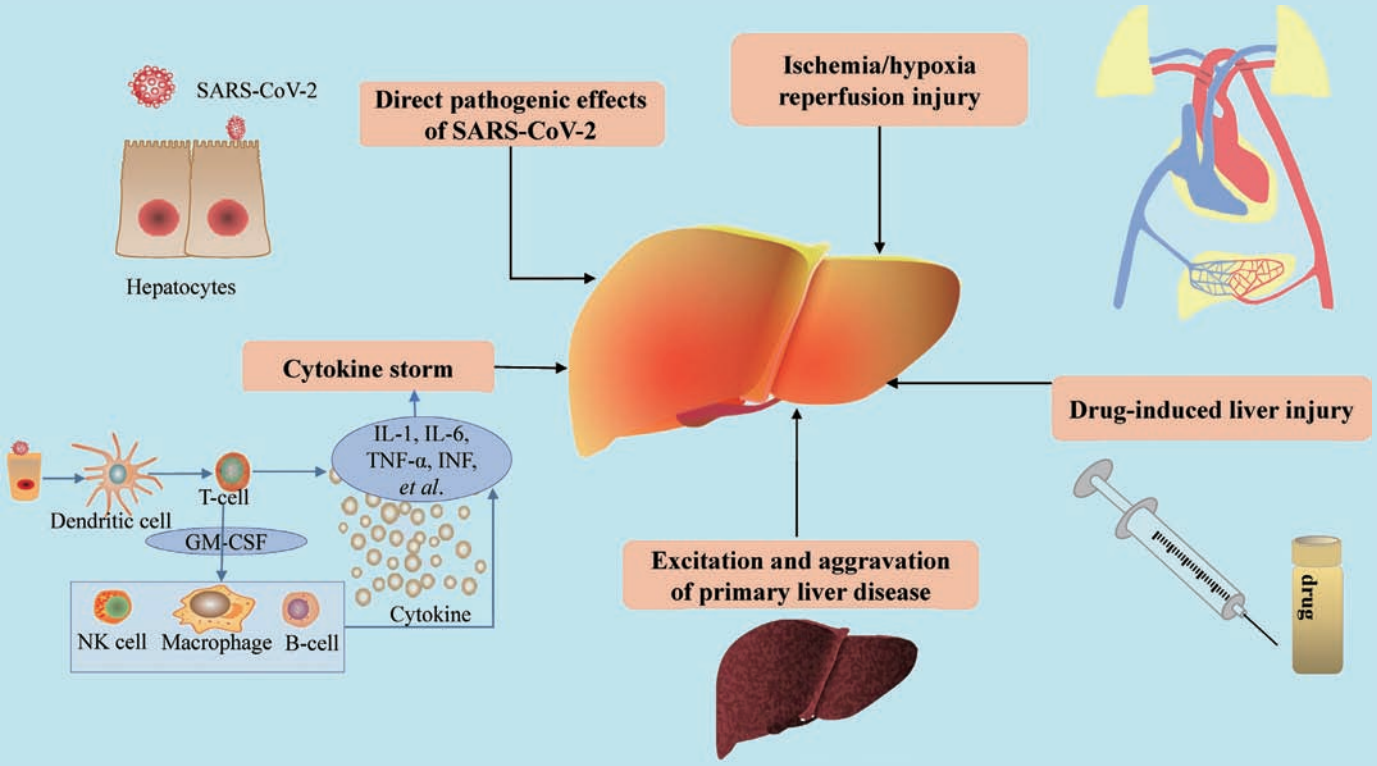
ISSN 2225-0719

2021

Volume 9 Issue 2
March / April

THE SECOND AFFILIATED HOSPITAL
CHONGQING MEDICAL UNIVERSITY

Journal of Clinical and Translational Hepatology



JCTH

OWNED BY THE SECOND AFFILIATED HOSPITAL OF CHONGQING MEDICAL UNIVERSITY
PUBLISHED BY XIA & HE PUBLISHING INC.
pISSN: 2225-0719; eISSN: 2310-8819
Frequency: Quarterly
Launch date: September 28, 2013 (Volume 1, Issue 1)

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Publisher

Xia & He Publishing Inc.
Website: www.xiahepublishing.com
E-mail: service@xiahepublishing.com
Postal Address: 14090 Southwest Freeway, Suite 300, Sugar Land, Texas, 77478, USA

Current Issue: Volume 9, Issue 2
Publication date: April 28, 2021

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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CONTENTS

2021 9(2):139–278

Editorials

A Simple and Rapid Method for Quantitative Detection of Hepatitis B Virus Drug-resistant Mutations
Ran Wang and Zhengde Xie 139

Very-early-stage Hepatocellular Carcinoma, Are We at Long Last on Route for Achieving Better Patient Outcomes?
Paulina Vidal-Cevallos and Norberto Chavez-Tapia 141

Origianl Articles

Detection of Hepatitis B Virus M204V Mutation Quantitatively via Real-time PCR
Jingjing Liang, Xinmiao Liang, Hong Ma, Leng Nie, Ying Tian, Guang Chen and Yu Wang 143

