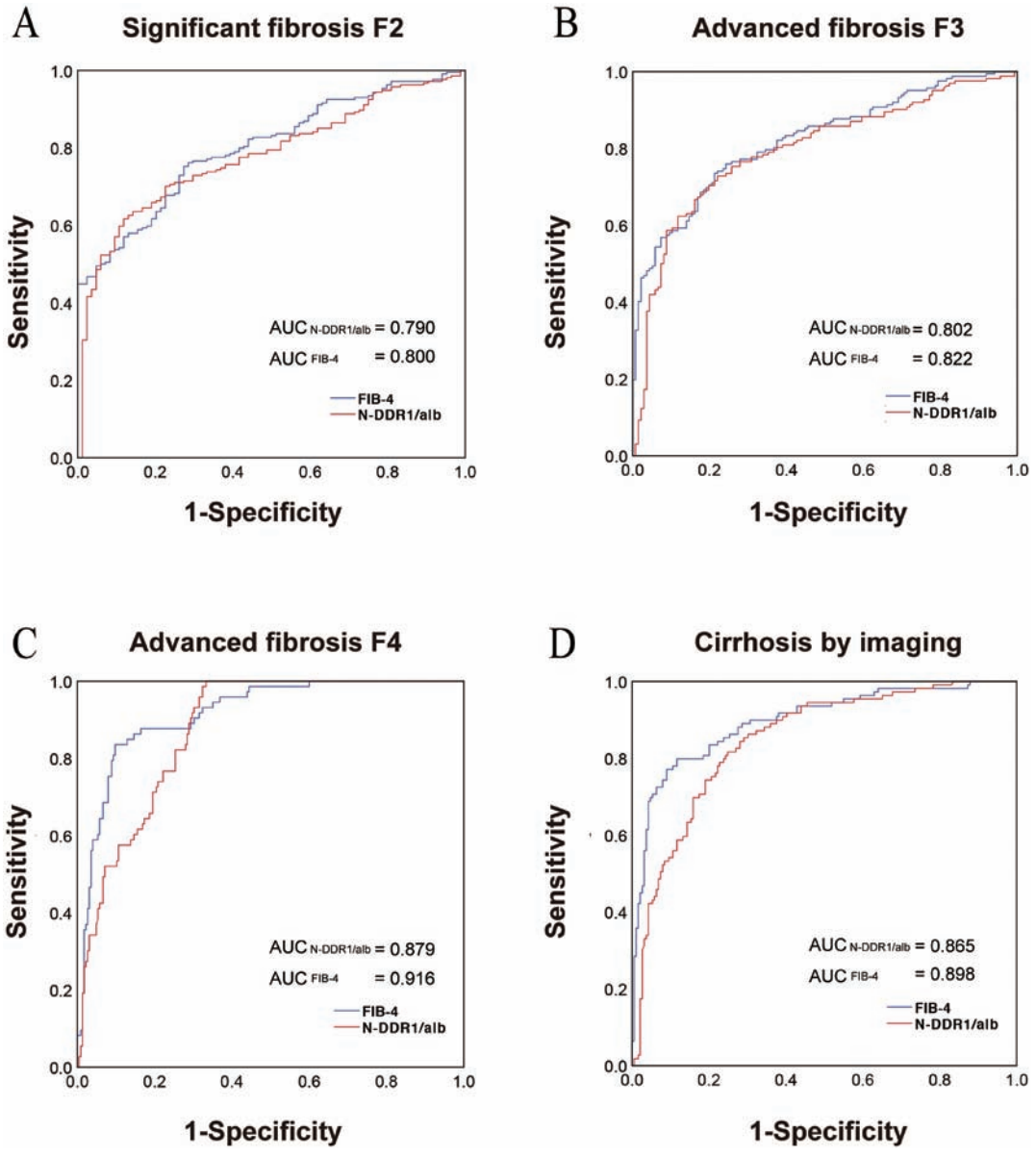

Fig. 4. Comparison of the diagnostic accuracy displayed as the AUC of the N-DDR 1/albumin ratio and FIB4 test for significant fibrosis (F ≥2) (A), advanced fibrosis (F ≥3) (B), fibrosis grade 4 (F 4) (C), and liver cirrhosis (D) according to imaging.

DDR1 levels with N-DDR1/albumin ratio, FIB4 test, and TE were 0.963 ($p \leq 0.001$), 0.380 ($p \leq 0.001$), and 0.453 ($p \leq 0.001$), respectively. The Spearman coefficient for the correlation of the N-DDR1/albumin ratio with the FIB4 test, and TE was 0.442 ($p \leq 0.001$) and 0.516 ($p \leq 0.001$), respectively.

Moreover, serum N-terminal DDR1 levels and N-DDR1/albumin ratio were independent of sex (median N-DDR1 levels [IQR]: male vs. female: 2,029.2 pg/mL [1,320.0; 2,642.2] vs. 2,219.5 pg/mL [1,375.9; 2,219.5], $p=0.623$; median N-DDR1/albumin ratio [IQR]: 51.6 [33.5; 67.1] versus 52.9 [32.6; 66.4], $p=0.819$). Additionally, the N-terminal DDR1 levels and N-DDR1/albumin ratio were independent of BMI category. In total, 65.8% ($n=196/298$) of patients had normal weight (BMI <25), 22.5% ($n=67/298$) were overweight ($25 \leq BMI \leq 30$), and 11.7% ($n=35/298$) conformed to the World Health Organization obesity criterion (BMI>30).[28] No significant difference was found among median N-terminal DDR1 levels (i.e. IQR) in normal weight, overweight and obese patients with corresponding values of 2,215.8 pg/mL (1,380.0; 2,688.4), 2,146.4 pg/mL (1,283.9; 2,544.1), and 1,938.6 ng/mL (1,262.9; 2,583.8), respectively ($p=0.687$). The median N-DDR1/albumin ratio (i.e. IQR) in normal weight, overweight and obese patients was 52.6 (33.5; 67.5), 50.7 (31.9; 63.1), and 51.6 (32.4; 66.5), respectively ($p=0.756$).

Discussion

DDR1 was identified as an attractive antifibrotic target that plays a vital role in LF.[29] Recent research focused on the diagnostic value and accuracy of DDR1 as a biomarker for

significant, advanced LF and cirrhosis compared to FIB4 and TE. The present study confirmed serum N-terminal DDR1 as an accurate biomarker of liver cirrhosis. Its diagnostic accuracy could be further increased by calculating the DDR1/albumin ratio, achieving an AUC of 0.879 for the diagnosis of F 4. Moreover, an AUC of 0.790, 0.802, 0.879, and 0.865 was achieved corresponding to histological fibrosis stages F ≥2, F ≥3, F 4 with liver biopsy as a reference method, and cirrhosis according to imaging techniques, respectively. With a cut-off of 55.6, a sensitivity, specificity, positive predictive value, and negative predictive value of 82.7%,76.6%, 67.4%, and 88.3% for the detection of cirrhosis was achieved. Notably, the serum N-terminal DDR1 level and DDR1/albumin ratio were independent of sex and BMI.

Transformation from healthy to pathological tissue may cause the ECM to become stiff, promoting myofibroblast activation and collagen deposition.[30] Collagen is the most abundant ECM component in the human body, and type I–V collagens can stimulate DDR1 activation.[20,21] Type I collagen, which is essential for the interstitial matrix structure, is predominantly produced by fibroblasts. According to the literature, the analysis of ECM components in patients with mild to moderate or severe LF showed a significant increase in type I collagen.[31–33] A previous study had shown that type I collagen promoted the shedding of DDR1 and the release of N-terminal fragments.[24] Therefore, type I collagen was used as a trigger to study the phenomenon of DDR1 shedding at the extracellular fragments in this study, which is schematically depicted in the accompanying graphic abstract. Similar to previous studies, the phenomenon of ectodomain shedding was confirmed in hepatocytes. Moreover, the shed fragment was also found in the serum. Indeed, a previous study demonstrated that a low level of DDR1 shedding occurs constitutively.[34] The present study validated collagen-induced DDR1 shedding in HEK293T cells transiently transfected with a DDR1 expression vector. Furthermore, it was found that the concentration of extracellular DDR1 fragments in the culture supernatant increased with the stimulation time after treatment with collagen I at 0, 2, 4, 6 and, 8 h, respectively. Subsequently, the cells for 8 h were 0, 25, 50, 75 μg/mL of collagen I. The results showed that as the collagen concentration was increased to 50 μg/mL, the extracellular DDR1 concentration was also increased. The previously reported saturation of collagen-stimulated DDR1 shedding can explain this phenomenon.[24] However, there was no similar phenomenon in the intracellular fragment of DDR1, which may be related to the strict regulation of the RTK family, because uncontrolled RTK activity leads to tumorigenesis.[24] The ligand-RTK complex is subject to endocytosis on the cell surface, which leads to the dissociation of the RTK from the ligand in the endocytic vesicle.[35–37] The ligand-receptor or both are subsequently degraded inside the cell to effectively terminate signaling.[35,37]

To investigate whether the shed extracellular DDR1 fragments can be detected in the blood, CCl4- and BDL-induced hepatic fibrosis models were established in mice. An obvious deposition of collagen I in the ECM of the liver was found, which is associated with the increase of LF. At the same time, the concentration of DDR1 in the serum of the mice concurrently increased with the degree of fibrosis. An exploratory study of clinical samples using immunohistochemistry also demonstrated the concurrent increase of collagen deposition and the serum levels of N-DDR1 with the aggravation of liver cirrhosis.

Activated fibroblasts are common cellular effectors of excessive fibrous ECM deposition in organs such as the lung, liver, kidney, and skin.[38] Furthermore, the expression of DDR1 is mainly limited to the epithelial cells of the aforementioned organs. Previous studies have shown that DDR1 plays a crucial role in the pathogenesis of renal fibrosis and glomerulosclerosis, which was further confirmed by *in vivo* experiments.[29] Researchers observed increased expression of DDR1 in bronchoalveolar lavage cells of patients with idiopathic pulmonary fibrosis.[39] Recently, single-cell sequencing of bronchial epithelial cells revealed increased expression of DDR1 in idiopathic pulmonary fibrosis patients, and the DDR1 small molecule inhibitor CQ-061was found to have antifibrotic and anti-inflammatory effects in bleomycin-induced idiopathic pulmonary fibrosis mice.[40] Consequently, patients with tumor, common kidney and lung diseases, osteoarthritis and atherosclerosis, or patients with a history of the above diseases were excluded. In this study, according to the medical history and physical examination results, 18 cases of chronic lung disease and 30 chronic kidney disease cases were excluded. The present study included 298 patients with chronic liver disease and 20 normal controls. A test cohort comprising 210 cases and health personnel was first investigated, followed by a validation cohort of 68 cases to confirm our findings. The results showed that the serum N-DDR1 levels and N-DDR1/albumin ratios were positively correlated with FIB4 and TE. The results of Spearman correlation statistical analysis showed that the degree of LF was correlated with serum N-DDR1 and DDR1/Alb ratio. The AUC confirmed that the serum N-DDR1/albumin ration and FIB-4 had similar diagnostic efficacy. Similar to DDR1, the receptor tyrosine kinase sAxl was reported to be a predictor of LF. However, DDR1 had better diagnostic effect than sAxl.[41] Previous studies have shown that TE was greatly affected by body weight, and obesity increased the difficulty of monitoring, affecting the accuracy of diagnosis.[41] However, body weight was not found to affect the serum DDR1/albumin ration in this study. Its repeated applicability and low cost are suitable as a screening parameter for advanced LF and cirrhosis, especially when TE is not available or applicable.

There are still some limitations in the existing research. The process of shedding and its significance are not yet entirely clear;[26] this study only demonstrated a correlation between shedding and serum N-terminal DDR1 levels. DDR1 is widely expressed in epithelial cells of the skin, lung, liver, kidney, intestine, colon, and brain. Regrettably, none of the available models include markers completely specific for LF, instead also reflecting hepatocyte damage or necrotic inflammatory activity and not only fibrosis.[42] Furthermore, due to the low rate of early screening and physical examination, the clinical specimens used in this study were mainly cirrhosis samples. Therefore, similar to the previous studies,[43] the best outcome was observed when differentiating fibrosis levels between patients with minimal or no fibrosis and patients with advanced fibrosis or cirrhosis, but the accuracy of diagnosing intermediate fibrosis was relatively poor. We tried to construct two different LF models to verify collagen-induced DDR1 shedding. However, most of the clinical samples used in this study were affected by hepatitis B virus-induced LF, which is common in Asians. Therefore, larger sample size is still needed for further validation.

In conclusion, this study indicated that serum N-terminal DDR1 levels could be used as a serological marker for diagnosing LF. Compared with invasive liver biopsy, serological markers have the advantages of inducing less damage, while being low cost and easy to repeat measurements. In practice, serum markers are often used in combination with other biomarkers or methods. Overall, serum N-terminal DDR1 may be an innovative serological diagnostic marker for LF.

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Conflict of interest

The authors have no conflict of interests related to this publication.

Author contributions

Study conception and design (WZ, ZhaZ, YuxZ), acquisition of data (YuxZ, YujZ), analysis and interpretation of data (YuxZ, ZhuZ, PF), drafting of the manuscript (YuxZ, YujZ), critical revision of the manuscript for important intellectual content (WZ, ZhaZ, HL), administrative, technical, or material support, study supervision (WZ, YC, HL).

Data sharing statement

All data are available upon request.

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Original Article

Development of a Novel Endovascular Brachytherapy Stent: A Proof-of-concept Study

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Abstract

Background and Aims: Endovascular implantation of iodine-125 (^{125}I) seeds strand combined with stent is an effective method of treatment for portal vein tumor thrombosis. The aim of this study was to develop a novel endovascular brachytherapy stent (EVB-Stent) and to evaluate its feasibility of use. ***Methods:*** An EVB-Stent was implanted into the main portal vein (MPV) in a live porcine model via the percutaneous transhepatic route. Blood samples were collected and tested before and after operation, as well as before euthanasia. Single-photon emission computed tomography (SPECT) combined with CT (SPECT/CT) scan were performed directly after operation and CT scan was performed 2 months after implantation. After the CT scan was performed, all animals were euthanized and histologically examined. ***Results:*** The novel stent was successfully positioned in all six pigs. No deterioration of liver function was observed during the 2-month follow-up period. SPECT/CT revealed the uniform distribution of radiation around the seeds strand, and the hottest spot was near the center of the MPV. The patency of the stented MPV was confirmed using CT scans. The tissue-accumulated absorbed dose was 31,822.11 mGy at 10 mm transversely away from the midpoint of the ^{125}I seeds strand, with a half-life of 59.4 days. Pathological examination results showed no significant atrophy or inflammation of adjunct liver tissue, and no obvious intima thickening or thrombosis were detected in the stented MPV. ***Conclusions:*** A liver porcine model was used to demonstrate that the transhepatic placement of a novel endovascular brachytherapy stent, EVB-Stent, is both technically feasible and safe.

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Keywords: Portal vein tumor; Tumor thrombus; Brachytherapy; Stent; Iodine-125 seeds strand.

Abbreviations: ^{125}I, iodine-125; EBRT, external beam radiotherapy; EVB, endovascular brachytherapy; HCC, hepatocellular carcinoma; MPV, main portal vein; MPVTT, main portal vein tumor thrombus; NBCA, n-butyl-2-cyanoacrylate; PVSI, portal vein stent implantation; PVTT, portal vein tumor thrombus; SPECT, single-photon emission computed tomography; TACE, transarterial chemoembolization.

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Introduction

Hepatocellular carcinoma (HCC) is the 7th most common malignancy and the fourth leading cause of cancer-related death worldwide.[1,2] Approximately 10–40% of HCC patients suffer portal vein tumor thrombus (PVTT) complication at the time of diagnosis.[3] Based on Barcelona Clinic Liver Cancer staging, sorafenib is the only evidence-based and recommended treatment option for patients with vascular invasion (stage C). However, several studies have shown only modest clinical efficiency when sorafenib monotherapy has been provided for HCC patients with PVTT.[4–6] In recent studies, transarterial chemoembolization (TACE) combined with sorafenib or radiotherapy has shown survival benefits for HCC patients with PVTT.[7–9] Although survival benefits have been provided through the adoption of these combinations, the survival duration of HCC patients with PVTT remains poor, which may be attributed to the obstructed blood flow in the PV. Owning to compromised PV blood supply, the interruption of hepatic arterial flow may result in severe ischemic liver damage.[10] Therefore, HCC patients with PVTT are generally considered as contraindicated for TACE.

In China, HCC patients with PVTT often receive treatment through multiple methods, such as surgery, external beam radiotherapy (EBRT), chemotherapy, and iodine-125 (^{125}I) seeds strand and stent implantation.[11] A recent multicenter study proved that patients with limited PVTT could benefit from liver resection.[12] However, patients with main PV tumor thrombus (MPVTT), can barely meet the requirements for liver resection. A systematic review and meta-analysis compared TACE plus EBRT with TACE alone for unresectable HCC.[13] The results of the study indicated that the pooled median survival for TACE plus EBRT-treated patients was significantly better than of TACE alone-treated patients. However, the pooled analysis also showed that compared with TACE alone, TACE plus EBRT significantly increased the occurrence of gastroduodenal ulcers, as well as elevated levels of alanine aminotransferase and total bilirubin. Since normal hepatic tissues show poor tolerance to radiation, it is also impos-

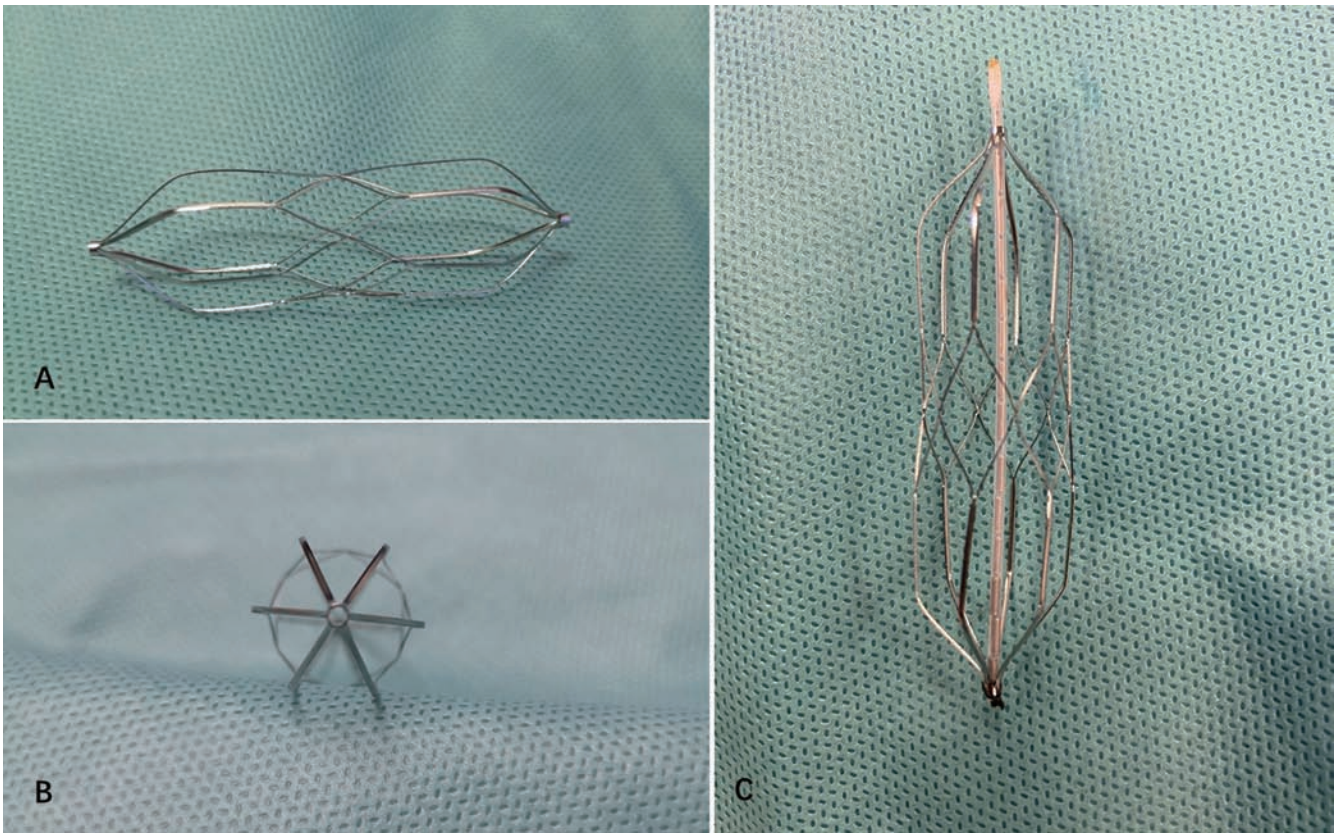

Fig. 1. Configuration and structure of the EVB-Stent system. (A) The EVB-Stent was made using a nickel titanium alloy and consisted of a cylindrical mesh structure with two rings at its two ends. Its length of its axis was 60 mm, with a diameter of 18 mm. (B) At the two tapered ends of stent, an ^{125}I seeds strand, which was a 4-Fr sterile plastic tube containing ^{125}I seeds, was inserted through the two rings and fixed to the stent using sterile sutures. (C) After assembly was completed, the novel stent equipped with an ^{125}I seeds stand was known as an EVB-Stent.

sible to increase the external irradiation dose provided for the treatment of PVTT. Endovascular brachytherapy (EVB) using ^{125}I seeds strand implantation could offer an adequate dose of radiation to exert a tumor killing effect with subtle damage during irradiation, leading to the relative protection of healthy tissues.[14] Animal models with vascular tumor thrombus have been established and employed to demonstrate the safety and efficacy of ^{125}I seeds strand in exerting an antitumor effect.[15,16] For patients with MPVTT, estimated overall survival was only 2 to 4 months.[17] Our previous studies have also established that TACE combined with ^{125}I seeds strand and portal vein stent implantation (PVSI) can prolong the overall survival of HCC patients with MPVTT.[18–21] Moreover, this method has been widely used for HCC patients with MPVTT in many tertiary hospitals in China.

Due to the eccentric location of ^{125}I seed strands in MPV, the uneven distribution of radiation in the PV was a potential deficiency that could compromise the efficacy of endovascular radiotherapy. Moreover, the usage of a higher dosage of ^{125}I seeds strand was restricted due to the close association to the vascular wall. Hence, even after undergoing ^{125}I seeds strand combined with stent MPV implantation, some patients may still suffer from re-occlusion of the PV owing to tumor thrombus progression. To further improve the antitumor effect of EVB using PV stent and ^{125}I seeds strand insertion, we introduced an innovative EVB-Stent, which is a biconical stent with an ^{125}I seed strand attached coaxially at the center of the stent. The aim of this study was to develop and evaluate the technical feasibility and safety of the EVB-Stent.

Methods

Assembling of the EVB-Stent

A self-expanding Nitinol stent (Hongpu Medical Device Corporation, Shanghai, China) with a mesh-like structure, was tapered into two rings at the proximal and distal ends. The stent used in this study was 18 mm × 60 mm in size. A 4-Fr sterile plastic tube (Boston Scientific Co., Marlborough, MA, USA) containing ^{125}I seeds was inserted through the rings at both ends and the seeds strand was fixed to the stent using sterile sutures (Fig. 1). The stent, once equipped with the ^{125}I seeds stand, was known as an EVB-Stent.

As described in our previous study,[19] Model 6711 ^{125}I seeds (XinKe, Shanghai, China) were encapsulated in a 4-Fr sterile plastic tube (Boston Scientific Co.) to assemble the seeds strand. The radioactivity of each ^{125}I seed was 25.9 MBq, with a half-life of 59.4 days. The principal photon emissions were 27.4–31.4 keV X-ray and 35.5 keV γ-ray. The half-value thickness of tissue for ^{125}I seeds was 17 mm, and the initial dose rate was 7 cGy/h. The effective irradiating range was 20 mm. The number of ^{125}I seeds was determined by the length of the plastic tube (L mm; N = L/4.5). In this study, the number of ^{125}I seeds used in each strand was 14.

Table 1. Comparison of laboratory test results pre-procedure and post-procedure

	Preoperative	One-week post-intervention	Two-month post-intervention	*p*
RBC as $\times 10^{12}$/L	7.2±0.3	6.7±0.6	7.0±0.5	0.261
HB as $\times 10^{9}$/L	116.8±9.4	107.8±13.9	111.3±10.3	0.352
WBC as $\times 10^{9}$/L	11.7±2.2	13.9±2.2	11.0±1.6	0.074
PLT as $\times 10^{9}$/L	514.2±82.8	507.1±77.4	513.8±67.3	0.984
TB in μmol/L	9.7±0.4	10.4±0.6	10.2±0.5	0.079
DB in μmol/L	6.5±0.4	7.2±0.5	7.0±0.5	0.053
AST in U/L	37.2±1.9	38.0±1.9	38.2±2.3	0.655
ALT in U/L	44.0±2.4	45.2±2.1	46.5±2.8	0.240
ALB in g/L	23.6±1.6	22.9±1.4	22.8±0.8	0.508

ALB, albumin; ALT, alanine aminotransferase; AST, aspartate aminotransferase; DB, direct bilirubin; HB, hemoglobin; PLT, platelet; RBC, red blood cell; TB, total bilirubin; WBC, white blood cell.

Animals

The animals selected for the experiment were six ordinary white pigs (weight 35–40 kg) provided by the Experimental Animal Center of our hospital. This study was approved by the institutional Animal Ethics Committee of our hospital. The animals were fasted for 12 h before operation. Intramuscular injection of xylazine hydrochloride (2–4 mg/kg) and diazepam (2 mg/kg) were used for sedation and anesthesia, respectively. Blood pressure, heart rate, and respiration rate were monitored during the procedure.

Interventional procedures

Under fluoroscopic and ultrasound guidance, the right intrahepatic PV was punctured using a 21G Chiba needle (Cook Medical Inc., Bloomington, IN, USA) and a 0.018-inch wire (Cook Medical Inc.) was inserted into the PV. A 6-F Neff Percutaneous Access set (Cook Medica Inc.) was introduced into the PV over the wire. Through the outer cannula of the Neff Percutaneous Access set, a 0.035-inch, 150 cm-long wire (Terumo, Tokyo, Japan) was manipulated across the MPV into the superior mesenteric vein, followed by the insertion of a 4-F pigtail catheter (Cook Medical Inc.). PV venography was performed via the pigtail catheter. After venography, the catheter was removed, and the outer cannula of the Neff Percutaneous Access set was replaced by a 7-Fr, 23 cm-long sheath (Cordis, Hialeah, FL, USA) over the wire. After 100 U/kg heparin (XinYi, Shanghai, China) was administered through the sheath, and the novel EVB-Stent was loaded into the sheath and pushed into the target main PV. The stent was deployed from the distal MPV into the proximal patent intrahepatic PV under fluoroscopic guidance. PV venography was repeated through the pigtail catheter to confirm the appropriate location and patency of the EVB-Stent. Finally, the transhepatic puncture track was occluded using n-butyl-2-cyanoacrylate (NBCA) (Compont, Beijing, China).

Radiation safety protection measures were followed by physicians during the ^{125}I seed preparation and PV implantation. Lead protective gloves were provided to animal house workers during the follow-up period. At the end of the study, the ^{125}I seeds were restored and processed properly.

Post-procedure management

After implantation, 100 U/kg heparin was subcutaneously administered once a day for 2 months. The animals were raised by conventional methods and monitored for the loss of appetite, vomiting, diarrhea, and weight loss after implantation. Blood samples were collected before implantation and at 1 week and 2 months after implantation, to determine liver function and blood toxicity. Single photon emission computed tomography (SPECT) combined with CT (SPECT/CT) scan was performed to evaluate the radiation distribution emitted by the ^{125}I seeds strand immediately after implantation.[21] Tissue accumulated absorbed doses 10 mm from the midpoint of the ^{125}I seeds strand were theoretically calculated using ^{125}I Radiation Field Distribution Calculation Software.[16] CT scan was performed at 2 months post operation, and three-dimensional (3D) reconstructions were made to evaluate the patency of the stented PV. The contrast agent used was Ultravist Injection (300 mgI/mL). Intravenous access was through the femoral vein, and the dose of the contrast agent was 80 mL (2.0 mL/kg) on average, at an injection rate of 4 mL/s, delay time of arterial phase enhancement of 15 s, and a portal venous phase enhancement delay time of 50 s. After CT scans were performed, the animals were sacrificed through intravenous injection of potassium chloride and necropsied, immediately. The stented MPV and adjacent organ tissue were harvested and fixed in 10% formalin for pathological examination. Hematoxylin and eosin staining were performed, to evaluate adjunct liver tissue change and to evaluate thrombus organization within the stented PV and neointima coverage of the PV.

Statistical analysis

Statistical analysis was performed using SPSS 22.0 (IBM Corp., Armonk, NY, USA) software. One-way analysis of variance was used to compare differences in hematological indices between the different time points. Measurement data are presented as mean±standard deviation. A two-sided *p*-value of <0.05 was considered statistically significant.

Results

The EVB-Stent was successfully implanted into the MPV of all six pigs without major complications. After stent placement, venography of the PV confirmed the proper location of the EVB-Stent in the MPV. All animals were in good condition without a loss of appetite, bleeding, weight loss, or death. Hematological indices taken throughout the examination period were summarized, and showed that no significant liver function deterioration or blood toxicity were detected (Table 1).

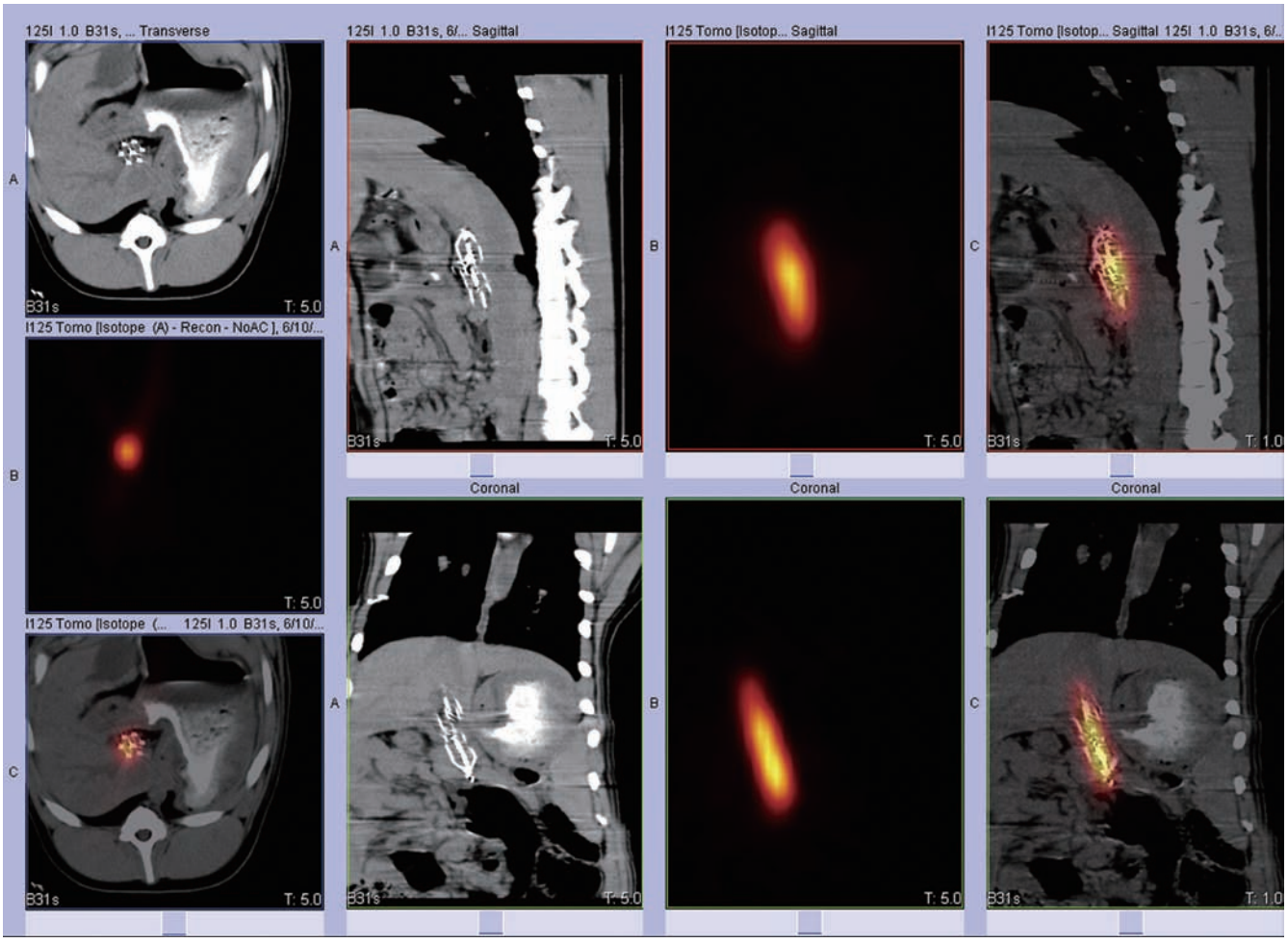

Fig. 2. Images obtained by performing a SPECT/CT scan 1 day after the procedure was performed. The EVB-Stent was implanted correctly in the MPV without displacement. The radiation emitted by the ^{125}I seeds was homogeneously distributed and completely covered the target lesion.

The SPECT/CT scan showed that the EVB-Stent had been correctly placed in the MPV without collapse, occlusion, or over-expansion. Radiation emitted by the ^{125}I seeds strand was distributed symmetrically in the MPV, and presented as a cylindrical shape that completely covered the targeted MPV (Fig. 2). Based on the ^{125}I Radiation Field Distribution Calculation software, the tissue accumulated absorbed dose was 31,822.11 mGy 10 mm transverse from the midpoint of the ^{125}I seeds strand, with a half-life of 59.4 days (Fig. 3). The three-dimensional reconstruction CT image showed that the stented vessel remained in position and that the ^{125}I seeds strand was tightly fixed to the center of the stent (Fig. 4). Additionally, no malposition, collapse, thrombosis, or stenosis occurred in the stented vessels, when observed using CT scans.

Necropsy and pathological examination results showed that none of the stents were covered or occluded at the two tapered ends by neointimal overgrowth in all six pigs (Fig. 5A). However, slight intimal hyperplasia and incomplete neointimal growth covered the tapered end near the hilar side of the stent in one pig (Fig. 5B). Hematoxylin-eosin staining revealed that the central grid of the EVB-Stent and even the densest part of the stent mesh were almost completely covered by neointimal growth, which resulted in the proper incorporation of the EVB-Stent, and its surface was smooth and free from tears, peelings, or injuries (Fig. 5C–D). No obvious abnormalities were found in nearby organs, including the liver parenchyma, duodenal wall, and pancreas, as examined through gross observations and pathological analysis (Supplementary Fig. 1).

Discussion

In this study, we demonstrated the technical feasibility of the percutaneous transhepatic placement of a novel radioactive stent, the EVB-Stent, in the MPV of a live porcine model. Furthermore, during the 2-month follow-up period, the stented PV remained in position in all cases, with minimal neointimal growth covering it. All these results present evidence that the EVB-Stent has the potential to be used for further clinical exploration.

PVTT is a prognostic factor for poorer overall survival among patients with HCC. Stent implantation promptly restores blood flow in the obstructed MPV and provides an opportunity for TACE to be applied for tumor lesions. However, in-stent stenosis can occur due to tumor growth and/or tumor thrombosis.[22] EVB along with ^{125}I seeds implantation, which can inhibit and prevent the progression of tumor thrombosis, has been provided an option to prolong stent patency.[21,23] In a previous study, better overall response rate as well as a significantly favorable level of survival

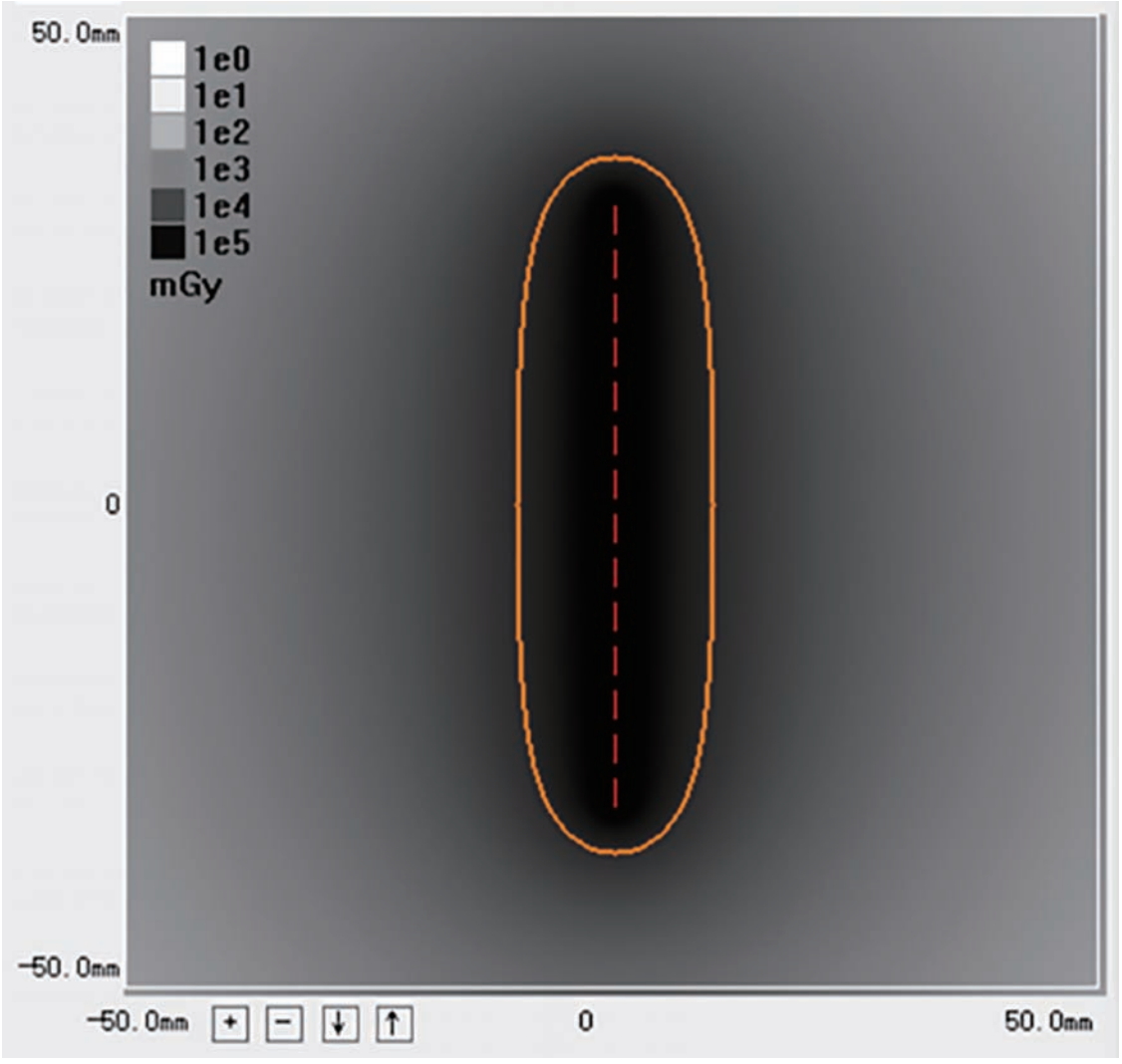

Fig. 3. Calculation of the accumulated absorbed dose presented by the ^{125}I seeds strand. The ^{125}I Radiation Field Distribution Calculation software showed the tissue accumulated absorbed dose 10 mm transversely away from the midpoint of the ^{125}I seeds strand. The yellow ellipses show the 31,822.11 mGy isodose curve.

were observed in patients who received TACE combined with EVB with ^{125}I seeds strand and stent placement.[24–26] Numerous studies have demonstrated that stent devices that are combined with EVB exert therapeutic efficacy in PVTT, unresectable malignant esophageal and biliary obstruction.[21,27,28] The eccentric distribution of the ^{125}I seeds strand may cause the delivery of an insufficient radiation dose to the contralateral blood vessel wall, thus compromising the therapeutic efficacy. Moreover, the eccentric distribution of the ^{125}I seeds in the MPV may limit the maximum dose of radiation delivered. Hence, we constructed a novel EVB-Stent to further improve the therapeutic efficacy of brachytherapy using ^{125}I seeds for MPVTT by improving the radiation dosage delivered. The ^{125}I seeds were presented as a sequential string coaxially at the center of the EVB-Stent, and the radiation emitted by the ^{125}I seeds was homogeneously distributed and covered the entire stent canal. To examine the safety and feasibility of the novel radioactive stent, we conducted an animal study through the percutaneous transhepatic PV deployment of the EVB-Stent in a live porcine model.

Previous experimental studies have proven that transhepatic puncture and catheterization in a porcine model can be technically feasible without bleeding complications.[29] In our study, ultrasound was used to assess intrahepatic vascular dissection and provided clear guidance for PV puncture. Moreover, owning to fine needle puncture, no bleeding or hematoma was observed after operation. A 7-Fr long sheath was used to create a transhepatic track and the EVB-Stent was released through the long sheath. The EVB-Stent could be easily introduced into the PV without any technical difficulties or challenges. Our previous experience showed us that the procedure used for the implantation of the ^{125}I seeds strand combined stent was complicated to some extent.[21] The outer cannula of the Neff set can be difficult to be delivered to the obstructed MPV and the release of the ^{125}I seeds strand can be complicated if the stent has already been inserted. In this study, the ^{125}I seeds were arranged linearly and continuously sealed into a 4-Fr sterile catheter to construct an ^{125}I seeds strand. Then, the strand was fixed at the center of the stent. Compared with previously used techniques, the transhepatic MPV deployment of the EVB-Stent performed in this study was relatively simple. Hence, we demonstrated the techni-

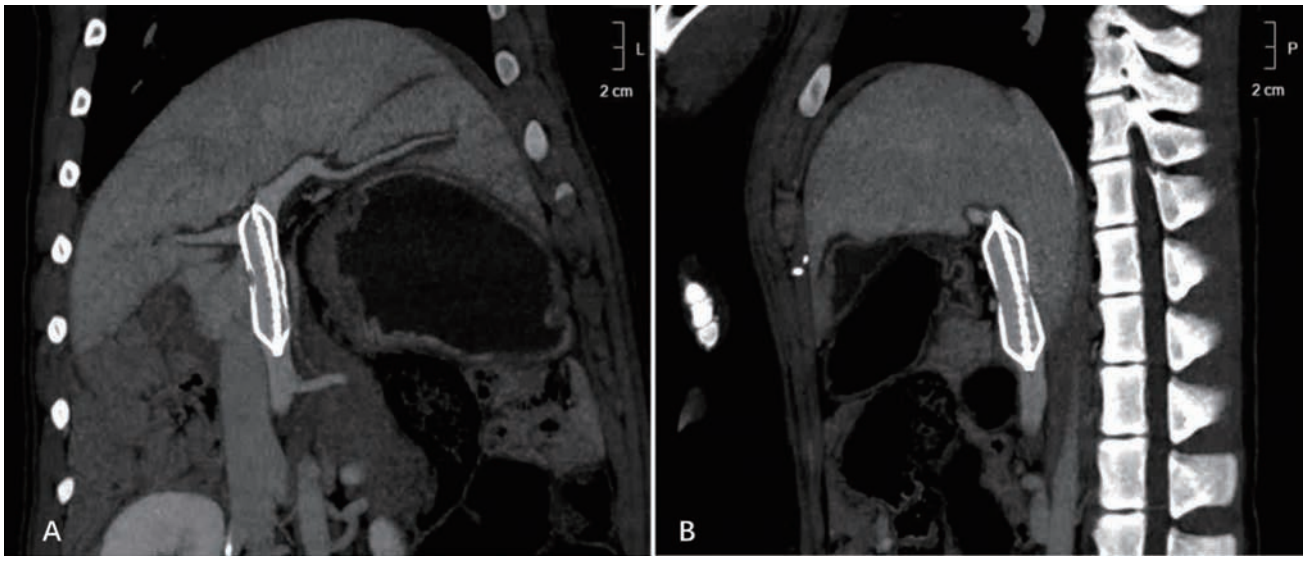

Fig. 4. CT images obtained 2 months after the operation was performed. Reconstruction of the CT image showed that the EVB-Stent expanded completely in the MPV, the ^{125}I seeds strand was fixed tightly at the center of the stent, and no thrombus was found in the entire PV.

cal feasibility of the transhepatic PV placement of an EVB-Stent into a pig model. However, its manipulative feasibility for HCC patients with MPVTT remains to be elucidated through clinical trials.

In this study, 14 ^{125}I seeds were encapsulated in a 4-Fr sterile plastic tube to create a seeds strand and then fixed at the center of the stent. SPECT/CT showed that the radiation emitted by the ^{125}I seeds was uniformly distributed in the PV. The overall isodose of radiation can exert an elongating tumor killing effect and minimize the irradiation of the surrounding normal tissue. Moreover, the potentially irregular and asymmetric radiation emitted by the ^{125}I seeds

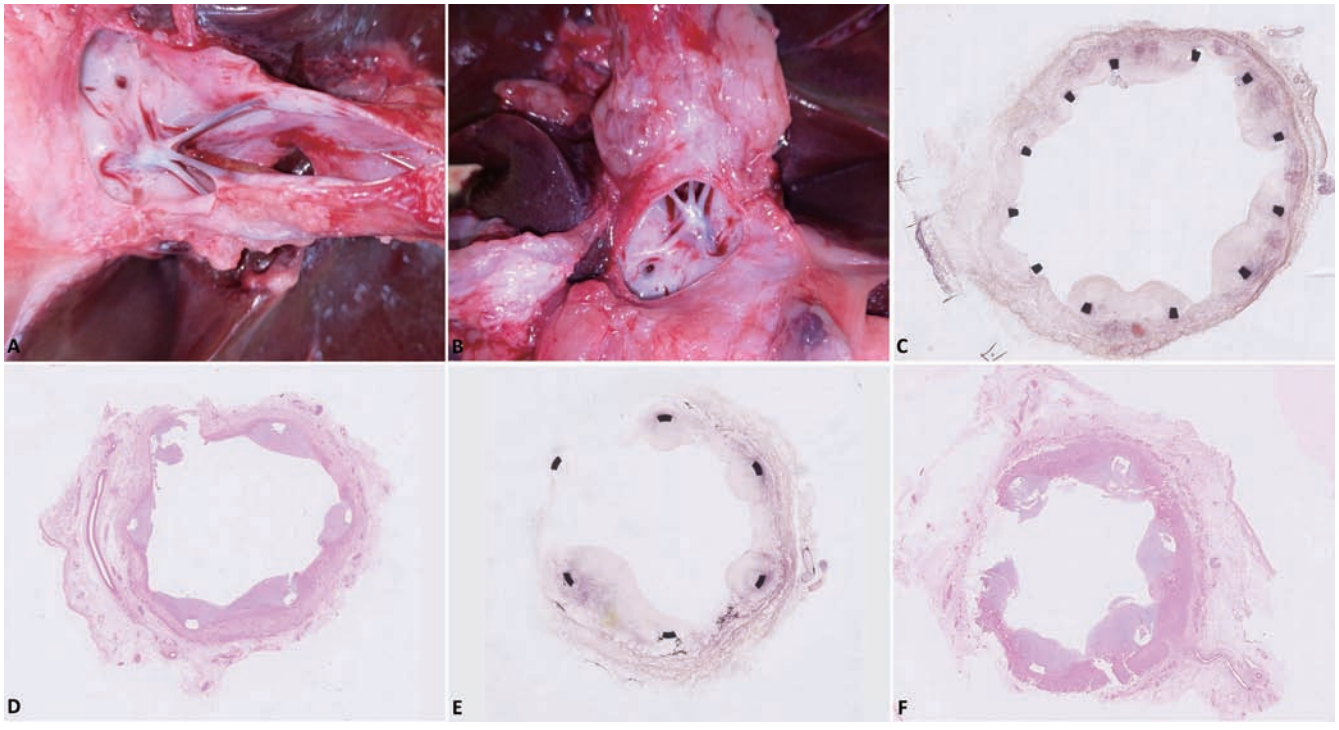

Fig. 5. Necropsy and pathological examination results obtained 2 months after the procedure. (A) There was no evidence of thrombosis in the stented portal vein and on both sides of the stent. The ^{125}I seeds strand was completely covered by neointima, without narrowing or occlusion. (B) In one pig, slight intimal hyperplasia and incomplete neointimal cover was observed at the tapered end of the YZP-Stent near the hilar end. (C, D) Hematoxylin and eosin staining showing the central grid section of the YZP-Stent and the densest section of the stent mesh was also almost completely covered by neointimal growth, which shows the proper incorporation of the YZP-Stent. Its surface was smooth and free from tears, peelings, or injuries. (E, F) At the tapered end of stent near the hilar end, necropsy and pathological imaging showed that there was a slight intimal hyperplasia but intimal overgrowth or neointimal growth that completely covered the stent was not observed.

strand for the eccentric location in the PV can be avoided and the potential heterotopia of implanted ^{125}I seeds strand can also be avoided. None of the animals were found to show signs of PV thrombosis after EVB-Stent placement during the 2-month follow-up period. On one hand, proper anticoagulant therapy with heparin is of great importance in preventing thrombosis. On the other hand, the anti-neointimal hyperplasia effect provided by the implantation of the ^{125}I seeds strand[30] allowed for a longer patency period to be achieved by the MPV stent. Moreover, the central location of the ^{125}I seeds strand in the stented PV contributed to full conformal radiotherapy implementation on the MPV. Hence, incomplete neointimal hyperplasia coverage of the stent was observed but without thickening or resulting in PV stenosis after 2 months of deployment of the EVB-Stent.

Studies have established that persistent low-energy ^{125}I irradiation therapy may keep tumor cells in the sensitive resting period, resulting in tumor cell apoptosis, which can induce epigenetic changes that reactivate silenced tumor suppressor genes, and damage to the DNA to kill cancer cells.[14,31] For end-stage patients, the combination of EVB and PV stent implantation may not only provide a long period in which symptoms of portal hypertension are relieved, but may also suppress the progression of tumor thrombus. Furthermore, compared with external radiotherapy, brachytherapy using ^{125}I seeds provides a high local dose close to the seeds and a steep fall in the dose provided to the surrounding tissues, which exerts an adequate tumor suppression effect with limited damage exerted onto the surrounding normal tissue.[15] Since ^{125}I seeds have a long half-life (59.4 days), a sustained level of radiation can be exerted to inhibit the replication of tumor cells and induce tumor cell apoptosis.[32] In this study, the EVB-Stent, which was used as a novel endovascular brachytherapy stent kit, provided three obvious advantages compared with its current usage. First, it may induce tumor cell apoptosis more effectively and inhibit the progression of tumor thrombus, since the gamma rays are evenly distributed at the center of the obstructed PV. Second, the support provided by the stent resulted in the ^{125}I seeds strand being firmly fixed at the center of the stent without displacement. Third, the central deployment of the radiative strand provided an opportunity to further improve the antitumor effect by increasing the dosage provided by the ^{125}I seeds without obvious damage to the PV wall.

There are several limitations in our study. First and foremost, only six pigs were used, which may introduce a case-by-case bias. Second, the use of healthy animals without tumor thrombus or portal hypertension can barely reproduce the complex environment in a real human diseased vessel in which a stent must be implanted. Finally, long-term results of the efficacy and safety of the EVB-Stent transhepatic PV implantation, as well as its impact on the vessel wall over longer periods of time, are still pending. The creation of an ideal animal model with PVTT remains a daunting challenge but is urgently required to demonstrate the antitumor efficacy and safety of the transhepatic PV placement of the EVB-Stent.

Conclusions

Mid-term preclinical results demonstrated the feasibility and safety of the percutaneous transhepatic MPV implantation of a novel EVB-Stent into a live porcine model. The implantation of the stent did not produce thrombosis or stenosis. Further studies using large samples of animals with or without PVTT are needed to further assess the efficacy and safety of this innovative stent before it can be considered suitable for clinical application.

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Conflict of interest

The authors have no conflict of interests related to this publication.

Author contributions

Study concept and design (JL, ZY), acquisition of data (ND, JM, YZ), analysis and interpretation of data (MY, WZ, ZZ), obtained funding (ZY), drafting of the manuscript (ND, JM), critical revision of the manuscript for important intellectual content (MY, WZ), administrative, technical, or material support, study supervision (JL).

Data sharing statement

All data are available upon reasonable request.

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Review Article

Alcohol and Metabolic-associated Fatty Liver Disease

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Abstract

The diagnosis of metabolic-associated fatty liver disease is based on the detection of liver steatosis together with the presence of metabolic dysfunction. According to this new definition, the diagnosis of metabolic-associated fatty liver disease is independent of the amount of alcohol consumed. Actually, alcohol and its metabolites have various effects on metabolic-associated abnormalities during the process of alcohol metabolism. Studies have shown improved metabolic function in light to moderate alcohol drinkers. There are several studies focusing on the role of light to moderate alcohol intake on metabolic dysfunction. However, the results from studies are diverse, and the conclusions are often controversial. This review systematically discusses the effects of alcohol consumption, focusing on light to moderate alcohol consumption, obesity, lipid and glucose metabolism, and blood pressure.

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Introduction

In 2020, the definition of metabolic-associated fatty liver disease (MAFLD) was proposed by Eslam *et al*.[1] Since then, clinical practice guidelines on MAFLD have been published by the Asian Pacific Association for the Study of the Liver.[2] An important significance of this definition is the "positive" criteria for the diagnosis of MAFLD, in contrast to a diagnosis of exclusion. More importantly, it is possible to diagnose MAFLD coexisting with liver injury caused by other reasons. The diagnosis of MAFLD is based on the detection of liver steatosis together with the presence of metabolic dysfunction, such as overweight or obesity, type 2 diabetes mellitus (T2DM), or clinical evidence of metabolic risk abnormalities.[1] The absence of alcohol intake limit is the prominent difference between the diagnostic criteria of MAFLD and the previous diagnostic criteria of non-alcoholic fatty liver disease. As is well known, a lack of ongoing or current consumption of significant amounts of alcohol was an important indicator in the latter.[3] However, the diagnosis of MAFLD is independent of the amount of alcohol consumed. Thus, it is possible to diagnose MAFLD coexisting with alcoholic-related liver disease (ALD).

Alcohol consumption is common in the general population. There are several common drinking patterns, including chronic heavy drinking,[4] light alcohol consumption, moderate alcohol consumption (MAC),[4,5] and binge drinking (Table 1). It has been well accepted that chronic heavy drinking is related with high risk of ALD and should be avoided. Compared with the chronic heavy drinking population, the non-heavy drinking population is much larger. Binge drinking, which is often related with serious social problems and deteriorative health problems, is another popular drinking pattern nowadays, especially among young people. Binge drinking could happen monthly or weekly, but it is different from chronic regular heavy drinking. The prevalence of binge drinking has significantly increased over the past two decades, with an average annual increase of 0.72% per year.[6] Binge drinking can coexist with MAC or regular heavy drinking, inducing antagonistic or synergistic effects.

Alcohol consumption and metabolic dysfunction are two main causes of chronic liver injury and can interact with each other. Early studies showed that MAC might be associated with improved dyslipidemia and reduced cardiovascular risk, indicating MAC may be related with restored metabolism. Several studies were conducted to investigate the role of light to moderate alcohol consumption (LMAC) in metabolic disorders. It has been demonstrated that alcohol and its metabolites have multiple effects on metabolic-associated factors, such as body weight, glucose and lipid metabolism, and the cardiovascular system. However, there is still no consensus on the effects of alcohol on metabolic-related diseases. At the same time, the above-mentioned metabolic abnormalities are the focus of MAFLD. This review systematically discusses the effects of alcohol consumption on obesity, lipid and glucose metabolism, and blood pressure, focusing on the effects of non-heavy alcohol consumption, to help better understand the relationship between alcohol consumption and MAFLD.

Alcohol consumption and overweight/obesity

Effects of alcohol consumption on body weight

There is a higher risk of overweight/obesity in chronic heavy

Keywords: Alcohol; Metabolic-associated fatty liver disease; Obesity; Insulin resistance; Hypertension.

Abbreviations: ADH, alcohol dehydrogenase; ALD, alcoholic-related liver disease; ALDH, aldehyde dehydrogenase; AMPK, 5′-AMP-activated protein kinase; BMI, body mass index; CI, confidence interval; CYP, cytochrome P450; DBP, diastolic blood pressure; HDL, high-density lipoprotein; HR, hazard ratio; IL, interleukin; IR, insulin resistance; LMAC, light to moderate alcohol consumption; MAC, moderate alcohol consumption; MAFLD, metabolic-associated fatty liver disease; mTOR, mechanistic target of rapamycin; mTORC1, mTOR complex 1; OR, odds ratio; SBP, systolic blood pressure; SREBP, sterol regulatory element-binding proteins; T2DM, type 2 diabetes mellitus; TG, triglyceride; TNF-alpha, tumor necrosis factor-α; WC, waist circumference.

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Table 1. Drinking patterns in this review

Drinking pattern		Definition
Chronic heavy drinking		Chronic alcohol consumption (generally more than 5 years) more than 60 g on one occasion[4]
Binge drinking		Alcohol consumption >40 g for women and >50 g for men within about 2 h[1]
Non-heavy drinking	MAC	Regular alcohol drinking <30-42 g/day for men and <20-28 g/day for women[4,5]
	Light alcohol consumption	Regular alcohol drinking <10-20 g/day for most studies

MAC, moderate alcohol consumption.

drinkers, mainly showing as higher body mass index (BMI) and increased waist circumference (WC).[7,8] A previous study showed a 17% higher risk for WC gain in men consuming 1,000 mL/day beer compared with those drinking less than 250 mL/day beer.[9] There seems to be a stronger connection between heavy drinking and increased body weight in men at different ages than in women.[8,10] In elderly men, greater BMI (+4.8%) and WC (+5%) were shown to be related to alcohol intake ≥50 g/day.[8] Energy from alcohol metabolism (7.1 kcal generated by 1 g ethanol) accounts for the increased total energy intake in heavy drinkers, partly contributing to increased body weight and BMI. Studies showed that alcohol intake of more than 3–5 drinks/day can dramatically increase the energy intake from alcohol.[7,8] More importantly, chronic heavy drinking has been proven to induce pancreatic β-cell dysfunction in human and animal models,[11,12] with decreased insulin-secretory ability and disrupted glucose homeostasis. Both increased energy intake and pancreatic β-cell dysfunction contribute to the pathogenesis of obesity in heavy drinkers. Heavy drinking-associated pancreatic β-cell dysfunction may play a more crucial role than increased energy intake in the development of MAFLD.

The effects of occasional binge drinking on body weight may be not obvious in the short term. However, frequent binge drinking could significantly increase the risk of becoming overweight and obese[13] and the risk of abdominal obesity in men.[14] On the one hand, frequent binge drinking has a similar effect as chronic heavy drinking as it involves increased energy intake. On the other hand, binge drinking could induce systemic insulin resistance (IR) by impairing hypothalamic insulin action, manifesting as suppressed hepatic glucose production and white adipose tissue lipolysis.[15] Besides, binge drinking is often accompanied by increased high-fat food intake and even binge eating,[16] indicating a much higher energy intake, thereby increasing the body weight. As a result, the increased energy intake and the glucose and lipid metabolism abnormalities induced by impaired insulin signaling eventually lead to increased body weight.

Clinical studies have shown that moderate drinking may help maintain normal weight and is associated with a lower prevalence of obesity than in non-drinkers,[17,18] showing as lower BMI values (by 1.34 kg/m^2),[19] a lower total abdominal fat volume, and less subcutaneous adipose tissue.[20] Among normal-weight middle-aged and older women, LMAC is associated with smaller weight gain and a lower risk of becoming overweight and/or obese compared to non-drinkers.[21] Similarly, LMAC does not increase the risk of increase in the BMI and WC in elderly men.[8] Moderate wine consumption (150 mL/day), as part of a Mediterranean diet, in persons with controlled diabetes does not promote weight gain or abdominal adiposity.[22] These results indicate a potentially beneficial effect of moderate drinking on maintaining a normal body weight in different populations, a different effect from that of heavy or binge drinking.

LMAC could regulate body weight through several mechanisms. Alcohol tends to increase appetite and food intake, probably through short-time reward effects of food and through the regulation of the expression of various neurotransmitters,[23,24] leading to increased total energy intake. However, the energy obtained from alcohol becomes part of the total daily energy intake in the long term; accordingly, the energy intake, excluding the calories from alcohol, decreases.[21] The reward effects of food gradually weaken and are even offset due to the reduction in total food intake or carbohydrate/fat intake.[23] Therefore, body weight probably does not increase significantly in chronic regular drinkers. Besides, MAC could decrease the body weight by improving IR, an opposite effect compared with heavy or binge drinking, showing as decreased body weight, decreased liver weight and triglyceride (TG) levels, and reduced glycemia and insulinemia in animal models.[25] Hence, LMAC drinkers tend to avoid significant body weight gain, in contrast to heavy drinkers.[26] Changes in body weight could be the result of an imbalance between (i) the regulation of the central nervous system and peripheral insulin function and (ii) energy use. However, the exact underlying mechanisms remain unclear, and more studies are required.

Combined effects of overweight/obesity and alcohol on liver

Heavy and binge drinking are often associated with high risk of fatty liver disease. Overweight/obesity can further promote the development of fatty liver disease.[27,28] Long-term obesity (longer than 10 years), especially abdominal obesity, is an important risk factor for alcoholic-related liver cirrhosis and alcoholic hepatitis[29,30] and is associated with an increased risk of 3-month mortality in alcoholic hepatitis (hazard ratio [HR]: 2.22, 95% confidence interval [CI]: 1.1–4.3).[31] A binge-like drinking pattern is independently associated with significant liver fibrosis progression in overweight/obese patients with MAFLD.[28] These results demonstrate that there are synergistic effects of high alcohol intake and of being overweight/obese on liver injury and an increased risk of fatty liver disease.

LMAC seems to play different roles in fatty liver disease. Studies showed that LMAC reduces the risk of fatty liver disease by 22.6% in general population,[32,33] and it reduces the risk of fatty liver disease by 31.3% in overweight and obese people.[33] Mild liver inflammation and fibrosis with a low risk of advanced liver fibrosis (stage F3/F4) were found in obese patients with MAC, compared with non-drinkers.[34–37] Our previous studies also showed that chronic MAC is related with alleviated liver fibrosis in a high-fat and high-cholesterol diet-induced liver fibrosis model, probably via reduced activation of Kupffer cells and hepatic stellate cells.[38] However, an increased risk of advanced liver cirrhosis in LMAC has been reported. A recent Asian population study showed that MAC reduced the risk of hepatic steatosis in overweight/obese individuals, while MAC increased the risk of advanced liver fibrosis (HR: 1.49, 95% CI: 1.33–1.66), as estimated by the fibrosis-4 index in overweight or obese individuals af-

ter a 15.7-year follow-up.[39] In another cross-sectional study among obese patients with T2DM, LMAC was found to be associated with an increased probability of advanced fibrosis in biopsy-proven MAFLD (odds ratio [OR]: 5.5–9.7, 95% CI: 1.05–69.6).[40] So far, the long-term impacts of LMAC on liver cirrhosis among obese people are still uncertain. More histological evidence is urgently needed to verify the role of LMAC in liver cirrhosis among obese people.

Adipose tissue can serve as another important source of proinflammatory factors that contribute to liver injury. Proteome analysis of serum inflammatory factors showed higher expression of chemokines (C-X-C motif and C-C motif ligands), interleukins (ILs), and tumor necrosis factor-alpha (TNF-α) in obese individuals than in non-obese controls; for example, CXCL 11 was markedly upregulated (by 40%) in obese patients and in adipose tissue in a murine model.[31] In adipose tissue, adipocytes can recruit immune cells (such as macrophages, neutrophils, and lymphocytes) and polarize them to their proinflammatory phenotypes to increase the production of proinflammatory cytokines, such as IL-1β, IL-6, IL-12, and TNF-α, and chemokines, promoting tissue inflammation. Macrophages of the proinflammatory M1 phenotype can induce adipocyte death, increasing the release of inflammatory mediators from adipocytes into the extracellular environment, which could recruit and polarize more macrophages.[41] In obese people, especially those with abdominal obesity, large amounts of subcutaneous adipose tissue and visceral fat could be important sources of inflammatory factors, which may enhance the effects of heavy drinking on the liver, leading to aggravated liver inflammation.

Cytochrome P450 (CYP) 2E1 is an important enzyme involved in many metabolic processes (including alcohol metabolism). CYP2E1 expression could be induced by alcohol, a high-fat or fructose diet, obesity, and drugs. Excessive CYP2E1 expression is associated with liver inflammation via intrahepatic and extrahepatic mechanisms. Elevated hepatic CYP2E1 mediates endoplasmic reticulum stress and oxidative stress in mitochondria, which contributes to the pathogenesis of ALD and MAFLD.[42] In the gut, CYP2E1-mediated oxidative and nitrative stress is related with gut leakiness and endotoxemia, contributing to liver lipid accumulation, increased proinflammatory cytokine production, and infiltration of macrophages in the liver.[43] In addition, CYP2E1-induced apoptosis under the coexistence of obesity and binge drinking is involved in liver injury.[44,45] Occasional or short-time binge drinking-induced liver injury could probably be restored by compensatory liver function. However, chronic frequent binge drinking or heavy drinking is not favorable for the recovery of the liver. Moreover, repeated inflammatory stimulation of the liver promotes the progression of liver fibrosis.[46] Meanwhile, binge eating and high fat intake during binge drinking lead to an increased fat accumulation in the adipose tissue, contributing to the secretion of proinflammatory factors.

Taken together, obesity- and alcohol-induced liver inflammation and fibrosis progression are probably related with interactions among the adipose tissue, the gut, and the liver. Heavy and binge drinking can result in the secretion of more inflammatory factors, contributing to the development of fatty liver disease. The relatively weak proinflammatory effects of LMAC, together with the potential role of LMAC in relieving IR, reduce the risk of fatty liver disease. However, in patients with long-term obesity or T2DM, the protective effects of LMAC may be overshadowed by the increased risk of liver fibrosis or cirrhosis.

Alcohol and lipid metabolism

Elevated plasma TG and decreased high-density lipoprotein (HDL)-cholesterol levels are two important indicators for the diagnosis of MAFLD. The liver is the main organ for both lipid and alcohol metabolism. Increased serum and hepatic TG concentrations are common in alcohol-drinking individuals and animals, including LMAC.[47–51] TG levels are significantly elevated in heavy drinkers compared with other drinkers and non-drinkers.[48] Similarly, binge drinking is associated with a significantly increased risk of elevated TG levels.[52] Binge drinking with a high-fat diet or chronic alcohol consumption can synergistically increase peripheral TG levels.[53,54] Mechanistic target of rapamycin (mTOR) signaling is considered to play fundamental roles in regulating lipid biosynthesis and metabolism in response to nutrition, showing as mTOR complex 1 (mTORC1) induced lipogenesis through its effect on sterol regulatory element-binding proteins (SREBP), inhibited breakdown of intracellular TG, and reduced fatty acid β-oxidation.[55] Recently, studies have demonstrated that mTORC1 is necessary for alcohol to activate hepatic lipogenesis through its effect on SREBP and to inhibit fatty acid β-oxidation, showing as enhanced mTORC1 activity in experimental animals and patients of ALD, characterized by an increase in mTOR-mediated phosphorylation and activity of S6K1, the downstream kinase of mTORC1. Importantly, the concomitant reduction of sirtuin 1 and DEPTOR, an inhibitor of mTOR kinase, signaling was linked to elevated lipogenesis and decreased fatty acid β-oxidation in human liver specimens with ALD. Inhibition of mTORC1 with rapamycin or DEPTOR overexpression ameliorated alcoholic steatosis and liver injury in animals,[56] indicating that inhibition of mTORC1 could be a therapeutic target in ALD in the future.

Elevated TG levels are related with alcohol and with enhanced expression levels of enzymes involved in lipid metabolism. During alcohol metabolism, ethanol is first metabolized to acetaldehyde by alcohol dehydrogenase (ADH) and then oxidized to acetic acid by aldehyde dehydrogenase (ALDH). In this process, the consumption of NAD^+ is increased and the generation of NADH is increased, resulting in a significant increase in the ratio of NADH:NAD^+. The increased ratio further promotes the synthesis of free fatty acids, inhibits fatty acid β-oxidation, and eventually leads to the accumulation of TG in hepatocytes.[57,58] Alcohol also upregulates the expression of fatty acid synthase[59] and SREBP-1c and downregulates acetyl-CoA carboxylase, the rate-limiting enzyme in fatty acid synthesis, and 5′-AMP-activated protein kinase (AMPK), the central regulator of fatty acid β-oxidation.[60,61] A net effect is enhanced fatty acid synthesis, further promoting the synthesis of TG. Long-term heavy alcohol consumption is also related to impaired adiponectin-sirtuin 1-AMPK signaling, a central signaling system controlling the lipid metabolism pathways,[62] thereby promoting hepatic steatosis. Therefore, higher amounts of alcohol intake seem more likely to show hepatic steatosis-promoting effects compared with lower amounts of alcohol intake. Besides, insulin is an important hormone involved in lipid metabolism. In the normal state, insulin helps maintain a dynamic balance of lipid metabolism by promoting the export of lipoproteins from the liver and inhibiting lipolysis in adipocytes to facilitate fat storage in adipose tissue.[63] Impaired insulin signaling and IR result in decreased serum insulin levels and dysfunction.[15,63] Consequently, the effects of insulin in the regulation of free fatty acids are attenuated, contributing to enhanced lipolysis in adipocytes and increased peripheral lipid levels.

HDL plays important roles in cholesterol efflux and reverse cholesterol transport. HDL-cholesterol dyslipidemia is considered to be a major independent risk factor for atherosclerotic cardiovascular disease.[64] Alcohol is positively related with HDL metabolism, as plasma HDL-cholesterol concentrations are increased in drinkers compared with non-drinkers.[65–67] Studies have shown elevated HDL-cholesterol

levels in MAC, together with increased apoprotein A-I levels (accounting for 70% of the total HDL protein mass), higher paraoxonase activity, and decreased cardiovascular risk due to its enhanced antioxidative properties.[68–72] The effects of heavy drinking on HDL seem inconsistent. Some studies observed increased HDL-cholesterol levels and enhanced cholesterol efflux potential in heavy drinkers,[66,73] while other studies showed declined HDL levels in patients with alcohol-related fibrosis and cirrhosis.[30,74] It is reasonable to assume that the onset of ALD may influence HDL metabolism. However, chronic heavy drinking with or without ALD was associated with a similar declined capacity of cholesterol efflux and reduced cholesterol uptake from peripheral blood in the hepatocytes,[74,75] suggesting that alcohol per se is responsible for its deleterious effects on cholesterol efflux and reverse cholesterol transport in heavy drinkers.

Serum HDL levels (quantity) reflect its antioxidant effect to some extent. More importantly, the capacity of cholesterol efflux and reverse cholesterol transport (quality) are two key factors in evaluating its antioxidant capacity. Intact hepatocyte structure and function are necessary for HDL metabolism. During the development of alcohol-related fibrosis and cirrhosis, hepatocytes are gradually depleted, and they become incompetent for lipid metabolism. HDL-cholesterol and total cholesterol levels in peripheral blood are probably not decreased or even increased in the early stage of ALD, partly due to a decline in HDL-mediated reverse cholesterol transport. However, lipid metabolism in the liver gradually weakens with the progression of ALD. Eventually, HDL and total cholesterol levels decrease,[76] with declined capacity of cholesterol efflux and reverse cholesterol transport. On the contrary, in LMAC, the liver function is often competent in lipid metabolism; so, higher HDL-cholesterol levels are probably the result of increased synthesis and reverse cholesterol transport, with increased antioxidative properties and capacity of cholesterol efflux, which may prevent lipid deposition in the vessel wall,[77] decreasing the risk of cardiovascular disease. However, more studies are needed to confirm these hypotheses.

Alcohol and T2DM

The quantity and function of insulin are crucial in maintaining the glycemic balance. Alcohol could cause pancreatic β-cell apoptosis[78] and dysfunction, decreasing insulin secretion, resulting in decreased circulating insulin levels.[79,80] With the increase of alcohol amount, the damage of β-cells is gradually aggravated. Heavy alcohol intake could reduce the insulin-secretory ability of pancreatic islets,[12] decrease glucokinase expression, and inhibit insulin receptor expression,[11] promoting the development of T2DM. On the contrary, LMAC seems to be related with lower fasting insulin levels, a similar effect to that observed in healthy people, who are often considered to be associated with higher insulin sensitivity,[81] showing as reduced fasting insulin concentrations by 19.2% and increased insulin sensitivity by 7.2% compared with non-drinkers.[82] The reasons for low insulin levels related with MAC may be different in men and women, demonstrating as higher clearance of insulin in men and lower secretion of basal insulin in women.[83] Presumably, heavy drinking may impair pancreatic β-cell function and disrupt insulin signaling pathways, contributing to the development of diabetes, while lower insulin levels in non-heavy drinkers seem helpful to maintain glycemic homeostasis. Binge drinking has been proven to be an independent risk factor for IR in MAFLD.[84] In terms of mechanism, binge drinking impairs hypothalamic insulin signaling and decreases insulin secretion, playing a central role in increasing the risk of IR and T2DM. In addition, peripheral insulin dysfunction might be involved in IR. More studies are needed to further verify these hypotheses.

Though some studies showed a positive relation between alcohol consumption and the risk of IR and T2DM,[85–87] most studies suggest reduced risks of T2DM in individuals with LMAC. According to a recent umbrella review, high-quality evidence shows that MAC (12–24 g/day) is negatively correlated with the incidence of diabetes.[88] Prospective and cross-sectional studies show a lower presence of IR and impaired glucose tolerance in obese individuals with MAC than in obese non-drinkers.[20,89] Another cross-sectional study showed that LMAC did not decrease the risk of T2DM in obese individuals,[90] indicating that the role of MAC in the regulation of glucose and lipid metabolism in obese people is controversial. Compared with women, men are more likely to benefit from LMAC.[91,92] Men with cardiovascular disease risk factors can benefit from long-term red wine consumption (40 g/day) in several aspects, including decreased plasma insulin levels, improved glucose homeostasis, and increased HDL-cholesterol levels.[69] Hence, LMAC seems to improve IR in individuals with a high risk of T2DM, especially in men. Interestingly, a reduced risk of T2DM in LMAC is often observed among regular drinkers. A study in Japan showed ~4 drinks per drinking day for 4–7 days weekly in men resulted in a lower risk of T2DM compared with non-drinkers.[93] In a large cohort study from Denmark, the lowest risk of T2DM was observed at 14 drinks/week in men and at 9 drinks/week in women. Compared with current alcohol consumers consuming <1 day per week, the consumption of alcohol for 3–4 days per week was associated with a significantly lower risk for diabetes in men.[94]

Alcohol may commonly impair pancreatic β-cell function. However, the risks of IR and T2DM are low in LMAC populations, as shown in several clinical studies, which is probably in part related with lifestyle. In a prospective cohort study with a 10-year follow-up in the Netherlands, individuals with LMAC (5.0–29.9 g/day for men and 5.0–14.9 g/day for women) exhibited a significantly lower risk of T2DM on the basis of one low-risk lifestyle behavior, and an approximately 40% reduced risk of T2DM on the basis of multiple low-risk lifestyle behaviors compared with non-drinkers.[95] Another randomized clinical trial showed that MAC with lifestyle modification reduced the incidence rate of diabetes in individuals at high risk of diabetes (including impaired glucose tolerance, elevated fasting glucose, or BMI ≥24 kg/m^2) after a 3-year follow-up.[80] As is well known, metabolic dysfunction is often related to unhealthy lifestyles, e.g., high-fat diet, lack of exercise, and smoking. In the above studies, a healthy lifestyle often includes an ideal body weight, a healthy diet, moderate exercise, no smoking, and reduced total energy intake, which are helpful in restoring normal metabolism. Additionally, MAC is considered as a healthy behavior. Thus, benefits from LMAC further improve metabolism on the basis of these healthy lifestyles.

The beneficial effects of MAC on insulin sensitivity are not fully understood. The expression of some molecules may change during MAC and further influence glucose and lipid metabolism. Adiponectin, an insulin-sensitizing adipokine, has been confirmed to play important roles in maintaining insulin sensitivity and suppressing fatty acid synthesis.[96] Hypoadiponectinemia is closely associated with IR in obesity and diabetes.[97,98] Diet-intervention studies in small groups of young and middle-aged men with or without IR have shown increased adiponectin concentrations after MAC intervention.[99,100] A large population study confirmed that adiponectin levels were higher in men with frequent MAC.[100] Alcohol-induced increases in adiponectin improve insulin sensitivity and glucose metabolism and decrease the risk of IR. Therefore, an improved IR and a decreased risk of T2DM in MAC may be the result of multiple factors, including proper drinking frequencies, low-risk lifestyle, and the

expression of molecules improving glucose metabolism.

Alcohol and blood pressure

Early studies have confirmed that chronic alcohol consumption affects blood pressure,[101] manifesting as increased blood pressure in drinkers.[102–105] The increased risk of elevated blood pressure is associated with the amount of alcohol consumed. However, the "threshold" amount is not quite clear. According to recent studies, the potential "threshold" could not be high, as individuals with alcohol consumption more than 100 g/week show elevated systolic blood pressure (SBP).[66] Chronic LMAC has a greater effect on awake blood pressure, increasing SBP and diastolic blood pressure (DBP) by about 2.5–3.0 mmHg and 2.0 mmHg, respectively, and a weaker effect on blood pressure during sleep, decreasing DBP by 2.0 mmHg.[106,107] Though a study showed a higher risk of hypertension in MAC individuals than in light drinkers,[67] the effect of chronic LMAC on hypertension, which has been investigated in human and animal models, is not that obvious.[66,108,109] A population-based prospective study even showed a significantly lower incidence of hypertension in participants with LMAC after a 20-year follow-up compared with non-drinkers.[110] Age is a well-known risk factor for blood pressure. Elderly people are often at higher risks of hypertension than young people. There is a similar effect of age on drinkers' blood pressure.[111] In young drinkers, elevated HDL-cholesterol levels (≥47 mg/dL) could be a protective factor for MAC, preventing significant increases in blood pressure.[102] In two studies, the incidence of hypertension was higher in individuals who consumed large amounts of alcohol (20–30 g/day or more).[102,103] In middle-aged men, an increased risk of hypertension, even in light drinkers (12 g/day), was observed, irrespective of the levels of HDL-cholesterol.[103] With the increase of age, alcohol consumption ≥140 g/week is associated with significantly increased SBP (5–12 mmHg) and DBP (3–6 mmHg) and an increased risk of hypertension (OR: 1.83, 95% CI: 1.40–2.40) in older men.[108] Therefore, age showed a similar negative relation with blood pressure in drinkers as in other populations. With increasing age, the protective effects of HDL-cholesterol and LMAC on blood pressure are gradually overshadowed by the increased risk of hypertension.

Both chronic heavy drinking and binge drinking (occasional or frequent) are related with an increased risk of hypertension in a dose-dependent manner, especially in men.[52,67,112] Blood pressure is temporarily reduced after binge drinking within approximately 2–3 h, but it could dramatically increase after 24 h.[113,114] An Asian cohort study showed that daily alcohol consumption exceeding 60 g/day significantly increased the risk of hypertension in men.[102] However, a recent population-based study on conventional epidemiology and genetic epidemiology showed that lower amounts of alcohol intake were related with increased blood pressure; SBP was increased by 4.8 mmHg (to a level of about 135 mmHg, 95% CI: 4.5–5.1) and DBP by 4.3 mmHg (95% CI: 3.7–4.9) in men with 280 g/week alcohol intake. In women with similar alcohol intake, SBP and DBP increased by 6.7 mmHg (95% CI: 4.3–9.0) and 3.8 mmHg (95% CI: 2.5–5.1), respectively.[66] Obviously, excessive alcohol intake is related with increased blood pressure, especially in male drinkers. Fortunately, the effects of alcohol consumption are reversible. A reduction in alcohol consumption could help to reduce blood pressure, especially in heavy drinkers. Individuals who drink six or more drinks per day could obtain a reduction in SBP by 5.5 mmHg and in DBP by approximately 4 mmHg if they reduce alcohol consumption by about 50%. Reductions in SBP and DBP are also achievable in other drinkers by reducing alcohol consumption, but to a lower degree.[115] Though effects on blood pressure have been observed for both LMAC and heavy drinking, heavy drinking results in much stronger increases, often reaching blood pressures above 140/90 mmHg, with a significantly higher risk of hypertension compared with other drinkers.

Evidence of the effects of alcohol consumption on blood pressure is not as strong in women as in men. According to a recent systematic review and meta-analysis, female drinkers only account for 14% in clinical trials,[115] indicating significant differences in gender distribution. Studies have indicated different effects of alcohol intake on blood pressure in female drinkers compared with male drinkers. Multivariate Cox proportional hazards analysis showed alcohol consumption was not necessarily associated with the risk of hypertension in women.[104] Though MAC could elevate SBP and DBP in premenopausal women, the increase in SBP was not more than 2 mmHg and that in DBP was not more than 1.4 mmHg.[116] Roerecke's systematic review and meta-analysis have shown different incidences of hypertension in men and in women who drank 1–2 drinks/day (relative $risk_{\text{women vs. men}}$=0.79; 95% CI: 0.67–0.93),[117] indicating that women with LMAC were less likely to suffer from hypertension. The increased risk of hypertension was more obvious in women who exceeded two drinks per day.[117] In older women, alcohol amounts below 140 g/week resulted in reductions in SBP of 3–5 mmHg and a reduced risk of hypertension (OR: 0.62, 95% CI: 0.53–0.72) compared with non-drinkers.[108] These results suggest that chronic, regular LMAC in women tends to exert protective effects on blood pressure compared with men. However, data on alcohol consumption in female drinkers are not sufficient, especially in heavy drinkers. More research is needed to explore the relationship between alcohol intake and blood pressure in women.

The main mechanisms underlying the effects of alcohol on blood pressure include a direct effect of alcohol, alcohol metabolic-associated enzymes, and alcohol sensitivity. ADH1B and ALDH2 are two important enzymes in alcohol metabolism. Genetic polymorphisms of ADH1B (rs1229984) and ALDH2 (rs671) are related with the elimination rate of alcohol, alcohol sensitivity, and drinking behavior. The ADH1B*2 allele carrier, with enhanced enzyme activity, is related with more rapid alcohol elimination[118,119] and, possibly, a reduced risk of hypertension and cardiovascular diseases.[119,120] ALDH2 polymorphisms are considered to be associated with alcohol sensitivity and drinking behavior. Enzyme activity is weakened or lost in ALDH2*1/*2 (G/A) and ALDH2*2/*2 (A/A) allele carriers, slowing the process of alcohol metabolism, with higher alcohol sensitivity compared with the wild-type ALDH2*1 (G/G) carriers. In women, LMAC without alcohol sensitivity further decreases SBP by 2 mmHg and is associated with a lower risk of hypertension (OR: 0.62, 95% CI: 0.53–0.72) compared with LMAC with alcohol sensitivity (OR: 0.71, 95% CI: 0.60–0.83). Similarly, in men with alcohol consumption of 140 g/week or more, SBP and DBP were much higher in those with alcohol sensitivity (145 mmHg and 82 mmHg, respectively) than in those without alcohol sensitivity (138 mmHg and 79 mmHg, respectively) and non-drinkers (133 mmHg and 76 mmHg, respectively).[108] The increased risk of hypertension in individuals with ALDH2 polymorphisms may be related with the rate of alcohol metabolism in part because slow elimination of alcohol enhances the effect of alcohol on blood pressure and partly reduces the blood pressure benefits of LMAC.

Limitations and expectations

Many studies show the beneficial effects of LMAC on metabolic functions. However, a recent combined analysis

showed a linear relationship between alcohol consumption and all-cause mortality, with an increase in all-cause mortality among those who consumed more than 100 g/week.[121] This dose is much lower than most guidelines' recommendations and also lower than what is considered a "moderate" amount in most studies. Therefore, drinking in patients should be cautiously guided, especially in those with metabolic dysfunctions. For obese patients with MAFLD and decompensated liver cirrhosis, any amount of alcohol consumption is related with an increased risk of hepatocellular carcinoma.[122,123] Alcohol drinking should be absolutely avoided in these patients.

Alcohol consumption is common in the MAFLD population and is related with metabolic dysfunction (Table 2). Interactions between alcohol and metabolic factors are complicated, and the benefits from non-heavy drinking may be reduced by other factors. A U-shaped or J-shaped relationship between alcohol consumption and the single component of metabolic dysfunction is common in many studies. In fact, there are probably more net-shaped relations among these factors than linear relations in real-world patients. As is well known, liver cirrhosis is an important stage during the development of chronic liver disease, and it is usually irreversible. Unfortunately, most studies on alcohol and MAFLD are focused on early-stage liver disease, and only a few studies focus on MAFLD with liver fibrosis or cirrhosis. Thus, the exact impacts of alcohol consumption (especially non-heavy drinking) on MAFLD and metabolic-associated impairments of target organs, complications, and even tumors are not quite clear. More research studies are needed to explore the long-term effects of alcohol consumption on end-stage MAFLD and metabolic syndrome, to fully understand the effects of alcohol consumption and guide patients who consume alcohol.