Safety and Efficacy of Physical Thermal Ablation Combined Sorafenib for Hepatocellular Carcinoma: A Meta-analysis
Mengdi Jin, Qiong Yu, Yahui Liu, Weiling Xu, Xueqi Fu and Bai Ji 149

Histone Deacetylase Inhibitors Romidepsin and Vorinostat Promote Hepatitis B Virus Replication by Inducing Cell Cycle Arrest
Yang Yang, Yu Yan, Zhen Chen, Jie Hu, Kai Wang, Ni Tang, Xiaosong Li and Zhi Zhou. 160

Development and Validation of a Metabolic-related Prognostic Model for Hepatocellular Carcinoma
Junyu Huo, Liqun Wu and Yunjin Zang 169

***UGT1A1*-related Bilirubin Encephalopathy/Kernicterus in Adults**
Jie Bai, Lu Li, Hui Liu, Shuang Liu, Li Bai, Wenyan Song, Yu Chen, Sujun Zheng and Zhongping Duan 180

Predictive Model of Ursodeoxycholic Acid Treatment Response in Primary Biliary Cholangitis
Yanyun Shu, Yuhu Song, Tao Bai, Xiaoli Pan, Haitao Shang, Ling Yang, Jin Ye and Fan Du 187

Associations of Hydroxysteroid 17-beta Dehydrogenase 13 Variants with Liver Histology in Chinese Patients with Metabolic-associated Fatty Liver Disease
Wen-Yue Liu, Mohammed Eslam, Kenneth I. Zheng, Hong-Lei Ma, Rafael S. Rios, Min-Zhi Lv, Gang Li, Liang-Jie Tang, Pei-Wu Zhu, Xiao-Dong Wang, Christopher D. Byrne, Giovanni Targher, Jacob George and Ming-Hua Zheng 194

Association of LDLR rs1433099 with the Risk of NAFLD and CVD in Chinese Han Population
Yi Han, Yongshuo Zhang, Shousheng Liu, Guangxia Chen, Linlin Cao and Yongning Xin 203

Clinical Validation of Global Coagulation Tests to Guide Blood Component Transfusions in Cirrhosis and ACLF
Madhumita Premkumar, Rohit Mehtani, Smita Divyaveer, Kamal Kajal, Anand V. Kulkarni, Syed Ahmed, Harmanpreet Kaur, Harpreet Kaur, Radhakrishna Dhiman, Ajay Duseja and Arka De 210

Review Articles

Hepatocellular Carcinoma: Downstaging to Liver Transplantation as Curative Therapy
Leana Frankul and Catherine Frenette 220

Gut Microbiota in Metabolic-associated Fatty Liver Disease and in Other Chronic Metabolic Diseases
Winston Hernández-Ceballos, Jacqueline Cordova-Gallardo and Nahum Mendez-Sanchez 227

Progress in the Clinical Features and Pathogenesis of Abnormal Liver Enzymes in Coronavirus Disease 2019
Haiyan Wu, Shuzhong Liu, Hesheng Luo and Mingkai Chen 239

Coronavirus Disease-2019 (COVID-19) and the Liver
Anshuman Elhence, Manas Vaishnav, Sagnik Biswas, Ashish Chauhan, Abhinav Anand and Shalimar 247

Cytokine Storm of COVID-19 and Its Impact on Patients with and without Chronic Liver Disease
Madhumita Premkumar and Chandan Kumar Kedarisetty 256

Case Reports

Checkpoint Inhibition in the Treatment of Unresectable, Advanced Lymphoepithelioma-like Hepatocellular Carcinoma
David J. Hermel, Emma Z. Du, Ray Lin, Catherine T. Frenette and Darren S. Sigal 265

Rapid Recovery in COVID-19 Patients with Chronic Hepatitis B Virus Infection Treated with Tenofovir Disoproxil Fumarate
Xiliu Chen, Di Liu, Dongliang Yang and Xin Zheng. 269

Letters to the Editor

Celiac Disease and Elevated Liver Enzymes: A Still Not Fully Defined Pathogenesis
Linda Beenet and Diego Tonesi 274

Favipiravir-induced Liver Injury in Patients with Coronavirus Disease 2019
Pramod Kumar, Anand Kulkarni, Mithun Sharma, Padaki Nagaraja Rao and Duvvuru Nageshwar Reddy . . . 276

Journal of Clinical and Translational Hepatology **2021** vol. 9(2) | 139–140
DOI: 10.14218/JCTH.2021.00135

Editorial

A Simple and Rapid Method for Quantitative Detection of Hepatitis B Virus Drug-resistant Mutations

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Received: 6 April 2021 | **Revised:** 10 April 2021 | **Accepted:** 12 April 2021 | **Published:** 16 April 2021

Citation of this article: Wang R, Xie Z. A simple and rapid method for quantitative detection of hepatitis B virus drug-resistant mutations. J Clin Transl Hepatol 2021;9(2):139–140. doi: 10.14218/JCTH.2021.00135.

Hepatitis B virus (HBV) is a small-enveloped virus enclosing a partially double-stranded DNA genome, belongs to the hepadnaviruses family.[1] To date, 10 genotypes (A–J) of HBV with distinct geographical distribution have been described, based on a divergence of at least 8% over the entire genomic sequence or >4% in the S gene sequence, with B and C being most prevalent and confined to Asia and Oceania.[2] HBV is a highly contagious pathogen that can lead to acute infection or chronic hepatitis B (CHB), cirrhosis and hepatocellular carcinoma (commonly known as HCC) in humans through immune anergy or upon immunosuppression.[3]

Although a global HBV vaccination program has been implemented in more than 200 countries and provided a significant decline in incidence of CHB, there are still approximately 292 million people worldwide suffering from CHB, with close to 1 million deaths occurring annually and maintaining the disease as a major global health problem.[4,5] Large-scale long-term prospective studies in the past decades have shown that antiviral nucleotide analogues (NAs) treatment of CHB patients may inhibit HBV replication without eliminating the virus, remitting HBV-related HCC in some patients and reducing associated morbidity and mortality but not completely.[6] Due to potent viral suppressive effects and good tolerance by patients taking the NAs for decades while experiencing limited side effects, these drugs have been widely used in the management of CHB treatment.[7]

Currently, NAs including lamivudine (LAM), entecavir (ETV), tenofovir disoproxil fumarate, telbivudine (LdT), adefovir dipivoxil, and tenofovir alafenamide are prescribed and available for CHB therapy in China.[8] All of these NAs represent the more preferred agents that work mainly by competitively inhibiting HBV DNA polymerase activity, with the incorporation of the natural endogenous intracellular nucleotides in assembled HBV DNA, causing DNA synthesis termination and suppressing viral replication.[9] In detail, LMT is often used in CHB patients with high HBV replication or decompensated liver disease; ETV and adefovir dipivoxil are not only suitable for patients in the acute phase of CHB, but also as alternative therapies for patients who have developed LAM resistance.

However, NA-associated resistance is a serious impediment to the treatment of CHB. For example, LAM was initially and extensively prescribed when resistance is impending. Resistance to LAM develops within 6 months of treatment, and emerges in ~20% after 1 year and at an accumulation rate of 70% following 5 years of treatment.[10] Moreover, resistance to LAM confers cross-resistance to ETV and LdT, which leads to lower antiviral efficacy and lower genetic barrier to the drugs.[11] Mutations targeting HBV polymerase/reverse transcriptase domains that are critical during viral replication, are responsible for conferring resistance to NAs.[12] High rates of HBV replication, combined with inadequately effective proofreading for HBV polymerase, is the basis for establishing mutations in the viral genome. Mutations that change the binding site between NAs and HBV represent the molecular mechanism underlying drug resistance. Mutation patterns such as M204V/I (primary resistance mutation) and L180M (secondary/compensatory mutation) were defined as joint resistance mutations across most genotypes of HBV and as involved in virological breakthrough or biochemical rebounds.[13,14] The M204V/I mutation in the C domain of polymerase represents one of the most common primary resistance mutations and directly results in high-level resistance to NAs, such as LAM, ETV, and LdT.[12,15] Thus, the monitoring and a high-speed feature detector of mutation for HBV drug resistance are necessary.

There are many types of laboratory tests that can be used to determine resistance mutations in the HBV reverse transcriptase region, with varying sensitivities. The most frequently used method for routine testing in clinical laboratories is PCR-based sequencing that is able to detect more than 20% of mutations among the total viral population.[11,16] Besides, PCR-restriction fragment length polymorphisms and reverse hybridization line-probe assays can consistently detect mutations present in 5% of the virus populations.[11] However, it should be pointed out that the emergence of HBV resistance mutations is silent and thus difficult to detect in a timely manner. Only until a certain

Abbreviations: CHB, chronic hepatitis B; ETV, entecavir; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; LAM, lamivudine; LdT, telbivudine; NAs, nucleotide analogs.

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number of drug-resistant mutations have accumulated can they be detected by the commonly used sequencing methods. Thus, the key disadvantage of this method comes from the low sensitivity; that is, the variant strain can only be found when it exceeds 20% of the HBV sequence. Moreover, the detection approach by PCR combined with sequencing is time-consuming and high-cost, and the related data analysis is more complicated. Nevertheless, this method is generally used as the gold standard for the detection of resistance mutations.

To counter the disadvantages of the methods above, Liang *et al.*[17] developed a real-time PCR assay aimed at monitoring the reaction process and quantitatively detecting HBV resistance mutations. Compared with the above-mentioned commonly used methods, this method has a streamlined operation process and can detect resistance mutations with a rate of less than 10%, whereas its disadvantage is that it can only detect known mutation sites by using designed probes and primers. The investigators tentatively chose M204V/I mutations to be detected due to the widespread application of LAM, ETV, LdT, etc., and thus to be more readily available for blood samples from NA-resistant patients. Methodologically, the samples were collected from patients who were treated with NAs and were clinically confirmed to have viral breakthrough, primary nonresponse, or poor response. Then, they designed primers and probes for M204V/I that were screened and confirmed with M204V/I-positive sera, in order to verify the specificity and sensitivity rates. Finally, a linear standard curve for quantification was obtained by use of a 10-fold dilution of the plasmid, and the formula was used to calculate the viral load within extracted DNA.[17] Real-time PCR can quantify the mutated gene with relatively accurate measurement in units of copies/mL, while the current method uses ab approximate description via the percentage of mutation.