Special attention should be paid to several populations. First is ex-drinkers and abstainers. The benefits from LMAC are often shown in current drinkers, usually accounting for the majority of participants in most studies. On the contrary, data on ex-drinkers and abstainers are not enough to analyze the impact of stopping alcohol consumption on metabolic factors well. Studies have shown changes of several metabolic factors after a period of abstinence, e.g., decreased HDL levels and visceral fat area and improved homeostasis, among moderate to heavy drinkers.[124,125] Thus, it is necessary to evaluate the effects of abstinence on metabolism and re-evaluate the effects of LMAC on metabolism after abstinence. Second is female drinkers. There are more male drinkers than female drinkers in the real world and in most clinical trials. Usually, the non-drinkers are mainly female, while the drinkers are mainly male in most studies. With increasing amounts of alcohol consumption, the proportion of males increases dramatically, and the heavy drinkers are almost all men, especially in large, population-based studies.[66,126,127] Thus, there is an almost inevitable sex bias because of the smaller female samples. As shown by some studies, the effects of alcohol are significantly different between male and female drinkers. There seems to be a much closer relationship between alcohol and male sex, including genetic epidemiological characteristics.[128] Therefore, more studies are needed to evaluate the role of alcohol consumption on metabolism in female drinkers. Third is patients with borderline high blood pressure. A criterion of SBP ≥140 mmHg and/or DBP ≥90 mmHg or special drug treatment is considered for the diagnosis of hypertension in many studies. According to the new definition of MAFLD, a blood pressure of ≥135/85 mmHg is considered a metabolic risk abnormality,[1] a more rigorous standard than the former. Therefore, the decreased risk of hypertension upon LMAC could possibly be overestimated in some individuals with other metabolic risk abnormalities. Individuals with blood pressure between 135/85 mmHg and

Table 2. Main clinical studies on alcohol and metabolic dysfunctions included in this review

		Author and year[REF]	Study type	Follow-up	Alcohol beverage	Amount	Population	Main indicators	Ralation type	Main results
Alcohol, obesity, and liver injury	1	Schütze M, 2009[9]	Prospective study	8.5 years	Beer	1,000 mL/day	7,876 men, 12,749 women	WC	U-shaped	Men consuming 1,000 mL/day beer were at 17% higher risk for WC gain compared with very light consumers.
	2	Inan-Eroglu E, 2020[19]	Cross-sectional study	–	Red wine; champagne and white wine; beer and cider; spirits; fortified wine	<14 units/week for women and <21 units/week for men	UK Biobank baseline data, n = 280,183, 48.3% female	BMI	–	Those drinking within the public health guidelines had a lower BMI by 1.34 kg/m^2 (95% CI 1.42–1.26 kg/m^2) compared to never drinkers.
	3	Wang L, 2010[21]	Prospective cohort study	12.9 years	Not mentioned	0 ~ ≥30 g/d	19,220 US women aged 39 years and above with baseline BMI of 18.5–25 kg/m^2	1. Overweight or obese (BMI ≥25 kg/m^2). 2. Obese (BMI ≥30 kg/m^2)	Almost linearly inverse relation	LMAC was associated with smaller weight gain and lower risk of becoming overweight and/or obese.

(continued)

Table 2. *(continued)*

		Author and year[REF]	Study type	Follow-up	Alcohol beverage	Amount	Population	Main indicators	Ralation type	Main results
	4	Golan R, 2017[22]	Randomized controlled study	2 years	Wine	150 mL/day	n = 48, alcohol-abstaining adults with well-controlled T2DM	Bodyweight and abdominal adiposity	–	Moderate wine consumption did not promote weight gain or abdominal adiposity.
	5	Naveau S, 1997[29]	Retrospective study	–	–	≥50 g/day	n = 1,604, patients with ALD. Biopsy-proven liver cirrhosis in most cases.	Liver histology	Synergistic effect	The presence of excess weight for at least 10 years was a risk factor for cirrhosis, acute alcoholic hepatitis, and steatosis.
	6	Kwon HK, 2014[34]	Cross-sectional study	–	–	≤40 g/week	n = 77, obese patients with liver biopsy-proven NAFLD	Liver histology, especially liver cirrhosis	Negative relation	Alcohol consumption of ≥24 gram-years was associated with less severe disease (fibrosis stage 3–4).
	7	Blomdahl J, 2021[40]	Cross-sectional study	–	–	<140 g/week	n = 86, obese patients with biopsy-proven NAFLD	Advanced liver cirrhosis	–	MAC was associated with advanced fibrosis. Patients with T2DM had the highest risk.
Alohol and lipid metabolism	8	Hiramine Y, 2011[48]	Cross-sectional study	–	Not mentioned	0 ~ ≥60 g/day	n = 9,886, men aged 30–69 years	Serum TG	U-shaped	Serum TG was lower in former drinkers than non-drinkers and other drinkers. Serum TG was highest in heavy drinkers than other drinkers.
	9	Sierksma A, 2002[72]	Randomized, controlled, cross-over study	–	Beer	40 g/day for men, 30 g/day for women	10 middle-aged men and 9 postmenopausal women	Apo A-I, HDL-cholesterol, and paraoxonase activity	–	Serum apo A-I, HDL-cholesterol, and paraoxonase activity were significantly increased during 3 weeks of MAC compared with no alcohol consumption.
Alcohol and diabetes	10	Crandall JP, 2009[80]	Randomized controlled study	3.2 years	Beer, wine, and hard liquor	<36 g/day, without heavy or binge drinking	n = 3,175, individuals at high risk of diabetes	Insulin secretion and risk of diabetes	Inverse association	Daily MAC was associated with lower insulin secretion and reduced risk of incident diabetes.

(continued)

Table 2. *(continued)*

		Author and year[REF]	Study type	Follow-up	Alcohol beverage	Amount	Population	Main indicators	Ralation type	Main results
	11	Davies MJ, 2002[82]	Randomized, controlled, cross-over study	8 weeks	Everclear in orange juice	0, 15, or 30 g/day	n = 51, healthy postmenopausal women	Insulin level and sensitivity, TG	Inverse association	Consumption of 30 g/day of alcohol reduced fasting insulin by 19.2% and TG by 10.3%, and increased insulin sensitivity by 7.2% compared with 0 g/day.
	12	Joosten MM, 2010[95]	Prospective study	10.3 years	Beer, wine, and spirits	0 ~ ≥30 g/day	n = 35,625, Dutch adults at low risk of diabetes	Risk of diabetes	Inverse association	On the basis of multiple low-risk lifestyle behaviors, LMAC (5.0–14.9 g/day for women; 5.0–29.9 g/day for men) was associated with ≈40% lower risk compared with abstinence.
Alcohol and hypertension	13	Rodrigues P, 2018[110]	Prospective study	Over 20 years	Beer, wine, and spirits	0 ~ ≥14 drinks/week (1 drink = 14 g of pure ethanol)	n = 2,368, individuals between 18 and 30 years of age	Incidence of hypertension	–	Incidence of hypertension was much lower in LMAC (<14 drink/wk) individuals.
	14	Mori TA, 2015[116]	Randomized controlled, cross-over study	4 weeks for each period	Red wine	146 or 218 g/week	n = 24, women aged 25 to 49 years	24-hour ambulatory blood pressure	Positive relation	High volume alcohol consumption was related with increased SBP (1.6–2 mmHg) and DBP (1.2–1.4 mmHg).
	15	Millwood IY, 2019[66]	Prospective study	About 10 years	Beer, wine, and spirits	0 ~ >420 g/week	n = 512,715 Chinese adults	Risk of hypertension		In individuals with per 280 g/week alcohol intake, SBP and DBP increased by 4.8 mmHg and 4.3 mmHg in men and by 6.7 mmHg and 3.8 mmHg in women, respectively.

BMI, body mass index; DBP, diastolic blood pressure; HDL, high-density lipoprotein; LMAC, light-to-moderate alcohol consumption; MAC, moderate alcohol consumption; NAFLD, non-alcoholic fatty liver disease; SBP, systolic blood pressure; TG, triglyceride; WC, waist circumference.

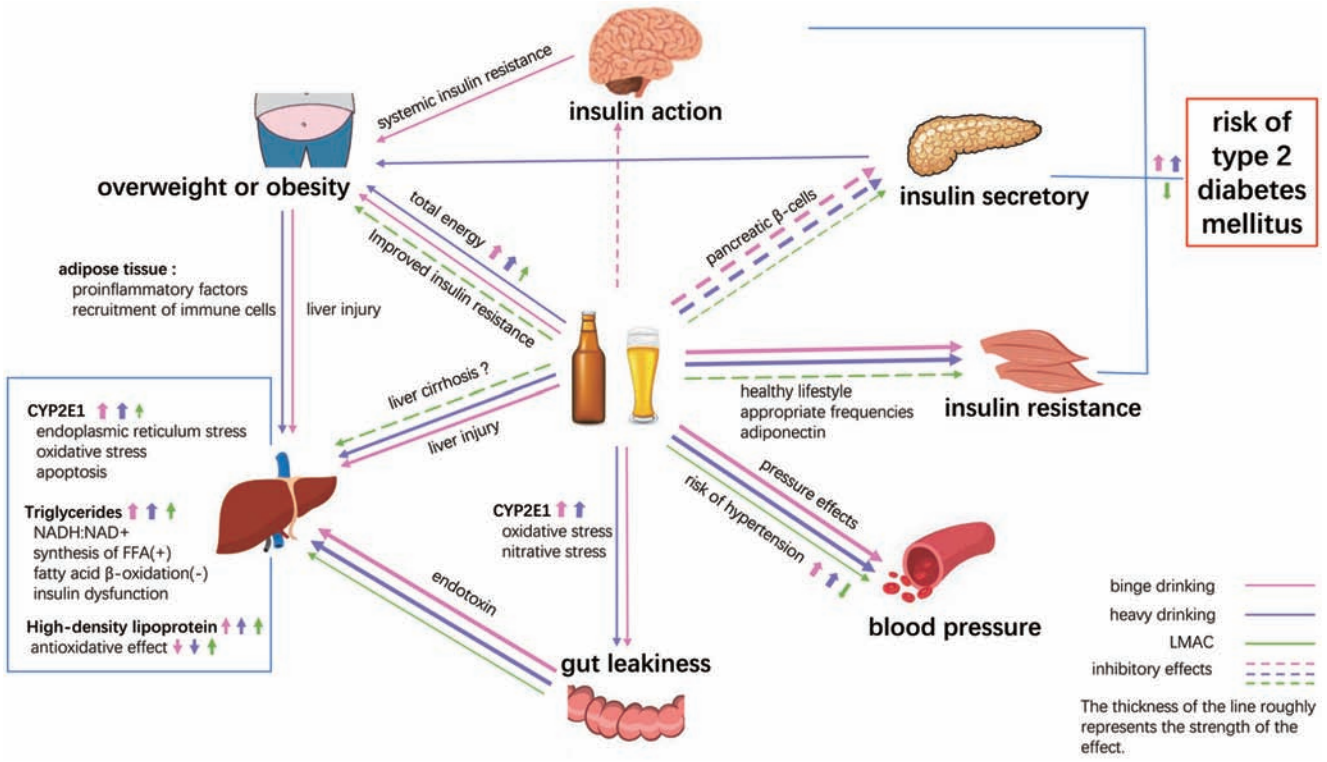

Fig. 1. Relationship between alcohol consumption and metabolic dysfunctions. *Obesity:* heavy drinking and binge drinking are associated with increased body weight and WC due to the obviously increased total energy intake and impaired peripheral or central insulin signaling pathways. LMAC is likely to maintain normal body weight mainly through improved IR. *Liver injury:* heavy or binge drinking in obese individuals exerts synergistic effects on liver injury. Liver-adipose tissue axis and liver-gut axis are two important mechanisms. The long-term effects of LMAC on liver cirrhosis are not quite clear. *Lipid metabolism:* alcohol is related with increased TG and HDL levels. However, the antioxidant effects of HDL are different, showing as enhanced antioxidative properties in LMAC and impaired antioxidative properties in heavy or binge drinkers with poor liver function. *T2DM:* heavy drinking and binge drinking are associated with increased risks of T2DM, mainly through impaired insulin signaling, decreased insulin secretory, and IR. Appropriate frequencies of LMAC, especially in combination with a healthy lifestyle, are related with improved IR and a decreased risk of T2DM, partly through regulating the expression of adiponectin. *Hypertension:* the effects of alcohol consumption on blood pressure are common. Heavy drinking and binge drinking are usually associated with significantly increased blood pressure and the risk of hypertension, while the risk of hypertension is lower in LMAC. ALD, alcoholic-related liver disease; CYP, Cytochrome P450; HDL, high-density lipoprotein; IR, insulin resistance; LMAC, light to moderate alcohol consumption; MAFLD, metabolic-associated fatty liver disease; T2DM, type 2 diabetes mellitus; TG, triglyceride; WC, waist circumference.

140/90 mmHg have shown an increased risk of metabolic dysfunctions.

In conclusion, alcohol drinking is closely related with metabolic dysfunction in several systems, such as the liver-gut axis, the liver-brain axis, the liver-pancreas axis, and the liver-adipose tissue axis (Fig. 1). LMAC combined with a healthy lifestyle may be helpful for maintaining metabolic homeostasis, while heavy drinking and binge drinking are two common dangerous drinking patterns that should be avoided. The new definition of MAFLD is a positive diagnosis of the disease with simple criteria. Though “alcohol” is excluded from the diagnosis, the relationship between MAFLD and alcohol is still close. Much attention should be paid to alcohol consumption during the management of MAFLD.

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Conflict of interest

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Author contributions

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Review Article

Drug-induced Fatty Liver Disease: Pathogenesis and Treatment

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Abstract

Metabolic dysfunction-associated fatty liver disease (commonly known as MAFLD) impacts global health in epidemic proportions, and the resulting morbidity, mortality and economic burden is enormous. While much attention has been given to metabolic syndrome and obesity as offending factors, a growing incidence of polypharmacy, especially in the elderly, has greatly increased the risk of drug-induced liver injury (DILI) in general, and drug-induced fatty liver disease (DIFLD) in particular. This review focuses on the contribution of DIFLD to DILI in terms of epidemiology, pathophysiology, the most common drugs associated with DIFLD, and treatment strategies.

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Introduction

Drug-induced liver injury (DILI) represents a significant health problem in the USA and many European countries.[1] In prospective and retrospective DILI studies,[2] the annual incidence has been reported as 2.7 per 100,000 people. Furthermore, in many countries, DILI has been associated with acute liver failure. The risk factors for DILI include numerous interrelated factors, such as advanced age, sex, drug dose, genetic factors, concomitant drugs, excessive alcohol consumption, nutrition, pre-existing liver disease, diabetes mellitus, human immunodeficiency virus infection, and kidney failure.[3] Historically, DILI has been divided into two types. Type 1 is dose-dependent and predictable, and type 2 results from idiosyncratic reaction. Type 2 is mostly dose-independent, and can be either allergic, immune-mediated, or non-allergic, nonimmune-mediated.[4] The diagnosis of DILI is determined by a temporal relationship between drug administration and increased levels of liver enzymes and/or alkaline phosphatase,[5,6] exclusion of other causes of liver damage, and rarely repeated drug challenge. There is no standardized clinical test for this condition.[5,7] Drug-induced cholestasis is induced when drugs disrupt bile acid transport by inhibiting liver transporters involved in bile flow.[6] Cholestasis can be also found in severe metabolic dysfunction-associated fatty liver disease (MAFLD) stages, alcoholic hepatitis and alcoholic cirrhosis.[8] Drug-induced cirrhosis is associated with drugs that cause fibrogenesis and production of extracellular matrix molecules.[9]

MAFLD is a new concept, proposed in 2020, that has been suggested to replace the term nonalcoholic fatty liver disease because it does not require the exclusion of alcoholic liver disease or viral hepatitis.[10,11] It is a more accurate term for people with fatty liver and those with dysmetabolism.[1,2] MAFLD is well known as a highly prevalent disease affecting a quarter of the world's adult population and is the main cause of chronic liver disease in Europe and USA.[11,12] Besides, with the very high prevalence of MAFLD and alcohol abuse worldwide, the relationship among any present study population and real-world populations is of concern.[10] The novel MAFLD criteria concentrate on the role of dysmetabolism in fat accumulation in the liver, that is the most frequent driver of fatty liver injury progression.[13,14] When fatty liver injury progresses due to preexisting MAFLD in combination with drug administration, it is defined as a dual-etiology fatty liver disease.[10] Recently, two studies have recommended that the MAFLD criteria are more efficient and better for perceiving patients with a higher risk of fibrosis, in contrast with nonalcoholic fatty liver disease criteria.[11,15] MAFLD is diagnosed in patients when they have the hepatic manifestation of metabolic syndrome, which is diagnosed when three or more of the following conditions are found: high glucose, hypertension, obesity, high triglyceride, and low high-density lipoprotein-cholesterol.[16] There are a growing number of clinical reports proposing that certain drugs can be more hepatotoxic in overweight patients with MAFLD, in contrast with lean patients.[17]

DILI in MAFLD appears in two particular clinical situations.[17,18] First, antibiotics such as piperacillin-tazobactam, telithromycin, and some analgesics and antipyretics, like

Keywords: Metabolic dysfunction-associated fatty liver disease; Drug-induced liver injury; Reactive oxygen species; Free fatty acids; Pharmacogenetics.

Abbreviations: DIFLD, drug-induced fatty liver disease; DILI, drug-induced liver injury; DILIN, Drug-Induced Liver Injury Network; DIS, drug-induced steatosis; DISH, drug-induced steatohepatitis; MAFLD, metabolic dysfunction-associated fatty liver disease; MASH, metabolic dysfunction-associated steatohepatitis; MRC, mitochondrial respiratory chain; mtFAO, mitochondrial fatty acid oxidation; PNPLA3, patatin-like phospholipase 3 gene; ROS, reactive oxygen species; UDCA, ursodeoxycholic acid; ULN, upper limit of normal; VLDL, very-low density lipoprotein.

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acetaminophen, can induce more serious and common acute liver injury. It appears that some drugs, like amiodarone and statins, do not induce hepatotoxicity more often in MAFLD patients.[17] Other drugs like antiretroviral agents, corticosteroids, and methotrexate appear to cause the alteration of simple fatty liver to nonalcoholic steatohepatitis or exacerbate necroinflammation, pre-existing steatosis, and fibrosis.[19,20] Some drugs can cause more serious acute liver injury in MAFLD because this illness is connected with the various modified activities of metabolizing enzymes such as cytochromes P450. Regarding the above-mentioned information, MAFLD is frequently connected with increased CYP2E1 activity and decreased CYP3A4 activity as well as with higher glucuronide formation. These enzymes are responsible for metabolism of, e.g., lorazepam and acetaminophen. More *in vitro* and *in vivo* research is required because the mechanisms wherewith drugs and xenobiotics are more hepatotoxic in MAFLD are not well known and more studies are a necessary in ensuring success in dealing with this issue, especially considering the worldwide epidemic of obesity.[21,22]

Drugs represent an alternative cause of fatty liver disease and the term that corresponds to this injury is drug-induced fatty liver disease (DIFLD). It is a specific form of DILI, characterized by intracellular lipid accumulation in hepatocytes with steatotic changes as the predominant histopathological pattern.[23,24] Although this histopathological finding is required for the diagnosis, the finding is not specific.[11] DIFLD is often accompanied by inflammation and oxidative stress, which leads to the development of drug-induced steatohepatitis (DISH).[25] Chronic liver injury leads to hepatocyte death, followed by the activation of stellate cells which finally results in liver tissue fibrosis. In addition, there are numerous drugs which can cause progression of steatohepatitis.[26] In 2015, Satapathy *et al*.[27] have shown that tamoxifen, an anti-estrogenic drug used in the treatment and prevention of breast cancer, was frequently associated with hepatic steatosis, although rarely with cirrhosis or steatohepatitis. Moreover, the authors emphasized that chronic exposure to amiodarone, 4, 4′-diethylaminoethoxyhexestrol and perhexiline maleate rarely led to cirrhosis.[27,28] It is known that phospholipidosis develops after prolonged treatment with these drugs, in a dose-dependent manner. However, it does not lead to steatohepatitis. Further investigations are needed to elucidate mechanisms by which drug-induced steatosis leads to steatohepatitis and consequently to fibrosis.

Buggey *et al*.[29] reported that amiodarone-induced acute and chronic liver injury without steatosis leads to necrosis and bridging fibrosis with early-stage cirrhosis. It is well known that amiodarone-induced hepatotoxicity has been characterized by histologic steatosis, phospholipidosis and fibrosis. However, in that case report, the histopathology showed an absence of steatosis and phospholipidosis, despite years of amiodarone ingestion. This suggests that lack of formerly accepted histopathologic findings, such as steatosis and phospholipidosis, should not exclude the diagnosis. This conclusion, however, requires further study and confirmation. Various other studies have confirmed the role of amiodarone in the induction of liver cirrhosis, with possible fatal outcomes.[30–32] Nevertheless, these adverse effects were found to be rare, with an incidence of 1–3%. A long-term surveillance for liver toxicity in high-risk patients using amiodarone has been suggested by numerous researchers.[30,31,33]

Most drugs capable of causing steatosis and steatohepatitis are known to have cationic amphiphilic structure.[34] These drugs are divided into three groups, including drugs that cause steatosis and steatohepatitis independently, such as amiodarone and perhexiline, drugs that can accelerate latent metabolic dysfunction-associated steatohepatitis (MASH), such as tamoxifen, and drugs that may cause sporadic events of steatosis/steatohepatitis, such as carbamazepine.[23] More details over the effects of these drugs on liver tissue will be discussed in the sections below.

Epidemiology of DIFLD

Recently, reported annual incidences of DILI have varied widely in population-based studies, from 2.7 to 19.1 cases per 100,000.[35] Accordingly, the true incidence of DIFLD in the general population remains unknown.[35] However, drug-induced steatosis (DIS) or drug-induced steatohepatitis (DISH) are generally rare but well-documented forms of DILI. According to the Drug-Induced Liver Injury Network (DILIN), approximately 27% of DILI cases have some form of steatosis with histological injury.[36] In the study of Kleiner *et al*.,[36] only one case was diagnosed with the predominant pattern of microvesicular steatosis, while the remaining cases showed a combination of macrovesicular steatosis with inflammation. Previously published descriptions of pathologic changes in DILI were used as the basis for the diagnostic classification in DILIN in the prospective study by Kleiner *et al*.[36,37] To define patterns of injury, standard hepatopathological diagnostic criteria were used.[38] Although this included a large proportion of DIFLD in DILI cases, the DILIN prevalence may be biased by the pre-existing presence of a fatty liver. The true data on DIFLD epidemiology might become clearer after eliminating diagnostic difficulties and deficiencies in systematic reporting.

Histology of DIFLD

DIFLD can present as pure macrovesicular or microvesicular steatosis or as DISH. Histologically, in macrovesicular steatosis, the accumulation of large lipid vesicles (mostly triglycerides) occurs in the hepatocyte, with the nucleus becoming consequently dislocated to the periphery of the cell.[36,39] As in other causes of steatohepatitis, aminotransferases are usually moderately increased.[40] The presence of triglycerides is associated with deterioration of mitochondrial fatty acid oxidation (mtFAO), decreased very-low density lipoprotein (VLDL) secretion, stimulation of *de novo* lipogenesis, direct activation of transcription factors, such as SREBP1c and PPARγ, and development of insulin resistance.[17,27,41–43] In microvesicular steatosis, the cytoplasm of hepatocytes is filled with numerous small lipid vesicles, and the nucleus remains in the center of the cell.[44] The severe impairment of mtFAO leads to increased esterification into triglycerides, which are known to be histologically related to microvesicular steatosis.[27,45] Steatohepatitis is characterized by lobular inflammation, balloon degeneration, hyaline Mallory bodies, and sometimes perisinusoidal fibrosis.[23,39,46] Additionally, mitochondrial dysfunction plays a key role in DIFLD, through the direct or indirect action of oxidative stress and increased production of reactive oxygen species (ROS) that mainly occur due to modification of the mitochondrial respiratory chain (MRC).[17,47] Microvesicular steatosis (drug-induced) is frequently the result of drug-induced damage to mitochondria.[48,49] This type of steatosis can start with small droplets of fat in the cytoplasm and then increase to macrovesicular steatosis characterized by large fat droplets that shifted the nucleus to the periphery. Frequently, macrovesicular steatosis can present with mixed large and small droplets.[50,51] Depending on the particular pathogenic mechanism of each lipotoxic drug, DIS/DISH can present as micro- or macrovesicular steatosis/steatohepatitis, but most cases start acutely with microvesicular injury.[52] The latency of DIFLD before clinical manifestations may vary.[24]

For DIS/DISH diagnosis, liver biopsy is the standard means for confirmation of hepatic cell injury and liver inflammation.[52]

Risk factors for occurrence of DIFLD

Some drugs cause progression of MAFLD to MASH or cirrhosis, and may also worsen the prognosis in patients with fatty liver.[17] This conversion to MASH appears to involve genetic and environmental factors.[17] MAFLD and obesity may enhance the risk of hepatotoxicity of various drugs.[18] The possible mechanisms by which certain drugs are able to accelerate progression of MAFLD include induction of oxidative stress, diminished mtFAO, increased *de novo* lipogenesis, and damaged egress of VLDL from liver cells.[53]

Most often, DIFLD is a product of direct impact of drugs on the liver, mostly associated with the extended intake of medications. For example, long-term administration of drugs, such as amiodarone, perhexiline and diethylaminoethoxyhexestrol, can lead to DISH. Furthermore, patients with additional risk factors, like obesity and cardiometabolic risks, are more prone to exacerbation of steatosis or steatohepatitis when irinotecan, tamoxifen and methotrexate are added to their therapy. Insulin resistance and hypertriglyceridemia in combination with antiepileptic drugs and steroids can also lead to steatohepatitis, MASH or DIFLD.[27] Fatty liver injury progression is related to factors such as insulin resistance, adipose tissue dysfunction, lipid aggregation, and oxidative and endoplasmic reticulum stress. Also, increased gut permeability and increased plasma endotoxin levels can be associated with fatty liver.[54–56]

Besides environmental risk factors, genetics also plays a significant role in the progression of simple steatosis.[57] Among patients with similar risk factors, large interindividual variability in phenotypic penetrance exists.[57] Various genetic, epidemiological and twin studies have shown a strong heritability of predisposition to MAFLD.[57] Apart from drugs, intrinsic (sex, age, ethnicity, liver, and renal condition) and other extrinsic (environmental chemicals, alcohol, diet, and drug-drug interactions) risk factors must be considered in any clinical algorithm associated with the fatty liver.[58] There is growing evidence for a genetic contribution to the development of MASH, even though environmental risk factors play a main role in the development of simple steatosis. In various (twin, epidemiological, and familial) studies, a large variability exists in phenotypic penetrance among people with related risk factors, and a powerful heritability of sensitivity to MAFLD has been noticed.[57] Studies on the role of genetics in DIFLD are still in the early phases, and more studies are needed to augment understanding of genetic variants and other risk factors in the progression of DIFLD and MAFLD.

Influence of pharmacogenetics on the risk for developing DIFLD

Alterations in genes involved in pharmacokinetics and pharmacodynamics are partially responsible for variations in drug response.[58] Part of an individual's predisposition for the development of side effects with high doses of certain drugs, like methotrexate or tamoxifen, can be explained by the patient's genetic makeup as well as pharmacogenetics. As mentioned before, methotrexate and tamoxifen are some of the drugs that can cause macrovesicular hepatic steatosis linked to DIFLD. In the context of high-dose methotrexate toxicity, it is important to emphasize that it is unpredictable, and interindividual variability is significant. The results from the previous studies on the pharmacogenetics of high doses of methotrexate differ, and are sometimes contradictory. This can be partly explained by significant differences in the pharmacogenetics of various populations.[39,59] Several genotypes have been associated with a higher risk of methotrexate toxicity, such as MTHFR 677TT (reduced activity of methylenetetrahydrofolate reductase which leads to diminished elimination of methotrexate), RFC-1 80G>A (reduced folate carrier 1, which is responsible for methotrexate entrance into the cells), and ABCB1 C3435TT (ATP binding cassette subfamily B member 1; reduced action of MDR1 and, therefore, slower elimination of methotrexate).[60] The metabolisms of 5-fluorouracil depends on the enzymatic activity of dihydropyrimidine dehydrogenase. Indeed, variants *2A or *13 of this enzyme are related to reduced metabolism of 5-fluorouracil, which can lead to serious side effects.[61] Genetic alterations in the patatin-like phospholipase 3 gene (PNPLA3) affect the plasma levels of hepatic enzymes and risk for MAFLD development,[62,63] including predisposition for fibrosis progression.[64,65] The above-mentioned polymorphism is a powerful predictor of inflammation, steatosis and fibrosis[66] but the role of PNPLA3 in DIFLD pathogenesis remains obscure.[27] Polymorphisms of PNPLA3 are strongly associated with ethnic and interindividual variations in liver fat content.[57] Hispanics were found to have a higher tendency to develop liver steatosis, unlike African-Americans.[67] In addition, twin studies suggest that about 60% of alanine transaminase variability may be ascribed to genetic factors.[68] Slow metabolizers for perhexiline, such as Caucasians, are at the greater danger of neuropathy and steatohepatitis. Perhexiline is catabolized by cytochrome P450 isoform 2D6 and has a long half-life due to the slow liver clearance in slow metabolizers.[69]

In recent years, the genetic factors of steatosis have been studied utilizing genome-wide association techniques. Further research in the area of pharmacogenomics is needed to better understand numerous possible gene polymorphisms that might be responsible for increasing risk of DIFLD development.

Drugs that cause DIFLD

Drugs shown to cause macrovesicular liver steatosis are glucocorticoids, amiodarone, methotrexate, estrogens, tamoxifen, nonsteroidal anti-inflammatory drugs, paracetamol, 5-fluorouracil, and metoprolol.[39,70–72] Drugs associated with microvesicular steatosis are valproic acid, tetracycline, aspirin, ibuprofen, zidovudine, and glucocorticoids.[24,44] Drugs associated with DISH are valproic acid, tamoxifen, perhexiline, amiodarone, and propranolol.[44,73] It is important to recognize the particular drugs that could cause acute liver damage on a fatty liver background or that could increase the danger of serious chronic liver disease. The hepatic accumulation of fat is not necessarily stable and DIS/DISH are reversible.[74] In many cases, it is difficult to elucidate whether the fatty liver disease is a direct result of an effect on hepatic cells or a consequence of a weight gain caused by the drugs such as antidepressants or antipsychotics. Pharmaceuticals that could induce the progression or exacerbate pre-existing fatty liver to MASH and fibrosis are shown in Table 1.[17]

Mechanisms of DIFLD development

The main mechanisms in the development of DIFLD are thought to include lipogenesis and generation of free radicals leading to oxidative stress induction in hepatocytes.[44,75] Kim *et al*.[76] showed that amiodarone caused an increase in short, medium- and long-chain acylcarnitines in the livers of

Table 1. Drugs specifically hepatotoxic in DIFLD, MAFLD and obesity

	Acute liver injury	Exacerbation of pre-existing fatty liver or MASH	Promoting the transition of pre-existing fatty liver into MASH, fibrosis, or cirrhosis
Drugs	Amiodaron, Aspirin, Acetaminophen, Ibuprofen, Isoflurane, Fosipronil, Halothane, Vitamin A, Valproat Acid, Tetracycline, Telithromycin, Piperacillin/tazobactam, NRTIs, Zalcitabin, Losartan, Omeprazole, Sorafenib, Ticlopidine, Troglitazone	Androgenic steroids, Benzbromarone, Corticosteroids, Irinotecan, Methotrexate, Tamoxifen, NRTIs, Pentoxifylline, Phenobarbital, Rosiglitazone, Tetracycline	Androgenic steroids, Benzbromarone, Corticosteroids, Irinotecan, Methotrexate, Tamoxifen

NRTIs, nucleoside reverse transcriptase inhibitors. Data from: Allard J *et al*.[17] Drug-induced liver injury in obesity and nonalcoholic fatty liver disease, EASL Clinical Practice Guidelines: Drug-induced liver injury.

rats, with the highest increases involving levels of acetylcarnitine. The most probable cause of these disturbances in liver tissue is the effect of amiodarone on mtFAO by blocking the activity of the carnitine palmitoyltransferase-1 enzyme, thereby directly inhibiting the mitochondrial β-oxidation of acyl-CoA to acetyl-CoA and by inhibiting complexes I and II of the MRC.[19,77] Another proven mechanism of amiodarone-induced DIFLD is triggering of *de novo* lipogenesis by augmenting the expression of genes sterol regulatory element-binding protein 1, thyroid hormone-inducible hepatic protein, ATP-citrate synthase, fatty acid synthase, and acyl-CoA desaturase, which are all involved in the process of lipogenesis.[78] Additionally, Anthérieu *et al*.[78] demonstrated *in vitro* that amiodarone administration led to overexpression of genes involved in formation of lipid droplets, namely perilipin-4 and adipose differentiation-related protein. Tamoxifen, like amiodarone, is a cationic amphiphilic compound that accumulates in liver tissue, causing liver injury.[34] Its toxic effect is also achieved by impairing the mtFAO and induction of *de novo* lipogenesis.[79] A possible mechanism for the induction of hepatic steatosis includes the upregulation of SREBP-1c and its downstream lipogenesis target genes.[24] Accumulation of triglycerides stimulates microsomal triglyceride transfer protein expression associated with VLDL assembly and secretion.[80] Several *in vivo* studies confirmed the role of oxidative stress in tamoxifen hepatotoxicity. Like amiodarone, it causes a reduction in liver glutathione levels, accumulation of oxidized form of glutathione, and lipid peroxidation.[75,81]

Methotrexate and especially its polyglutamated metabolite are both stored in hepatocytes and exert hepatotoxic effects.[82] Several mechanisms are proposed for the hepatotoxic effect of methotrexate, including hampering of folate entry to mitochondria, which leads to mitochondrial dysfunction and generation of ROS and finally induction of caspase-dependent apoptosis.[54,83,84] Another possible mechanism of hepatotoxicity is disruption of the intestinal epithelial barrier by methotrexate, which then leads to leaky gut syndrome, and the progression of fatty liver injury.[34,54] 5-Fluorouracil, irinotecan, and l-asparaginase, all exert their hepatosteatotic effects by impairing mtFAO and enhancing ROS accumulation in hepatocytes.[20,85] Valproate, a branched-chain fatty acid, disrupts the mtFAO, leading to the accumulation of triglycerides and steatosis.[44] Valproate in its free acid form can serve as a substrate for mtFAO pathways, competing with other free fatty acids. After entering the hepatic mitochondria, it conjugates with coenzyme A and causes a deficiency in that enzyme.[44] Chronic valproate administration increases the progression of a pre-existing fatty liver disease by inducing systemic insulin resistance and weight gain.[86,87] Tetracycline is well known for causing DIFLD. Mechanisms for this toxic effect include inhibition of mtFAO, inhibition of MTP enzyme (which results in accumulation of VLDL), decrease in the expression of several genes involved in mtFAO (peroxisome proliferator-activated receptor alpha, carnitine palmitoyltransferase I, and fatty acid-binding protein 1), and enhancement of ROS generation by activation of the transcription factor ATF4 (which up-regulates CYP2E1; specifically, by doxycycline and minocycline).[34,41,88,89] Nucleoside reverse transcriptase inhibitors, such as zidovudine, didanosine, stavudine, tenofovir and abacavir, are capable of inhibiting human DNA polymerase γ, leading to the decrease in mitochondrial DNA replication.[90,91] Consequently, oxidative stress and accumulation of fat occur.[90,91] All the above-mentioned mechanisms involved in DIFLD development are summarized in Table 2.

Current and future directions in the treatment of DIFLD

A fairly common recommendation for the management of DILI and potential manifestation of DIFLD is the withdrawal of the potential offending agent. Timely exclusion of the problematic drugs can lead to full recovery; up to 95% of patients show improvement but a few will still develop chronic liver disease.[92] Criteria of withdrawal of the drugs causing DILI were published in 2009 by the Food and Drug Administration[93] and are summarized in the following guidelines as follows: alanine aminotransferase or aspartate aminotransferase are >8 upper limit of normal (ULN), >5 ULN (for the period of 2 weeks), >3 ULN combined with international normalized ratio >1.5 and total bilirubin >2 ULN or levels of alanine aminotransferase/aspartate aminotransferase higher than 3, but followed with nausea, fever, fatigue, vomiting, rash, tenderness or pain (right upper abdominal quadrant) and potential eosinophilia.[92] If there is no adequate replacement for the hepatotoxic drug, then the dose should be adjusted in order to manage the primary disease, especially in intrinsic DILI.[92]

Glucocorticoids are used sometimes to treat DILI and DIFLD, but only after a serious risk-benefit assessment. They are beneficial in patients who show notable signs of autoimmunity or hypersensitivity, even after drug withdrawal.[92] Ursodeoxycholic acid (UDCA) has a hepatoprotective effect (including for cholangiocytes), stimulatory effect on hepatobiliary secretion, and prevents cellular apoptosis, as described in 15 DILI patients.[94] The effectiveness of UDCA in DILI cases lies in its improvement of the liver function abnormalities and relieving symptoms such as fatigue, pruritus and jaundice,[95–97] significantly improving liver tests[98] and possibly delaying liver transplantation.[99,100] Beneficial effects of UDCA have been shown in cohort studies and case reports after administration of the following drugs that cause liver injury, namely chlorpromazine, cyclosporine, amoxicillin-clavulanate, ticlopidine, flucloxacillin, paraquat, and methotrexate.[97,101–105] Rarely, individual case reports have supported the therapeutic properties of UDCA. One of those is a pediatric report of amoxicillin/clavulanic acid toxicity 4 years after the liver transplantation. Amelioration

Table 2. Drugs that cause DIFLD and proposed mechanisms responsible for their toxicity

Drugs that cause DIFLD	Proposed mechanisms
Amiodarone	Blockage of CPT1 enzyme activity, blockage of mtFAO, increase in acetylcarnitine levels, inhibition of MRC I and II complexes. Trigger of *de novo* lipogenesis by augmenting SREBP1, THRSP, ACLY, FASN, SCD1 PLIN4, ADFP genes' expression. Reduction in GSH levels
Tamoxifen	Impairment of the mtFAO, induction of *de novo* lipogenesis by upregulation of SREBP1c and its downstream genes. Stimulation of MTP expression and VLDL assembly and secretion. Reduction in GSH levels
Methotrexate	Effect on mitochondrial activity by hampering of folate entry into mitochondria, generation of ROS, disruption of the intestinal epithelial barrier
5-Fluorouracil, irinotecan, l-asparaginase	Impairment of mtFAO and enhancement of ROS accumulation in hepatocytes
Valproate	Competition with other FFAs for mtFAO, decrease in CoA levels. Induction of systemic insulin resistance and weight gain
Tetracycline	Inhibition of MTP enzyme, decrease in the PAARα, CPTI and FABP1 genes' expression, which are all involved in mtFAO. Enhancement of ROS generation by activation of ATF4
NRTIs	Inhibition of human DNA polymerase γ, decrease in mitochondrial DNA replication, induction of oxidative stress

ACLY, ATP-citrate synthase; ADFP, adipose differentiation-related protein; ATF4, transcription factor 4; CoA, coenzyme A; CPT1, carnitine palmitoyltransferase-1; CPTI, carnitine palmitoyltransferase I; FABP-1, fatty acid-binding protein 1; FASN, fatty acid synthase; FFA, free fatty acid; GSH, glutathione; MTP, microsomal triglyceride transfer protein; PLIN4, perilipin-4; SCD1, stearoyl-CoA desaturase; SREBP1, sterol regulatory element-binding protein 1; THRSP, thyroid hormone-inducible hepatic protein.

of the amiodarone-induced hepatotoxic effect was achieved with antioxidants such as N-acetyl-cysteine and vitamins C and E.[75] Further clinical trials on humans are needed to confirm these observations.

Conclusions

DIFLD remains a great challenge for researchers and clinicians because of the lack of adequate diagnostic tools and numerous underlying pathophysiologic mechanisms involved. Therefore, many cases of DIFLD are unrecognized or confirmation of diagnosis occurs in later irreversible stages of liver disease. Elucidation of various pathways by which specific drugs cause DIFLD represents a step forward in the development of appropriate therapy. It is important to emphasize that drug withdrawal or dose adjustment are so far the best therapeutic recommendation when it comes to DILI/DIFLD cases. Nevertheless, some treatments, such as UDCA for cholestasis, have shown benefit in the early stages.[98] However, the field needs more studies, especially in the use of pharmacogenetics to predict and avoid DILI, and in identifying individuals who may benefit from pharmacological interventions.

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Conflict of interest

The authors have no conflict of interests related to this publication.

Author contributions

Conceived of and designed the article, and critically revised the manuscript (MS, TOK, VN), obtained funding, and provided administrative, technical and material support (MS), performed literature searches and wrote the manuscript (VN, LK, KD, KB), updated the text of the manuscript (TOK, NRL, SV, GYW), performed figure generation (TOK), and performed critical revision of the manuscript for important intellectual content (MS, GYW).

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Review Article

Hepatocellular Carcinoma and the Role of Liver Transplantation: A Review

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Abstract

Hepatocellular carcinoma (HCC) is one of the leading causes of cancer deaths worldwide and liver transplantation (LT) is the only potentially curative treatment. Over the years, Milan criteria has been used for patient selection. There is ongoing research in this field with introduction of new biomarkers for HCC that can help guide future treatment. Furthermore, newer therapies for downstaging of the tumor are being implemented to prevent dropout from the transplant list. In addition, combination therapies for better outcome are under investigation. Interestingly, the concept of living-donor LT and possible use of hepatitis C virus-positive donors has been implemented as an attempt to expand the organ pool. However, there is a conflict of opinion between different centers regarding its efficacy and data is scarce. The aim of this review article is to outline the various selection criteria for LT, discuss the outcomes of LT in HCC patients, and explore future directions of LT for HCC. Therefore, a comprehensive PubMed/MEDLINE review was conducted. To expand our search, references of the retrieved articles were also screened for additional data. After selecting the studies, the authors independently reviewed them to identify the relevant studies. After careful evaluation 120 studies relevant to out topic are cited in the manuscript. Three tables and two figures are also included. In conclusion LT for HCC has evolved over the years. With the introduction of several expanded criteria beyond Milan, the introduction of bridging therapies, such as transcatheter arterial chemoembolization and radiofrequency ablation, and the approval of newer systemic therapies, it is evident that there will be more LT recipients in the future. It is promising to see ongoing trials and the continuous evolution of protocols. Prospective studies are needed to guide the development of a pre-LT criteria that can ensure low HCC recurrence risk and is not overly stringent, clarify the role of LDLT, and determine the optimal bridging therapies to LT.

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Keywords: Hepatocellular carcinoma; Liver transplantation; Model for end-stage liver disease; Trans-arterial radioembolization; Locoregional therapies.
Abbreviations: HCC, Hepatocellular Carcinoma; LT, Liver Transplantation; DAA, Direct acting antivirals; MT, Metro ticket; OS, Overall Survival.
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Introduction

Hepatocellular carcinoma (HCC) constitutes greater than 80% of all primary liver cancers worldwide.[1] It is the sixth most common cancer and the third leading cause of cancer-related deaths.[2] In the US, from 1999 to 2016, the age-adjusted death rate due to HCC has increased annually by 2.1% (1.9% to 2.3%, $p<0.001$), with increased incidence in all 50 states.[3] Liver transplant (LT) remains one of the most curative treatment options for HCC. According to the Scientific Registry of Transplant Recipients (commonly referred to as the SRTR), in 2019, HCC was the primary diagnosis for 10.6% of waitlist candidates.[4] The deceased-donor transplant rate for candidates with HCC exception points remained higher than those without HCC exception (94.3 vs. 58.3 per 100 waiting list-years). Also, compared with 2018, the deceased-donor transplant rate among patients without HCC exception increased from 50.5 to 58.3 (per 100 waiting list-years). Interestingly, deceased-donor liver transplant (DDLT) recipients with a primary diagnosis of HCC had 5-year survival rates comparable to other disease etiologies (75.2%) but living-donor liver transplant (LDLT) recipients with HCC demonstrated worse 5-year survival rates (61.8%).[4] The prognosis of HCC depends on the tumor burden as well as the underlying liver function. Therefore, LT is an attractive option, especially in patients with HCC and cirrhosis. With the availability of living donor LT, an additional benefit is potential reduction in transplant wait times.