According to the results, Liang *et al.*[17] confirmed probes 204-2-VP2 and 204-2-IP2 with a positive rate of 100% and the false positive rate of 0%. Furthermore, the amplification curves showed a highly linear relationship between Ct values and the amount of serially diluted plasmid DNA for the primers and probes (R^2=0.996, slope=−3.723), indicating an appropriate quantitative detection of M204V/I. The limit of detection, sensitivity, and specificity were 10^3 copies/mL, 92.86%, and 100%, respectively. Meanwhile, the assay presented good reproducibility and accuracy.

In conclusion, a rapid and accurate assay is urgently needed to monitor HBV DNA polymerase/ reverse transcriptase gene mutations in patients undergoing NA treatment. The features of M204V/I detection for the pilot assay by real-time PCR provided high-efficiency, cost-effectiveness, and convenience, representing a high generalizability to quantitatively detect multiplex mutations in the target genes. This method is expected to present the association between specific mutations and the phenotypic resistance of an isolate with that mutation during the early stages of NA treatment.

Funding

RW was funded by the National Natural Science Foundation of China (82002130) and the Open Research Fund Program of the State Key Laboratory of Virology of China (2021IOV002); ZX was funded by the National Natural Science Foundation of China (82072266) and the National Major Science & Technology Project for Control and Prevention of Major Infectious Diseases in China (2017ZX10103004-004).

Conflict of interest

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

Author contributions

Article conception and design (RW and ZX), drafting of the manuscript (RW), critical revision of the manuscript (ZX).

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Journal of Clinical and Translational Hepatology **2021** vol. 9(2) | 141–142
DOI: 10.14218/JCTH.2021.00137

Editorial

Very-early-stage Hepatocellular Carcinoma, Are We at Long Last on Route for Achieving Better Patient Outcomes?

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Received: 12 April 2021 | **Accepted:** 16 April 2021 | **Published:** 23 April 2021

Citation of this article: Vidal-Cevallos P, Chavez-Tapia N. Very-early-stage hepatocellular carcinoma, are we at long last on route for achieving better patient outcomes? J Clin Transl Hepatol 2021;9(2):141–142. doi: 10.14218/JCTH.2021.00137.

Hepatocellular carcinoma (HCC) represents ~90% of the primary liver cancer cases, and 90% of HCC cases occur in patients with chronic liver disease.[1] It also represents the sixth most common cancer (4.7%) and the third leading cause of cancer-related death globally (8.3%).[2] More troubling, the global incidence and mortality rates have been increasing since 1990.[3] In the United States, the highest average annual percentage change (known as AAPC) reported between 2000 and 2012 involved individuals between 55 and 59 years of age (AAPC: 8.9%; 95% confidence interval: 7.1–10.7%).[4]

The current European Association for the Study of the Liver (commonly known as EASL) guidelines advise treatment assignment according to tumor stages following the Barcelona Clinic Liver Cancer (BCLC) staging system.[5] The concept of very-early or stage 0 classification was not introduced until the 2003 BCLC modification.[6] Surgical resection or image-guided ablation are the first-line therapies recommended in these set of patients.[5] The reported overall survival (OS) at 5 years after surgical resection is 71.1%, with a 5-year recurrence rate of 43.3%.[7] In patients excluded from surgery with Child class A, the reported 5-year survival rate is 61% and 5-year recurrence rate is 81%.[8]

Since patients with HCC BCLC stage 0 and A are deemed curable, such high recurrence rates are rather dismal and attempts are being made to improve patient outcomes. An interventional review explored chemotherapy, chemoembolization, internal radiation, and retinoids as neoadjuvant or adjuvant therapy after surgical resection and did not find enough evidence of their efficacy,[9] and hence are not currently advised.

Sorafenib, a multikinase inhibitor that targets receptor tyrosine and serine/threonine kinases to inhibit tumor growth and angiogenesis,[10] was shown in a phase II trial to have efficacy in patients with non-operable HCC.[11] A phase III double-blind placebo-controlled trial was then conducted and showed that sorafenib conferred a median OS of 10.7 months (hazard ratio: 0.69; 95% confidence interval: 0.55–0.87; $p<0.001$).[12] It was in the second BCLC modification, published in 2008, that sorafenib was incorporated as a first-line treatment option for BCLC stage C patients.[13]

A very large multicenter, phase III, double-blind, placebo-controlled trial was conducted to assess the efficacy and safety of sorafenib as an adjuvant therapy for both surgical resection and local ablation; results were disheartening, as no efficacy was found.[14] However, other smaller trials have shown more promising results.

A meta-analysis[15] was conducted to answer this question. Overall, the combined therapy showed significantly higher 1-, 2- and 3-year survival rates and an odds ratio of 2-year recurrence of 0.40 (95% confidence interval: 0.18–0.87). It is to be noted that the rate of adverse events was also higher for the combination therapy group, especially for that of hand-foot syndrome.

The results of this meta-analysis should be analyzed with caution. First, only three out of the fifteen studies were randomized controlled trials (commonly referred to as RCTs). Furthermore, the largest RCT[14] conferred great heterogeneity to the results. This was also the only trial which included some non-Asian patients, and HCC etiology may vary in different geographical regions, making results not applicable to all populations.

Some new light has been shed over the question of how to improve OS and decrease recurrence rates in candidates for potentially curative treatments. Nevertheless, the question remains largely unanswered, and a recommendation to include adjuvant treatment with sorafenib in the treatment of stage 0 or Child A patients cannot yet be made. High-quality RCTs, including diverse populations and with long-term follow-up are needed.

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Journal of Clinical and Translational Hepatology **2021** vol. 9(2) | 143–148
DOI: 10.14218/JCTH.2020.00118

Original Article

Detection of Hepatitis B Virus M204V Mutation Quantitatively via Real-time PCR

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Received: 15 November 2020 | **Revised:** 25 January 2021 | **Accepted:** 30 January 2021 | **Published:** 16 March 2021

Abstract

Background and Aims: Drug-resistant DNA mutations of the hepatitis B virus (HBV) affect treatment response in chronic hepatitis B patients. We have established a new, sensitive, specific, accurate and convenient real-time PCR method to detect HBV mutations quantitatively. ***Methods:*** Blood samples were collected from patients showing viral breakthrough, primary nonresponse, or poor response during treatment, and mutations were detected via direct sequencing to assess our method. A plasmid containing the M204V mutation was synthesized and standard curves plotted. ***Results:*** The determination coefficient for linear correlation between Ct and log plasmid copy numbers was 0.996, where Ct value was −3.723log (DNA concentration) +48.647. Coefficients of variation indicated good reproducibility. Correctness was within tolerable bias. Limit of detection was 10^3 copies/mL. Specificity, accuracy, positive predictive value and negative predictive value were 92.86%, 100%, 96.88%, 100% and 94.74%, respectively. ***Conclusions:*** These results show that our method can be used to detect HBV M204V mutations with the advantages of sensitivity, specificity and efficiency, providing a new choice for monitoring drug resistance.

Citation of this article: Liang J, Liang X, Ma H, Nie L, Tian Y, Chen G, *et al*. Detection of hepatitis B virus M204V mutation quantitatively via real-time PCR. J Clin Transl Hepatol 2021; 9(2):143–148. doi: 10.14218/JCTH.2020.00118.

Introduction

Hepatitis B virus (HBV) infection remains a global disease, with significant morbidity and mortality.[1,2] One of the main treatment options for patients infected with chronic hepatitis B (CHB) is the nucleotide analogs (NAs). Currently, NAs approved for clinical use in China include lamivudine (LAM), adefovir dipivoxil (ADV), entecavir (ETV), telbivudine (LdT), tenofovir disoproxil fumarate (TDF) and tenofovir alafenamide (TAF).[3] However, antiviral resistance is the most important factor in the failure of hepatitis B treatment.

The occurrence of drug resistance may lead to recurrence of hepatitis, and even cause progression of the disease, including virological breakthroughs, biochemical rebounds, hepatitis condition aggravation, and even hepatic failure.[4,5] Long-term drug resistance monitoring of patients with CHB who are taking LAM showed the proportion of hepatitis recurrence increased significantly compared to patients without drug resistance; in addition, the incidence of compensation cirrhosis was increased in patients with long-term drug resistance.[6,7] Not only for its impact on hepatitis, the occurrence of drug resistance will also lead to an increase in the incidence of hepatitis B-related liver cancer. A meta-analysis showed that the incidence of liver cancer in patients with LAM resistance is significantly higher than in treatment-naive patients (42/594 vs. 126/3,287, p=0.001).[8] Thus, the occurrence of drug resistance mutations not only limits the choice of treatment options and increases the cost of treatment but is also closely related to the progression and prognosis of the disease.

Antiviral drug-resistant HBV variants occur spontaneously in CHB patients following exposure to NAs.[9,10] Genotypic antiviral resistance refers to the presence of unique nucleotide mutations in drug target genes, which are the HBV polymerase genes that have been shown to be associated with antiviral resistance during HBV treatment with NAs. There are two types of mutations associated with drug resistance: primary resistance mutations and compensatory mutations. Primary resistance mutations directly lead to a decrease in the sensitivity to the drug, while the compensatory mutations can restore or enhance the replication of virus. The mutations rtM204V/I represent one of the most common primary resistance mutations in hepatitis B patients, which directly decrease the susceptibility to NAs, especially to LAM and LdT.[11–15]

LAM is the most widely used and longest-serving antiviral drug, but it also has the highest resistance rate. Recent studies have indicated that the percentage of LAM-associated resistance mutations that appear after 1 year

Keywords: HBV DNA; Drug-resistance mutation; Real-time PCR; DNA sequencing.