Epidemiology

HCC is the fifth leading cause of death in the USA amongst men and the ninth amongst women.[5] Its incidence has in-

creased over the years, and as per the Surveillance Epidemiology and End Results (commonly referred to as the SEER) database, it is estimated that in 2020 it contributed to 2.4% of all cancers and 5% of all cancer deaths. Based on cases from 2013 to 2017, after age adjustment, the reported incidence of liver and intrahepatic bile duct cancer was 9/100,000 men and women each year, rising an average of 1.7% per year between 2008 and 2017. American Indian/Alaskan native men showed the highest incidence (21.6/100,000), followed by Hispanic males (20.3/100,000). Cancer was most frequently diagnosed (35.3%) in the 55–64 years age group, with the highest mortality (29.9%) occurring in the 65–74 age group. Age-adjusted death rates rose an average of 1.7% each year between 2009 and 2018.[6]

Distribution of HCC varies across the globe. Per the 2020's International Agency for Research on Cancer (commonly referred to as the IARC) report conducted by the World Health Organization (commonly referred to as the WHO) in 2020, the incidence (72.5%), mortality (73.3%), and 5-year prevalence (73.6%) of HCC is highest in Asia. This is likely due to hepatitis B virus (HBV) being endemic to Asia. Europe and Africa follow in second and third place.[7]

Risk Factors

Viral

Up to 90% of HCC cases can be attributed to hepatitis B and C.[8] Globally, approximately 240 million people have chronic HBV infection, and 130–150 million have chronic hepatitis C virus (HCV) infection. HBV has been projected to cause 20 million deaths between 2015 and 2030.[9] High viral DNA levels, high alanine aminotransferase levels, HBV genotype, older age, male sex, and active hepatitis are risk factors for HCC progression. Although 70–90% of the HCC cases arise from HBV cirrhosis, HBV can also cause HCC in the absence of cirrhosis.[10] Comparatively, HCV is associated with a 15- to-20-fold increased risk of HCC, with the 25- to 30-year risk of cirrhosis being 15% to 35%.[11] Interestingly, hepatitis D virus when coinfected with HBV results in severe hepatitis and is reported to have oncogenic properties leading to HCC.[12]

Direct acting antivirals (DAAs) for HCV have dramatically increased sustained virological response (SVR), that helps to change the course of the disease.[13] Surprisingly, there has been some concerns that DDAs may result in unexpected increase in HCC occurrence in patients with HCV.[14] However, recent studies have shown that DAA treatment is not associated with a higher risk of HCC in patients with cirrhosis and chronic HCV infection.[15] In fact, they have a protective effect.[16] Thus, supporting the argument that earlier studies might have been subject to selection bias by attributing high risk patients in the DAA group or there might be pre-existing microscopic undetectable tumors. Therefore, DAAs are a valuable prospect in patients with underlying HCV that might aid in preventing the progression towards HCC and ultimately lowering the transplant burden.

Host

Susceptibility to HCC is influenced by factors such as male sex, older age, diabetes, smoking, alcohol consumption, and genetics. Heavy alcohol intake (>50–70 g/day) has a synergistic effect with HCV and HBV[17] and presumably accelerates the progression to cirrhosis. Similarly, a meta-analysis by Chuang *et al*.[18] concluded that cigarette smoking appears to interact with both HBV and HCV and increases HCC risk, separate from its independent carcinogenic effect. Aflatoxin exposure is another risk factor, whereby highly hepatocarcinogenic metabolites are secreted by certain *Aspergillus* molds commonly present in staple cereals (such as those made from corn, peanuts, and soybeans) when stored in damp conditions. Aflatoxins exhibit tumorigenic properties via mutating the tumor suppressor gene p53. Exposure is prevalent in HBV endemic areas (Sub-Saharan Africa and Eastern Asia).

Nonalcoholic fatty-liver disease (NAFLD) / non-alcoholic steatohepatitis (NASH)

Individuals with obesity and diabetes experience a higher incidence of HCC than those without these comorbidities. A meta-analysis conducted by Larsson *et al*.,[19] in Europe, the USA and Asia, concluded that overweight and obese individuals have an increased relative risk of developing HCC (1.07, 95% confidence interval [CI]: 1.01–1.15 and 1.85, 95% CI: 1.44–2.37, respectively). Similarly, another meta-analysis by El-Serag *et al*.[20] reported a significant association between HCC and diabetes, independent of alcohol use or viral hepatitis. The obesity epidemic and insulin resistance are closely associated with the rising prevalence and severity of NAFLD/NASH, which causes hepatic fibrosis and leads to end-stage liver disease. A meta-analysis comprising of 88 studies from 22 countries reported global prevalence of NAFLD is 25.24% with pooled regional incidence of NAFLD from the West to be estimated around 28 per 1,000 person-years (95% CI: 19.34–40.57).[21] The annual incidence of HCC in NAFLD patients was 0.44 per 1,000 person-years (95% CI: 0.29–0.66), whereas for NASH the annual HCC incident rate was 5.29 per 1,000 person-years (95% CI: 0.75–37.56).[21] Similarly, a population-based study in the USA has shown that metabolic syndrome is significantly associated with an increased risk of HCC (odds ratio: 2.13; 95% CI: 1.96–2.31, $p<0.0001$).[22] Furthermore, the cumulative incidence of HCC in patients with NASH cirrhosis ranges from 2.4% over 7 years to 12.8% over 3 years.[23] Moreover, some studies have demonstrated that HCC can occur in patients who have NASH without cirrhosis.[24]

Surveillance

The aim of screening is early detection of tumor so it may be treated while still having a good prognosis. Cirrhosis is the fundamental risk factor for HCC and is found in 85–95% of HCC.[25] Subsequently, approximately 2–4% of patients with cirrhosis develop HCC annually.[26] Several surveillance guidelines for HCC have been developed across the globe. The American Association for the Study of Liver Diseases (commonly known as AASLD) recommends screening of adults with cirrhosis, using ultrasound (US) with/or without alpha-fetoprotein (AFP) every 6 months.[27] Once a lesion is detected, either multiphasic computed tomography (CT) or multiphasic magnetic resonance imaging (MRI) is recommended. It is recommend against screening of patients with Child-Pugh class C cirrhosis, unless they are on the transplant waiting list and the routine biopsy reveals indeterminate nodules.[27] Though the European Association for the Study of the Liver (commonly known as EASL) guidelines are similar to those of the AASLD, except that they are more aggressive in their surveillance and recommend to start screening in patients with bridging fibrosis (Metavir F3) but without AFP.[28] In comparison, the Chinese guidelines recommend mandatory AFP testing and a diagnostic diameter threshold of 2 cm (compared to 1 cm by EASL and

Table 1. Outcome of trials for systemic therapy

Study	Drug	Control	OS in months	HR (95% CI)
SHARP[37]	Sorafenib (TKI)	Placebo	10.7 vs. 7.9	0.69 (0.55–0.87)
Asia-Pacific[38]	Sorafenib (TKI)	Placebo	6.5 vs. 4.2	0.68 (0.50–0.93)
REFLECT[40]	Lenvatinib (TKI)	Sorafenib	13.6 vs. 12.3	0.92 (0.79–1.06)
RESORCE[41]	Regorafenib (TKI)	Placebo	10.6 vs. 7.8	0.63 (0.50–0.79)
CELESTIA[42]	Cabozantinib (TKI)	Placebo	10.2 vs. 8.0	0.76 (0.63–0.92)
REACH-2[43]	Ramucirumab (VEGRFI)	Placebo	8.5 vs. 7.3	0.71 (0.53–0.95)
IMbrave150[44]	Atezolizumab (CPI) and bevacizumab (VEGRFI)	Sorafenib	At 12 months 67.2% vs. 54.6%	–

CPI, check point inhibitor; TKI, tyrosine kinase inhibitor; VEGRFI, vascular endothelial growth factor inhibitor.

AASLD).[29] This discrepancy in guidelines is likely due to the cost effectiveness in the population that is being screened.

Prevention

Primary prevention is defined as avoiding the initiation of the disease process. Global vaccination against HBV is an excellent example of primary prevention. In Taiwan, due to the vaccination program initiated in early 1980s, the changes in age and sex-adjusted rate ratios for individuals aged 5 to 29 years led to in decreased HCC incidence by more than 80% till the early 2000s.[30] In addition, to avoid HBV and HCV transmission by blood contamination, practices of disposable needles and syringes use, adequate sterilization of equipment, and wearing gloves to handle wounds and blood products have been implemented.[31] Furthermore, alcohol abstinence and smoking cessation should be encouraged. A recent liver cancer pooling project consisting of 14 USA-based prospective cohort studies determined that smoking at baseline is associated with an increased risk of HCC (hazard ratio [HR]: 1.86, 95% CI: 1.57–2.20). Also compared to non-drinkers, heavy alcohol consumption (>7 drinks/day) was associated with an 87% increased HCC risk (HR: 1.87, 95% CI: 1.41–2.47).[32] Lifestyle modification to mitigate the development of metabolic syndrome is another reasonable intervention since obesity and diabetes are also linked to HCC.[32] Though data is limited, medications such as statins and metformin have shown a protective effect against HCC.[33,34] Secondary prevention is early detection and prevention of worsening disease. It can be achieved with agents such as interferon and antivirals (for example in cases of HBV infection) that can prevent viral replication and help achieve sustained virological response.[35]

Treatment options

Table 1 shows the results of treatment trials and Figure 1 shows the systemic therapy treatment algorithm.

Barcelona Clinic Liver Cancer (commonly referred to as BCLC) staging has been adopted worldwide as the background of HCC treatment. Patients with early-stage HCC are effectively treated with LT, radiofrequency ablation (RFA), or surgical resection. Individuals with intermediate stage with intrahepatic multifocal HCC benefit from liver-directed treatments, such as transcatheter arterial chemoembolization (TACE). Many transplant centers now accept patients with HCC patients who have been successfully down-staged by liver-directed therapy.[36]

Systemic therapy is recommended for advanced-stage HCC. After the Sorafenib Hepatocellular Carcinoma Assessment Randomized Protocol (referred to as 'SHARP') trial in 2008, sorafenib became the first approved systemic therapy for HCC.[37] It is an oral multi-kinase inhibitor of tyrosine kinase receptors, including vascular endothelial growth factor

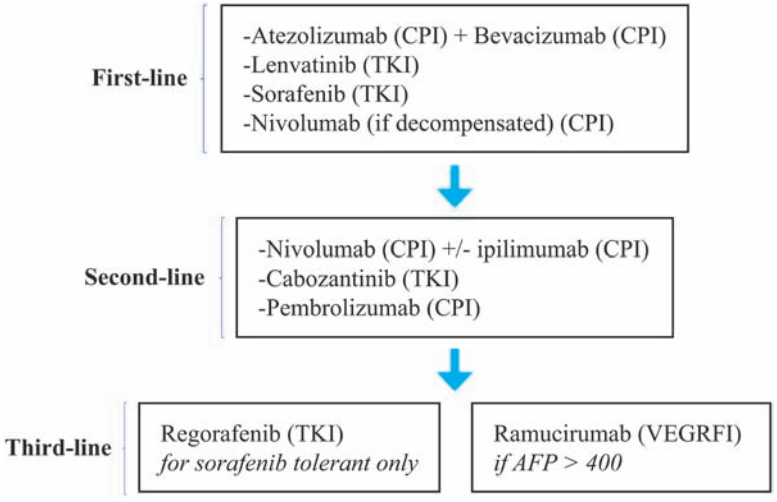

Fig. 1. Systemic therapy of HCC. CPI, check point inhibitor; HCC, Hepatocellular Carcinoma; TKI, tyrosine kinase inhibitor; VEGRFI, vascular endothelial growth factor inhibitor.

Table 2. Different criteria for liver transplantation

Criteria	Detail
MILAN[48]	1 lesion ≥2 cm and ≤5 cm OR up to three lesions, each ≥1 cm and ≤3 cm. No evidence of vascular invasion or extrahepatic metastases
UCSF[49]	Solitary tumor ≤ 6.5 cm or ≤ 3 tumors, with the largest ≤ 4.5 cm
Up-to-seven[51]	7 as total of the size of the largest lesion in cm and number of lesions. No vascular invasion
Toronto criteria[53]	No upper limit on size and number of lesions. No extrahepatic metastases, evidence of venous or biliary tumor thrombus cancer-related symptoms

receptors (commonly referred to as VEGFRs) and platelet-derived growth factor receptor (PDGFR)-β, which is associated with neovascularization and cell proliferation. Its benefit was confirmed by an Asia-Pacific study.[38] Analysis of these trials showed that sorafenib was of the greatest benefit to patients with HCV etiology, without extrahepatic spread and low neutrophil-to-lymphocyte ratio.[39] In 2018, lenvatinib, which is also a multi-kinase agent, proved to be non-inferior to sorafenib in the REFLECT trial for advanced HCC.[40] It did improve secondary endpoints, such as time to progression, progression-free survival and quality of life. It was effective in patients with AFP >200 ng/mL and less effective in patients with HCV etiology, in Western countries, and without extrahepatic spread. Currently, both are approved as first-line systemic agents. More recently, based on successful trials, three more multi-kinase inhibitors (regorafenib, cabozantinib and ramucirumab) were approved as second-line agents, after demonstrating success in trials.[41–43] Regorafenib is a multi-kinase inhibitor that was used in patients who tolerated sorafenib but showed radiological progression. Its use resulted in median survival of 10.6 months compared to 7.8 months with the placebo group (HR: 0.63, $p<0.0001$).[41] Unlike the trial for regorafenib, the drug cabozantinib was studied in patients who failed up to two previous systemic treatments, including prior immunotherapy. It produced favorable results in patients ≥65 years of age, with AFP ≥400 ng/mL, with extrahepatic spread, of non-Asian population, and with HBV etiology.[42] Although, median overall survival (OS) was only 1.2 months, ramucirumab showed benefit in patients with baseline AFP ≥400 ng/mL.[43] The IMbrave-150 trial, which included the combination of atezolizumab, a programmed death ligand 1 (commonly referred to as PD-L1) inhibitor, and bevacizumab, a monoclonal antibody targeting the vascular endothelial growth factor, demonstrated superiority over sorafenib[44] and the combination is now approved as first-line chemotherapy. This resulted in significantly longer OS and progression-free survival (OS at 12 months was 67.2% [95% CI: 61.3–73.1] with atezolizumab-bevacizumab and 54.6% [95% CI: 45.2–64.0] with sorafenib).[44] For patients without liver decompensations, these are now the preferred first-line agents for advanced HCC. Immunotherapy has further expanded treatment options for advanced hepatocellular carcinoma, and programmed cell death 1 (commonly referred to as PD-1) inhibitors nivolumab and pembrolizumab have received accelerated approval in USA.[45] With such advancement of systemic treatment options in recent years, several clinical trials are underway examining use of systemic treatments in intermediate stage disease. Two clinical trials involving a combination of immunotherapy and tyrosine kinase inhibitors are ongoing, specifically examining their potential as neoadjuvant treatments prior to LT and with a primary outcome of recurrence-free survival after LT.[46,47] In future years, such trials will likely significantly transform the treatment paradigm.

Selection criteria for LT

Selecting patients with HCC for LT and prioritizing them on the transplant waitlist has long been decided based on the Milan criteria (MC). This is defined as: (a) single tumor with a diameter ≤5 cm; OR (b) no more than three tumors, each ≤3 cm in size; and (c) no vascular invasion; and (d) no extrahepatic involvement. This is the earliest criteria that set standards for our current transplant protocol.[48] In patients transplanted using MC, the survival rate was 75% and the rate of recurrence-free survival was 83%.[48] As these results were comparable to individuals with benign disease, the MC was accepted worldwide. However, with concerns that the MC was too restrictive and excluded patients who might have benefited from LT, other criteria have been introduced. One of these is the University of San Francisco California (commonly known as the UCSF) criteria, which was introduced in 2001. This is defined as: a) solitary tumor ≤6.5 cm or ≤3 nodules with each lesion ≤4.5 cm; and b) total maximum diameter ≤8 cm. It showed comparable survival of 75.2% at 5 years.[49] A study published in 2007 on 467 transplants showed similar 5-year survival in patients meeting MC and UCSF criteria by preoperative imaging (79% vs. 64%; $p=0.061$) and explant pathology (86% vs. 71%; $p=0.057$).[50] However, survival beyond UCSF criteria was below 50%. Thus, with studies like this showing similar results, Mazzaferro *et al*.,[51] who introduced MC criteria, expanded it and proposed "up-to-7 criteria", defined as: the sum of the tumor number and the size of the largest tumor (in cm) not larger than 7. Patients without microvascular invasion, but who fell within the up-to-seven criteria, had a 5-year OS of 71.2%. In comparison, the survival rate was 48% in patients with microvascular invasion.[51] In addition to increased mortality, the presence of microvascular invasion is not assessable before transplantation. This limits the routine application of up-to-seven criteria.

Criterion by different countries, such as the extended Toronto criteria (no restrictions on tumor size or number), with 5-year survival of 68%, and the Kyoto criteria (tumor ≤10 nodules, all ≤5 cm and a serum des-gamma-carboxy prothrombin (commonly referred to as DCP) level ≤400 mAU/mL) with 5-year survival rate of 86.7% have been proposed.[52,53] However, the MC is still the gold standard for a successful LT and is used to assess the validity of other suggested criteria. Table 2 presents the different criteria.[48,49,51,53]

Metro ticket (MT) prognostic model

MT is a predictive model that was introduced in 2009 from a European cohort of patients.[51] It predicts 3-year and 5-year survival post-transplant using radiological data. The MT calculator only incorporates tumors >10 mm diameter, with a maximum of 10 nodules. Additionally, MT can also predict 5-year survival in patients who undergo transplant. This considers tumor size, number and the presence or absence of microvascular invasion and, therefore, can only be calculated from explant pathology. Raj *et al*.[54] validated this model in their study, where the predicted and observed out-

Table 3. Summary of some of the studies included in the manuscript

Reference	Country	Study Design	Samples, *n*	Median age	Median biological MELD score	Recurrence % at last follow-up, *n*/N	Survival % (1 or 5 year)	Follow-up in years
Mazzaferro *et al*. (1996)[48]	Italy	Prospective	48	52	Child-Pugh used	8.3 (4/48)	94% (1)	2.16
Yao *et al*. (2001)[49]	USA	Prospective	70	54	Child-Pugh used	11.4 (8/70)	73% (5)	5
Duffy *et al*. (2007)[50]	USA	Prospective	467	56.6	NA	21.2 (99/467)	82 (1), 52 (5)	6.6
Mazzaferro *et al*. (2009)[51]	Multi-national	Retrospective	1,556	55	NA	20.0 (311/1,556)	62 (4.4)	4.4
Ito *et al*. (2007)[52]	Japan	Retrospective	125	55	15	16 (20/125)	68.3 (5)	2.41
Sapisochin *et al*. (2016)[53]	Canada	Prospective	243	Within MC (57.9), exceeded MC (60.4)	Within MC (11), exceeded MC (10)	Within MC (16.1, *n*=20), exceeded MC (25.6, *n*=22)	Within MC 78 (5), exceeded MC 68 (5)	5

MELD, model for end-stage liver disease.

comes were within 95% CIs. In a larger single-center study comprised of 230 patients, MT accurately predicted patients with microvascular invasion and no invasion.[55] However, there was a high discrepancy in the 23 cases with macrovascular invasion, where the predicted 5-year survival rate was 43.5%, whereas the observed 5-year survival rate was only 8.7%.[55] This is one of the draw backs, as the MT calculator does not consider the difference in microvascular or macrovascular invasion and might need a revision. Recently, the MT calculator was revised and AFP was added.[56] Thus, with the additions of some more important parameters, accurate prediction could be made. As MT provides continuous survival probabilities, accurate prediction will be helpful for transplant centers to prioritize their transplant list.

LT Evolution and Outcomes

Table 3 summarizes some of the studies on this topic.

Introduction of MELD

Early studies of LT for HCC showed a 70% to 80% range for 5-year mortality rate.[57,58] This led to the introduction of strict allocation criteria such as the MC in 1996 and various other scoring systems. However, despite these advancements, patients with HCC remained on the waiting list longer than candidates without HCC, resulting in less than 5% LT for HCC in the USA from 1997–2002.[59] Thus, in 2002, the United Network for Organ Sharing (commonly referred to as the UNOS) adopted the model for end-stage liver disease (MELD) score for allocation. The MELD score is an objective predictor of 3-month mortality without LT and is calculated using serum bilirubin, creatinine, and international normalized ratio for prothrombin time (INR).[60] There have been several modifications to MELD based on different parameters and it is noteworthy to mention the MELD-sodium (Na) score. In cirrhotic patients, hyponatremia leads to portal hypertension, which is an independent predictor of survival at 3 and 12 months.[61] Thus, addition of Na to the MELD improves its predictive accuracy, especially for patients with lower range MELD scores, helping them to get prioritized on the transplant list. However, when the MELD score increases, serum Na contributes much less to increasing mortality prediction.[62] Furthermore, serum Na can change with the use of diuretics and intravenous hypotonic fluids. Thus, limiting the use of MELD-Na. Therefore, in order to promote equal allocation of donor organs between HCC and non-HCC patients on the waiting list, MELD exception points are given to HCC candidates. Initially, 24 points were assigned to stage 1 tumors (1 nodule <2 cm) and 29 points to stage 2 tumors (1 nodule 2–5 cm or 2 or 3 nodules each ≤3 cm). It was subsequently revised in 2005, when no points were assigned for stage 1 tumors and 22 points for stage 2 tumors (Table 4) with incremental increase in points over time.[63] This resulted in a rise from 5% to 26% LT for HCC from 2002–2007.[59] This criterion changes periodically and most recently in the UNOS regulation, with the candidate receiving a MELD score that is 3 points below the median MELD at transplant for liver recipients at least 18 years-old in the donation service area where the candidate is registered. However, If the candidate's exception score would be higher than 34 based on this calculation, the candidate's score will be capped at 34.[64]

Table 4. Changes in MELD score over time

Stage	Original MELD score	2005 MELD score	2018 MELD policy pointers
First stage: one tumor <2 cm	24	0	Upon initial registration candidate should be at least 18 years of age and will be assigned the calculated MELD
Second stage: one tumor 2–5 cm or two to three tumors not >3 cm	29	22	Initial exception request in 6 months for 3 points below the median MELD at transplant in donation service area, and subsequent requests every 3 months

MELD, model for end-stage liver disease.

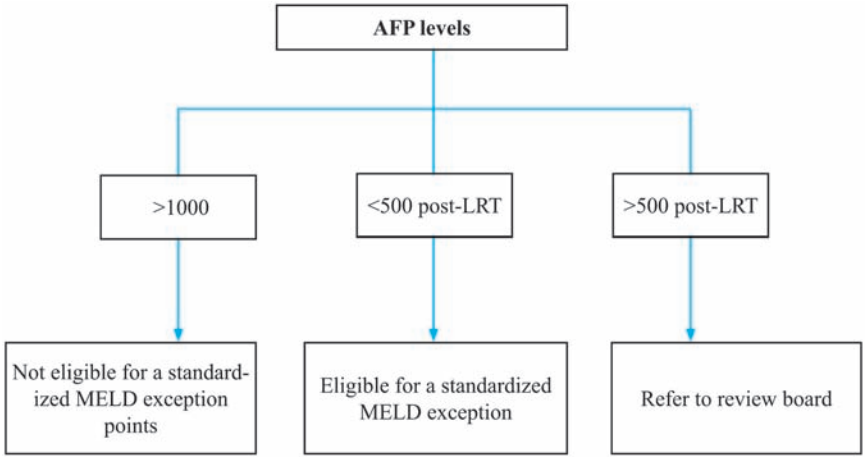

Fig. 2. Algorithm for selecting candidates for LT based on AFP levels. AFP, alpha-fetoprotein; LRT, locoregional therapy; MELD, model for end-stage liver disease.

Biomarkers and role of liver biopsy for HCC

Treatment of HCC is a moving target and there is ongoing research on predictive biomarkers that can set a standard for treatment. There are various prognostic markers, such as AFP, lens culinaris agglutinin-reactive fraction of α-fetoprotein (AFP-L3) and DCP that are being used for surveillance and diagnostic purposes. AFP has been commonly used in conjugation with US for HCC surveillance. Similarly, AFP-L3 predicts tumor recurrence and poor outcome. Cheng *et al* performed a meta-analysis and determined that high pre-treatment serum AFP-L3 suggested poor OS (HR: 1.65, 95% CI: 1.45–1.89, $p<0.00001$) and disease-free survival (DFS) (HR: 1.80, 95% CI: 1.49–2.17, $p<0.00001$) of HCC.[65] Furthermore, subgroup analysis revealed that pre-treatment AFP-L3 may have significant prognostic value in HCC patients, even with low AFP concentration. Interesting, DCP was once believed to be a useful predisposing clinical parameter for the development of portal vein thrombosis.[66] However, in addition, it is now thought to be a useful recurrence predictive factor, indicating 5-fold increased risk of HCC recurrence after LT.[67] Likewise, AFP >1,000 ng/mL among patients with HCC either within or beyond MC is associated with a very high risk of HCC recurrence and poor survival after LT.[68] A recent national policy has been recently implemented by UNOS, in which patients with HCC and AFP >1,000 ng/mL are deprived of HCC exception points. These patients are required to show a decrease in AFP to <500 ng/mL with locoregional therapy (LRT) before they can proceed with LT (Fig. 2).[69]

Biomarkers for HCC therapy, such as with sorafenib, have also been investigated. Sorafenib is an oral multikinase inhibitor that acts on VEGFR-2/3, PDGF-R, Flt3 and c-Kit, and the mitogen-activated protein kinases/extracellular signal-regulated kinase (commonly known as MAPK/ERK) pathway. Initially, it was thought that levels of phosphorylated-ERK may be a biomarker for the therapy.[70] However, this potential was not confirmed and there is still no validated prognostic or predictive markers of response to sorafenib.[71] More recently, there has been a lot of research on the potential use of microRNAs (commonly referred to as miRNAs), long non-coding RNAs (commonly referred to as lncRNAs), and circular RNAs (commonly referred to as circRNAs) as diagnostic and therapeutic biomarkers.[72] However, results are limited, warranting more prospective studies.

Liver biopsy for HCC can be challenging, as there is a risk of bleeding (3–4%) and chance of seeding (2.7%).[73,74] Although imaging alone is sufficient in cirrhotic patients, especially if the tumor is >1 cm. However, in non-cirrhotic patients, biopsy is strongly recommended by some international guidelines, such as that of the EASL.[28] Liver biopsy is not only helpful for correct diagnosis or proper staging; it can also be used for detection of therapeutic targets. However, as only small tissue samples can be obtained, there is a chance to miss tumor heterogeneity or dynamic tumor progression. Therefore, the non-invasive method known as liquid biopsy is becoming popular, where tumor components such as circulating tumor cells (also referred to as CTCs), circulating tumor DNA (commonly referred to as ctDNA) and miRNAs are analyzed from body fluids (blood, cerebral spinal fluid, etc.).[75]

Bridging therapies

The SRTR registry shows an increase in the number of new waitlist registrants (11,844 in 2018 vs. 11,514 in 2017 vs. 11,340 in 2016 and 10,636 in 2015) and a continued increase in the transplant rate (54.5 per 100 waitlist-years in 2018 vs. 51.5 per 100 waitlist-years in 2017) for patients with HCC.[76] While on the waiting list, candidates are prone to tumor growth, resulting in going beyond the transplant criteria and an eventual 12 month dropout probability of 25%.[77] Therefore, bridging therapies are offered to patients, which help in downstaging of the tumor. Amongst them, LRTs like the TACE, transarterial radioembolization (TARE), transarterial embolization (TAE) and RFA are most commonly used. Kulik *et al*.[78] carried out a meta-analysis of 63 studies on bridging therapies. The subgroup analysis compared TACE vs. RFA vs. multiple therapies and showed dropout from the waiting list to have a relative risk (95% CI) of 0.212 (0.027–1.650) vs. 1.434 (0.793–2.594) vs. 0.131 (0.038–0.449) and recurrence post-LT of 1.74 (0.49–6.15) vs. 0.745 (0.069–8.003) vs. 1.49 (0.826–2.7). Currently, there is heterogenicity amongst the studies and most of the data are from single centers. More multicenter randomized controlled trials (RCTs) are needed to further explore this branch of transplantation.

TAE

This technique uses particulate and liquid materials for embolization, which target hepatic vessels and thereby lead to cell necrosis via ischemia. It is commonly known as “bland”

embolization, as the particles do not have chemotherapeutic or radioactive functions. Cone-beam CT is used to make sure that only the target lesion is embolized.[79] A RCT comparing drug-eluting beads (DEBs)-TACE with TAE showed that DEB-TACE resulted in better local response, fewer recurrences, and a longer time to progression than TAE.[80] However, a meta-analysis comparing TAE to conventional-TACE (c-TACE) showed no significant difference in OS.[81] Thus, TAE is a promising option compared to conservative treatment and, as it is devoid of systemic toxicity (using no chemotherapeutic agent), it can be used more confidently in patients with borderline liver function.

TACE

This technique helps to cut blood supply to the neoplastic cells via embolization and chemotherapeutic drugs. Currently, it is the standard of treatment for intermediate (BCLC stage B) HCC. The most commonly used chemotherapy agent in TACE is doxorubicin.[82] A RCT showed a 2-year survival rate of 63% in patients with advanced HCC who received TACE compared to 27% survival among the conservative management group.[83] The c-TACE technique had a limitation of systemic toxicity. Therefore, the use of DEBs, which are non-absorbable embolic microspheres charged with cytotoxic agents, was introduced. Burrel *et al*.[84] reported a median survival of 48.6 months with the use of DEB-TACE. Currently, there is no clear evidence on the superiority of DEB-TACE over c-TACE. Lammer *et al*.[85] performed a RCT comparing the two therapies and reported that the DEB group had higher rates of complete response, objective response, and disease control compared with the c-TACE group (27% vs. 22%, 52% vs. 44%, and 63% vs. 52%, respectively). However, superiority was not established (p=0.11). Irrespective, DEB use was associated with improved tolerability, with a significant reduction in serious liver toxicity (p<0.001) and a significantly lower rate of doxorubicin-related side effects (p=0.0001).[85]

TARE

In this technique, the microspheres contain a radioactive element, yttrium-90 (Y-90), which undergoes beta decay and generates free radicles. This hinders the cell's repair mechanisms, leading to cell death.[79] This technique is particularly helpful in patients with portal vein thrombosis (PVT), who experience reduced embolic effect with other techniques. Following performance of a clinical trial using TARE, Salem *et al*.[86] reported OS of 17.2 months amongst Child-Pugh A cirrhotic patients with PVT, decreasing to 5.6 months among Child-Pugh B cirrhotic with PVT. In another study, regression of PVT was reported with the use of Y-90.[87] TARE has also shown to result in better quality of life scores compared to TACE.[88] However, when compared to sorafenib, trials have shown no difference in OS with TARE.[89,90] Nevertheless, as it does not have systemic effects like sorafenib, it is an attractive option in selected patients.

Ablation

Amongst the ablation techniques for HCC, RFA and microwave ablation (MWA) are the most commonly used. They are considered as valuable option in very early-stage disease (i.e. BCLC 0).[91] One study found that the 5-year survival rate in patients who had RFA pre-transplant was approximately 70%.[92] RFA is valuable in targeting smaller lesions but is prone to the heat-sink effect. Therefore, MWA can be used alternatively, as it targets multiple tumor sites with higher energy. Shibata *et al*.[93] reported equivalent therapeutic effects and complication rates for RFA and MWA. Similarly, a meta-analysis by Tan *et al*.[94] showed no significant difference between MWA and RFA regarding complete ablation, local recurrence, DFS, OS, and major complications. Thus, these ablation techniques can be used interchangeably based on center-specific experience, but there remains a need for more prospective studies. In conclusion, although data are scarce, patient survival (79% vs. 75%, p=0.03) and graft survival (76% vs. 71%, p=0.03) at 3 years post-LT indicates more benefit for HCC patients receiving ablative therapy vs. those not receiving locoregional treatment.[95]

Stereotactic body radiotherapy (SBRT)

This technique delivers high-dose radiation in small fractions and with great precision. The Asia-Pacific Primary Liver Cancer Expert meeting (referred to as APPLE), an association of liver cancer experts in the Asia-Pacific region, has recommended application of SBRT for early-stage or small-sized HCC.[96] Prospective data are limited, but studies have demonstrated 3-year OS up to 70% and 5-year OS up to 64% for tumors <5 cm.[97,98] Recently, a phase 2 multicenter trial found 3-year local control rate of 95%, progression-free survival of 36% and OS of 76%.[99] Furthermore, evaluation of gastroduodenal toxicity by esophagogastroduodenoscopy was performed before and 2 months after SBRT, and showed no significant difference.[99] Thus, this is considered a safe option. Sapisochin *et al*.[92] compared SBRT, TACE and RFA as a bridge to LT and reported no significant difference in dropout rate, OS from listing, or LT in any of the groups. Therefore, it is another option for patients with borderline liver function. However, more RTCs are needed to compare SBRT with other treatment modalities for HCC.

Combination therapy

Treating HCC can be challenging with monotherapy, and therefore the concept of combination therapy was introduced to increase OS. Although improved time to progression has been shown in studies combining systemic (sorafenib) with LRT (TACE), results on OS are contradictory.[100,101] Combination of LRTs, for example TACE and SBRT, have resulted in reduction in local recurrence and improved OS.[102] However, when TACE-RFA dual therapy was used, the response to TACE-RFA appeared to be similar to that of RFA but better than that of TACE monotherapy.[103] Similarly, combination therapy of PD-L1 inhibitor and a monoclonal antibody have shown longer OS.[44,47] Furthermore, there are ongoing trials involving combination of immunotherapy and tyrosine kinase inhibitors.[46,47] Thus, once we have more data, we will be more confident with the optimal treatment combinations for HCC.

LDLT

Currently, there is a growing demand for LT in HCC patients. In the USA, over 18,000 people await transplantation annually and only approximately 5,000 organs are available.[104] This has led to the suggestion of LDLT to meet the growing demand and reduce waitlist time. A meta-analysis carried out by Liang *et al*.[105] showed comparable results in terms of patient survival (5 years, OR: 0.64, 95% CI: 0.33–1.24), recurrence (5 years, OR: 1.21, 95% CI: 0.44–3.32), and recurrence-free survival rates (5 years, OR: 1.11, 95% CI:

0.70–1.77)) in patients undergoing LDLT vs. DDLT for HCC. In comparison, another meta-analyses comparing LDLT vs. DDLT showed overall hazard ratios for DFS as 1.59 (95% CI: 1.02–2.49, p=0.041) and the OS as 0.97 (95% CI: 0.73–1.27, p=0.81).[106] While this may suggest a worse DFS after LDLT, there may be a selection bias with limited assessment of tumor biology from the shorter waiting period of LDLT.

Likewise, a recent meta-analysis including 39 studies with 38,563 patients showed LDLT to be comparable in requirement for red blood cell transfusion, perioperative mortality, length of hospital stays, re-transplantation rate, HCV recurrence rate, and HCC recurrence rate with DDLT. Cold ischemia time was shorter, and duration of recipient operation was longer in LDLT. The postoperative intra-abdominal bleeding rate was lower in LDLT recipients (OR: 0.64, 95% CI: 0.46–0.88, p=0.006), but this did not decrease the perioperative mortality. LDLT was associated with significantly higher biliary (OR: 2.23, 95% CI: 1.59–3.13, p<0.00001) and vascular (OR: 2.00, 95% CI: 1.31–3.07, p=0.001) complication rates and better OS (1 year: OR: 1.32, 95% CI: 1.01–1.72, p=0.04; 3 years: OR: 1.39, 95% CI: 1.14–1.69, p=0.0010; and 5 years: OR: 1.33, 95% CI: 1.04–1.70, p=0.02).[107] Subsequent studies, including the Adult-to-Adult Living Donor Liver Transplantation Cohort Study (known as the A2ALL), did not find a significant difference in the 5-year post-transplant survival between LDLT and DDLT.[108] Therefore, with the current evidence, it is clear the survival of patients with HCC undergoing LDLT is not significantly impacted.

Eligibility criteria for LDLT currently used in the Johns Hopkins LT program

- Patients with HCC diagnosed by imaging according to the MC and biological MELD of ≤25. Bridging therapy may or may not be required.
- Patients beyond the MC, who have undergone down-staging should have MELD of ≤25, with no extrahepatic disease or vascular invasion, AFP of ≤500 or have well-differentiated lesion on biopsy. Bridging therapy may or may not be required.

Recurrence after transplant

Despite the strict criteria used for LT, tumor recurrence is expected in 15–20% of HCC patients who have undergone LT, with 75% of the recurrence occurring during the first 2 years after the LT.[109] A systemic review consisting of 61 studies showed recurrence rate of 16% at median time of 13 months post-transplant.[110] Early recurrence is thought to originate from micrometastasis. Also, patients beyond the MC prior to LT have higher rates of tumor recurrence. There is also a discrepancy between radiology and pathology results. A recent case series showed that approximately one-third of patients were within MC on explant pathology when they were all within MC according to imaging findings.[111] Other factors such as vascular invasion, degree of tumor differentiation, tumor stage and AFP levels also play an important role in recurrence. The OS after HCC recurrence is approximately 1 year. Surgical resection of localized HCC recurrence and systemic treatments for controlling extrahepatic spread of HCC recurrence have been shown to be associated with the higher survival rates.[110] Despite the advances in systemic treatments with immunotherapy, immunotherapy is not recommended in the post-transplant setting, due to graft failure and high mortality.[112] Recently, some serum markers such as AFP, neutrophil-to-lymphocyte ratio and platelet-to-lymphocyte ratio have been proposed in addition to morphological criteria to predict recurrence.[113] Furthermore, the Risk Estimation of Tumor Recurrence After Transplant (also known as the RETREAT) score, consisting of AFP levels, microvascular invasion and number/diameter of viable tumor, has been introduced.[114] However, data are limited and better biomarkers for prediction of HCC recurrence after LT are needed. Furthermore, the role of immunosuppressive therapy post-LT cannot be underestimated; although, calcineurin inhibitors (CNIs) are considered the main agents for use. These agents are used in combination with mammalian target of rapamycin inhibitors (commonly referred to as mTORi), such as sirolimus and everolimus, which represents an alternative immunosuppressive regimen. Unfortunately, a phase 1 RTC where everolimus was used in combination with sorafenib did not show improvement in OS.[115] This trial did not proceed to phase II, as they participants were unable to reach an antiproliferative dose of everolimus due to cirrhosis. Another phase II multicenter trial showed that everolimus resulted in severe adverse events without any added benefit of progression-free survival.[116] Its counterpart, sirolimus, has been associated with increased mortality rates.[117] A metanalysis comprising 42 studies showed that patients on everolimus had significantly lower recurrence rates of HCC, compared with those on sirolimus or CNIs (4.1% vs. 10.5% vs. 13.8%, respectively, p<0.05).[118] However, these results are biased, as everolimus-treated recipients had shorter follow-up period (13 vs. 30 vs. 43.2 months) and more frequently had been transplanted for HCC within MC (84% vs. 60.5% vs. 74%, respectively, p<0.05).[118] Nevertheless, studies have shown that everolimus used in combination with CNIs post-LT allows for decreased doses of CNIs and improvement in kidney function.[119,120] However, in light of the current limited evidence, everolimus is not used routinely as part of a treatment protocol and its use is center-specific.

Post-transplant Surveillance

Follow-up of transplant recipients is essential, as to ensure their health and identify potential complications. Per the SRTR report published in 2019, graft failure occurred in 6.6% of DDLT recipients at 6 months and 8.9% at 1 year for transplants performed in 2018. In addition, within 1 year, 12.3% of liver transplant recipients in 2017–2018 experienced at least one episode of acute rejection and 1% of adult liver recipients developed post-transplant lymphoproliferative disorder over 5 years.[76] Thus, timely follow-up is mandatory. Considering the poor outcome associated with HCC recurrence after LT, strict HCC surveillance after LT is recommended. Unfortunately, there is no standardized protocol worldwide regarding the type and frequency of post-LT cross-sectional imaging in surveillance of HCC LT recipients. At our center, postoperative HCC surveillance usually consists of contrasted cross-sectional imaging with CT or MRI with AFP measurement every 3 months for the first year and every 6 months for the second and third years.[111]

Conclusions

LT for HCC has evolved over the years. With the introduction of several expanded criteria beyond MC, the introduction of bridging therapies (such as TACE and RFA), and the approval of newer systemic therapies, it is evident that there will be more LT recipients in the future. It is promising to see ongoing trials and the continuous evolution of protocols. Prospective studies are needed to guide the development of a pre-LT criteria that can ensure low HCC recurrence risk and not be overly stringent, clarify the role of LDLT and de-

termine the optimal bridging therapies to LT.

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Conflict of interest

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Author contributions

Study concept and design (HM, AT, AG, RK), acquisition of data (HM, AT, AG, SYL), drafting of the manuscript (HM, AT, PST, MG, SAA, SYL), and critical revision of the manuscript for important intellectual content (AG, MG, SAA, PST, RK, AKK, CS).

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Review Article

Novel Agents in the Management of Hepatic Encephalopathy: A Review

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Abstract

Hepatic encephalopathy is an often devastating complication of chronic liver disease, associated with high mortality and increased burden on patients and healthcare systems. Current agents (such as nonabsorbable disaccharides and oral antibiotics) are often only partially effective and associated with unpleasant side effects. With our improved understanding of the pathophysiology of hepatic encephalopathy, multiple treatment modalities have emerged with promising results when used alone or as an adjunct to standard medications. The mechanisms of these agents vary greatly, and include the manipulation of gut microbial composition, reduction of oxidative stress, inhibition of inflammatory mediators, protection of endothelial integrity, modulation of neurotransmitter release and function, and other novel methods to reduce blood ammonia and neurotoxins. Despite their promising results, the studies assessing these treatment modalities are often limited by study design, sample size, outcome assessment heterogeneity, and paucity of data regarding their safety profiles. In this article, we discuss these novel agents in depth and provide the best evidence supporting their use, along with a critical look at their limitations and future directions.