Abbreviations: ADV, adefovir dipivoxil; CHB, chronic hepatitis B; ETV, entecavir; HBV, hepatitis B virus; LAM, lamivudine; LdT, telbivudine; NAs, nucleotideanalogs; TAF, tenofovir alafenamide; TDF, tenofovir disoproxil fumarate.

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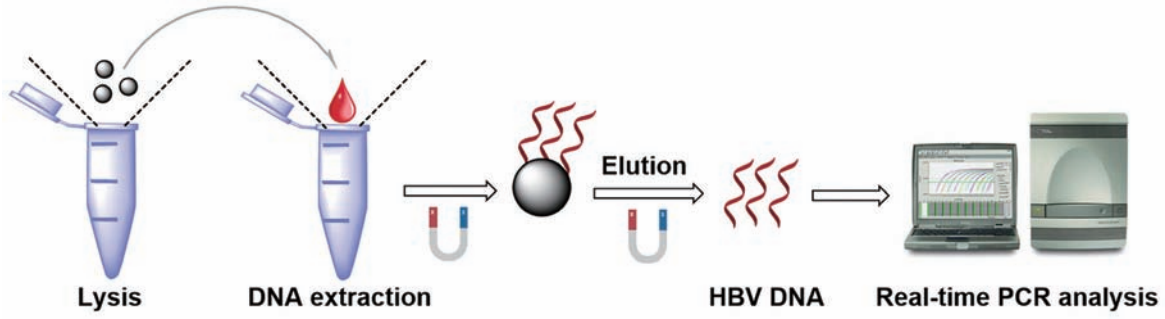

Fig. 1. Process of real-time PCR based on magnetic nanoparticles for detecting HBV DNA.

may vary from 7% to 30%.[15–17] The accumulation rate of LAM-associated drug resistance following 5 years of therapy is 70%.[7] As a recommended first-line drug, ETV is also widely used in clinical practice. In addition to a background of the mutations rtM204V/I, the other mutations, such as rtI169T, rtS184G, rtS202I and rtM250V, are associated with the emergence of ETV resistance.[18–21] A retrospective study conducted in China from 2009 to 2016 demonstrated that 73.7% of male CHB patients developed HBV genotypic resistance mutations to NAs, and that ETV-associated resistance increased to 17.1% in 2016.[22] Moreover, rtM204V combined with some other mutations may lead to a resistance to TDF.[23] Due to the widespread use of the above-mentioned drugs, drug-resistant specimens are the most readily available. Therefore, we chose mutations rtM204V/I to be detected.

Real-time PCR, a relatively new quantitative testing technique, includes a new platform that allows the initial concentration of the sample template to be estimated. In contrast to conventional PCR, it uses fluorescent dyes or specific fluorescent labeled probes to monitor the reaction process and illustrate the amount of DNA present, in real time, at each cycle of amplification. Two methods are used to calculate the initial DNA concentration: absolute quantitation and relative quantitation. Relative quantitation is used to determine the relative changes in expression in a similar target nucleic acid sequence and the correction sample. Absolute quantitative analysis determines the absolute value of a nucleic acid sequence in a sample.[24–28] Real-time PCR is widely used in molecular diagnostics to detect and identify bacteria and viruses. In addition, because real-time PCR is quantifiable, it is used to evaluate disease progression and efficacy of antiviral/antibiotic therapies.

The current study developed a real-time PCR assay aimed at quantitatively detecting HBV mutations. The schematic diagram of this work is shown in Fig. 1.

Methods

Sample preparation

We collected blood samples from patients treated with NAs, especially LAM and ETV, showing viral breakthrough (defined as a confirmed increase in HBV DNA levels of more than 1 log10 copies/mL compared to the lowest HBV DNA level on-therapy), primary nonresponse (defined as less than 1 log10 copies/mL decrease in the HBV DNA level from baseline following 12 weeks of therapy), or poor response (defined as more than 1 log10 copies/mL decrease in the HBV DNA level from baseline but detectable following at least 12 months of therapy) in response to NAs at the Beijing Friendship Hospital between 2015 and 2019. The exclusion criteria were as follows: co-infection with other hepatitis viruses or/and human immunodeficiency virus; poor treatment compliance; and patients who refused to participate in the experiment.

The study was approved by the Medical Ethics Committee of Beijing Friendship Hospital, affiliated with Capital Medical University.