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Introduction

Hepatic encephalopathy (HE) is a serious and common complication of liver dysfunction, encompassing a broad spectrum of neurocognitive and psychomotor dysfunction ranging from disorientation to coma.[1] It is classified into three major subtypes, based on the underlaying etiology, as follows: type A, resulting from acute liver failure; type B, resulting from portosystemic shunt; and type C, resulting from liver cirrhosis.[2] HE, especially due to liver cirrhosis, is associated with significant mortality, reaching up to 64% at 1 year.[3] In addition to the high mortality rate, HE imposes a great burden on various aspects of patient lives and healthcare systems.[4] The management of HE starts with identifying and treating any precipitating cause, especially in patients with chronic liver diseases who may develop acute HE secondary to infection, bleeding, etc. Currently, several medications are utilized to treat HE, with a primary focus on decreasing ammonia production and absorption, such as by lactulose and rifaximin. Newer therapies are emerging and currently under study for the management of HE targeting traditional mechanisms of ammonia clearance in addition to novel mechanisms related to altering gut microbiome, reducing inflammation and oxidative stress, protecting endothelial integrity, and modifying neuronal responses (Fig. 1). In this article, we aim to review the management of HE, starting with the efficacy and limitations of traditional agents with a focus on the evidence supporting newer therapies in HE (Table 1).

Efficacy and limitations of traditional agents in management of HE

Lactulose and lactitol

Lactulose (beta-galactosidofructose) and lactitol (beta-galactosidosorbitol) are synthetic nonabsorbable disaccharides (NADs) that are given orally or rectally in patients with HE, in order to trap ammonia in the gut, thereby limiting intestinal absorption. Lactulose and lactitol are not absorbed due to the absence of a hydrolytic disaccharidase in the small intestine. This permits entry into the colon, where they undergo bacterial fermentation by colonic flora, resulting in an acidification of the luminal contents. Because of this acidity, ammonia (NH_3) is converted to ammonium (NH_4^+) which cannot be absorbed, thus trapping ammonia within the colon and resulting in excretion in feces.[5,6] In addition, the hyperosmolar properties of lactulose and lactitol exert cathartic effects which reduce gastrointestinal transit time available for ammonia absorption.[7] Other potential mechanisms that have been described include increasing total fecal nitrogen excretion due to increased stool mass[8] and reducing the formation of toxic fatty acids and ammo-

Keywords: Hepatic encephalopathy; Liver cirrhosis; Ammonia.

Abbreviations: AASLD, American Association for the Study of Liver Diseases; CGA-HE, Clinical Global Assessment of Hepatic Encephalopathy; CI, confidence interval; EASL, European Association for the Study of the Liver; FMT, fecal microbiota transplantation; GABA, gamma aminobutyric acid; GPB, glycerol phenylbutyrate; HAS, hepatic encephalopathy scoring algorithm; HE, hepatic encephalopathy; HR, hazard ratio; LOLA, L-ornithine L-aspartate; NAD, non-absorbable disaccharides; NNT, number needed to treat; OR, odds ratio; PAA, phenylacetic acid; PBA, phenylbutyric acid; PEG, polyethylene glycol; PHES, Psychometric HE-score; RBANS, Repeatable Battery for the Assessment of Neuropsychological Status; RCT, randomized clinical trial; RR, relative risk; SCFA, short-chain fatty acid; SOC, standard of care; TIPS, transjugular intrahepatic portosystemic shunt; WHS, West Haven scale.

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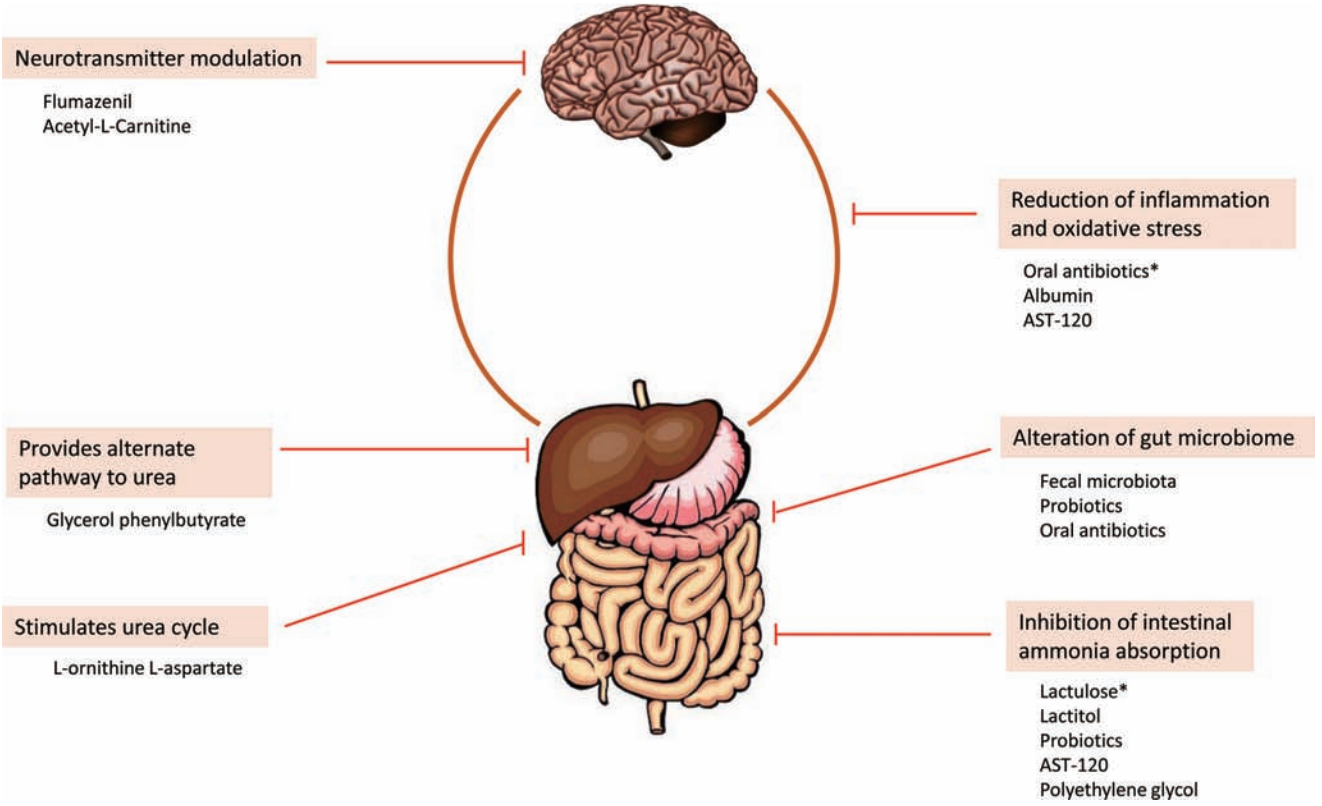

Fig. 1. Postulated mechanisms of medications used or being studied for treatment of hepatic encephalopathy. *These agents are approved by the U.S. Food and Drug Administration for the treatment of hepatic encephalopathy

nia in the colon.[9] However, the most commonly used NADs to treat and prevent HE have been reported to have variable efficacy in randomized-controlled trials (RCTs).

In a recent systematic review and meta-analysis of RCTs (2016), treatment with NADs compared to placebo or no intervention was associated with improvement in HE in ~1/3 of patients (relative risk [RR]: 0.63, 95% confidence interval [CI]: 0.53-0.74, number needed to treat [NNT]: 4), and reduced mortality by half (RR: 0.49, 95% CI: 0.23-1.05, NNT: 100). These benefits were more pronounced in overt HE compared to minimal HE.[10] Studies comparing lactulose to lactitol showed no differences in HE outcomes.[11,12] Despite the consistent results showing benefit of NADs in reducing HE and its related mortality, these RCTs did not assess the confounding effect of factors precipitating HE since strategies directed at management of precipitating factors may improve HE with or without NADs. In addition, none of the prevention RCTs reported data on quality of life. Furthermore, the use of NADs was associated with increased risk of nonserious adverse events, such as bloating, diarrhea and nausea.[10] These adverse events are likely to affect patient tolerability and compliance.[13,14] In addition, the treatment effects on HE improvement (RR: 0.63) and mortality (RR: 0.49) from this meta-analysis indicate that a large proportion of patients with HE did improve despite treatment with NADs.[10] Lactulose is Food and Drug Administration-approved and guideline-recommended (American Association for the Study of Liver Diseases [AASLD] 2014) for treatment and prevention of HE.

Oral antibiotics

Rifaximin is the most common oral antibiotic used to treat and prevent HE, usually as an adjunct therapy added to NADs. Because rifaximin is minimally absorbed, it is concentrated in the gastrointestinal tract, which in turn alters gut microbiota composition and function, affects bile acid levels and composition, and exerts anti-inflammatory action and alters neurotoxin levels, all of which are implicated in the pathogenesis of cirrhosis complications.[15] The efficacy of rifaximin was evaluated in a meta-analysis of five RCTs comparing rifaximin and NADs for treatment of HE. In that study, rifaximin had similar efficacy to NADs but with better tolerability.[16] A subsequent placebo-controlled RCT evaluated the efficacy of rifaximin in prevention of future episodes of HE among patients with history of HE who were in remission. Compared to placebo, rifaximin reduced the incidence of breakthrough HE and future hospitalization involving HE by more than half.[17] In that trial, however, more than 90% of patients received concomitant lactulose. A subsequent trial compared the efficacy of rifaximin plus lactulose vs. lactulose alone in resolution of overt HE. The combination therapy was more effective in reversal of HE (76% vs. 50.8%, $p<0.004$) and resulted in significant reduction of mortality (23.8% vs. 49.1%, $p<0.05$) and length of hospital stay (5.8±3.4 vs. 8.2±4.6 days, $p=0.001$). A subsequent, more recent meta-analysis confirmed the benefit of rifaximin in treatment and prevention of HE in addition to its benefit on mortality reduction.[18] The 2014 Practice Guideline by the AASLD and the European Association for the Study of the Liver (EASL) recommended the use of rifaximin as an add-on therapy to lactulose for prevention of HE recurrence.[19]

There are multiple problems with the trials assessing the use of rifaximin,[20] such as confounding effects of transjugular intrahepatic portosystemic shunt (TIPS) and surgical

Table 1. Characteristics of novel agents for the treatment of HE

Agent	Mechanism of action	Current stage of research	Route and dose used in clinical studies	Methods of assessing neuropsychiatric outcomes used in clinical trials	Effect shown in clinical studies	Main limitations of published trials	Examples of ongoing trials in HE[Ω]
FMT	Altering gut microbiome by replacing potentially pathogenic taxa with beneficial, SCFA-producing taxa	Two phase 1 RCTs	90 cc enema once in one trial, and 15 FMT tablets once in the other trial	PHES, EncephalApp Stroop	No serious adverse events associated with the use of enema FMT, including no bacterial infections. - Enema FMT was associated with a reduced number of hospitalizations due to liver-related complications. Also, improvement in cognitive outcomes between baseline and post-treatment in the enema FMT group but none among those undergoing SOC. Oral FMT safe and well-tolerated, associated with enhanced microbial diversity, and favorable changes in antimicrobial protein expression and intestinal inflammatory markers, along with improved performance on cognitive scores	Enema FMT: small sample size; confounding effect of pre-FMT antibiotics; the control arm being SOC instead of placebo antibiotics or autologous FMT; short-term follow up; no significant change in microbiome diversity. Oral FMT: small sample design; single-blind design; low power to assess efficacy	NCT03420482, NCT03796598, NCT04014413
Probiotics	Reduction of ammonia-producing bacteria, and decreasing ammonia absorption in the gut	21 RCTs; 1 Cochrane systematic review and meta-analysis	Oral; variable	WHS, PHES, TMT, NCT, SDT, MRS, BAER	Compared to placebo, probiotics improve recovery and may lead to improvements in overt hepatic encephalopathy, quality of life, and plasma ammonia concentrations. No effect on all-cause mortality. No difference in adverse events. Compared to lactulose, benefit is uncertain due to limitations	High risk of bias. Outcome heterogeneity. Different types of probiotics used in different trials	NCT04787276, NCT04243148, NCT04175392, NCT03863730

(continued)

Table 1. *(continued)*

Agent	Mechanism of action	Current stage of research	Route and dose used in clinical studies	Methods of assessing neuropsychiatric outcomes used in clinical trials	Effect shown in clinical studies	Main limitations of published trials	Examples of ongoing trials in HE[Ω]
Albumin	Neutralizes reactive oxygen species, inhibits inflammatory mediators and reduces endothelial dysfunction and vasodilation	4 RCTs, meta-analysis of cohort and RCT	Short-term: 1-1.5 g/kg/day for 1-10 days. Long-term: 40 g twice weekly for 2 weeks, and then 40 g weekly for up to 18 months. Other: 20% albumin infusion up to 14 days or discharge to raise albumin level to ≥30 g/L	WHS	Initial trials and meta-analysis showed reduction in mortality, incidence of HE, HE recovery, hospital length of stay compared to placebo or standard of care. Recent trial (reference 54) showed no benefit of albumin over SOC when targeting albumin level ≥30 g/L	Open-label randomized trials. Most RCTs have small sample size. Non-blinding of outcome assessment. Cost effectiveness. Some trials with short-term follow-up	NCT03585257, NCT02401490
AST-120	Reduction of blood ammonia and oxidative stress by limiting absorption of neurotoxins and hepatotoxins	2 RCTs	Varied per trial; 6-12 (oral) sachets per day divided in 2-4 doses	WHS, HESA; RBANS, PHES, CGA-HE	Reduction of serum ammonia compared to placebo, reduction of diarrhea and flatulence compared to placebo. No difference on neurocognitive outcomes	Small number of patients. Study design allowed for improvement of neurocognitive outcomes even prior to randomization limiting its ability to detect true differences	None
Acetyl-L-carnitine	Reduction of serum ammonia by increasing ureagenesis; enhancement of neurotransmitter, protein and phospholipid synthesis	5 RCTs; 1 Cochrane systematic review and meta-analysis	2 g (oral) twice daily	TMT, SDMT, AVL, MMSE, EEG, BDI, STAI, VOT, Digit Cancellation Time, EMQ, COWAT, EEG, NPT	Individual studies showed improving neurological findings; reduction of serum ammonia level and improvement in performance on neuropsychological testing; improvement in energy levels, general functioning and well-being, and reduction of anxiety and depression; reduction of physical and mental “fatigue”; and improvement of cognitive deficits and EEG findings. Meta-analysis showed a reduction of blood ammonia among participants receiving acetyl-L-carnitine; however, the certainty of this finding was low due to limitations	Small number of participants, high risk of bias, and low power for detection of meaningful differences between the treatment groups; no reporting of mortality or serious adverse events between the groups	None

(continued)

Table 1. *(continued)*

Agent	Mechanism of action	Current stage of research	Route and dose used in clinical studies	Methods of assessing neuropsychiatric outcomes used in clinical trials	Effect shown in clinical studies	Main limitations of published trials	Examples of ongoing trials in HE[Ω]
GPB	Increased urinary excretion of ammonia	1 pilot study; 1 phase 2 RCT	6 mL (oral) twice daily	WHS	GPB reduced the number of patients with HE events, time to first event, total events, HE hospitalizations, and blood ammonia levels. Benefit was sustained after controlling for rifaximin use. No difference in serious adverse events between the two groups	Small sample size. More patients in the GPB group exited the study prematurely. 1/3 patients were taking rifaximin at the time of randomization	None
Flumazenil	Neurotransmitter modulation through competitive inhibition of GABA-A receptors	12 RCTs; 1 Cochrane systematic review and meta-analysis	Varied per trial; intravenous flumazenil at a total daily dose 0.2-6.5 mg	NCT, BAER, GCS, EEG, NPT	No on all-cause mortality, no difference in serious adverse events. Flumazenil was associated with improvement of HE	Small sample size in individual studies. High risk of bias Cross-over design; relapse rate not assessable. Short-term follow up	None
PEG	Reduce ammonia absorption by exerting a cathartic effect and reducing transit time	3 RCTs	4 L	HESA, WHS	PEG (alone or in combination with lactulose) is more effective than lactulose at improving HE at 24 h and associated with decreased length of stay. No difference in blood ammonia levels. No difference in serious adverse events	Small sample size. Single-center experiences. Non-blinding. Short-term follow up	NCT04436601, NCT03987893

[Ω]Clinicaltrials.gov website was searched for clinical trials relevant to hepatic encephalopathy and the agent of interest. AVL, auditory verbal learning test; BAER, brain-auditory evoked response; BDI, Beck depression inventory; COWAT, controlled oral word association test; EEG, electroencephalogram; EMQ, everyday memory questionnaire; MMSE, mini mental state examination; NCT, number connection test; NPT, neuropsychiatric test; SDMT, symbol digit modalities test; SDT, serial dotting test; STI, state trait anxiety inventory; TMT, trails making test; VOT, Hooper visual organization test.

portosystemic shunts,[17] randomization imbalance,[21,22] lack of benefit in high risk populations (such as in prevention of HE in those undergoing TIPS),[23] and absence of objective HE scales in outcome assessment in some of the studies.[17] Despite these limitations, rifaximin is believed to be the best agent for use in combination with lactulose to maintain remission in patients with recurrent HE.[19] Other antibiotics have been studied in management of HE; such as neomycin, metronidazole and vancomycin.[24–27] Their use is limited by inconsistent data and concerns regarding toxicity and adverse effects.[19] Rifaximin is Food and Drug Administration-approved and guideline-recommended (AASLD 2014) for treatment and prevention of HE.

L-ornithine L-aspartate (LOLA)

Ammonia detoxification is achieved by two main pathways in periportal hepatocytes: 1) urea synthesis in zone 1 and 2) glutamine synthesis in zone 3.[28] LOLA is a combination of endogenous amino acids that are metabolized in periportal and perivenous hepatocytes, where L-ornithine is utilized as a substrate in the urea cycle and acts as an activator of carbamoyl phosphate synthetase, the rate limiting enzyme of the urea cycle. Ammonia is also incorporated with glutamate to form glutamine catalyzed by glutamine synthase. The latter process also takes places in skeletal myocytes.[28] Multiple RCTs have studied the efficacy of intravenous and oral LOLA compared to placebo for treatment of HE (such as lactulose). Meta-analyses of these trials showed consistent reductions in ammonia levels and clinical improvement of HE.[29] Clinical trials assessing the efficacy of LOLA showed that it is at least comparable (sometimes superior) to other interventions (such as lactulose or oral antibiotics), in addition to being well-tolerated and associated with improvement in quality of life.[29,30] Despite these benefits, the trials assessing the efficacy of LOLA suffer from several biases related to inadequate blinding, incomplete data, selective reporting, and pharmaceutical funding.[31] In addition, there is no evidence to support the use of LOLA in patients with acute liver failure.[32] LOLA is available and used routinely for management of HE in Europe. However, it is not available in the USA. Intravenous LOLA is not Food and Drug Administration-approved but is recommended by the guideline (AASLD 2014) as an alternative or additional agent for HE not responsive to conventional therapy.

Other therapies of HE

Mechanism of actions and critique of the evidence

Fecal microbiota transplantation (FMT): It has been shown that the gut microbial profile of cirrhotic patients with HE is different from those without HE or normal controls. Although this difference in the gut microbiome is in part driven by standard of therapy used in treatment of cirrhosis and HE (such as oral antibiotics, NADs, and acid suppressants) which can affect the gut microbiome composition,[33] cross-sectional data of stool metagenomics have revealed that certain metagenomic species are overexpressed or underexpressed in decompensated compared to compensated cirrhosis.[33] Additionally, gut dysbiosis has been shown to predict poor outcomes in HE.[34] Specifically, HE patients were found to have a lower prevalence of short-chain fatty acid (SCFA)-producing families, such as *Lachnospiraceae* and *Ruminococcaceae*, and increased prevalence of potentially pathogenic *Enterobacteriaceae*.[34,35]

Using this microbial profile, Bajaj and colleagues[36] were able to obtain stool specimens from a single healthy donor with the highest relative abundance of *Lachnospiraceae* and *Ruminococcaceae*. Frozen-then-thawed FMT units prepared from the single donor were instilled by enema and retained for 30 m in patients with HE after a 5-day course of broad spectrum antibiotics (metronidazole, ciprofloxacin, and amoxicillin) aimed to decrease host bacterial burden and make the colonic environment more receptive to colonization from the donor microbiota. In this safety and open-label RCT involving 20 patients with cirrhosis and recurrent HE, who were randomized 1:1 to either FMT or standard-of-care (including lactulose and rifaximin), there was no serious adverse event associated with the use of FMT, including no bacterial infections. Additionally, the FMT was associated with a reduced number of hospitalizations due to liver-related complications, and there was a significant improvement in cognitive outcomes between baseline and post-treatment in the FMT group but none among those undergoing standard of care (SOC). This trial had several limitations, including a small sample size, confounding effect of pre-FMT antibiotics, control arm being SOC instead of placebo antibiotics or autologous FMT, and short-term follow up (up to 20 days). Additionally, there was no significant change in microbiome diversity, as assessed by 16S rRNA sequencing.[36]

In another phase 1, randomized, single-blind, placebo-controlled safety trial, Bajaj and colleagues[37] studied the use of oral FMT capsules in patients with cirrhosis and recurrent HE. FMT capsules were prepared from the same healthy donor with the relative high abundance of *Lachnospiraceae* and *Ruminococcaceae* used in their previous enema trial, and were given at a dose of 15 capsules at one time. This trial was unique because all subjects underwent esophagogastroduodenoscopy and sigmoidoscopy for mucosal biopsies before and after FMT treatment. Twenty patients already on lactulose/rifaximin were enrolled (randomized 1:1 to either FMT capsules or placebo capsules); FMT appeared to be safe, well-tolerated and associated with enhanced microbial diversity, and to provide favorable changes in antimicrobial protein expression and intestinal inflammatory markers, along with improved performance on cognitive scores. Another, ongoing phase 2 RCT is underway to further investigate the safety and benefit of aggressive gut microbial manipulation using FMT oral capsules.[38] At this time, FMT is not Food and Drug Administration-approved nor mentioned by the guidelines (AASLD 2014) yet as a treatment in HE.

Probiotics

A probiotic is conventionally defined as “a preparation of or a product containing viable, defined microorganisms in sufficient numbers, which alter the microflora (by implantation or colonization) in a compartment of the host and by that exert beneficial health effects in this host”.[39,40] Although probiotics are often bacterial microorganisms, most commonly *Lactobacillus* or *Bifidobacterium*, yeasts are also used. Because of the variety of microorganisms in probiotics, various species or strains may confer a variety of health benefits, and disease-specific probiotics exist. In HE, as discussed above, it has been shown that the alteration of gut microbiome plays an important role in neurocognitive outcomes in patients with cirrhosis. Probiotics are hypothesized to benefit patients with HE through reduction of harmful, ammonia-producing bacteria, and decreasing ammonia absorption in the gut by affecting different aspects of the gastrointestinal environment (including enzymatic composition, epithelial permeability, acidic environment and nutritional status of the gut).[41]

Evidence supporting the use of probiotics in HE comes

from a comprehensive Cochrane systematic review and meta-analysis of 21 trials published prior to June, 2016 involving 1,420 patients comparing a probiotic to either placebo (14 trials) or lactulose (7 trials). When probiotics were compared to placebo or no treatment, this review found no effect in all-cause mortality. However, there was moderate-quality evidence that probiotics improve recovery and may lead to improvements in overt HE, quality of life, and plasma ammonia concentrations. When antibiotics were compared to lactulose, the benefits were uncertain because of the very low-quality evidence. Importantly, no reports of septicemia related to the use of probiotics were found.[41] The review highlighted several limitations in the included trials, including high risk of bias, outcome heterogeneity, and different types of probiotics used.[41] In these studies, VSL#3 (containing four species of *Lactobacilli*, three of *Bifidobacteria* and *Streptococcus thermophilus*) was the most commonly used probiotic product in the clinical trials. Probiotics use in HE remains under study and multiple clinical trials assessing other strains are underway to investigate its benefits in patients with cirrhosis.[42–45] At this time, probiotics are not Food and Drug Administration-approved nor guideline-recommended (AASLD 2014) for the treatment of HE, though they are mentioned as possible alternative therapy pending further study.

Albumin

Synthesized in the liver, albumin is known to decrease in patients with progressive liver disease and cirrhosis. Intravenous albumin administration has been shown in experimental studies to neutralize oxygen-reactive species, inhibit inflammatory mediators and reduce endothelial dysfunction and vasodilation in patients with liver cirrhosis, in addition to its oncotic, volume-expanding effect on the circulation[46–48] in patients with cirrhosis. Albumin has been shown to improve response to diuretics, prevent circulatory dysfunction after large-volume paracentesis and to have a role in prevention and treatment of hepatorenal syndrome.[49] The benefit of albumin administration in the prevention and treatment of HE was studied in few clinical trials with promising results.

A multicenter, double-blind small RCT involving 56 cirrhotic patients with acute HE who had been randomized to receive intravenous albumin (1.5 g/kg on day 1 and 1.0 g/kg on day 3) vs. isotonic saline, in addition to usual treatment (laxatives and oral antibiotics), showed that there was no significant differences in the percentage of patients with short-term resolution of HE (at day 4). However, there was a significant reduction in mortality at day 90 (69.2% vs. 40%, p=0.02).[50] In 2017, Sharma and colleagues[51] randomized 120 patients with overt HE to receive lactulose (30–60 mL three times a day; goal 2–3 semisoft stools per day) plus albumin (1.5 g/kg/day) or lactulose alone, and treatment was continued until recovery of HE or for a maximum of 10 days. The combination therapy resulted in more patients achieving complete recovery of HE by day 10, as assessed by West Haven scale (WHS) (75% vs. 53.3%, p=0.03), shorter hospital stay 6.4±3.4 vs. 8.6±4.3 days, p=0.01), lower mortality (18.3% vs. 31.6%, p=0.04), in addition to significant reductions in levels of IL-6, IL-18, TNF-alpha and endotoxins but not levels of arterial ammonia. There was no difference in side effects related to drug therapy. The main limitations of that study included the small sample size, open-label design, and absence of concomitant rifaximin use, which is known to reduce short-term mortality.

The value of long-term albumin administration was investigated in the ANSWER study, a multicenter, randomized, open-label trial that assigned 440 patients with cirrhosis and uncomplicated ascites resistant to diuretic therapy to receive either standard medical therapy or standard medical therapy plus albumin (40 g twice weekly for 2 weeks, and then 40 g weekly) for up to 18 months. Although HE assessment was not the main goal in that study, it was assessed as a secondary end point. At the study completion, 18-month survival was higher in the standard medical therapy plus albumin group (77% vs. 66%, p=0.028), and there was decreased incidence in grade 3–4 HE (odds ratio: 0.48, 95% CI: 0.37–0.63, p<0.001). In addition, albumin treatment decreased the future need for therapeutic paracentesis, renal dysfunction, hyponatremia, hyperkalemia, bacterial infections, hepato-renal syndrome, and hospital length of stay.[52] Given the concerns regarding costs of albumin administration, cost-effective analysis in that study showed a favorable cost-effective ratio, likely attributed to better quality of life and fewer hospital admissions in the albumin group. Despite its impressive results, the study had several limitations, the main being its open-label design, which may have led to patients receiving albumin to be seen more frequently than patients in the other group. Additionally, although outcome assessors were from an independent nonprofit consortium, they were not blinded to the treatment allocations and may have introduced bias. A more recent, single-center retrospective propensity-matched analysis involving 2,868 patients and meta-analysis of nine cohort and prospective trials showed that albumin administration was associated with reduced incidence and improvement of overt HE in addition to lowering in-hospital mortality.[53]

Another recent, randomized, multicenter, open-label trial was conducted in the UK and studied whether targeting an albumin serum level ≥30 g/L would reduce the risk of infections, renal dysfunction and death in patients with decompensated cirrhosis.[54] In that study, 777 patients hospitalized with decompensated cirrhosis (~20% of which admitted for HE) and serum albumin <30 g/L were randomized to receive daily infusions of 20% albumin for 14 days or until discharge (whichever comes first) vs. SOC; patients in the SOC group were allowed to receive albumin infusions when indicated (such as hepatorenal syndrome, peritonitis or large-volume paracentesis). At the conclusion of the trial, the primary end-point (new infection, kidney dysfunction, or death between days 3 and 15 after the initiation of treatment) did not differ significantly between the groups (adjusted odds ratio: 0.98; 95% CI 0.71–1.33). Furthermore, subgroup analysis of the primary outcome in those hospitalized for HE did not reveal significant benefit (adjusted OR 0.91; 95% CI 0.44–1.86). The study concluded that targeting albumin level ≥30 g/L is not beneficial compared to SOC in patients with decompensated cirrhosis, and called into question the utility of albumin in patients with decompensated cirrhosis; however, it was limited mainly by its open-label design and short-term follow up. Albumin is currently being evaluated in other ongoing trials.[55–57] At this time, albumin is not Food and Drug Administration-approved nor guideline-recommended (AASLD 2014) for the treatment of HE, though it is mentioned as possible alternative therapy pending further study.

AST-120

AST-120 is a synthetic activated carbon microsphere that has a large surface area with a high nonspecific adsorptive capacity. AST-120 has limited gastrointestinal absorption, which adds to its ability to trap neurotoxins and hepatotoxins in the gut.[58] The ability of AST-120 to reduce blood ammonia levels and reduce oxidative stress has been shown previously in rat models of cirrhosis[58,59] and renal failure.[60] AST-120 was studied in a phase-2, multicenter RCT that

evaluated the efficacy and safety of AST-120 in the treatment of low-grade HE. The study included 41 patients who were randomized to receive either AST-120 (2 g sachets four-times per day) or lactulose (titrated to 2–3 soft stools per day) for 4 weeks. The primary end-point was defined as ≥1-point reduction in the WHS of HE over 4 weeks. Secondary endpoints were changes in the Hepatic Encephalopathy Scoring Algorithm (HESA), venous ammonia, and tolerability. At the study completion, the primary endpoint at week 4 was similar between treatment groups (38.1% vs. 35.0%, AST-120 vs. lactulose); secondary endpoints were also similar. However, diarrhea and flatulence occurred less frequently in the AST-120 group.[61,62] One of the major limitations of this study was the low number of patients and the absence of a placebo arm, prompting a placebo-controlled "AST-120 Used to Treat Mild Hepatic Encephalopathy" (ASTUTE) clinical trial,[63] a multicenter, double-blind RCT that randomized 148 patients with compensated cirrhosis to receive either dose-ranging oral AST-120 (2 or 4 g three times per day) vs. placebo.[64] The primary endpoint was neurocognitive improvement defined as a change in the global summary score of Repeatable Battery for the Assessment of Neuropsychological Status (RBANS) at 8 weeks compared to baseline; secondary endpoints included Psychometric HE-score (PHES), Clinical Global Assessment of HE (CGA-HE), and frequency of occurrences of overt HE and hospitalization. At study completion, there was no difference in RBANS scores between baseline and 8 weeks for all groups, and there were no differences in secondary endpoints. However, all groups had improvement in RBANS score between the time of screening and baseline visits (at 1 week), even before randomization. Thus, the study was strongly confounded by its design, allowing for improvement in neurocognitive scores prior to randomization. Interestingly, venous ammonia levels significantly improved in treatment groups (but not in placebo) independently of neurocognitive changes.[63,64]

At the time of this article preparation, there are no known ongoing clinical trials evaluating the use of AST-120 for the treatment of HE. AST-120 is currently being used and actively studied in treatment of progressive of chronic kidney disease.[65] At this time, AST-120 is not Food and Drug Administration-approved nor guideline-recommended (AASLD 2014) for the treatment of HE.

Acetyl-L-carnitine

Carnitine is an essential nutrient that is important for fatty acid transfer across the inner mitochondrial membrane, especially in hepatocytes.[66] The metabolism of carnitine has been shown to be impaired (and serum carnitine levels reduced) in patients with chronic liver diseases. Acetyl-L-carnitine is an ester of carnitine that is endogenously produced within mitochondria and peroxisomes in the liver, brain and kidney by the enzyme acetyl-L-carnitine transferase.[67] The role of acetyl-L-carnitine in the treatment of HE is postulated to be related to reduction of serum ammonia by increasing ureagenesis[67] in addition to enhancing the production of acetylcholine in the brain (by facilitating the uptake of acetyl-coenzyme A) and stimulating protein and phospholipid synthesis, all of which increase cellular energy production and reduce neuronal toxicity in patients with HE.[66,67]

Most of the data on the use of acetyl-L-carnitine comes from small RCTs; although, the individual RCTs suggested a benefit of acetyl-L-carnitine compared to placebo in improving neurological findings.[68] Reduction of serum ammonia level and improvement in performance on neuropsychological testing,[69] improvement in energy levels, general functioning and well-being, and reduction of anxiety and depression,[70] reduction of physical and mental "fatigue",[71] and improvement of cognitive deficits and EEG findings[71] in these studies were limited by small number of participants, high risk of bias, and low power for detection of meaningful differences between the treatment groups. A recent Cochrane systematic review and meta-analysis assessing these five RCTs that collectively randomized 398 participants to oral or intravenous acetyl-L-carnitine vs. placebo concluded the studies to be underpowered for the treatment effect, with a high risk of bias.[72] Meta-analysis of these trials showed a reduction of blood ammonia among participants receiving acetyl-L-carnitine. However, the certainty of this finding was low due to limitations in study design and execution of the trials. Importantly, none of these trials assessed all-cause mortality and differences in serious adverse events. Adverse events of acetyl-L-carnitine were poorly reported, making the potential harms of acetyl-L-carnitine remain currently unknown.[72] More highly powered and adequately designed clinical trials are needed to assess the efficacy and safety of acetyl-L-carnitine compared to placebo and current standard of therapy prior to the implementation of its widespread use. At this time, acetyl-L-carnitine is not Food and Drug Administration-approved nor guideline-recommended (AASLD 2014) for the treatment of HE.

Glycerol phenylbutyrate (GPB)

GPB is a nitrogen-binding agent consisting of three phenylbutyric acid (PBA) molecules joined to glycerol by an ester linkage. It is currently approved in the USA and Europe for use in urea cycle disorders in patients with chronic hyperammonemia who cannot be managed by dietary protein restriction and/or amino acid supplementation alone.[73–76] Phenylacetic acid (PAA), the major metabolite of PBA, is conjugated with glutamine (which contains two molecules of nitrogen) by acetylation in the liver and kidneys to form phenylacetylglutamine (PAGN) which is easily excreted by the kidneys, providing an alternate vehicle for nitrogen waste excretion and reducing blood ammonia level.[74,76,77] A pilot, open-label dose-ranging study involving 15 patients with cirrhosis and HE patients showed that oral GPB (6 mL) twice a day was tolerated and resulted in significant lowering of blood ammonia concentrations.[78] This study was followed by a phase 2, randomized, double-blind trial enrolling 178 cirrhosis patients with history of recurrent HE who received either GPB (6 mL twice daily for 16 weeks) vs. placebo (1:1 randomization). Compared to placebo, GPB reduced the number of patients with HE events (21% vs. 36%, $p=0.02$), time to first event (hazard ratio [HR] = 0.56, $p<0.05$), total events (35 vs. 57, $p=0.04$), HE hospitalizations (13 vs. 25, $p=0.06$), and blood ammonia levels ($p=0.04$).[79] There was no difference in serious adverse events between the two groups. The study was limited by small sample size, and more patients in the GPB group exited the study prematurely, which could result in a lower HE event rate in the treatment arm. However, the authors showed that the treatment effect remained in a time-to-event survival analysis performed to account for dropouts.[79] Additionally, 59 patients (33%) were taking rifaximin at the time of randomization, likely indicating more refractory disease. However, the treatment benefit was sustained after controlling for rifaximin use. At this time, GPB is not Food and Drug Administration-approved nor guideline-recommended (AASLD 2014) for the treatment of HE, though it is mentioned as possible alternative therapy pending further study.

Flumazenil

Flumazenil is a competitive inhibitor at the benzodiazepine

binding site on the gamma aminobutyric acid (GABA)-A receptor. It is most commonly used in benzodiazepine overdose and reversal of anesthesia.[80] Several studies have shown that patients with HE have an up-regulation of GABA-A receptors and increased GABAergic tone.[81,82] Because GABA is the main inhibitory neurotransmitter in the central nervous system, this upregulation of GABAergic neurons is postulated to be responsible, at least in part, for the neurocognitive manifestations of HE. A number of clinical trials assessed the effects of flumazenil in patients with HE. However, these trials were individually relatively small and included cross-over designs that limited the interpretability of clinically meaningful outcomes.[83] A Cochrane systematic review and meta-analysis of 12 RCTs involving 842 patients comparing flumazenil vs. placebo reported that there was no effect of flumazenil on all-cause mortality. However, flumazenil was associated with an improvement of HE, and with no difference in serious adverse events. The main limitation of these studies was the short follow-up time which ranged from a few minutes to 2 weeks in these trials. However, follow up was less than 1 day in the majority of the studies, limiting any overreaching conclusions about the benefit of flumazenil on long-term cognitive outcomes.[83] Other limitations include high risk of bias in the majority of the studies, and cross-over designs in individual studies limiting the ability to estimate the risk of HE relapse.[83] Because of this limited duration of action and no effect on mortality, flumazenil is not routinely used for the treatment of HE until warranted by further trial data. At this time, flumazenil is not Food and Drug Administration-approved nor guideline-recommended (AASLD 2014) for the treatment of HE, though it is mentioned as possible therapy in select cases pending further study.

Polyethylene glycol

Polyethylene glycol 3350-electrolyte solution (PEG) is a cathartic agent postulated to improve outcomes in HE by reducing gastrointestinal transit time available for ammonia absorption. This cathartic effect is somewhat similar to that exerted by NADs due to their unabsorbed hyperosmolar characteristics. However, unlike lactulose and lactitol, PEG does not have the carbohydrate load that reduces stool pH and is not metabolized by colonic bacteria.[84] Published in 2014, the HELP study (Hepatic Encephalopathy: Lactulose vs. Polyethylene Glycol 3350-Electrolyte Solution) was the first RCT to compare PEG (4-L dose) vs. lactulose in 50 patients with cirrhosis admitted for HE. PEG was found to be associated with a higher incidence of HE improvement assessed by improvement in HESA scores at 24 h (91% vs. 52%, $p<0.01$), and with a shorter median time to improvement in HE (1 vs. 2 days, $p=0.01$). There was no difference in serious adverse events, although the PEG group experienced more diarrhea and the lactulose group experienced more bloating.[85] Ammonia levels in that study did not correlate with improvement in HE scores.

A more recent RCT similarly compared PEG with lactulose for treatment of overt HE in 100 patients with post-hepatitis C cirrhosis admitted for HE. At study completion, PEG was associated with a higher incidence of HE improvement on HESA scores compared to lactulose (94% vs. 72%), along with a reduced time needed for HE resolution and length of hospital stay, and no differences in serious adverse events.[86] Combining lactulose with PEG might be helpful, which was assessed in a non-inferiority trial that randomized 40 patients with cirrhosis and HE to receive either lactulose alone (20–30 g orally or 200 g enema) or a similar dose of lactulose plus PEG (280 g in 4 L of water orally as a single dose in 30–120 m). Combination therapy (PEG plus lactulose) was more effective than lactulose alone in improving HESA scores at 24 h and was associated with reduced length of hospital stay and with no significant differences in blood ammonia levels or serious adverse events.[87] The main limitations of these trials include the small sample size, being limited to single-center experiences, non-blinding of the studies, and absence of long-term outcomes. There are multiple ongoing trials assessing the benefit of PEG in HE.[88,89] At this time, polyethylene glycol is not Food and Drug Administration-approved nor guideline-recommended (AASLD 2014) for the treatment of HE, though it is mentioned as possible alternative therapy pending further study.

Agents such as L-ornithine phenyl acetate, sodium benzoate, and zinc have been studied but lack sufficient evidence of efficacy to be recommended.[90–92]

Conclusions

The management of HE is complex and requires clinicians to be updated on the most recent advances in prevention and treatment. Older therapies (such as NADs and oral antibiotics) remain the first line of treatment according to current guidelines. However, multiple new agents have been developed and are being used for the treatment of HE. These agents are in various stages of research and some require further study prior to routine use in clinical practice. Because of several limitations in the existing literature, future research should focus on large-scale clinical trials with adequate design, sample size, elimination of biases, reporting of adverse events, and standardization of treatment outcomes.

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Conflict of interest

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Author contributions

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Review Article

COVID-19 and Indirect Liver Injury: A Narrative Synthesis of the Evidence

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Abstract

The liver is frequently affected by severe acute respiratory syndrome-coronavirus 2 (SARS-CoV-2) infection. The most common manifestations are mildly elevated alanine aminotransferase and aspartate aminotransferase, with a prevalence of 16-53% among patients. Cases with severe coronavirus disease 2019 (COVID-19) seem to have higher rates of acute liver dysfunction, and the presence of abnormal liver tests at admission signifies a higher risk of severe disease during hospitalization. Patients with chronic liver diseases also have a higher risk of severe disease and mortality (mainly seen in patients with metabolic-associated fatty liver disease). Several pathways of damage have been proposed in the liver involvement of COVID-19 patients; although, the end-cause is most likely multifactorial. Abnormal liver tests have been attributed to the expression of angiotensin-converting enzyme 2 receptors in SARS-CoV-2 infection. This enzyme is expressed widely in cholangiocytes and less in hepatocytes. Other factors attributed to liver damage include drug-induced liver injury, uncontrolled release of proinflammatory molecules ("cytokine storm"), pneumonia-associated hypoxia, and direct damage by the infection. Hepatic steatosis, vascular thrombosis, fibrosis, and inflammatory features (including Kupffer cell hyperplasia) are the most common liver histopathological findings in deceased COVID-19 patients, suggesting important indirect mechanisms of liver damage. In this translational medicine-based narrative review, we summarize the current data on the possible indirect mechanisms involved in liver damage due to COVID-19, the histopathological findings, and the impact of these mechanisms in patients with chronic liver disease.

Keywords: COVID-19; SARS-CoV-2; Liver hepatitis; Liver injury; Novel coronavirus.

Abbreviations: ACE2, angiotensin-converting enzyme 2; ALT, alanine aminotransferase; AST, aspartate aminotransferase; COVID-19, coronavirus disease 2019; DILI, drug-induced liver injury; FIB-4, fibrosis-4 score; GGT, gamma-glutamyl transferase; GM-CSF, granulocyte-macrophage colony-stimulating factor; HBV, hepatitis B virus; IFN, interferon; IL, interleukin; LPV/R, lopinavir/ritonavir; MAFLD, metabolic-associated fatty liver disease; MERS, Middle East respiratory syndrome; NAFLD, nonalcoholic fatty liver disease; OR, odds ratio; PSI, pneumonia severity index; SARS-CoV-2, severe acute respiratory syndrome-coronavirus 2; SIRS, systemic inflammatory response syndrome; TNF, tumor necrosis factor.

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Introduction

The severe acute respiratory syndrome-coronavirus 2 (SARS-CoV-2) infection and related coronavirus disease 2019 (COVID-19) was first described in December 2019 in patients with severe pneumonia in Wuhan, Hubei Province, China.[1] In less than 3 months, SARS-CoV-2 infections had spread rapidly from Wuhan city to the entire country, and then on to more than 191 countries worldwide. The World Health Organization declared COVID-19 a global pandemic in March 2020.[2] The disease quickly became a great burden for health systems and focused worldwide research on therapies against this disease.[3,4] To date, SARS-CoV-2 has infected more than 60 million individuals and caused 1.4 million deaths worldwide. Patients with COVID-19 usually present with fever and respiratory symptoms.[5–9] However, patients can either behave asymptomatically[10,11] or have extrapulmonary involvement, even multiorgan failure. Gastrointestinal symptoms are evident in 11.4–18% of patients[12,13] and are associated with a potential higher risk of hospitalization.[14] These include anorexia, diarrhea (13%), nausea/vomiting (10%), and abdominal pain (8%). In some patients, it may even be their chief complaint.[13–15]

The liver is the second most common organ affected in COVID-19, after the lung. The most frequent manifestation is a mildly elevated alanine-aminotransferase (ALT) and aspartate-aminotransferase (AST). Elevation of alkaline phosphatase, gamma-glutamyl transferase (GGT), and hepatic steatosis is also commonly observed in patients who tested positive for SARS-CoV-2.[16–18] Several pathways have been proposed as a cause of liver involvement in COVID-19. SARS-CoV-2 activates angiotensin-converting enzyme 2 (ACE2) receptors; therefore, abnormal liver tests could be explained by the presence of ACE2 in endothelial cells of the liver and the biliary epithelium.[19–21] Other mechanisms include drug-induced liver injury (DILI), cytokine storm, pneumonia-associated hypoxia, and even direct damage by the infection.[22,23] This review aimed to summarize the current data on the possible indirect mechanisms involved in liver damage due to COVID-19.

General mechanisms of damage in COVID-19

The pathogenic process in COVID-19 begins when the virus binding to ACE2 in the target cell via receptor in the viral capsule,[19,24,25] allowing inoculation and multiplication. ACE2 is expressed mainly in the lung's epithelia; however, it is also present in the liver, gastrointestinal tract, and vascular endothelium.[26] Usually, the inoculation of the virus in the pulmonary epithelium leads to severe pulmonary, and later, systemic inflammation. On the other hand, some patients will develop a systemic inflammatory response syndrome (SIRS), which is characterized by a "cytokine storm" (an uncontrolled release of proinflammatory molecules). These patients have increased levels of tumor necrosis factor (TNF)-α, interleukin (IL)-2, IL-6, IL-7, and IL-10. There is also an increase in other inflammatory biomarkers, such as granulocyte-macrophage colony-stimulating factor (GM-CSF), interferon (IFN)-γ, inducible protein-10, monocyte chemoattractant protein-1, and macrophage inflammatory protein-1-α. Patients also present lymphopenia (mainly CD4+ and CD8+ T cells),[9,27,28] altered coagulation, and an increase of D-dimer, troponin, and N-terminal pro-B-type natriuretic peptide serum levels.[29–31] Some of these dysfunctions have been associated with significantly increased mortality.

Liver and COVID-19

In the liver, ACE2 is expressed mainly in cholangiocytes and less in hepatocytes.[26] However, bile duct injury has been reported, albeit rarely, in COVID-19 patients. In contrast, elevated ALT and AST levels have been extensively reported. Abnormal liver enzymes were first reported by Chen *et al*.[6] in Wuhan. In that descriptive study, a total of 43 out of 99 (43.4%) cases had mildly increased ALT and AST serum levels, with only 1 case having high levels of aminotransferases (ALT of 7,590 U/L and AST of 1,445 U/L). Several subsequent studies described the prevalence of high ALT and AST serum levels as being between 16–53% for all patients.[32]

Also, patients with severe COVID-19 seem to have higher liver dysfunction rates, and patients with abnormal liver tests at admission have a higher risk of progressing to severe disease during hospitalization.[21] In a large study by Chuan *et al*.,[5] conducted in China and including 1,099 patients from 552 different hospitals in 30 provinces, AST/ALT was elevated in 18.2–19.8% of patients with mild disease and 28.1–39.4% with severe disease. Similarly, Huang *et al*.[9] demonstrated an elevated AST in 62% of patients in intensive care units compared with 25% of non-intensive care unit patients.[15,21,33] In a study from New York by Richardson *et al*.,[34] 2.1% (56/5,700) of patients developed acute hepatic injury (defined as an elevation in AST or ALT of >15 times the upper limit of normal), which correlated with older patients and higher mortality (53 of them died). In contrast, in patients with subclinical disease, abnormal liver tests are rare (AST 8.7% and ALT 8.9% of patients).[35] Despite being frequent, changes in liver enzymes are usually mild, transitory, and do not impact the majority of patients.[16]

Liver damage indirectly related to COVID-19

Several liver damage mechanisms in patients with COVID-19 have been proposed, although the end-cause is most likely multifactorial. While the increase in liver enzymes could be the consequence of COVID-19 binding to ACE2 in the liver's endothelial cells and the biliary epithelium,[19–21] liver involvement is likely due to other, more indirect, pathways such as DILI, "cytokine storm", and pneumonia-associated hypoxia.[22,23]

SIRS

SIRS can be caused by infection, drugs, and other factors and is characterized by an acute and uncontrolled increase in the level of a large number of proinflammatory cytokines, also called the "cytokine storm". Many of which are produced by the liver. These inflammatory mediators in severe cytokine storms usually include IFNs, TNFs, ILs, and chemokines.[36] Among these, IL-6 is one of the critical components in SIRS,[37] as it is a multi-effective cytokine taking part in different signal transduction pathways, including classical signal transduction, trans-signal transduction, trans-presentation, and the JAK-STATA, RAS-RAF, SRC-YAP-NOTCH, and AKT-PI3K pathways. Thereby, IL-6 can promote T cell population expansion, activation, and differentiation of B cells, which increases antibodies consequently. It also regulates the acute phase response and has a hormone-like effect on lipid metabolism, insulin resistance, mitochondrial activity, and regulates the neuroendocrine system. This contributes to essential biological functions, including immune regulation.[38] In hepatocytes, IL-6 is a potent inducer of acute-phase reactive proteins. It can induce hepatocytes to synthesize acute-phase reactive protein at the gene transcription level, especially serum amyloid A and C-reactive protein.

Equally owing to its unique anatomical location, the liver is highly exposed to circulating antigens, endotoxins, inflammatory signals, and viral particles, which reach the liver either from the systemic circulation via arterial blood or the gastrointestinal tract through the portal vein.

SARS-CoV-2 binds to pulmonary epithelial cells and can directly induce multiple proinflammatory signals via Toll-like receptors and activation of cytotoxic T cells (powerfully activating the natural and cellular immunity).[39,40] After SARS-CoV-2 infection, cytotoxic T cells are rapidly activated, producing GM-CSF, IL-6, and other proinflammatory factors. Later GM-CSF activates CD14+/CD16+ inflammatory monocytes, which produce more IL-6 and other proinflammatory factors (Fig. 1). On the other hand, viral-specific CD8+ T cells, generated in response to a viral infection restricted to sites outside the liver (as in COVID-19), can trigger T cell-mediated hepatitis in the absence of viral antigens in the liver via activation of Kupffer cells. The recruitment of CD8+ effector T cells to the liver in response to the viral infection may be part of the liver damage's pathophysiology during cytokine storm.[41]

Huang *et al*.,[9] in a study of 41 hospitalized patients in China, described high levels of proinflammatory cytokines, including IL-2, IL-6, IL-7, G-CSF, inducible protein-10, monocyte chemoattractant protein-1, macrophage inflammatory protein-1-α, and TNFα in severe COVID-19 cases. Likewise, lymphopenia has been described in patients with COVID-19, particularly in severe cases.[42,43] These findings suggested a relationship between lymphopenia and "cytokine storm" in the pathogenesis of COVID-19, as previously described in SARS and Middle East respiratory syndrome (MERS).[44–46] In patients with COVID-19, the presence of lymphopenia, increased neutrophil count, and higher plasma levels of many innate cytokines have been associated with a higher risk of severe COVID-19.[9,42,47]

This excessive or uncontrolled release of proinflammatory molecules ("cytokine storm") leads to immune damage to other organs, acute respiratory distress syndrome,[34] respiratory failure, shock, and multiple organ failure.[48,49] The apoptosis and necrosis release damage-associated mo-

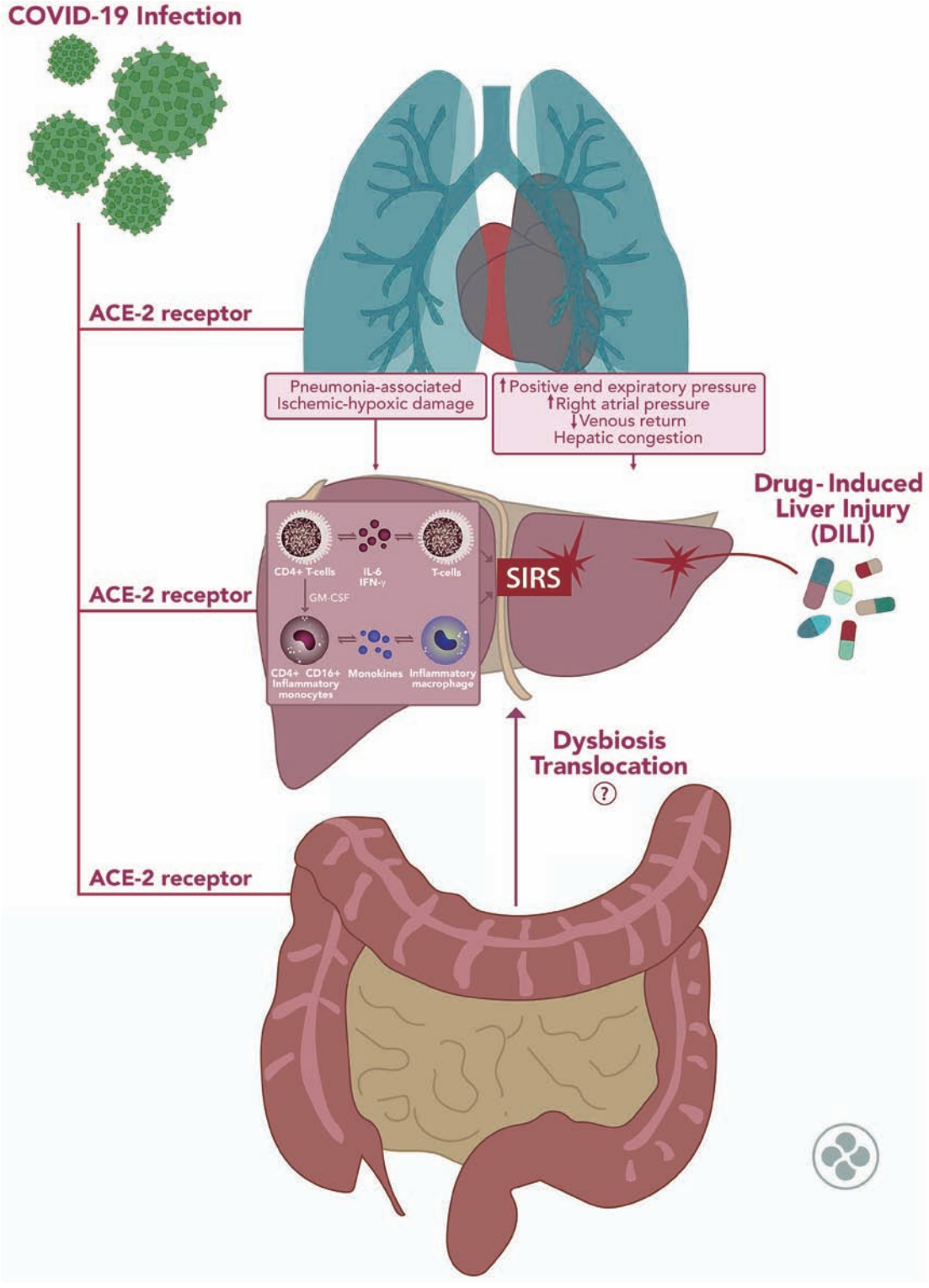

Fig. 1. Mechanisms involved in the pathogenesis of liver damage in patients with COVID-19 infection. The pathogenic process in COVID-19 begins when the virus binding to ACE2 in the target cell via receptor in the viral capsule. Some patients develop SIRS characterized by a "cytokine storm". The activated T cells produced GM-CSF, IL-6, and other proinflammatory factors. The inflammatory monocytes CD14+CD16+ respond to GM-CSF, producing a larger amount of IL-6 and other proinflammatory factors. Other factors such as hepatic ischemia, hypoxia-reperfusion dysfunction, and DILI probably perpetuate and induce more significant damage. Other mechanisms of damage, including intestinal abnormalities, have been raised (abnormal permeability, dysbiosis, viral translocation); however, without clear evidence yet. ACE2, angiotensin-converting enzyme 2; COVID-19, coronavirus disease 2019; DILI, drug-induced liver injury; GM-CSF, granulocyte-macrophage colony-stimulating factor; IL, interleukin.

lecular patterns and inflammatory signals can interact with Toll-like receptors, increasing the inflammatory response. Also, the T lymphocyte depletion cannot control the viral infections, activating multiple inflammatory signaling pathways, macrophage activation, and more secondary inflammatory reactions.[39,40] In severely ill COVID-19 patients, this mechanism of damage has been proposed as vital in the disease's evolution and mortality. This also explains why immunosuppressive therapies (such as corticosteroids or monoclonal antibodies) have been proposed. Tocilizumab (a humanized monoclonal antibody that targets IL-6) has been proposed as a possible specific treatment for cytokine storm in COVID-19. However, to date, this therapy has not demonstrated clinical benefit.[50]

Two other studies using artificial liver support systems (consisting of plasma exchange, plasma adsorption, blood/plasma filtration, and other blood purification modules) has been used to treat patients with COVID-19, with positive results. In one retrospective study, 23 patients with COVID-19 on corporeal extracorporeal membrane oxygenation were examined and found to have lower levels of IL-6 post treatment.[51] Interestingly, the results exhibited that the levels of IL-10 were not significantly reduced after treatment. This is important, as IL-10 is produced by many different cell populations including hepatocytes, sinusoidal endothelial cells, Kupffer cells, hepatic stellate cells and liver associated lymphocytes.[52] This cytokine has immunoregulatory functions and improved levels of IL-10 exert protective effects on the hepatocyte.[53] Changes of inflammation-related indicators, including white blood cell count, neutrophil count, lymphocyte count, C-reactive protein, and procalcitonin, showed a downward trend after artificial liver support treatment. Conversely, the lymphocyte count was reversely increased. In another study by Guo *et al*.[54] of 12 patients, the use of artificial liver blood purification systems was also associated with lower and sustained decrease of cytokines. However, it did not correlate with a significant improvement of liver enzymes. It is of note that in both of these studies, the decrease in cytokines was associated with improvement of clinical parameters such as APACHE II, Pneumonia Severity Index (PSI), sequential organ failure assessment and oxygenation index.

There is a direct link between COVID-19 and cytokine storms (an uncontrolled release of proinflammatory signals), which could correlate to disease severity. This phenomenon has multiple effects on immune regulation and may cause multiple organ failure, including the liver. This could also be worsened by the inability to mount a contra-inflammatory response by the liver. The use of artificial liver blood purification systems could have a positive impact in severe cases.

Hypoxia-reperfusion injury

As stated before, COVID-19 primarily affects the lungs, and many patients present with hypoxia. In newer studies, hemoglobinopathy and cell iron overload might additionally have a possible role. Two potential pathophysiological mechanisms have been proposed: acute respiratory distress syndrome[34] caused by SARS-CoV-2 and its interaction with hemoglobin through CD147, CD26, and other receptors located on erythrocyte or blood cell precursors. Hepcidin-mimetic action of a viral spike protein can also induce ferroportin blockage.[55] Other mechanisms, such as hypoperfusion caused by hemodynamic changes, may cause hepatic ischemia and hypoxia-reperfusion injury.[23,56,57]

In patients with acute cardiac failure, the decrease in systemic arterial pressure leads to an acute reduction in hepatic arterial perfusion, producing hepatocellular hypoxia.[58] Hepatic venous congestion resulting from heart failure can also cause hypoxic damage to the hepatocyte.[59] In mechanically ventilated patients (where high positive end-respiratory pressure is used), similar hemodynamic alterations have been described, mainly due to increased intra-abdominal pressure.[60,61] Studies in this context show abnormal liver enzymes, but their meaning is unclear.[61]

The hepatic ischemia and hypoxia-reperfusion dysfunction lead to lipid accumulation in hepatocytes until cell death. Hypoxia also induces an increase in reactive oxygen species, and their peroxidation products act as a second messenger activating redox-sensitive transcription factors and amplifying the release of multiple proinflammatory cytokines.[62] Mitochondrial damage probably also has a role in liver damage.[63,64]

DILI

In addition to the indirect mechanisms already exposed, DILI likely has an important role in liver injury in patients with COVID-19. Some findings show that DILI may be present in patients with COVID-19 during autopsy examinations (described as moderate microvascular steatosis and hepatic inflammation).[65] This finding could be due to the widespread use of hepatotoxic drugs in patients with COVID-19, such as acetaminophen, antivirals (i.e. oseltamivir, abidol, and lopinavir/ritonavir), corticosteroids, immunemodulators, and antibiotics.[32,66,67] Acetaminophen has been widely used to manage symptoms, and its hepatotoxic effects are well-known; however, no studies have assessed its role in liver damage in COVID-19 patients.

In a study of the incidence of adverse drug reactions in COVID-19 patients in China, based on a Hospital Pharmacovigilance System, the prevalence was 37.8%. The most prominent was drug-induced gastrointestinal disorders (23%) and liver system disorders (13.8%). Length of stay (odds ratio (OR): 2.02), number of drugs used in the hospital (OR: 3.17), and underlying diseases (OR: 2.07) were independent risk factors for having an adverse reaction. In this study, these were mainly associated with lopinavir/ritonavir (LPV/R) (63.8%) and umifenovir (18.1%).[68]

In a meta-analysis by Kulkarni *et al*.,[65] which included 107 articles (20,874 patients), the global incidence of DILI in COVID-19 patients was 25.4%. The highest incidence of DILI was associated with patients using remdesivir (15.2%) and LPV/R (37.2%).[69]

Remdesivir, a nucleotide analog prodrug that inhibits viral RNA polymerases, has shown *in vitro* activity against SARS-CoV-2 and had promising results in some clinical studies.[70] In a multicenter study of 53 individuals that received remdesivir, 23% (12/53) of patients developed elevated aminotransferases, and two discontinued the drug due to the same reason.[70] In another double-blind, placebo-controlled, multicenter trial in Hubei, China, that included 158 patients using remdesivir, the total incidence of adverse effects was reported at 66%. This included hyperbilirubinemia (10%) and AST elevation (5%). Remdesivir was stopped early because of adverse events in 18 patients (12%).[71] Nevertheless, recent studies have determined that remdesivir use was not associated with statistically significant clinical benefits.[72]

LPV/R are combined protease inhibitors approved for use against human immunodeficiency virus infection that has also been used in COVID-19 patients. The most frequently reported adverse effects of LPV/R is hyperbilirubinemia, followed by elevated aminotransferases.[21,73,74] A retrospective, single-center study of 148 patients with confirmed SARS CoV-2 in Shanghai found that 48% of them had developed abnormal liver biochemistries a week after admission. Of

those, 58% had received LPV/R. In a retrospective study of 417 patients with confirmed COVID-19 in Shenzhen, China, the use of LPV/R was associated with an increase of liver injury (7 times higher odds of abnormal liver biochemistries).[21] In another retrospective cohort study of patients treated with oral arbidol and LPV/R, 68.7% of them showed elevated bilirubin levels (mean of top bilirubin was 25.26 μmol/L). Interestingly, some studies have reported a similar prevalence of patient adverse drug reaction using placebo vs. LPV/R.[73,75] Similar to that described in remdesivir, the use of LPV/R has not shown to have a clinical benefit.[73]

Hydroxychloroquine has been linked to arrhythmias due to QTc prolongation,[76] but preliminary data did not associate this treatment with significant liver abnormalities. A probable benefit was reported in patients with COVID-19. However, recent reports have shown that hydroxychloroquine does not reduce the viral load of the virus nor does it have any clinical benefit.[77,78] In a systematic review including four randomized controlled trials, ten cohort studies, and nine case series, liver injury was not reported.[79] Similar results were observed in a double-blind, randomized, phase IIb clinical trial with 81 adult hospitalized patients.[76] Despite this, hydroxychloroquine has significant drug-drug interactions, particularly with anti-rejection immunosuppressants.[22]

Other drugs such as tocilizumab commonly produce liver enzyme elevation but are only rarely linked with severe liver injury.[22] Nevertheless, tocilizumab treatment has been associated with an increased risk of hepatitis B virus (HBV) reactivation. A prospective study of patients with rheumatoid arthritis treated with tocilizumab combined with conventional synthetic disease-modifying drugs showed an increased risk of HBV reactivation.[80]

It is important to consider that antibiotics are the most common type of drugs that have been reported as a cause of DILI. In severe COVID-19 patients, antibiotics are widely used and probably have an essential role in liver injury.[81]

Other mechanisms of liver damage

As previously stated, the main target of SARS-CoV-2 is the lung via ACE2 receptors, which are also present in cholangiocytes and hepatocytes,[26,82] meaning that during a SARS-CoV-2 infection, the liver could also be directly targeted. It is notable that despite the extensive ACE2 expression of cholangiocytes, specific abnormalities of bile duct chemistries are rare.[5] However, cholestasis has been observed. In a recent case report, three young adults without preexisting chronic liver disease underwent liver biopsies due to prolonged and severe cholestasis during recovery from critical COVID-19 that required mechanical ventilation. Of note, each patient had severe but acute aminotransferase elevations, in line with previously stated biochemical alterations common in COVID-19 patients. After cardiopulmonary and renal recovery, they developed persistent cholestasis associated with jaundice. Their biopsies exhibited moderate portal and periportal fibrosis, with focal fibrotic obliteration of terminal hepatic veins in one case. All three cases showed extensive degenerative cholangiocyte injury, including necrosis. Furthermore, there was necrosis of the cholangiocyte epithelial layer of terminal bile ducts and marginal ductules and microvascular changes. These changes are similar to secondary sclerosing cholangitis of the critically ill patient that could be superimposed due to direct injury to cholangiocytes after exposure to SARS-CoV-2. Although further evidence is needed (as *in situ* hybridization and immunohistochemistry for SARS-CoV-2 was negative in two of the three patients), these findings point to the unique susceptibility of the liver to COVID-19.[83]

Recent studies have observed SARS-CoV-2 viral particles in the cytoplasm of hepatocytes. The majority of viral particles were noted to harbor a complete envelope with corona-like spikes, suggesting that SARS-CoV-2 can enter and replicate in hepatocytes.[84] This may drive hepatocyte apoptosis[85] via caspase-dependent pathways,[86] and translocation from the gut lumen into the liver via portal flow.[23] These findings were also described in the SARS and MERS diseases.[32]

Pathological findings of COVID-19 disease

Several reports have described multiple liver histopathological findings in COVID-19 patients, including hepatic steatosis, congestion of hepatic sinuses, and inflammatory features. A recent systematic review of autopsies from patients with COVID-19 reported that the most frequent histopathological features were hepatic steatosis (55.1%), venous outflow obstruction (36.4%), congestion of hepatic sinuses (34.7%), vascular thrombosis (29.4%), fibrosis (20.5%), necrosis (15.4%), Kupffer cell hyperplasia (13.5%), portal inflammation (13.2%), and lobular inflammation (11.6%).[87]

The high prevalence of steatosis can be partially explained by the population's baseline characteristics (risk factors of severe COVID-19 are similar to the risk factors associated with steatosis).[88] Also, metabolic-associated fatty liver disease (MAFLD) is independently associated with more severe COVID-19 disease. DILI and cytokine storm can also contribute to the development of hepatic steatosis.[89] Vascular thrombosis was frequently observed due to endothelial dysfunction (endotheliitis), a procoagulant state, and direct vascular injury of the disease, all mechanisms described as part of the pathophysiology of severe COVID-19 disease.[65,84,90–98]

Congestion and necrosis may be explained by circulatory dysfunction, heart failure, and ischemia due to multiorgan failure. Finally, some of these findings have also been described in SARS and MERS patients and may be related to the ongoing systemic inflammatory process and sepsis, affecting the liver (portal inflammation, lobular inflammation, and Kupffer cell hyperplasia or proliferation).[85,91–95] To date, no specific histologic indicator of direct infection in the liver tissue (i.e. viral cytopathic effect) has been reported.

Liver damage in pre-existing liver disease

Patients with pre-existing liver disorders, such as liver cirrhosis and hepatocellular carcinoma, are considered to have a higher susceptibility for any kind of infection and sepsis secondary to impaired host defense. The prevalence of chronic liver diseases is between 0.6% and 1.4% in patients hospitalized for COVID-19.[34,99,100] These patients are at high risk of severe disease (up to 60% develop a severe disease) and higher mortality (even reaching 18%).[101] Also, SARS-CoV-2 infection causes higher liver injury in chronic liver disease patients, decompensation in 20% of cases with cirrhosis, and worsening clinical outcomes of already unstable patients.[102]

Among cases with chronic liver diseases and COVID-19, the relationship between MAFLD and COVID-19 has been the most studied. The first evidence was established by Qian *et al*.,[103] in an early study in China that included 324 COVID-19 patients, 21.6% of the subjects had MAFLD (diagnosed by computed tomography scan), and the prevalence of MAFLD was higher among patients with severe COVID-19. Later Ji *et al*.[104] studied 202 patients admitted for COVID-19 and with the diagnosis of MAFLD (established through hepatic steatosis index >36 points and/or by abdominal ultrasound).

They concluded that patients with MAFLD had a higher risk of disease progression, a higher likelihood of abnormal liver function from admission to discharge, and longer viral shedding time than patients without MAFLD. Subsequently, other studies have described similar results with higher mortality in patients with MAFLD, obesity, and those over 60 years of age.[88] Similarly, a multicenter retrospective study by Zheng *et al*.[105] further validated this information.

Furthermore, patients with MALFD had an OR for severe COVID-19 of 2.3, and for obese patients with MALFD it was an OR of 6.32 compared to non-obese patients. This is believed to be caused by liver fat, and associated inflammation could exacerbate the virus-associated cytokine storm, leading to worsening COVID-19. Other studies found that increasing liver fibrosis measured by NAFLD Fibrosis Score and Fibrosis-4 (FIB-4) scores was linked to increased severity of disease in COVID-19 patients. Moreover, liver fat has been independently linked with an increased risk of testing positive for COVID-19.[106]

Likely, the presence of already activated inflammatory pathways in patients with MAFLD is associated with more severe SIRS development when they are infected with SARS-CoV-2.[107] The increased ACE2 expression on hepatocytes of patients with MAFLD[108] and paired hepatic innate immune system in these patients are potential mechanisms that would explain the increased risk of severe COVID-19 in patients with MAFLD.[109]

Autoimmune liver disease, treated with immunomodulatory or immunosuppressive drugs, could increase the risk of complications associated with COVID-19. In an Italian study based on 148 clinical telephone interviews, the incidence of SARS-CoV-2 infection in patients with autoimmune hepatitis was similar to the general population, and the prevalence of severe COVID-19 was low.[110] Since there are no adequate studies to define the real risk, the patients with autoimmune hepatitis on immunosuppressive treatment should be considered at high risk for severe COVID-19.[36,111]

Finally, according to initial reports concerning co-infection with COVID-19 and other viruses, chronic infection with HBV does not seem to confer a worse prognosis in patients with SARS-CoV-2.[5] However, it is necessary to pay carefully attention to the use of immunosuppressors (high-dose glucocorticoids or tocilizumab) as therapy for COVID-19, due to the possible risk of virus reactivation.[112]

Management

Although we aimed to review the mechanism of liver damage in COVID-19, it is important to mention how current guidelines have addressed this issue and the implications of COVID-19 for patients with previous liver diseases. Due to the pandemic's novel nature, there is still little evidence regarding this topic, and most recommendations are based on expert consensus.[113] These include recommending that patients with cirrhosis or other liver diseases minimize their risk of contracting COVID-19 through general preventive measures, such as hand hygiene, social distancing, use of telemedicine visits for ongoing disease management, and reducing exposure to health services if possible. It is also recommended that clinicians decrease routine laboratory and imaging surveillance frequency when the associated risk is deemed to be low and delay non-urgent procedures. As the pandemic prolongs in time, this recommendation must be addressed more fully in newer updates and the impact on mortality on patients isolated from care studied. On the other hand, early admission for patients with COVID-19 who also have advanced liver disease, especially with other risk factors, is recommended.[36,114] Another important topic will be to assess the effectiveness and security of the available vaccines against SARS-CoV-2 in this particular population.

As discussed above, there are few drugs with clinically significant impact against COVID-19. To date, remdesivir is the only approved medical treatment for COVID-19 as of October 2020. Elevated liver biochemistries are not currently a contraindication for its use, although it is not recommended in patients with an ALT ≥5 times the upper limit of normal. The Federal Drug Administration in the USA also suggests that clinicians should perform hepatic laboratory testing in all patients before and while receiving remdesevir and consider discontinuing if ALT levels increase to greater than 10 times the upper limit of normal or is accompanied by signs or symptoms of liver inflammation.[72,115,116] As stated above, there is consensus that more research is needed to validate remdesivir as an effective treatment against COVID-19. Furthermore, current studies have shown that it may lead to DILI. Therefore, it is unclear whether there is clinical benefit in the use remdesivir to prevent or treat direct liver damage due to SARs-Cov-2 and more studies are necessary.[117]

Other data from randomized trials overall support the role of glucocorticoids for severe COVID-19. In a meta-analysis of seven trials that included 1,703 critically ill patients with COVID-19, glucocorticoids reduced 28-day mortality compared with standard care or placebo (32 vs. 40 percent, OR: 0.66, 95% confidence interval: 0.53–0.82) and were not associated with an increased risk of severe adverse events.[118] For patients on glucocorticoids, such as patients with autoimmune hepatitis, therapy should not be abruptly discontinued but should be used at the lowest dose possible to control the underlying disease, regardless of COVID-19 exposure or infection status.[119] This is why guidelines suggest an individualized approach in patients with autoimmune hepatitis and COVID-19, based on the severity of infection, patient comorbidities, the severity of liver disease, and the existing medication regimen. The goal of medication adjustment is to reduce immunosuppression during active viral replication, so as to lower the risk of COVID-19-related complications while balancing the risk of disease flare.[36] The general strategy includes maintaining treatment in asymptomatic patients and dose reduction in moderate to severe COVID-19. This must be balanced with the possible benefits of dexamethasone for moderate to severe COVID-19.

As previously mentioned, reactivation of HBV infection has been observed in patients treated with glucocorticoids and tocilizumab. Thus, HBV prophylaxis may be indicated when initiating these therapies. Certain antiviral therapies have shown a greater risk for developing DILI. However, there are no contraindications to initiating or continuing specific antiviral therapy for HBV or HCV infection in patients with or without COVID-19.[36]

Due to the pandemic, transplant programs worldwide have been severely impacted, with fewer number of transplantations performed. There is also conflicting data regarding the risk of severe COVID-19 in recipients.[120,121] Furthermore, there is insufficient data as to how immunosuppressive medications should be adjusted. SARS-CoV-2 infections have been reported in patients receiving different types of antirejection therapy.[122] Guidelines have extrapolated from their practices of managing other viruses, such as Epstein-Barr virus, cytomegalovirus, and BK viruses. This follows that for transplant recipients without COVID-19, maintenance immunosuppression is continued without adjustment or in some patients reduces the antimetabolite component of immunosuppression (e.g., mycophenolate or azathioprine), although clear guidelines must be issued. For patients with acute T cell-mediated (cellular) rejection of the liver allograft, the approach to management, including high-dose glucocorticoids for patients with moderate to

severe rejection, has not changed.[36] In transplant recipients with COVID-19, adjustments to immunosuppression are individualized, based on COVID-19 severity, the specific regimen used, time post-transplant, and allograft rejection risk. Experts recommend reducing immunosuppression in patients with moderate to severe COVID-19 (e.g., those requiring hospitalization).

Finally, specific management in scenarios like an acute liver failure in critically ill patients or hepatic carcinoma and other issues such as when to suspend or resume chronic therapies escape the scope of this review. It is of note that the indirect impact of the pandemic on patients isolated from their healthcare providers is yet unknown.

Conclusions

The liver is the second most common organ affected in COVID-19. Liver injury caused during COVID-19 infection is likely multifactorial, such as the use of potentially hepatotoxic drugs, systemic inflammatory response, respiratory distress syndrome-induced hypoxia, and direct damage. Indirect damage probably plays a prominent role in seriously ill patients and patients with chronic liver disease.

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Conflict of interest

The authors have no conflict of interests related to this publication.

Author contributions

FI, GA, JPA and LAD conceived and designed the review, all authors collected the data and contributed to the interpretation. All the authors participated in drafting the article and revising it critically for important intellectual content, and all the authors gave final approval of the version submitted.

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Guideline

Guidelines for Prevention and Treatment of Chronic Hepatitis B

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Abstract

To achieve the goal of the World Health Organization to eliminate viral hepatitis as a major public health threat by 2030, the Chinese Society of Infectious Diseases and the Chinese Society of Hepatology convened an expert panel in 2019 to update the guidelines for the prevention and treatment of chronic hepatitis B (CHB). The current guidelines cover recent advances in basic, clinical, and preventive studies of CHB infection and consider the actual situation in China. These guidelines are intended to provide support for the prevention, diagnosis, and treatment of CHB.

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A Chinese expert panel led by the Chinese Society of Infectious Diseases and the Chinese Society of Hepatology developed the first edition of the guidelines for the prevention and treatment of chronic hepatitis B (CHB) in 2005, which were updated in 2010 and 2015, respectively. Over the past 4 years, significant progress has been made in basic and clinical research on chronic infection with the hepatitis B virus (HBV), both at home and abroad. The guidelines were updated again to standardize the prevention, diagnosis, and treatment of CHB and help to meet the goal to eliminate viral hepatitis as a major public health threat by 2030 that was proposed by the World Health Organization (WHO) in 2016.

The present guidelines aim to help clinicians make informed decisions for the prevention, diagnosis, and treatment of CHB; however, they are not intended to be mandatory standards and will probably not cover and solve all the issues associated with the diagnosis and treatment of CHB. Therefore, clinicians should use the best clinical evidence and their expertise, experience, and available medical resources to develop a comprehensive and reasonable diagnosis and treatment plan that addresses individual needs.

The quality of evidence in this guideline is divided into three levels: A, B, and C, and the strength of the recommendations is categorized into two levels, 1 and 2, as given in Table 1. (revised according to the Grades of Recommendation, Assessment, Development, and Evaluation classification system).

Epidemiology and prevention

Epidemiology

HBV is a global epidemic; however, its prevalence varies significantly between different regions. According to WHO, approximately 257 million people live with chronic HBV infection, 68% of whom live in Africa and the Western Pacific.[1] In 2015, approximately 887,000 people died from HBV infection-related diseases worldwide, and cirrhosis and hepatocellular carcinoma (HCC) accounted for 52% and 38% of the deaths, respectively. The prevalence of hepatitis B surface antigen (HBsAg) in the general population in Southeast Asia and the Western Pacific was 2% (39 million cases) and 6.2% (115 million cases), respectively. The endemicity of HBV in Asia is heterogeneous, and most of the region has a moderate to high prevalence of HBV infection, except for in a few low endemic areas.

In 2014, the Chinese Center for Disease Control and Prevention conducted a national seroepidemiological survey of hepatitis B among people aged 1–29 years. The results showed that the prevalence of HBsAg in people aged 1–4, 5–14, and 15–29 years was 0.32%, 0.94%, and 4.38%, representing a decrease of 96.7%, 91.2%, and 55.1%, respectively compared with the 1992 survey.[2] It was estimated that the current prevalence of HBsAg in the general population was from 5% to 6% in China, and approximately

Keywords: Hepatitis B; Chronic; Guidelines; Prevention; Treatment.

Abbreviations: ACLF, acute-on-chronic liver failure; ADV, adefovir dipivoxil; AFP, alpha-fetoprotein; ALP, alkaline phosphatase; ALT, alanine aminotransferase; APRI, AST to platelet ratio index; AST, aspartate aminotransferase; cccDNA, covalently closed circular DNA; CHB, chronic hepatitis B; CT, Computerized tomography; DAA, direct-acting antiviral; ETV, entecavir; GFR, glomerular filtration rate; GGT, γ-glutamyl transferase; HBV, hepatitis B virus; HBcAg, hepatitis B core antigen; HBeAg, hepatitis B e antigen; HBsAg, hepatitis B surface antigen; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; HIV, human immunodeficiency virus; IFN, interferon; Ig, immunoglobulin; LAM, lamivudine; LdT, telbivudine; MRI, Magnetic resonance imaging; PT, prothrombin activity time; PTA, prothrombin activity; TAF, tenofovir alafenamide fumarate tablets; TE, transient elastography; TDF, tenofovir disoproxil fumarate; ULN, upper limit of normal; WHO, World Health Organization.

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Table 1. Evidence level and recommendation strength

Level	Descriptions
Level of evidence	
High quality (A)	Further research will probably not change confidence in this assessment.
Medium quality (B)	Further research will probably have a significant impact on confidence in this assessment.
Low quality (C)	Further research will probably affect and change this assessment.
Recommended level	
Strong (1)	Quality of the evidence, possible outcomes, and potential results of prevention, diagnosis and treatment are all considered, with expected benefits outweighing estimated costs.
Weak (2)	Quality of evidence varies substantially, which results in a less certain recommendation, with expected benefits not necessarily outweighing estimated costs.

Terminology	Definition
Chronic HBV infection	HBsAg, or HBV DNA seropositive, or both for ≥6 months.
CHB	Chronic liver disease caused by persistent HBV infection for ≥6 months.
HBV reactivation	Increase in HBV DNA ≥1 lg IU/mL from baseline, or detection of HBV DNA in patients with negative baseline HBV DNA, or HBsAg seroconversion from negative to positive in HBsAg-negative/anti-HBc-positive patients who receive immunosuppressive therapy or chemotherapy.
HBeAg clearance	Loss of HBeAg in HBeAg-positive patients.
HBeAg seroconversion	Loss of HBeAg and anti-HBe seroconversion from negative to positive in HBeAg-positive patients.
Resolved hepatitis B	Patients have a history of acute or CHB but are currently negative for HBsAg, positive or negative for anti-HBs, positive for anti-HBc, with undetectable HBV DNA and normal serum ALT levels.
Virological breakthrough	Increase in HBV DNA >1 lg IU/mL from nadir during treatment, or reversion to positive following conversion to negative, with or without elevated ALT, in patient with good compliance to NAs therapy, as confirmed 1 month later using the same reagent.
Virological relapse	Serum HBV DNA >2,000 IU/mL following the withdrawal of treatment in patients with virological response, confirmed 1 month later.
Drug resistance	
Genotypic resistance	Genetic mutations that confer resistance to HBV detected during antiviral therapy.
Phenotypic resistance	Decreased susceptibility (determined by *in vitro* testing) to antiviral drugs, which is associated with genotypic resistance.
Cross-resistance	Drug-resistant mutations that arise for one antiviral drug that can show resistance to other antiviral drugs (either one or several).
Multidrug resistance	Drug resistance to at least two different classes of NAs.

ALT, alanine aminotransferase; NAs, nucleos(t)ide analogs; HBeAg, hepatitis B e-antigen; anti-HBe, hepatitis B e-antibody; CHB, chronic hepatitis B; HBV, hepatitis B virus.

70 million people were chronically infected by HBV, which included approximately 20–30 million patients with CHB.[3]

HBV is transmitted from mother-to-child or through blood (which includes minor wounds on the skin and mucous membranes) and sexual contacts. Mother-to-child transmission is the most common route of transmission in China, which accounts for 30–50% of HBV infections.[4] It mainly occurs during the perinatal period through exposure to blood and body fluids of HBV-positive mothers. Maternal HBV DNA levels are closely correlated with the risk of HBV infection in infants. Mother-to-child transmission is more probable to occur in children born to HBeAg-positive mothers with high HBV DNA levels.[5] HBV is mainly transmitted through blood and sexual contact in adults. Patients with a history of drug injection, immunosuppressive therapy, blood transfusion or on hemodialysis, patients with hepatitis C virus (HCV) or human immunodeficiency virus (HIV) infection, family members of HBsAg-positive people, healthcare workers and public safety workers at risk of occupational exposure to blood or body fluids in the work environment, prisoners, and diabetic patients who have not been vaccinated against hepatitis B have a higher risk of HBV infection.[6] Due to the strict screening for HBsAg and HBV DNA in blood donors and the adoption of safe injection practices, transmission through blood transfusion or blood products is rare. In addition, transmission could occur through damaged skin or mucous membranes, such as pedicures, tattoos, piercings, accidental exposure of healthcare workers during their work, and sharing of razors or toothbrushes.[6] Unprotected sexual contact with persons infected with HBV carries a high-risk of HBV infection, in particular, for those with multiple sexual partners and who are homosexual men.[7]

HBV does not spread through the respiratory or digestive tracts. Therefore, HBV cannot be transmitted via normal exposure in schools, workplaces, and other group settings, for example, working in the same office (which includes sharing

computers and other office supplies), contact through shaking hands and hugging, living in the same dormitory, dining in the same restaurant, toilet sharing, and other nonblood exposure contacts. No epidemiological or experimental studies have found that HBV can be transmitted via blood-sucking insects (e.g., mosquitoes and bedbugs).[8]

Prevention

Protecting susceptible people: HBV vaccination is the most effective measure to prevent HBV infections. Vaccination mainly targets newborns, followed by infants,[9] previously unvaccinated children and adolescents aged <15 years, and high-risk groups.[7–10]

The hepatitis B vaccine is administered in a series of three doses; the first dose is received immediately after birth, the second after 1 month, and the final dose at 6 months. The HBV vaccine should be administered as soon as possible after birth. The vaccine is administered by intramuscular injection into the deltoid muscle of the upper arm or the anterolateral aspect of the thigh for newborns and the middle deltoid muscle of the upper arm for children and adults. Newborns who suffer from severe diseases, such as very low birth weight, severe birth defects, severe asphyxia, and respiratory distress syndrome, should receive the first dose of hepatitis B vaccine as soon as possible after their vital signs have stabilized.

The dosing regimen for hepatitis B vaccine for newborns is: (1) 10 μg of recombinant yeast hepatitis B vaccine per injection, regardless of whether the mother is HBsAg-positive or not, and (2) recombinant Chinese hamster ovary (commonly known as CHO) cell hepatitis B vaccine, 10 μg for newborns of HBsAg-negative mothers or 20 μg for newborns of HBsAg-positive mothers per injection.

It is recommended that adults should be vaccinated with three doses of 20 μg recombinant yeast hepatitis B vaccine or 20 μg recombinant CHO cell hepatitis B vaccine. For immunocompromised people or non-responders, the dose (e.g., 60 μg) and the number of injections should be increased; for those who do not respond to the three-dose series (0, 1, and 6 months), one additional dose of 60 μg or three doses of 20 μg hepatitis B vaccine could be administered. Serum anti-HBs should be tested 1–2 months after the second-round vaccination. If there was no response, another dose of 60 μg recombinant yeast hepatitis B vaccine should be administered. The protection conferred by vaccination with hepatitis B vaccine generally lasts for ≥30 years in responders.[11] Therefore, anti-HBs monitoring or booster immunization is not required for the general population; however, anti-HBs should be monitored for high-risk groups or immunocompromised people. Another dose of the hepatitis B vaccine should be administered if anti-HBs levels were <10 mIU/mL.