Process for the detection system

DNA extraction: HBV DNA was extracted using nano-magnetic beads according to the following steps: nano-beads and heated lysis buffer were mixed in a 70 °C water bath to dissolve insoluble substances, and then mixed evenly before use. A 100 μL serum sample was added to a new centrifuge tube and mixed with 400 μL of cracking buffer solution, and was heated for 10 m at 70°C and mixed every 2–3 min. Subsequently, 300 μL of binding buffer and 20 μL of magnetic beads were added to the above centrifuge tube in turn, and mixed. The mixture was left to stand at room temperature for 5 m, with mixing once every 2 m. Next, 200 μL each of cleaning buffers I, II and III were added to the centrifuge tube, respectively, a magnet was used to adsorb the nano-beads for 30 s, and the supernatant was discarded. Centrifugation at high speed was used to separate the nano-beads from the mixture, followed by extraction. Next, 50 μL eluting buffer was added to the centrifuge tube, heated to 70°C and left for 5 m at 70°C. Nano-beads adsorbed on the magnets and supernatant fluid containing DNA were transferred to an RNA-free centrifuge tube and stored at −20°C for further use.

Primers and probes of M204V

Primers and probes were designed by the Wawasye Nanotech Company (Wuhan, China) and synthesized by Sangon Biotech (Shanghai, China). The probes were quenched using BHQ1 or MGB at the 3′-end (Table 1). Better probes were selected by subjecting the positive serum, containing the mutant gene of HBV polymerase M204V previously confirmed by Sanger sequencing, to a series of tests.

Preparation of the reaction system

We blended 12.5 μL of SYBR Premix (2x) with 0.5 μL of forward primer, 0.5 μL of reverse primer, 0.1 μL of specific probe and 9.4 μL of deionized water as a reaction system. Subsequently, we added 2 μL of template DNA to the experimental group and 2 μL of deionized water to the control

Table 1. Primer and probe sequences with their respective dye and quencher

	Target gene	Name	Sequences
Primer		primer forward	5′-GCACTTGTATTCCCATCCCATCAT-3′
		primer reverse	5′-AGCAAAGCCCAAAAGACCCACAAT-3′
Probe	M204	204-2-P	5′-FAM-TCTGTACAACATCTTGAGTCCCTT-BHQ1-3′
	M204	204-2-CP	5′-FAM-TRAACCCTAATAAAACCAAACGTTGG-BHQ1-3′
	M204V	204-2-VP	5′-FAM-CATCATCCACATARC-BHQ1-3′
	M204I	204-2-IP	5′-FAM-CCACATCATCAATATA-BHQ1-3′
	M204V	204-2-VP2	5′-FAM-CATCATCCACATARC-MGB-3′
	M204I	204-2-IP2	5′-FAM-CCACATCATCAATATA-MGB-3′

group in order to obtain a final reaction volume of 25 μL each.

PCR amplification detection of mutations in 204 HBV nucleotides

Amplifications were processed using the FAMTM/SYBR® Green channel of the SLAN®-96P Real-Time PCR System (Shanghai Hongshi Medical Technology, Shanghai, China). Cycling conditions were as follows: 5 m at 95°C, followed by a two-step cycling stage of 40 cycles of 15 s at 95°C and 60 s at 59°C. Samples were considered positive when presenting a typical amplification curve with a Ct value of ≤35. Analyses of samples with Ct values of 35 and 38 were repeated.

Plasmid design and standard curve protraction

In order to obtain standard curves for the purposes of quantification and estimation of efficacy of the new real-time PCR protocol, a specific plasmid containing the M204V mutation was designed and synthesized by Sinogene Biotech (Beijing, China). Standard curves based on a 10-fold dilution of the plasmid were plotted. The formula [DNA (copies/μL)=6.02×10^{23} (copies/mol)×DNA concentration (μg/μL)/[DNA length (bp)×660(daltons/bp)] was used to calculate the DNA load. Next, we analyzed linear relationships based on amplification results. Log DNA concentration (horizontal axis; X) was plotted against Ct values (vertical axis; Y).

Results and discussion

We enrolled 32 CHB patients with drug-resistant gene mutations. The patients' information is shown in Table 2.

We designed two different probes for 204 nucleotide mutations, to ensure the presence of a mutation, and subsequently designed two specific probes each for M204V or M204I. The specificity and sensitivity of the probes were confirmed via the amplification of positive serum samples, which had been verified as containing the M204V/I mutation via Sanger sequencing. We repeated a portion of the experiments on the same specimen in order to verify its accuracy and repeatability. An acceptance criterion of Ct <38 was used. According to the results of 16 positive serum samples, the 204-2-P probe was more specific and sensitive. The true positive rates were 100% and 62.4%, respectively. Both 204-2-VP and 204-2-IP were abandoned due to the absence of a fluorescent signal. The specificity and sensitivity of probes 204-2-VP2 and 204-2-IP2 were confirmed. The positive rate of detection was 100% and the false positive rate was 0%.

Amplification curves of the 10-fold dilution series of the plasmid are shown in Fig. 2. The efficiency of quantitative real-time PCR was confirmed via plasmid-based curves. The plasmid DNA concentration was 2,000 ng/μL and DNA length was 2,980 bp. We introduced relevant parameters to the formula, stated above, to obtain the initial DNA load. The serial dilution curves indicated that the relationship between Ct value and log DNA concentration was linear (Fig. 3). The linear coefficient of determination (R^2) was 0.996, indicating a significant linear relationship for the quantitative real-time PCR. The slope of the standard curve was −3.723.