Women who have not been infected with HBV can safely receive the hepatitis B vaccine during pregnancy.[12,13] In addition to the standard vaccination procedure, the accelerated vaccination schedule (0, 1, and 2 months) has been proven to be feasible and efficacious.[14]

Accidentally-exposed people refer to those whose damaged skin or mucous membranes have encountered the blood or body fluids from HBsAg-positive or HBsAg-unknown sources, or who have been stabbed by needles that are contaminated by such blood or body fluids.

Management of the sources of infection: People who have been identified as HBsAg-positive for the first time should be reported to local disease control and prevention centers, as required, if they meet the reporting standards for infectious diseases. In addition, it is recommended that their family members are tested for serum HBsAg, anti-HBs, and anti-hepatitis B core (HBc). Susceptible people should be vaccinated against HBV. The infectivity of HBV-infected people is mainly determined by serum HBV DNA levels, instead of serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), and bilirubin levels. It is recommended that HBV infection markers should be tested during health examinations and medical activities that do not involve school admission or job recruitment. The aim is to facilitate early diagnosis and early treatment and to mitigate the harm caused by HBV infection. For the follow-up of people with chronic HBV infection, refer to section XV, "Monitoring and follow-up management of people with chronic HBV infection." Individuals with chronic HBV infection should avoid sharing dental appliances, razors, syringes, and blood collection needles. In addition, they are not allowed to donate blood, organs, or sperm, and it is suggested that they receive regular medical follow-ups. Their family members or sexual partners should be vaccinated against hepatitis B as soon as possible.

Blocking transmission routes: The promotion of safe injection (which includes blood collection needles and tools for acupuncture and moxibustion) is critical and standard precaution principles for hospital infection management should be followed. The equipment used in service industries, including hairdressing, shaving, pedicuring, puncturing, and tattooing, should be strictly disinfected. People whose sexual partners are HBsAg-positive should receive the HBV vaccine or use condoms. When the health condition of the sexual partner is unknown, condoms must be used to prevent HBV and other blood-borne or sexually transmitted diseases. For pregnant women who are HBsAg-positive, amniocentesis should be avoided whenever possible to ensure the placenta is intact and to minimize the chance of the newborn's exposure to maternal blood.

Recommendation 1: Hepatitis B vaccination for newborns

1. Infants born to HBsAg-negative mothers should receive 10 μg of recombinant yeast hepatitis B vaccine as soon as possible within 12 h of birth, the second dose at 1 month, and the third dose at 6 months (A1).
2. Infants born to HBsAg-positive mothers should receive 100 IU of hepatitis B immunoglobulin (HBIG) and concurrent recombinant yeast HBV vaccine (10 μg) at different sites as soon as possible within 12 h of birth, followed by the second and third doses of HBV vaccine at 1 and 6 months, respectively. Infants born to HBsAg-positive mothers should be tested for HBsAg and anti-HBs 1–2 months after the third dose of hepatitis B vaccine. If HBsAg is negative and anti-HBs levels are <10 mIU/mL, three additional doses of hepatitis B vaccine can be given according to the 0, 1, and 6 months immunization program. If HBsAg is positive, it means that the vaccination has failed, and the infants should be monitored regularly (A1).
3. Premature infants and low-weight infants born to mothers with unknown HBsAg status should receive the first dose of hepatitis B vaccine and HBIG as soon as possible within 12 h of birth; after 1 month, three doses of hepatitis B vaccine can be administered according to the 0, 1 and 6 month immunization program (A1).
4. Newborns who are vaccinated with hepatitis B vaccine and HBIG within 12 h of birth can breastfeed from HBsAg-positive mothers (B1).

Recommendation 2: Catch-up vaccination should be administered for children who have not been vaccinated with the hepatitis B vaccine or who have not completed the full vaccination series. The interval between the first dose and the second dose should be ≥28 days, and the interval between the second dose and the third dose should be ≥60 days (A1).

Recommendation 3: For immunocompromised or non-responsive adults, the vaccination dose (e.g., 60 μg) and

the number of doses should be increased; for those who do not respond to the three-dose procedure (0, 1, and 6 months), one additional dose of 60 μg or three doses of 20 μg hepatitis B vaccine should be administered, and serum anti-HBs should be tested 1–2 months after the second-round vaccination. If there is still no response, another dose of 60 μg recombinant yeast hepatitis B vaccine should be administered (A1).

Recommendation 4: People who are accidentally exposed to HBV should be treated as follows:

1. Gently squeeze around the wound to drain blood from the wound, rinse the wound with 0.9% NaCl solution, and then treat the wound with disinfectant (A1).
2. HBV DNA and HBsAg should be tested immediately and rechecked after 3–6 months (A1).
3. No treatment is required for those who have been vaccinated with hepatitis B vaccine and are known to be positive for anti-HBs (≥10 mIU/mL). For those who have not been vaccinated against hepatitis B or those who have been vaccinated against HBV but have anti-HBs levels <10 mIU/mL or unknown anti-HBs levels, 200–400 IU HBIG and concurrent hepatitis B vaccine (20 μg) should be administered immediately at different sites, followed by second and third doses of hepatitis B vaccine (20 μg) 1 and 6 months later (A1).

Recommendation 5: HBsAg, anti-HBc, and anti-HBs should be screened for during health examinations or medical treatments that do not involve school admission or job recruitment. HBsAg, anti-HBc, and anti-HBs should be screened in high-risk groups, pregnant women, and patients who receive antitumor treatment (chemotherapy or radiotherapy) or immunosuppressive agents or direct-acting antiviral (DAA) therapy for HCV, and in HIV-infected people. Hepatitis B vaccination is recommended for those who are negative for all the three HBV markers (B1).

Etiology

HBV belongs to the Hepapadnaviridae family. It is an enveloped DNA virus with a genome length of approximately 3.2 kb and contains a partially double-stranded circular DNA. Its genome encodes HBsAg, HBc antigen (HBcAg), hepatitis B e antigen (HBeAg), viral polymerase, and HBV X protein (HBx). HBV is highly resistant but can be inactivated at 65°C for 10 h, at 100°C for 10 min, or by high-pressure steam. In addition, HBV can effectively be inactivated by ethylene oxide, glutaraldehyde, peroxyacetic acid, and iodophors.

HBV enters liver cells by binding to sodium-taurocholate cotransporting polypeptide (commonly referred to as NTCP) on the liver cell membrane.[15] After HBV invasion of liver cells, the partially double-stranded circular DNA uses the negative strand as a template and extends the positive strand into the cell nucleus to repair the nick in the positive strand, which forms a covalently closed circular DNA (cccDNA). cccDNA plays an important role in chronic infection because it has a long half-life and is difficult to completely remove from the body. HBV can integrate into the host genome. HBV uses cccDNA as a template to transcribe viral mRNAs with different lengths, which include the 3.5 kb pregenomic RNA (commonly referred to as pgRNA) that can be released into the peripheral blood. Serum HBV RNA levels can reflect the activity of cccDNA in liver tissue and might be related to virological responses and the prognosis of patients.[16–18] HBV can be divided into at least nine genotypes, from A to I;[19] genotypes B and C are the most prevalent in China. The incidence of mother-to-child transmission in HBV patients with type B and C was higher than that of other genotypes, and genotype C is associated with earlier progression to HCC. HBV genotypes are associated with disease progression and responses to interferon-α (IFN-α) therapy.[20–22] The response rate of HBeAg-positive patients to IFN-α treatment was higher in type B than in type C and higher in type A than in type D.[23]

Natural history and pathogenesis

Natural history

The natural history of HBV infection depends mainly on the interaction between the virus and the host. The age when HBV infection is acquired is one of the most critical factors that determine whether the HBV infection will become chronic. The risk of chronic HBV infection in newborns and infants aged <1 year is 90%.[24] Most people with HBV infection in China are infected during the perinatal period or infancy. The world has achieved significant success in blocking HBV mother-to-child transmission.[25] A universal immunization program that combines hepatitis B vaccine and HBIG has been adopted for newborns of HBsAg-positive mothers in China; however, approximately 5–7% of newborns are still infected with HBV due to mother-to-child transmission. This occurs in 7–11% of HBeAg-positive pregnant women and 0–1% in HBeAg-negative pregnant women.[26,27]

In general, the natural history of chronic HBV infection is divided into four phases based on its natural progression,[28–30] namely the immune tolerance phase (chronic HBV carrier state), immune clearance phase (HBeAg-positive CHB), immune control phase (inactive HBsAg carrier state), and reactivation phase (HBeAg-negative CHB) (Table 2). For more details, refer to section IX, "Clinical Diagnosis". Not all patients with chronic HBV infection will experience all four phases. Patients who are infected with HBV during adolescence and adulthood usually experience no immune tolerance phase and directly enter the immune clearance phase.

Spontaneous HBeAg seroconversion might occur during the immune clearance phase, with an annual incidence of 2–15%. Patients with age <40 years, elevated ALT, HBV genotype A and genotype B had higher incidence.[28,31] After HBeAg seroconversion, the HBsAg clearance rate was 0.5–1.0% annually.[32] Ten years after HBsAg disappeared, cccDNA was still detectable in the liver of approximately 14% of these patients.[33] Patients with either age >50 years, cirrhosis, or concomitant HCV or hepatitis D virus (HDV) infections can still progress to HCC even if HBsAg has disappeared; however, the incidence rate is low.[34]

The annual incidence of cirrhosis in CHB patients without antiviral therapy is 2–10%,[35] and risk factors include host (older age, male, age >40 years when HBeAg seroconversion occurs, and persistently elevated ALT levels[36,37]), the virus (HBV DNA >2,000 IU/mL), persistently positive HBeAg status,[38] genotype C, coinfection with HCV, HDV, or HIV, and other liver injury-inducing factors (e.g., alcohol or obesity).[35] The annual incidence of decompensated cirrhosis from compensated cirrhosis is 3–5%, and the 5-year survival rate of the patients with decompensated cirrhosis is 14–35%.[35] The annual incidence of HCC in patients with HBV infection without cirrhosis is 0.5–1.0%.[35] The annual incidence of HCC in patients with cirrhosis is 3–6%.[39–41] Also, cirrhosis, diabetes, immediate relatives with HCC, high serum HBsAg levels, and exposure to aflatoxin are all associated with a high incidence of HCC.[35,42–46] Lower HBsAg levels often suggest that hosts have exerted good immune control over HBV replication and infection. Studies have shown that even if HBeAg is negative and HBV DNA levels are low, patients with higher HBsAg levels (≥1,000 IU/mL) are still at a higher risk of HCC, regardless of genotype B or C.[45,46]

Table 2. Stages of chronic HBV infection

Markers	Immune tolerance phase (chronic HBV carrier status)	Immune clearance phase (HBeAg-positive CHB)	Immune control phase (inactive HBsAg carrier status)	Reactivation phase (HBeAg-negative CHB)
		HBV serological markers		
HBsAg (IU/mL)	$>1\times10^4$	+	$<1\times10^3$	+
anti-HBs	–	–	–	–
HBeAg	+	+	–	+/–
anti-HBe	–	–	+	+/–
anti-HBc	+	+	+	+
HBV DNA (IU/mL)	$>2\times10^7$	$>2\times10^4$	$<2\times10^3$	$\geq2\times10^3$
ALT	Normal	Persistent or recurrent increase	Normal	Persistent or recurrent increase
Liver pathology	No obvious necroinflammation or fibrosis	Obvious necroinflammation, or fibrosis, or both	No or mild inflammation, with varying degrees of fibrosis	Obvious necroinflammation, or fibrosis, or both

CHB, chronic hepatitis B; HBV, hepatitis B virus; HBeAg, hepatitis B e antigen; HBsAg, hepatitis B surface antigen.

Pathogenesis

The pathogenesis of chronic HBV infection is complicated and is not fully understood. HBV does not directly kill liver cells. The immune response caused by the virus is the main mechanism that leads to liver cell damage and necroinflammation. Persistent or recurrent necroinflammation is an important factor for patients with chronic HBV infection progressing to cirrhosis and even HCC.

The nonspecific (innate) immune response plays an important role in the initial stages of HBV infection and initiates the subsequent specific (adaptive) immune response.[47,48] HBV uses its proteins, such as HBeAg and HBx, to interfere with antiviral signaling pathways that involve Toll-like receptors and retinoic acid-inducible gene I-like receptors; therefore, the level of the nonspecific immune response is suppressed. CHB patients often present with decreased frequencies of myeloid dendritic cells (mDCs) and plasmacytoid dendritic cells (pDCs) in the peripheral blood. The ability of mDCs to mature and the ability of pDCs to produce IFN-α are significantly impaired, which make it more difficult for the body to eliminate the virus and induce HBV-specific T lymphocytes, which impedes viral elimination.

The HBV-specific immune response plays a leading role in HBV clearance.[49] Major histocompatibility complex class I-restricted $CD8^+$ cytotoxic T lymphocytes can induce the apoptosis of infected hepatocytes and can secrete IFN-γ and suppress the expression and replication of HBV genes in liver cells in a noncytolytic fashion.[50] During chronic infection, HBV-specific T cells are susceptible to apoptosis. Their ability to produce cytokines and proliferate is significantly impaired, and their functions become exhausted, which might be one of the mechanisms that lead to persistent HBV infection.[51] Currently, it is believed that there are large amounts of HBsAg in serum and liver tissue, and the lack, or dysfunction, or both of HBsAg-specific cytotoxic T lymphocytes is an important cause of immune tolerance in patients with chronic HBV infection.[52]

Laboratory tests

Serological testing for HBV

Traditional serum markers for HBV include HBsAg, anti-HBs, HBeAg, anti-HBe, anti-HBc, and anti-HBc immunoglobulin (Ig)M. Serum HBsAg can be produced by cccDNA-derived mRNA or an HBV DNA sequence that is integrated into the human genome. HBsAg positivity indicates HBV infection. Anti-HBs is a protective antibody, and anti-HBs positivity indicates immunity to HBV, which can be seen in people with a resolved hepatitis B infection or who have been inoculated with the hepatitis B vaccine. Anti-HBc-IgM positivity is mostly found in patients with acute hepatitis B. Anti-HBc-IgM is usually mildly positive in patients with reactivation of chronic HBV infection. The main subtype of anti-HBc is IgG. The IgG subtype is positive in most HBV cases, whether the virus is eliminated or not.

Recently, the quantitative detection of HBsAg has been widely used in clinical practice. Its level is indicative of the stage of the disease and the risk of disease progression. In addition, it can be used to guide the use of recombinant human IFN and peginterferon-α (peg-IFN-α).

Virological testing for HBV

HBV DNA quantification: This is mainly used to assess the level of virus replication in HBV-infected patients. In addition, it can be used as a crucial component to select the indications and assess the efficacy of antiviral therapy. During antiviral therapy, obtaining a sustained virological response could significantly control the progression of cirrhosis and lower the risk of HCC.[53,54] The quantitative detection of HBV DNA utilizes real-time quantitative polymerase chain reaction; however, the detection limit varies between manufacturers' reagents.

HBV genotyping: To date, at least nine HBV genotypes (A to I) and one undefined genotype (J) have been identified. Some genotypes are further divided into subtypes. The detection of HBV genotypes could help to predict the efficacy of IFN and determine the disease prognosis.[55–58]

Detection of resistant mutants: HBV is a highly mutable virus. During reverse transcription and replication, a mutation might occur to one or more nucleotides during replication, due to the lack of proofreading ability in RNA polymerase and reverse transcriptase. HBV can naturally mutate during chronic persistent infection. Viral mutation can be induced by antiviral treatment. In both cases, mutated HBV might become less susceptible to antiviral drugs.[59]

The appropriate detection of drug-resistant mutant strains could help clinicians to identify drug resistance and adjust the treatment plan as required. Commonly used clinical tests for drug resistance include gene sequencing that uses reverse transcriptase and reverse hybridization-based line probe assays (INNO-LIPA kit).

Detection of new HBV markers

Anti-HBc quantification (qAnti-HBc): A novel double-antigen sandwich enzyme-linked immunosorbent assay (commonly known as ELISA) can quantitatively determine serum anti-HBc levels. In natural history studies, the quantitative (q)anti-HBc levels in patients during the immune clearance and reactivation phases were significantly higher than those in the immune tolerance and low replication phases.[60,61] The baseline qAnti-HBc levels of HBeAg-positive CHB patients could predict the efficacy of peg-IFN-α and nucleos(t)ide analogues (NAs).[62,63] In addition, qAnti-HBc levels are strongly correlated with ALT levels and are associated with the degree of necroinflammation in liver tissue in patients with normal ALT levels.[64]

HBV RNA quantification: HBV RNA levels are related to the transcriptional activity of cccDNA in liver cells, and further study is required to assess the risk of recurrence after withdrawal of NAs.[65,66] Its use is limited, because the detection methods used by different research teams are different.

Hepatitis B core-related antigen (HBcrAg): This is a composite marker that contains HBcAg, HBeAg, and p22. It is related to the transcriptional activity of cccDNA in liver cells. Studies have examined its usefulness to determine disease progression, predict the antiviral efficacy of peg-IFN-α and NAs, and assess the risk of HBV recurrence and HCC development with the discontinuation of treatment.[67–70]

Serum biochemical testing[71]

ALT and AST: Serum ALT and AST levels can partially reflect the degree of liver cell injury. An increase in ALT levels in patients with long-term virus suppression requires further tests to identify the possible causes.[72]

Total bilirubin: This is related to the production, uptake, metabolism, and excretion of bilirubin. Bilirubin elevation is caused by liver cell damage, intrahepatic and extrahepatic biliary obstruction, abnormal bilirubin metabolism, and hemolysis. In patients with liver failure, total bilirubin levels might be >171 μmol/L or could increase by >7.1 μmol/L daily.

Serum albumin: This reflects the synthetic functions of the liver. Patients with cirrhosis and liver failure present with decreased serum albumin levels. In addition, serum albumin levels are affected by nutritional status.

Prothrombin activity time (PT), prothrombin activity (PTA), and international normalized ratio: These indicators reflect the synthetic functions of coagulation factors in the liver and are valuable to predict disease progression and prognosis.

Serum γ-glutamyl transferase (GGT): The serum GGT in healthy people mainly comes from the liver. This enzyme is significantly increased in alcoholic liver disease, drug-induced liver disease, cholangitis, and intrahepatic and extrahepatic cholestasis.

Serum alkaline phosphatase (ALP): This is not a liver-specific indicator, although cholestasis can stimulate ALP synthesis. If ALP levels are elevated, elevated GGT or ALP isoenzyme levels are required to confirm whether ALP elevation has occurred in the liver. The dynamic changes in ALP levels are often used clinically to assess disease progression, prognosis, and efficacy.

Alpha-fetoprotein (AFP) and its isoform AFP-L3: AFP is an important indicator for the diagnosis of HCC. The magnitude and dynamic changes in AFP increase, and its correlation with the increase and decrease of ALT and AST levels require special attention. In addition, clinical manifestations and liver imaging examinations need to be carried out to offer a comprehensive analysis.[73]

Protein induced by vitamin K absence or antagonist-II (PIVKA-II): This is known as des-γ-carboxy prothrombin (DCP) and it is another important indicator for the diagnosis of HCC, and can be used to complement AFP.[74]

Noninvasive tests for liver fibrosis

AST to platelet ratio index (APRI)

APRI is an index that has been developed based on the data from chronic HCV infections to assess the degree of HCV-related liver fibrosis. The formula for APRI is as follows: [upper limit of normal (ULN) of AST/AST×100]/platelet count ($\times 10^9$/L). An APRI score ≥2 indicates the presence of cirrhosis and an APRI score <1 suggests the absence of cirrhosis in adults. However, recent studies have suggested that this index is less accurate to evaluate the degree of HBV-related liver fibrosis.[75–77]

Fibrosis 4 (FIB-4) score

FIB-4 was developed based on the data from chronic HCV infections and is used to assess the degree of HCV-related liver fibrosis. The formula for FIB-4 is as follows: age (years)×AST (IU/L)/[platelet count ($\times 10^9$/L)×√ALT (IU/L)]. A FIB-4 value ≥3.25 or a Metavir score ≥F3 suggest the presence of liver fibrosis; a FIB-4 value <1.45 suggests the absence of liver fibrosis. Recent studies have shown that an FIB-4 value ≥0.25 has a 97% specificity for the diagnosis of fibrosis in people with chronic HBV infection,[76] and an FIB-4 value ≤0.70 has a negative predictive value of 96% for the exclusion of hepatitis B cirrhosis in people aged >30 years.[78]

Other indicators

Extracellular matrix components, such as hyaluronic acid, type III procollagen peptide, type IV collagen, and laminin, can indicate the presence of liver fibrosis, but no generally accepted cutoffs are available for clinical application. GGT to platelet ratio (GPR) [=GGT/GGT ULN/platelet count ($\times 10^9$/L)×100] and red cell distribution width-platelet ratio (RPR) [=red cell distribution width (%)/platelet count ($\times 10^9$/L)] are composed of routine lab indicators, but stable diagnostic cutoffs need to be defined.[79,80] Serum Golgi glycoprotein 73 (commonly referred to as GP73) used in combination with AST and GGT levels can reflect moderate to severe liver inflammation.[81] Serum chitinase 3-like protein 1 (commonly referred to as CHI3L1 or YKL-40) can predict moderate to severe liver fibrosis in patients with normal or mildly elevated ALT levels.[82,83]

Liver stiffness measurement

Liver stiffness can be measured using transient elastogra-

phy (TE), ultrasound-based acoustic radiation force impulse (ARFI), and magnetic resonance elastography (MRE). ARFI includes two techniques, namely point shear wave elastography (p-SWE) and 2D shear wave elastography (2D-SWE). ARFI and MRE are still undergoing clinical research.

TE has been approved for use in the USA, Europe, and the Asia-Pacific region. It can accurately identify advanced liver fibrosis and early cirrhosis,[84,85] but its results are susceptible to liver necroinflammation, cholestasis, and severe steatosis. TE results should be interpreted in combination with ALT and bilirubin levels.[86–88] The combined use of TE and other serological indicators could improve diagnostic accuracy.[84,89–91] A multicenter study carried out by Chinese researchers suggested a cutoff of 21.3 kPa for the diagnosis of hepatitis B cirrhosis (specificity: 95%; positive likelihood ratio: 8.5), a cutoff of 12.4 kPa for the diagnosis of advanced liver fibrosis (specificity: 95%; positive likelihood ratio: 11.8), a cutoff of 9.1 kPa for diagnosis of significant liver fibrosis (specificity: 95%; positive likelihood ratio: 6.4); a cutoff value of 8.2 kPa for the exclusion of cirrhosis (sensitivity: 95%; positive likelihood ratio: 0.07), and a cutoff value of 5.8 kPa for the exclusion of advanced liver fibrosis (sensitivity: 95%; positive likelihood ratio: 0.10).[92] For guidance on the clinical application of TE, see "Expert consensus on the use of transient elastography for diagnosing liver fibrosis (2018 update)."[85]

Imaging diagnosis

The aim of imaging examination in patients with chronic HBV infection is to monitor clinical progression by the identification of signs of cirrhosis and portal hypertension, and to detect and differentiate space-occupying lesions to make an early diagnosis of HCC.[93,94]

Abdominal US

Abdominal US is the most commonly used liver imaging technique, because it offers a noninvasive, inexpensive, real-time imaging solution that is easy to repeat. US can demonstrate the size, shape, and parenchymal echogenicity of the liver and spleen, and can determine the calibrator and blood flow of the portal, splenic, and hepatic veins. In addition, it can identify the presence and severity of ascites which suggests the presence of cirrhosis and portal hypertension. Regular US surveillance is essential for the identification of early HCC. Contrast-enhanced ultrasonography can be used to better differentiate the nature of space-occupying lesions. The downside of US is that the image quality and results are susceptible to several factors, such as equipment performance, gas in the gastrointestinal tract, and the operator's skills.

Computerized tomography (CT)

In patients with CHB, CT is mainly used to investigate the imaging changes of the liver to determine the presence of cirrhosis and portal hypertension and identify/differentiate the space-occupying lesions. Contrast-enhanced multiphase CT scanning has a high sensitivity and specificity for the diagnosis of HCC.

Magnetic resonance imaging (MRI)

MRI is a preferred imaging modality for the liver due to its avoidance of radiation exposure. In general, MRI with enhanced multiphase scans and with hepatobiliary-specific contrast agents is outperformed to contrast-enhanced CT in differentiating benign from malignant space-occupying lesions in the liver.

Pathological diagnosis

The aim of liver biopsy in people with chronic HBV infection is to evaluate the degree of necroinflammation and fibrosis, to determine the presence or absence of cirrhosis, to exclude other liver diseases, thereby providing information on the diagnosis, prognosis, and efficacy assessment.

The main pathological features of CHB are portal and periportal necroinflammation and fibrosis. The inflammatory cells infiltrating the portal area are predominantly lymphocytes with a few plasma cells and macrophages. The aggregation of inflammatory cells often destroys the limiting plate and leads to interface hepatitis (previously called piecemeal necrosis). Degeneration, necrosis (e.g., spotted, bridging, and confluent necrosis) and apoptosis of hepatocytes can be seen in the lobules. Ground glass hepatocytes and apoptotic bodies can be formed by apoptotic hepatocytes, which is proportional to inflammation activity. Chronic necroinflammation in the liver can cause the excessive deposition of extracellular matrix, especially collagen, which results in fibrosis that is manifested by varying degrees of portal fibrous expansion and fibrous septum formation. Masson's trichromatic staining and reticulin staining can help to determine the degree of liver fibrosis and lobular disarray. Then, cirrhosis develops, which is, by definition, the combination of diffuse fibrosis and regenerative nodules (pseudolobules). In addition, immunohistochemical staining for HBsAg and HBcAg as well as *in situ* hybridization or PCR for HBV DNA or cccDNA in liver tissue can be exploited.

The scoring systems developed by Knodell, Scheuer, Metavir, or Ishak are widely used to grade hepatic necroinflammation and stage fibrosis in persons with chronic HBV infection.[95–98] The Laennec system further subclassifies Metavir stage 4 (cirrhosis) into stages 4A, 4B, and 4C, based on the size of the regenerative nodules and the thickness of fibrous septa.[99] In addition, Chinese researchers have proposed a histopathological grading and staging system for viral hepatitis B.[100] Comparisons of the various grading and staging systems are shown in Tables 3 and 4.

Computer image analysis can be used to determine the collagen proportional area of the stained sections of liver tissue. Quantitative assessment of liver fibrosis (qFibrosis) based on two-photon second harmonic generation can automatically measure the collagen area and the morphological features in unstained liver tissue sections, with high reproducibility and accuracy.[101] Chinese researchers recently developed a P-I-R classification for liver fibrosis. Based on the width and shape of fibrous septa, this system qualitatively subdivides fibrosis with an Ishak score ≥3 into predominantly progressive (P), intermediate (I), and predominantly regressive (R) liver fibrosis. Therefore, the merit of this classification is to judge the dynamic trend of liver fibrosis evolution.[102]

Clinical diagnosis

According to the results of serological, virological, biochemical, imaging, and pathological tests as well as other auxiliary examinations, chronic HBV infection can be classified into:

Table 3. Comparisons of major grading standards for liver inflammation

Knodell's scoring system				Scheuer's scoring system		
Score	**Periportal inflammation with or without bridging necrosis**	**Intralobular degeneration and focal necrosis**	**Portal inflammation**	**Score**	**Portal/periportal activity**	**Intralobular activity**
0	None	None	None	0	None or minimal	None
1	Mild piecemeal necrosis	Mild (acidophilic bodies, ballooning degeneration or scattered foci, or both of hepatocellular necrosis in <⅓ of lobules or nodules)	Mild (a few inflammatory cells in <⅓ of portal tracts)	1	Portal inflammation alone	Inflammation but no necrosis
3	Moderate piecemeal necrosis (involving <50% of the circumference of most portal tracts)	Moderate (involving ⅓–⅔ of lobules or nodules)	Moderate (increased inflammatory cells in ⅓–⅔ of portal tracts)	2	Mild piecemeal necrosis	Focal necrosis or acidophilic bodies
4	Marked piecemeal necrosis (involving >50% of the circumference of most portal tracts)	Marked (involving >⅔ of lobules or nodules)	Marked (dense inflammatory cells in >⅔ of portal tracts)	3	Moderate piecemeal necrosis	Marked focal cell damage
5	Moderate piecemeal necrosis plus bridging necrosis	–	–	4	Marked piecemeal necrosis	Damage includes bridging necrosis
6	Marked piecemeal necrosis plus bridging necrosis	–	–			
10	Multilobular necrosis	–	–			

Wang Tailing's scoring system			Ishak's scoring system				
Score	**Portal/periportal area**	**Intralobular area**	**Score**	**Portal inflammation**	**Periportal or periseptal interface inflammation**	**Focal necrosis, apoptosis, or focal inflammation**	**Confluent necrosis**
0	No inflammation	No inflammation	0	None	None	None	None
1	Portal inflammation	Degeneration and a few necrotic foci	1	Mild, involving some or all portal areas	Mild (focal, involving few portal areas)	≤1 foci per 10× objective	Focal confluent necrosis
2	Mild piecemeal necrosis	Degeneration, spotted or focal necrosis or acidophilic bodies	2	Marked, involving some or all portal areas	Mild/moderate (focal, involving most portal areas)	2–4 foci per 10× objective	Zone 3 necrosis in some areas
3	Moderate piecemeal necrosis	Degeneration, severe necrosis, or bridging necrosis	3	Moderate to severe, involving all portal areas	Moderate (continuous around <50% of tracts or septa)	5–10 foci per 10× objective	Zone 3 necrosis in most areas
4	Marked piecemeal necrosis	Bridging necrosis involving multiple lobules	4	Severe, involving all portal areas	Severe (continuous around >50% of tracts or septa)	>10 foci per 10× objective	Zone 3 necrosis and occasional portal-central bridging necrosis
			5	–	–	–	Zone 3 necrosis and multiple portal-central bridging necrosis
			6	–	–	–	Panacinar or multiacinar necrosis

–, not applicable.

Table 4. Comparisons of major staging standards for liver fibrosis

Knodell's scoring system		Scheuer's scoring system		Metavir's scoring system		Wang Tailing's scoring system		Ishak's scoring system	
0	No fibrosis	0	No fibrosis	0	No fibrosis	0	No fibrosis	0	No fibrosis
1	Fibrous expansion of portal areas	1	Fibrous expansion of portal areas	1	Fibrous expansion of portal areas, without fibrous septa	1	Fibrous expansion of portal areas	1	Fibrous expansion of some portal areas, with or without short fibrous septa
2	–	2	Periportal fibrosis or portal-portal fibrous septa but intact architecture	2	Fibrous expansion of portal areas, with few fibrous septa	2	Periportal fibrosis, with fibrous septa but intact architecture	2	Fibrous expansion of most portal areas, with or without short fibrous septa
3	Marked bridging (portal-portal as well as portal-central)	3	Fibrosis with architectural distortion but no obvious cirrhosis	3	Numerous septa, with architectural distortion, but no cirrhosis	3	Fibrous septa with architectural distortion but no obvious cirrhosis	3	Fibrous expansion of most portal areas with occasional portal-portal bridging
4	Cirrhosis	4	Probable or definite cirrhosis	4	Cirrhosis	4	Early or definite cirrhosis	4	Fibrous expansion of most portal areas with marked bridging (portal-portal as well as portal-central)
								5	Marked bridging (portal-portal, or portal-central, or both) with occasional nodules (incomplete cirrhosis)
								6	Probable or definite cirrhosis

–, not applicable.

Chronic HBV carrier state

This is also known as HBeAg-positive chronic HBV infection.[103,104] Patients are in the immune tolerance phase and are generally younger. They are positive for HBeAg, with high levels of HBV DNA (usually $>2\times10^7$ IU/mL) and serum HBsAg (usually $>1\times10^4$ IU/mL). However, serum ALT and AST levels are persistently normal (three follow-ups within 1 year, ≥3 months apart), and liver biopsy shows no obvious necroinflammation or fibrosis. In the absence of liver biopsy, factors such as age, HBV DNA level, HBsAg level, noninvasive liver fibrosis tests, and imaging examination should be considered to help make a diagnosis.

HBeAg-positive CHB

Patients are in the immune clearance phase and are seropositive for HBsAg and HBeAg. HBV DNA levels are high (usually $>2\times10^4$ IU/mL). ALT levels are persistently or intermittently abnormal, or liver biopsy reveals obvious necroinflammation, or fibrosis, or both (≥G2/S2).

Inactive HBsAg carrier state[105,106]

This is also known as HBeAg-negative chronic HBV infection. Patients are in the immune control phase. They are positive for HBsAg and anti-HBe and negative for HBeAg. HBV DNA levels are <2,000 IU/mL, and HBsAg levels are <1,000 IU/mL. ALT and AST levels are persistently normal (three follow-ups within 1 year, ≥3 months apart). Imaging examination shows no signs of cirrhosis. Liver biopsy shows a histological activity index score <4, or lesions are mild using other semi-quantitative scoring systems.

HBeAg-negative CHB

This is the reactivation phase. Patients are positive for HBsAg and persistently negative for HBeAg, which is often accompanied by anti-HBe positivity. HBV DNA levels are usually ≥2,000 IU/mL. ALT levels are persistently or intermittently abnormal, or liver biopsies show obvious necroinflammation, or fibrosis, or both (≥G2/S2).

Occult HBV infection (OBI)[107]

Patients are negative for HBsAg in serum but positive for HBV DNA in serum or liver tissue, or both. A total of 80% of OBI patients might be seropositive for anti-HBs, and anti-HBe or anti-HBc, or both, which is designated as seropositive OBI; however, 1–20% of OBI patients are seronegative for all serological indicators, which is designated as seronegative OBI. Its mechanism has not been determined. One possible explanation is that HBsAg disappears after apparent (acute or chronic) HBV infection, and HBV DNA levels are usually very low in the serum or liver tissue, without obvious liver tissue damage. Another possibility is mutations in the S gene region of HBV, which makes HBsAg undetectable by currently available commercial kits. Serum HBV DNA levels are usually high, which might be accompanied by significant liver histopathological changes. These patients can transmit HBV to recipients through blood transfusion or organ transplantation and might experience reactivation of

HBV if they are immunosuppressed.

Hepatitis B cirrhosis[108,109]

The diagnosis of hepatitis B cirrhosis should meet the first and the second criteria of the following pathological diagnosis or the first and the third criteria of the following clinical diagnosis.

1. Patients are HBsAg-positive, or HBsAg-negative, anti-HBc positive and have a clear history of chronic HBV infection (presence of HBsAg >6 months), and other causes have been excluded.
2. Liver biopsy suggests cirrhosis.
3. Patients meet ≥2 of the following five criteria, and noncirrhotic portal hypertension has been excluded: (1) imaging examination shows signs of cirrhosis, or portal hypertension, or both; (2) endoscopy shows esophagogastric varices; (3) liver stiffness measurements suggest cirrhosis; (4) blood biochemical tests show reduced albumin levels (<35 g/L), or prolonged PT, or both (>3 s longer than controls); and (5) routine blood tests show platelet count $<100\times10^9$/L.

Clinically, cirrhosis is divided into the compensated stage and decompensated stage based on the presence or absence of serious complications, such as ascites, esophagogastric variceal bleeding, and hepatic encephalopathy: (1) compensated cirrhosis; patients who are pathologically or clinically diagnosed with cirrhosis but have never had serious complications, such as ascites, esophagogastric variceal bleeding, or hepatic encephalopathy, could be diagnosed as having compensated cirrhosis and most of them have Child-Pugh A liver function; and (2) decompensated cirrhosis; if patients with cirrhosis develop serious complications, such as ascites, esophagogastric variceal bleeding, or hepatic encephalopathy, they are diagnosed as decompensated cirrhosis.[110] Most of them have Child-Pugh B or C liver function.

Recently, to more accurately predict the progression, death risk, or treatment effect of patients with cirrhosis, some researchers have suggested dividing cirrhosis into five stages,[111] between which stages 1 and 2 are compensated cirrhosis, and stages 3–5 are decompensated cirrhosis: stage 1, no varicose veins or ascites; stage 2, varicose veins, no bleeding or ascites; stage 3, ascites, no bleeding, with or without varicose veins; Stage 4, bleeding, with or without ascites; and stage 5, sepsis.

Due to the advances in antiviral therapy, many patients with decompensated cirrhosis could be reversed to compensated cirrhosis following treatment. The reversal is characterized by improved liver cell function, such as higher albumin levels, shorter PT than before, the disappearance of serious complications, such as ascites and hepatic encephalopathy, and long-term survival without liver transplantation. This phenomenon is called recompensation of cirrhosis; however, there is no accurate definition and uniform diagnostic criteria for this concept.

Treatment goals

Treatment goals are to maximize long-term inhibition of HBV replication,[6,112,113] alleviate the degree of hepatocyte inflammation, necrosis, and hyperplasia of liver fibrous tissue, delay and reduce the occurrence of liver failure, decompensation of liver cirrhosis, HCC and other complications, and to improve the quality of patients' life as well as prolong their survival time. For eligible patients, the therapy aims are "clinical cure".

Clinical cure (or functional cure):[6,112,114–116] HBsAg remains negative (with or without the presence of anti-HBs), HBV DNA is undetectable, and liver biochemical indicators are normal following withdrawal of the treatment. However, because cccDNA remains in the nuclei of liver cells, patients are still at risk of HBV reactivation and HCC development.

Indications for antiviral therapy

Patients should be assessed for the risk of disease progression to determine whether to start antiviral therapy based on a comprehensive analysis of serum HBV DNA levels, ALT levels, the severity of liver disease, as well as their age, family history, and concomitant diseases.[6,112,113] Dynamic assessment is more meaningful than a single test (Fig. 1).

For patients with chronic HBV infection with positivity for serum HBV DNA, antiviral therapy is indicated if their ALT levels are persistently abnormal (>ULN) and other causes of ALT elevation have been excluded.

Other causes of ALT elevation include infection by other pathogens, drug-induced liver injury, alcoholic hepatitis, nonalcoholic steatohepatitis, autoimmune liver disease, and systemic diseases that involve the liver. In addition, the possibility of drug-induced temporarily normal ALT levels should be ruled out.

For patients with clear evidence of cirrhosis, antiviral treatment should be initiated if HBV DNA can be detected, regardless of ALT levels and HBeAg status. For patients with decompensated cirrhosis, antiviral therapy is indicated if HBV DNA is undetectable but HBsAg is positive.

Patients with seropositive HBV DNA and normal ALT levels are at a high risk of disease progression and antiviral therapy is indicated if they meet any of the following criteria: (1) obvious liver inflammation on liver biopsy (≥G2) or fibrosis (≥S2); (2) persistently normal ALT levels (tested every 3 months for 12 months) but with a family history of cirrhosis or liver cancer, and >30 years of age; (3) persistently normal ALT levels (tested every 3 months for 12 months), no family history of cirrhosis or liver cancer, but >30 years of age with noninvasive tests for liver fibrosis or liver biopsy revealing obvious liver inflammation or fibrosis; a (4) HBV-related extrahepatic manifestations (e.g., glomerulonephritis, vasculitis, polyarteritis nodosa, and peripheral neuropathy).

Recommendation 6: If serum HBV DNA is positive, ALT levels are persistently abnormal (>ULN), and other causes have been excluded, antiviral therapy is indicated (B1).

Recommendation 7: Antiviral therapy is indicated for HBV-related compensated cirrhosis patients with positive serum HBV DNA and HBV-related decompensated cirrhosis patients with positive HBsAg (A1).

Recommendation 8: If patients are seropositive for HBV DNA with normal ALT levels, antiviral therapy is indicated when they meet any one of the following criteria: (1) liver biopsy suggests obvious inflammation, or fibrosis, or both (G ≥2, or S ≥2, or both) (A1); (2) a family history of HBV-related cirrhosis or liver cancer and age >30 years-old (B1); (3) noninvasive tests or liver biopsy revealing obvious liver inflammation or fibrosis in those with persistently normal ALT levels and age >30 years-old (A1); and (4) HBV-related extrahepatic manifestations (e.g., HBV-related glomerulonephritis) (B1).

NAs treatment

Efficacy and safety of NAs

Entecavir (ETV): Numerous studies have shown that the

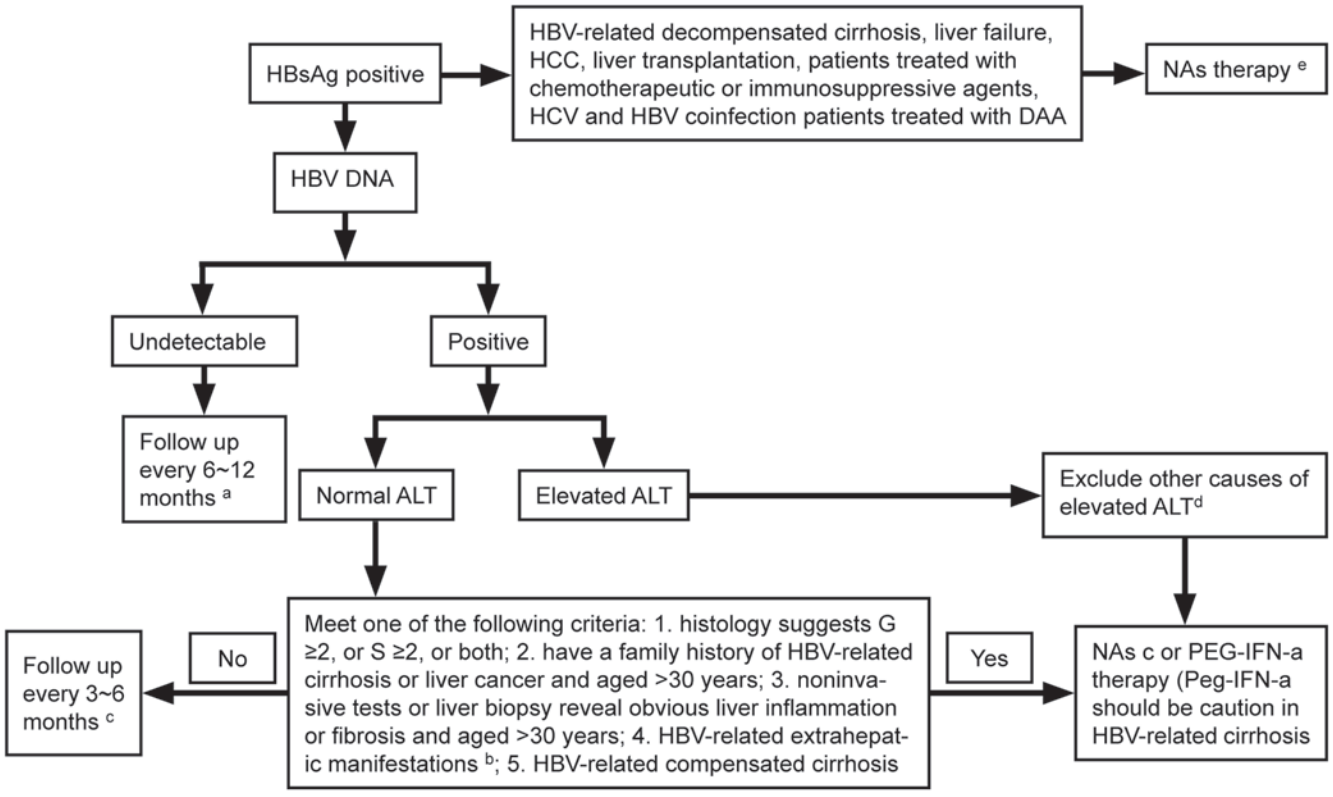

Fig. 1. Flow chart of antiviral therapy for patients with chronic HBV infection. (a) Follow-up tests: virological test, liver biochemical test, AFP test, PIVKA-II test, abdominal ultrasound, and liver stiffness measurement. (b) HBV-related extrahepatic manifestations: glomerulonephritis and vasculitis. (c) Follow-up criteria for patients with HBV-related decompensated cirrhosis during NA treatment: perform a routine blood test, liver biochemical test, renal function test, virological test, and test for blood ammonia, AFP, and PIVKA-II, and abdominal ultrasound every 3 months. If necessary, perform enhanced CT or MRI. (d) Other causes of ALT elevation: infection by other pathogens, history of drug or poison use, history of alcohol use, lipid metabolism disorders, autoimmune disorders, liver congestion or vascular diseases, inherited metabolic liver disease, systemic diseases. (e) NAs-ETV, TDF, TAF. HbsAg, hepatitis B surface antigen; HBV, hepatitis B virus; ALT, alanine aminotransferase; HCC, hepatocellular carcinoma; NAs, nucleos(t)ide analogs; peg-IFN-α, pegylated interferon-α.

use of ETV is safe and highly effective to suppress virus replication and reduce liver inflammation.[117–119] Long-term treatment with ETV can improve histological changes in patients with cirrhosis,[120,121] significantly reduce the incidence of cirrhosis-related complications and HCC, and reduce liver-related and all-cause mortality.[53,122] The 5-year cumulative probability of ETV resistance was 1.2% in treatment-naïve patients with CHB but increased to 51% in CHB patients who were resistant to lamivudine (LAM).[123]

Tenofovir disoproxil fumarate (TDF): Multicenter clinical studies of TDF treatment for CHB patients have shown that it can strongly inhibit virus replication and has low rates of resistance.[124,125] A study of TDF usage for 8 years showed that there were 41 cases of virological breakthroughs, 29 cases (70%) of which were due to poor compliance. In total, 59% of patients with virological breakthroughs continued to receive TDF treatment and achieved virological responses. Further nucleic acid sequencing did not identify TDF-related resistance.[126] Long-term treatment with TDF can significantly improve liver histology and reduce the incidence of HCC.[127,128]

In total, 90 CHB patients who were ETV resistant and serum HBV DNA levels >60 IU/mL were equally randomized to receive TDF alone or in combination with ETV for 48 weeks. Clearance of HBV DNA (<15 IU/mL) was achieved in 73% and 71%, respectively. HBV DNA levels decreased by 3.66 lg IU/mL and 3.74 lg IU/mL from baseline, respectively. Overall, six and three patients retained their baseline resistance mutations in TDF alone and in combination with ETV, respectively. Both regimens demonstrated a favorable safety profile.[129] Several studies into TDF therapy for patients previously treated with NAs for 48–168 weeks showed that TDF resulted in virological responses in 70–98% of LAM-resistant, adefovir dipivoxil (ADV)-resistant, ETV-resistant, or multidrug-resistant patients. In addition, virological response rates increased during treatment.[129–139]

Tenofovir alafenamide fumarate tablets (TAF): In a global phase III clinical trial, 581 HBeAg-positive CHB patients (which excluded patients with decompensated cirrhosis) received TAF treatment for 48 weeks. HBV DNA levels were <29 IU/mL in 64% of patients and ALT levels returned to normal in 72% of patients. HBeAg seroconversion occurred in 10% of patients, and HBsAg disappeared in 1% of patients. After treatment for 96 weeks, HBV DNA levels were <29 IU/mL in 73% of patients, and ALT levels returned to normal in 75% of patients. HBeAg seroconversion occurred in 18% of patients and HBsAg disappeared in 1% of patients. In 285 HBeAg-negative CHB patients (which excluded patients with decompensated cirrhosis) that received TAF treatment for 48 weeks, HBV DNA levels were <29 IU/mL in 94% of patients and ALT levels returned to normal in 83% of patients. No patients experienced HBsAg loss. Following treatment for 96 weeks, HBV DNA levels were <29 IU/mL in 90% of patients and ALT levels returned to normal in 81% of patients. HBsAg loss occurred in <1% of patients.[140–142]

During the 96-week treatment, headache (12%), nausea (6%), and fatigue (6%) were the most common ad-

Table 5. Recommendations for salvage therapy for resistance to NAs

Types of resistance	Recommended drugs
LAM- or LdT-resistant	Switch to TDF or TAF
ADV-resistant, never used LAM or LdT before	Switch to ETV, TDF, or TAF
ADV-resistant and LAM/LdT-resistant	Switch to TDF or TAF
ETV-resistant	Switch to TDF or TAF
ETV-resistant and ADV-resistant	ETV combined with TDF, or ETV combined with TAF

LAM, lamivudine; LdT, telbivudine; ADV, adefovir dipivoxil; ETV, entecavir; TDF, tenofovir disoproxil fumarate; TAF, tenofovir alafenamide fumarate.

verse events.[142] Following 96 weeks of treatment, patients that received TAF had significantly smaller decreases in bone mineral density than those that received TDF in the hip (−0.33% vs. −2.51%; $p<0.001$) and lumbar spine (−0.75% vs. −2.57%; $p<0.001$), as well as a significantly smaller median change in estimated glomerular filtration rate (eGFR) (−1.2 vs. −4.8 mg/dL; $p<0.001$).

Other drugs: Telbivudine (LdT) can improve eGFR but it is associated with high rates of resistance.[113] LdT exhibits favorable efficacy and safety in blocking mother-to-child transmission (refer to the section "Recommendations on antiviral therapy for special populations").

Selection of NAs

Antivirals with high potency and low risk of resistance (ETV, TDF, and TAF) should be the preferred NAs for treatment-naïve patients. ADV and LAM are not recommended for antiviral therapy for HBV-infected patients.

For patients who are being treated with nonpreferred drugs, it is recommended that they switch to antivirals with high potency and low risk of resistance to further reduce the risk of resistance. For patients that receive ADV, it is recommended that they switch to ETV, TDF, or TAF. For patients that receive LAM or LdT, it is recommended that they switch to TDF, TAF, or ETV. For patients that are resistant to LAM or LdT, it is recommended that they switch to TDF or TAF. For patients that are resistant to ADV, it is recommended that they switch to ETV, TDF, or TAF.[143] For patients that are being treated with ADV in combination with LAM/LdT, it is recommended that they switch to TDF or TAF.

Prevention and management of NA resistance

Treatment-naïve patients: Potent antivirals with minimal resistance, such as ETV, TDF, and TAF are recommended.

During treatment: HBV DNA levels should be quantified regularly to detect virological breakthroughs and initiate save therapy as soon as possible (Table 5). For patients who develop resistance to NAs, IFN-α is associated with low response rates.

Monitoring of NA treatment

Detection of the following relevant indicators at baseline before treatment: (1) major biochemical markers, such as ALT, AST, bilirubin, and albumin; (2) major virological and serological markers, such as HBV DNA quantification, HBsAg, HBeAg, and anti-HBe; (3) blood routine serum creatinine levels, blood phosphorus levels, and renal tubular function should be tested if required; (4) noninvasive tests for liver fibrosis, such as liver stiffness measurement; and (5) when ETV and TDF are used in patients with creatinine clearance <50 mL/min, the doses of both drugs should be adjusted. There is no recommended dose for TAF when it is used in patients with creatinine clearance <15 mL/min who are not receiving hemodialysis. In other cases, no dose adjustment is required.

Patient compliance: It should be closely monitored, which includes dosage, method of use, missed medication or self-discontinuation, to ensure that patients understand the risks that might result from unwarranted discontinuation and improve their compliance.

Prevention and management of rare adverse events: Although NAs are generally safe and well-tolerated, serious adverse events such as renal insufficiency (TDF, ADV), hypophosphatemic bone disease (TDF, ADV), myositis/rhabdomyolysis (LdT), and lactic acidosis (ETV, LdT) can occur in rare cases, and require attention. It is recommended that physicians make detailed inquiries into the medical history before treatment to reduce the risk. Patients should be closely monitored if they present with significant increases in serum levels of creatinine, creatine kinase, or lactate dehydrogenase during treatment, which could be accompanied by clinical manifestations, such as general deterioration, myalgia, muscle weakness, and bone pain. If they are diagnosed with renal insufficiency, myositis, rhabdomyolysis, or lactic acidosis, the existing regimen should be withdrawn and changed to other treatment interventions.

Monitoring and management of drug resistance: The use of potent antiviral drugs with minimal resistance has resulted in significantly reduced rates of resistance that could arise from long-term treatment of NAs. If HBV DNA levels increased >2 lg IU/mL from nadir during treatment and the potential of poor compliance has been ruled out, salvage therapy should be initiated promptly, and drug resistance should be tested for.

IFN-α treatment

Peg-IFN-α and IFN-α have been approved for CHB treatment in China.

Regimens and efficacy of peg-IFN-α therapy

Initial monotherapy with peg-IFN-α: In several multicenter, randomized, controlled clinical trials, HBeAg-positive CHB patients were treated with peg-IFN-α-2a or peg-IFN-α-2b for 48 weeks (180 g/week). HBV DNA levels were <2,000 IU/mL in 30% of the patients and HBeAg seroconversion occurred in 30.75–36.3% of patients at week 24 after drug discontinuation (or 68.4% of patients with HBsAg <1,500 IU/mL at week 12 of treatment and baseline ALT >2×ULN). In addition, HBsAg seroconversion occurred in 2.3–3% of patients at week 24 after drug discontinuation; HBsAg clearance was achieved in 11% of patients after 3 years of drug withdrawal.[113,144–146] In HBeAg-negative patients with chronic HBV infections (60% Asians) treated with peg-IFN-

α-2a for 48 weeks, HBV DNA levels were <2,000 IU/mL in 43% and 42% of patients at 24 and 48 weeks after drug withdrawal, respectively. HBsAg clearance was achieved in 3%, 8.7%, and 12% of patients 24 weeks, 3 years, and 5 years after drug withdrawal.[113,146]

At week 24 of peg-IFN-α therapy, if HBV DNA levels decreased by ≤2 lg IU/mL and HBsAg levels were >20,000 IU/mL in HBeAg-positive patients or decreased by <1 lg IU/mL in HBeAg-negative patients, it is recommended to stop peg-IFN-α treatment and switch to NAs therapy.[112,116,146]

Combined treatment of peg-IFN-α and NAs: The combined use of peg-IFN-α in eligible CHB patients treated with NAs could achieve clinical cure in some patients.[116,146] HBsAg clearance was high after combined treatment in patients with low levels of HBsAg (<1,500 IU/mL) before treatment and who experienced a rapid decline of HBsAg levels during treatment (HBsAg <200 IU/mL or a decrease of >1 lg IU/mL at week 12 or 24).[147–151] However, further research is required to determine baseline conditions, the optimal course of treatment, and sustained virological responses of combination therapy.

Peg-IFN-α further reduces the incidence of HBV-related liver cancer: In a study of CHB patients that were treated with peg-IFN-α or ETV, a 5-year follow-up showed that none of the patients treated with peg-IFN-α developed HCC within 5 years; however, two and one patients treated with ETV developed HCC at years 4 and 5, respectively. There was no significant difference in the cumulative HCC incidence between the observed and the predicted cases for patients treated with ETV (p=0.36).[152] In another retrospective study that included 682 patients treated with NAs and 430 patients treated with IFN-α alone or in combination with NAs, a total of 31 patients developed HCC at a median follow-up of 5.41 years and the 10-year cumulative incidence of HCC was significantly lower in patients treated with IFN-α than those treated with NAs (2.7% vs. 8.0%, p<0.001).[146,153] The role of peg-IFN-α in reducing the incidence of HBV-related liver cancer requires further research.

Predictive factors for the efficacy of peg-IFN-α-based antiviral therapy

Pretreatment predictors: HBV DNA levels $<2\times10^8$ IU/mL, high ALT levels (2–10×ULN) or liver tissue necroinflammation ≥G2, genotype A or B, low baseline HBsAg (<25,000 IU/mL),[6,112,113,146,154,155] high levels of anti-HBc at baseline (qAnti-HBc),[62,63] signal transducer and activator of transcription 4 (commonly known as STAT4) rs7574865 polymorphism at baseline[156] are predictors for favorable outcomes of IFN-based therapy. HBV DNA levels and HBsAg levels at 12 weeks of peg-IFN-α therapy and their dynamic changes could be used to predict the efficacy of peg-IFN-α.[146]

Management of adverse events of peg-IFN-α therapy[6,112,113]

Influenza-like syndrome: If patients complain of fever, headache, myalgia, or fatigue, peg-IFN-α could be injected before sleep or taking nonsteroidal anti-inflammatory agents along with the IFN-α.

Myelosuppression: If neutrophil count $\leq0.75\times10^9$/L and/or platelet count $<50\times10^9$/L develops, peg-IFN-α dosage should be reduced and cell counts retested after 1–2 weeks. If cell counts return to normal, the initial dosage should be restored. If neutrophil count $\leq0.5\times10^9$/L or platelet count $<25\times10^9$/L, or both, the peg-IFN-α should be discontinued. Granulocyte colony-stimulating factor (commonly known as G-CSF) or granulocyte-macrophage colony-stimulating factor (commonly known as GM-CSF) could be used for those with significantly decreased neutrophil counts.

Mental disorders: If patients experience depression, delusions, severe anxiety, or other mental disorders, peg-IFN-α should be immediately withdrawn, and patients could seek consultations with psychiatrists and psychologists if required.

Autoimmune diseases: Some patients might develop autoantibodies, and few patients develop thyroid disease, diabetes, decreased platelet count, psoriasis, vitiligo, rheumatoid arthritis, and systemic lupus erythematosus-like syndrome. In such cases, patients should have consultations with physicians with related expertise, and treatment should be discontinued for patients with severe symptoms.

Other rare adverse events: For other rare adverse events, which include retinopathy, interstitial pneumonia, hearing loss, kidney damage, and cardiovascular complications, peg-IFN-α therapy should be discontinued.

Contraindications for peg-IFN-α therapy[6,112,113]

Absolute contraindications: Pregnancy or intention to be pregnant in the short-term, history of mental illness (e.g., history of schizophrenia or severe depression), uncontrolled epilepsy, decompensated cirrhosis, uncontrolled autoimmune diseases, severe infection, retinal disease, heart failure, chronic obstructive pulmonary diseases, and other underlying diseases.

Relative contraindications: These include thyroid disease, a history of depression, uncontrolled diabetes, hypertension, and heart disease.

Recommendation 9: HBeAg-positive CHB patients should be treated with ETV, TDF, or TAF. If HBV DNA levels are below the detection limit, ALT levels return to normal, and HBeAg seroconversion occurs after 1 year of treatment, patients should receive consolidation treatment for at least 3 years (followed up every 6 months). Treatment could be discontinued if HBV DNA remains undetectable, and ALT normalization and HBsAg loss are maintained. A longer duration of treatment might reduce recurrence (A1).

Recommendation 10: HBeAg-positive CHB patients could be treated with peg-IFN-α. At week 24 of treatment, if HBV DNA decrease <2 lg IU/mL and HBsAg levels >20,000 IU/mL, it is recommended that peg-IFN-α therapy should be discontinued and changed to NA treatment (A1). For patients who responded effectively, the course of treatment is 48 weeks, and it might be extended according to the patient's condition but it should not exceed 96 weeks (B1).

Recommendation 11: HBeAg-negative CHB patients should be treated with ETV, TDF, or TAF. Discontinuation and subsequent follow-up are advised if HBsAg loss and undetectable HBV DNA are achieved (A1).

Recommendation 12: HBeAg-negative CHB patients could be treated with peg-IFN-α. At week 12 of treatment, if HBV DNA decreased <2 lg IU/mL or HBsAg decreased <1 lg IU/mL, it is recommended that peg-IFN-α treatment should be discontinued and changed to NA treatment (B1). For patients who responded effectively, the course of treatment is 48 weeks, and it might be extended according to the patient's condition, but it should not exceed 96 weeks (B1).

Recommendation 13: For HBV-related compensated cirrhosis patients, long-term treatment with ETV, TDF, or TAF, or peg-IFN-α therapy is recommended. The adverse events of peg-IFN-α must be closely monitored (A1).

Recommendation 14: For HBV-related decompensated cirrhosis patients, long-term treatment with ETV or TDF is recommended (A1). TAF therapy could be used if necessary (C1).

Other treatments

Anti-HBV therapy can reduce the incidence of HBV-related complications, HBV-related liver cancer, and improve the survival rate of patients, which is the most important treatment for chronic HBV infection. In addition, other treatment options include anti-inflammatory, antioxidant, hepatoprotective, anti-fibrosis, and immunomodulatory interventions.

Anti-inflammatory, antioxidant, and hepatoprotective treatment

The necroinflammation of liver cells caused by HBV infection is an important pathological and physiological process of disease progression. Glycyrrhizic acid, silymarin, polyunsaturated lecithin, and bicyclol have anti-inflammatory, antioxidant, and hepatoprotective effects, and might reduce inflammatory damage of the liver. For patients with obvious liver inflammation or significantly elevated ALT levels, these agents could be used where appropriate, but it is not advised to use more than two of them in combination.

Antifibrosis treatment

Several antifibrosis Chinese medicine prescriptions, such as Anluohuaxian wan, Fufang Biejiaruangan tablets, Fuzhenghuayu capsules, have shown protective effects against fibrosis in animal experiments and clinical studies.[157–161] They could be used where appropriate for patients with obvious fibrosis or cirrhosis. However, multicenter randomized controlled studies are required to further define their treatment course of and long-term efficacy.

Monitoring and follow-up management of people with chronic HBV infection[6,112,113]

Management of chronic HBV carriers and inactive HBsAg carriers

Because chronic HBV carriers are in the immune tolerance phase, they do not have or only have mild inflammatory activity in the liver, patients in this phase respond poorly to antiviral treatment. Therefore, antiviral therapy is not recommended. However, it is emphasized that some patients in the immune tolerance phase might enter the immune clearance phase and develop hepatitis flares. Inactive HBsAg carriers are in the immune control phase, but they might progress to HBeAg-negative CHB and are at risk of developing HCC in long-term follow-up.

Therefore, for chronic HBV carriers and inactive HBsAg carriers, it is recommended that they should undergo routine blood tests, biochemical tests, virological tests, AFP tests, abdominal ultrasound, and noninvasive tests for liver fibrosis every 6–12 months. A liver biopsy is recommended if necessary. Antiviral therapy should start if they meet the indications for such treatment.

Monitoring during antiviral therapy

Regular monitoring during antiviral therapy aims to monitor the efficacy of antiviral therapy, compliance of patient, drug resistance, and adverse events.

Patients treated with peg-IFN-α: Routine blood tests (every 1–2 weeks in the 1st month of treatment, then once a month after measurements become stable), liver biochemical tests (once a month), thyroid function and blood glucose tests (once every 3 months), quantitative tests of HBV DNA, HBsAg, HBeAg and anti-HBe (every 3 months), liver stiffness measurement (every 6 months), abdominal ultrasound and AFP tests (every 6 months for patients without cirrhosis, and every 3 months for patients with cirrhosis). Enhanced CT or enhanced MRI for early detection of HCC may be performed if necessary.

NAs: Routine blood tests, liver biochemical tests, HBV DNA quantification, serological markers, and liver stiffness measurement should be performed every 3 to 6 months; abdominal ultrasound and AFP test should be performed every 6 months for patients without cirrhosis, and every 3 months for patients with cirrhosis. Enhanced CT or enhanced MRI for early detection of HCC may be performed if necessary. For patients treated with TDF, blood phosphorus levels and renal function should be tested every 6 to 12 months. Early renal tubular injury may be monitored if feasible.

Follow-up after the end of antiviral therapy

The purpose of follow-up of patients after the end of treatment is to evaluate the long-term efficacy of antiviral therapy, monitor disease progression and the development of HCC. Therefore, regardless of patient's response to antiviral treatment, biochemical tests of the liver, and HBV DNA quantification should be performed once a month within the first 3 months after drug withdrawal, every 3 months thereafter, and every 6 months one year after drug withdrawal. Patients without cirrhosis need to undergo abdominal ultrasound and AFP test every 6 months, and patients with cirrhosis need to be tested every 3 months, and if necessary, enhanced CT or enhanced MRI may be performed for early detection of HCC.

Recommendations on antiviral therapy for special populations

Patients with poor response

CHB patients: After treatment with ETV, TDF, or TAF for 48 weeks, if HBV DNA is $>2\times10^3$ IU/mL, and excluding compliance and detection errors, NA-based treatment regimens should be adjusted (those using ETV could be switched to TDF or TAF,[162,163] and those using TDF or TAF could be switched to ETV), or combination therapy could be used (ETV combined with TDF or TAF). Peg-IFN-α therapy can also be used in combination.

Patients with HBV-related cirrhosis: After treatment with ETV, TDF, or TAF for 24 weeks, if HBV DNA is $>2\times10^3$ IU/mL, and excluding compliance and detection errors, NA-based treatment regimens could be adjusted (those using ETV could be switched to TDF or TAF, and those using TDF or TAF could be switched to ETV), or combination therapy might be used (ETV combined with TDF or TAF).

Patients treated with chemotherapeutic or immunosuppressive agents

Chemotherapy or immunosuppressive therapy in CHB patients might cause HBV reactivation and might lead to liver failure or even death in severe cases. HBV reactivation occurs after antitumor therapy in approximately 20–50% of HBsAg-positive, anti-HBc-positive tumor patients, and in 8–18% of HBsAg-negative, anti-HBc-positive tumor pa-

tients.[164,165] Prophylactic antiviral therapy can significantly reduce the incidence of hepatitis B reactivation.[166,167] ETV, TDF, or TAF, which are potent antivirals with minimal resistance, are recommended.[168–170]

All patients that receive chemotherapy or immunosuppressive therapy should be routinely screened for HBsAg and anti-HBc before starting treatment.

HBsAg-positive patients should be given NA therapy (usually 1 week) before or when they start receiving immunosuppressive or chemotherapeutic agents.[6,171,172]

In addition, HBsAg-negative, anti-HBc-positive patients need prophylactic antiviral therapy if they are positive for HBV DNA;[112] if HBV DNA is negative, ALT levels, HBV DNA, and HBsAg should be monitored every 1–3 months. Antiviral therapy should be initiated immediately when HBV DNA or HBsAg becomes positive.[112,173]

For HBsAg-negative and anti-HBc-positive patients, the use of B-cell monoclonal antibodies or hematopoietic stem cell transplantation is associated with a high-risk of HBV reactivation.[174,175] The prophylactic use of antiviral drugs is recommended.[112,165,168,176,177]

For patients with CHB or cirrhosis that are treated with chemotherapy and immunosuppressants, the course of NA treatment, follow-up monitoring, and drug withdrawal are the same as those for patients with CHB or cirrhosis that receive no chemotherapy or immunosuppressants. For patients with chronic HBV infection in the immune tolerance or immune control phases, or HBsAg-negative, anti-HBc-positive patients that require the prophylactic use of NAs, treatment with ETV, TDF, or TAF should be continued for 6–12 months after the end of chemotherapy and immunosuppressive therapy.[6,168,178]

For patients that receive B-cell monoclonal antibodies or undergo hematopoietic stem cell transplantation, NAs could be discontinued at least 18 months after the end of immunosuppressive therapy.[179,180] HBV could recur or even worsen after NAs are withdrawn. Patients should be followed up for 12 months, during which HBV DNA should be monitored every 1–3 months.

Pregnancy

Women of childbearing age or women that are preparing to become pregnant should be screened for HBsAg, and HBV DNA should be tested for HBsAg-positive patients.[181] Peg-IFN-α treatment should be initiated before pregnancy if antiviral therapy is indicated, and antiviral therapy should be completed 6 months before pregnancy. Reliable contraceptive measures should be taken during treatment. If peg-IFN-α is not indicated or fails, TDF could be used for antiviral therapy. For patients diagnosed with CHB for the first time during pregnancy, treatment indications are the same as those for other CHB patients, and TDF could be used for antiviral therapy. Pregnant women with CHB who started taking antiviral drugs before or during pregnancy should continue antiviral therapy after delivery, and based on virological responses, decide whether to continue the original treatment regimen or change to other NAs or peg-IFN-α.

For patients with unexpected pregnancy during antiviral therapy, it is recommended that they continue their pregnancy if they are taking TDF. Patients do not have to terminate the pregnancy if they are taking ETV, and it is suggested that they switch to TDF instead. If pregnant patients are receiving IFN-α treatment, they should be informed (and their family members) of the associated risks, and whether or not to continue the pregnancy should be decided by patients themselves; if the pregnancy choice is to continue, patients should change to TDF treatment.

High levels of serum HBV DNA is a high-risk factor for mother-to-child transmission. If HBV DNA levels are $>2\times10^5$ IU/mL in the second and third trimesters of pregnancy,[182] the patients should be informed of the associated risks and their consent should be obtained to start antiviral therapy at 24–28 weeks of the pregnancy using TDF or LdT.[183,184] Breastfeeding is not a contraindication for TDF therapy.[185,186] Pregnant women taking NAs at the immune tolerance phase could discontinue NAs immediately after delivery or continue for another 1–3 months. Approximately 17.2–62% of patients might develop hepatitis flares after discontinuation, which usually occurs within 24 weeks.[187–189] Postpartum monitoring is needed. Blood biochemical indexes for the liver and HBV DNA should be rechecked 4–6 weeks after delivery. If the biochemical markers for the liver are normal, they should be followed up every 3 months until 6 months after delivery. If patients develop hepatitis flares, antiviral therapy is indicated.

Fertility issues for male patients that receive antiviral therapy: Male patients treated with IFN-α might consider having children 6 months after drug withdrawal. No evidence is currently available that indicates NA therapy harms sperm in male patients that receive NA therapy. Male patients should be informed of the potential risks when they consider having children.

Pediatric patients

If children with HBV infection are in the immune tolerance phase, antiviral therapy is not considered. Antiviral therapy should be initiated as soon as possible for children with chronic hepatitis or cirrhosis. Antiviral therapy for children with CHB can significantly inhibit HBV DNA replication and increase the chance of ALT normalization and HBeAg seroconversion.[190] However, the safety and resistance issues associated with long-term treatment should be concerned.[112,191,192]

The drugs approved by the USA's Food and Drug Administration (FDA) for the treatment of pediatric patients include IFN-α (aged ≥1 year), ETV (aged ≥2 years), and TDF (aged ≥2 years and body weight ≥10 kg).[6,190] China has approved TAF for adolescents (aged ≥12 years and body weight ≥35 kg). Peg-IFN-α-2a could be used in CHB children aged ≥5 years.[6]

HBeAg-positive CHB patients with elevated ALT levels should be treated with a finite course of IFN-α or peg-IFN-α-2a therapy to achieve HBeAg conversion.[178,193] Alternatively, they could be treated with ETV, TDF, or TAF. The recommended dose of IFN-α for pediatric patients is 3–6 million U/m^2, three times a week. The maximum dose should not exceed 10 million U/m^2, and the recommended duration of treatment is 24–48 weeks. Peg-IFN-α-2a should be dosed at 180 μg/1.73 m^2, with a treatment course of 48 weeks.[194] The doses of ETV, TDF, or TAF refer to the recommendations of the USA's FDA and WHO, and drug labels (Table 6).[8,190,193]

For children with CHB or cirrhosis that receive therapy with IFN-α or peg-IFN-α-2a but fail to achieve HBeAg seroconversion or HBeAg-negative status, NAs should be used for treatment.[178]

Patients with renal injury

High-risk factors for renal injury include ≥1 of the following: decompensated cirrhosis; eGFR <60 mL/min; poorly controlled hypertension; proteinuria; uncontrolled diabetes; active glomerulonephritis; concomitant use of nephrotoxic drugs; or history of solid organ transplantation.[112] When there are high-risk factors for renal injury, the changes in

Table 6. Recommended doses of NAs for children

Drug	Body weight (kg)	Dose (mg/d)
ETV (aged ≥2 years, and body weight ≥10 kg; use adult dose if body weight >30 kg)	10–11	0.15
	>11–14	0.20
	>14–17	0.25
	>17–20	0.30
	>20–23	0.35
	>23–26	0.40
	>26–30	0.45
	> 30	0.50
TDF		
Aged ≥2 years and body weight ≥17 kg, who can take tablets orally	17–22	150
	22–28	200
	28–35	250
	≥35	300
Aged ≥2 years and body weight ≥10 kg; those who cannot take tablets orally can be given powder using a special spoon (40 mg/spoon)	10–12	80 (2 spoons)
	12–14	100 (2.5 spoons)
	14–17	120 (3 spoons)
	17–19	140 (3.5 spoons)
	19–22	160 (4 spoons)
	22–24	180 (4.5 spoons)
	24–27	200 (5 spoons)
	27–29	220 (5.5 spoons)
	29–32	240 (6 spoons)
	32–34	260 (6.5 spoons)
	34–35	280 (7 spoons)
	≥35	300 (7.5 spoons)
TAF (aged ≥12 years)	≥35	25

ETV, entecavir; TAF, tenofovir alafenamide fumarate; TDF, tenofovir disoproxil fumarate.

renal function should be monitored during the use of any NA. If ADV or TDF is used for treatment, serum creatinine and blood phosphorus levels should be monitored regularly, regardless of the presence or absence of high-risk factors for renal injury.[195,196]

For patients with chronic kidney disease, renal insufficiency, or who receive renal replacement therapy, ETV or TAF is recommended as the first-line anti-HBV therapy; LdT could also be chosen for antiviral therapy, where appropriate. ADV or TDF is not recommended.[142,197] When TAF is used in patients without HIV coinfection, no dose adjustment is necessary when eGFR is ≥15 mL/min; however, the doses of other NAs currently on the market need to be adjusted when eGFR is <50 mL/min. Dosing adjustments should refer to drug labels.

ETV or TAF could be used as prophylactic or therapeutic drugs for HBsAg-positive patients that receive kidney transplantation. Because of the increased risk of rejection, kidney transplant patients should avoid IFN-α or peg-IFN-α therapy.

HBV-related glomerulonephritis could be treated with NA therapy, and ETV or TAF is recommended.[198]

For patients that have been treated with ADV or TDF, it is recommended that they should change to ETV or TAF if kidney or bone disease occurs, or if other high-risk factors are involved.[197]

Recommendation 15: For CHB patients, after 48 weeks of treatment with ETV, TDF, or TAF, of HBV DNA is $>2\times10^3$ IU/mL, NAs treatment regimens should be adjusted when excluding compliance and detection errors. Those using ETV could change to TDF or TAF, and those using TDF or TAF could change to ETV, or combination therapy might be used (ETV combined with TDF or TAF) (C2). Peg-IFN-α therapy could be used in combination (B1).

For patients with HBV-related cirrhosis, after treatment with ETV, TDF, or TAF for 24 weeks, of their HBV DNA is $>2\times10^3$ IU/mL, and excluding compliance and detection errors, NAs treatment regimens could be adjusted. Those using ETV could change to TDF or TAF, and those using TDF or TAF could change to ETV, or combination therapy might be

used (ETV combined with TDF or TAF) (C2).

Recommendation 16: All patients that receive chemotherapy or immunosuppressive therapy should be routinely screened for HBsAg and anti-HBc before starting treatment (A1). For HBsAg-positive patients, antiviral therapy should be given 1 week before or when they start to receive immunosuppressive therapy or chemotherapy (A1), and ETV, TDF, or TAF should be used (B1). For HBsAg-negative and anti-HBc-positive patients, antiviral therapy with ETV, TDF, or TAF is recommended if they receive B-cell monoclonal antibodies or undergo hematopoietic stem cell transplantation (B1).

Recommendation 17: When patients with chronic HBV infection attempt pregnancy or have antiviral indications during pregnancy, TDF could be used when informed consent has been obtained (B1).

Recommendation 18: For patients with unexpected pregnancy during antiviral therapy it is recommended that they continue their pregnancy if they are taking TDF. Patients do not have to terminate the pregnancy if they are taking ETV and it is suggested that they change to TDF instead (B1). If patients are receiving IFN-α treatment, they (and their family members) should be informed of the associated risks, and whether or not to continue the pregnancy should be decided by patients themselves; if they decide to continue, they should change to TDF treatment (C2).

Recommendation 19: If HBV DNA levels are $>2\times10^5$ IU/mL during the second and third trimesters of pregnancy, patients should be informed of the associated risks and could start antiviral therapy at 24–28 weeks of pregnancy using TDF or LdT when they give informed consent (A1). Pregnant women in the immune tolerance phase should stop medication immediately after delivery or after 1–3 months of treatment. Breastfeeding is not a contraindication for TDF treatment (C2). Blood biochemical indexes for the liver function and HBV DNA levels should be tested at least every 3 months until 6 months after delivery after drug withdrawal. Patients that develop hepatitis flares should start antiviral therapy immediately (A2).

Recommendation 20: For children with advanced liver disease or cirrhosis, antiviral therapy should be given in time, but safety and resistance issues associated with long-term antiviral treatment should be considered. IFN-α treatment could be considered for children aged ≥1 year, ETV or TDF for children aged ≥2 years, peg-IFN-α-2a for children aged ≥5 years. and TAF treatment for children aged ≥12 years (A1).

Recommendation 21: For patients with chronic kidney disease, renal insufficiency, or who receive renal replacement therapy, ETV or TAF is recommended as the first-line anti-HBV therapy. Alternatively, LdT could be used for antiviral therapy, where appropriate. ADV or TDF is not recommended (B1). The changes in renal function should be monitored during the use of any NAs for CHB patients at a high-risk of kidney injury. For patients that have been treated with ADV or TDF, it is recommended that they switch to ETV or TAF if they develop kidney or bone disease or present with other high-risk factors (B1).

Patients with coinfection of HBV and HCV

All people that are HBsAg-positive should be screened for anti-HCV. If they are positive, they need further testing for HCV RNA. Those who test positive for HCV RNA should receive DAA therapy. These patients are at risk of HBV reactivation. Therefore, it is recommended that they receive antiviral therapy with ETV, TDF, or TAF during and within 3 months after the end of anti-HCV therapy and they should be closely monitored.[112]

In addition, HBsAg-negative and anti-HBc-positive patients are at risk of HBV reactivation during treatment of hepatitis C with DAAs. Serum HBV DNA levels and HBsAg should be monitored monthly. If HBsAg seroreversion occurs, antiviral therapy is recommended.[112]

Recommendation 22: When a DAA is used to treat HCV in patients with coinfection of HCV and HBV, NA treatment should be administered to prevent HBV reactivation if HBsAg is positive. NA treatment could be withdrawn 12 weeks after the end of DAA treatment (B2). HBsAg-negative, anti-HBc-positive patients should be closely monitored for HBV DNA and HBsAg levels during DAA therapy. If HBsAg seroreversion occurs, NA therapy is recommended (B2).

Patients with coinfection of HBV and HIV

Antiretroviral therapy (commonly referred to as ART) should be initiated as soon as possible if there is no contraindication for anti-HIV therapy, regardless of $CD4^+T$ lymphocyte count. HIV/HBV co-infected patients should be treated for both viral infections at the same time. The highly active anti-retroviral therapy (HAART) regimen should include two drugs active against HBV, preferably TDF or TAF + LAM or emtricitabine (FTC) (TDF+FTC and TAF+FTC are available as a mixture). HBV-related markers, such as HBV DNA, liver biochemical indicators, and liver imaging should be monitored during treatment. For HIV/HBV co-infected patients, a treatment regimen for hepatitis B that contains only one NA against HBV is not recommended (TDF, LAM, ETV, LdT, ADV).[199,200]

The following should be noted for patients with renal insufficiency: (1) if the creatinine clearance rate is <60 mL/min, avoid use of TDF; and (2) if the creatinine clearance rate is between 30 mL/min and 50 mL/min, TAF+ (FTC or LAM) could be considered in the regimen. However, TAF has not been approved for use yet in patients with a creatinine clearance rate <30 mL/min. Finally, (3) when TDF/TAF cannot be used, ETV should be added to the HAART regimen. For pregnant women with HIV/HBV co-infection, the recommended regimen should include LAM (FTC)+TDF.[201]

Recommendation 23: For people who are co-infected with HBV and HIV, it is recommended that a combination of antiviral drugs effective for both HIV and HBV be chosen (A1).

Patients with HBV-related liver failure

Patients with HBV-related acute, subacute, acute-on-chronic, and chronic liver failure are associated with high mortality. If HBsAg is positive, antiviral therapy is indicated.

Anti-HBV therapy can improve the long-term prognosis of HBV-related acute-on-chronic liver failure (ACLF).[202,203] Several clinical studies have confirmed that both ETV and LAM can effectively reduce the mortality of ACLF.[204,205] Meta-analyses have shown that ETV is superior to LAM for the treatment of HBV-related ACLF.[205,206] Small cohort clinical studies have found that HBV-related ACLF can benefit from the use of LdT and TDF.[207,208] Compared with TDF, TAF can reduce nephrotoxicity and maintain antiviral efficacy,[142] but no definite clinical evidence is available to support the use of TAF for liver failure. Early rapid reduction of HBV DNA levels is key to treatment.[204,207] If HBV DNA levels can be reduced by 2 lg IU/mL within 2–4 weeks, survival can be improved.[206,207] Fast-acting NAs with high potency and minimal resistance (ETV, TDF, or TAF) should be chosen for antiviral therapy.[209] Antiviral therapy should be continued for long periods after patients with liver failure recover.

Recommendation 24: For patients with HBV-related

acute, subacute, acute-on-chronic, and chronic liver failure, it is recommended that they should receive antiviral therapy with ETV, TDF, or TAF if they are positive for HBsAg (A1).

Patients with HBV-related HCC

HBV DNA-positive HCC patients that receive anti-HBV treatment could reduce the recurrence of HCC after surgery and improve the overall survival rate.[210–216] Fast-acting NAs with high potency (ETV, TDF, or TAF) should be chosen for antiviral therapy. Patients without contraindications could use IFN-α.

HCC patients that are HBsAg-positive but HBV DNA-negative might experience HBV reactivation when undergoing liver resection, transcatheter arterial chemoembolization, radiotherapy, or systemic chemotherapy.[217–221] ETV, TDF, or TAF are recommended for antiviral therapy.

Recommendation 25: Patients with HBV-related HCC are recommended to use ETV, TDF or TAF for antiviral therapy if they are positive for HBsAg (A1).

Liver transplant patients

For patients that undergo liver transplantation for HBV-related diseases (including liver failure and HCC), anti-HBV regimens should be rationally selected to reduce the risk of reinfection of HBV in the transplanted liver. The design of treatment regimens mainly depends on the major risk factor for reinfection, i.e. HBV DNA levels before transplantation.

A negative HBV DNA quantification test before transplantation suggests a low risk of reinfection. High potency NAs with minimal resistance, such as ETV, TDF, or TAF, should be used as early as possible before surgery to prevent HBV reactivation. There is no need to use HBIG after surgery.[222,223] Positive HBV DNA testing before transplantation suggests a high-risk of reinfection. NAs with high potency and minimal resistance should be used as early as possible before surgery to reduce HBV DNA levels; HBIG should be injected intravenously during the anhepatic phase. After surgery, low-dose HBIG should be used in combination with NAs for 0.5–1.0 year, followed by the use of NAs alone.[222,224,225] A recent study found that a shortened course of HBIG was effective in patients treated with ETV.[226] If patients have already used other NAs, HBV DNA should be closely monitored to detect drug resistance and adjust the regimen if necessary. In addition, hepatitis B vaccination was reported to prevent recurrence after liver transplantation, but its clinical use remains controversial.[227]

Recommendation 26: For patients that undergo liver transplantation for HBV-related infections, it is recommended that they start antiviral therapy with ETV, TDF, or TAF before transplantation if they are positive for HBsAg (A1).

Unsolved clinical issues

1. Identify and evaluate new markers that could be used to differentiate the various stages of the natural course of chronic HBV infections.
2. Define the value of new serum markers, such as anti-HBc levels during the treatment planning for patients with normal ALT levels.
3. Define the value of noninvasive tests for liver fibrosis in initiating treatment, evaluating the efficacy, and predicting long-term outcome.
4. Assess the effect of long-term treatment with different NAs on the reversal of cirrhosis and the incidence of HCC.
5. Identify clinical indicators and biological markers that could guide the safe discontinuation of NAs.
6. Develop innovative drugs aimed at a clinical cure (functional cure) and evaluate their synergy and combined use with existing drugs.
7. Use real-world data (e.g., long-term follow-up cohorts or medical insurance databases) to evaluate the safety, efficacy, and cost-effectiveness of marketed drugs and provide evidence for clinical and public health decisions.
8. Innovate the management of CHB, enhance the ability to detect, diagnose, and cure CHB, and reduce mortality related to hepatitis B.

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Conflict of interest

The authors have no conflict of interests related to this publication.

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Biographies of the Editors-in-Chief

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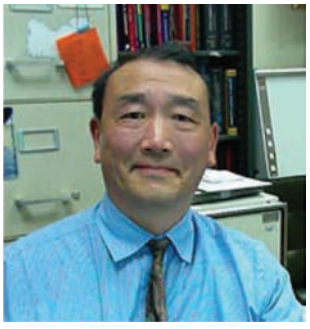
Prof. Ren, is the President, Director [Key Laboratory of Molecular Biology of Infectious Diseases (Ministry of Education of China), Medical Imaging Department, Liver and Viral Hepatitis Research Institute], Leader and Distinguished Super Specialist Consultant [Division of Infectious Diseases (one of the national key discipline in China), Department of Internal Medicine] of the Second Affiliated Hospital of Chongqing Medical University. In addition, he is also the Vice-Chairman and Group Head of the Chinese Society of Hepatology, Chinese Medical Association.

Prof. George Y. Wu (Comprehensive Editor-in-Chief)



Prof. Wu obtained his MD and PhD degree at Albert Einstein College of Medicine in 1976. He was a resident at Harlem Hospital Center from 1976 to 1979. He worked as a postdoctoral fellow at Albert Einstein College of Medicine from 1979 to 1982. From 1983, he worked as Assistant Professor and then Professor at University of Connecticut School of Medicine. He is now the Director of Hepatology Section, Division of Gastroenterology-Hepatology. Dr. Wu's awards include the following: Research Prize awarded by the American Liver Foundation in 1982; Industry Research Scholar Award from the American Gastroenterological Association for 1985 to 1988; Gastroenterology Research Group Young Scientist Award from the American Gastroenterological Association in 1990; Herman Lopata Chair in Hepatitis Research from 1992 to date; Scientific Award from the Chinese American Medical Society in 1992; He was elected to membership in exclusive societies: American Society for Clinical Investigation in 1989; Association of American Physicians in 1995; and Top Doctor in the U.S. awarded by U.S. News and World Report in 2011. He has published about 180 peer-reviewed academic articles, 11 books, and is series editor for Clinical Gastroenterology book series by Springer-Nature, and is Senior Associate editor of J. Digestive Diseases.

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