We diluted plasmid concentrations to 6.12×10^6, 6.12×10^5, and 6.12×10^4 copies/mL to determine the reproducibility of real-time PCR. Six repetitions of the dilution series were analyzed. The coefficients of variation of log DNA concentration were 0.43%, 0.69%, and 0.58%, respectively, which indicate good reproducibility.

The accuracy of real-time PCR was determined via a 10-fold dilution series ranging from 6.12×10^6 to 6.12×10^2 copies/mL. The plasmids were diluted using a mixture of serum samples from patients with CHB. The average log DNA concentration of five replicates tested simultaneously under similar operating conditions was estimated. The biases were −0.174, −0.085, 0.005, and 0.06, respectively, which indicated good correctness.

In order to determine the limit of detection, the plasmids were diluted to 10^6, 10^5, 10^4, 10^3, 10^2, and 10 copies/mL. A Ct value <38 and a standard deviation of 0.5 were considered as the reference standard. The coefficients of variation of log DNA concentration were 0.43%, 0.69%, 0.58%, 2.79%, 5.54%, and 4.89%, respectively. The standard deviations of log DNA concentration were 0.03, 0.04, 0.03, 0.11, 0.16, and 0.12, respectively. The positive rates of detection were 100%, 100%, 100%, 100%, 83.3%, and 50%, respectively. Thus, the limit of detection for real-time PCR was determined to be 10^3 copies/mL.

A comparative analysis of 32 serum samples from CHB patients suspected of showing signs of drug-resistance was conducted. Both real-time PCR and direct sequencing protocols indicated that 13 out of the 32 samples (40.625%) were M204V-positive. One sample (3.125%) was identified as M204V-positive by direct sequencing, whereas the real-time PCR results could not be evaluated. Eighteen samples (56.25%) were identified as M204V-negative by both real-time PCR and direct sequencing. None of the samples were found to be M204V false positives. Direct sequencing is considered as the gold standard. Overall, the sensitivity of real-time PCR for detecting the M204V mutation was 92.86% (13/14), with a specificity of 100%.

Table 2. Detailed information of the 32 patients

No.	Sex	Genotype	HBV viral load, copies/mL	Mutations
1	Male	C	2.03×10^5	M204V, L180M
2	Male	B	1.317×10^5	M204V, L180M
3	Male	C	1.2×10^3	M204V, L180M
4	Female	C	1.4×10^4	M204I, L180M
5	Male	B	2.5×10^4	M204I, L180M
6	Male	B	3.03×10^2	M204I
7	Female	B	1.99×10^2	M204V, L180M, T184A
8	Female	C	4.33×10^3	M204V, M204I
9	Male	C	4.33×10^3	M204V, M204I, L180M
10	Male	C	2.3×10^7	M204V, L180M
11	Female	C	1.8×10^5	M204I
12	Female	C	10^7	M204I
13	Male	C	3.03×10^3	M204V, L180M, S202G, T184A
14	Female	B	4.79×10^3	M204V, L180M, S202G
15	Male	C	3.6×10^7	M204V, L180M, T184L
16	Female	C	2.2×10^7	M204V, L180M, T184A
17	Male	B	6.4×10^3	A181T, N236T
18	Female	B	$4.\ 3\times10^5$	A181V
19	Female	B	3.07×10^5	A181T
20	Male	C	1.68×10^4	A181T, A181V, N236T
21	Male	C	8.5×10^4	A181V
22	Male	C	2.2×10^5	M204V, L180M
23	Male	C	1.07×10^3	M204V, L180M
24	Male	C	1.8×10^3	M204V, L180M
25	Male	C	4.25×10^7	M204I, L180M
26	Female	B	3.51×10^3	M204I, L180M
27	Male	C	1.45×10^3	M204I
28	Male	C	4.2×10^4	A181T, N236T
29	Male	C	2.09×10^3	A181V
30	Male	B	5.2×10^2	A181T
31	Female	B	2×10^3	A181T, A181V, N236T
32	Female	B	1.29×10^3	A181V

Conclusions

The current study established a practical quantitative real-time PCR that monitors HBV DNA polymerase gene mutations in patients undergoing treatment with NAs, with particular reference to the M204V mutation. Compared with direct sequencing, quantitative real-time PCR is effective, low-cost, and convenient. It does not involve complex and expensive instruments. Furthermore, our quantitative detection system, which gathers a fluorescence signal from each cycle, is rapid, as each DNA test requires only 6–7 m. Moreover, the limit of detection is similar to that of direct sequencing. This new method is able to monitor HBV DNA variations quantitatively, thus providing a new method that monitors drug resistance during the early stages of therapy and assesses the relationship between genetic mutations and phenotypic resistance.

Tests that determine the limit of detection are insufficient, requiring many further tests to confirm the limitation. Further assays are required to develop systems designed to monitor other mutations, and also to establish a system for multiplex detection purposes.

Funding

This work was supported by funds from the Beijing Municipal Science & Technology Commission (No. Z171100 001017037) and the Chinese National Science and Technology Major Project (No. 2017ZX10105015002).

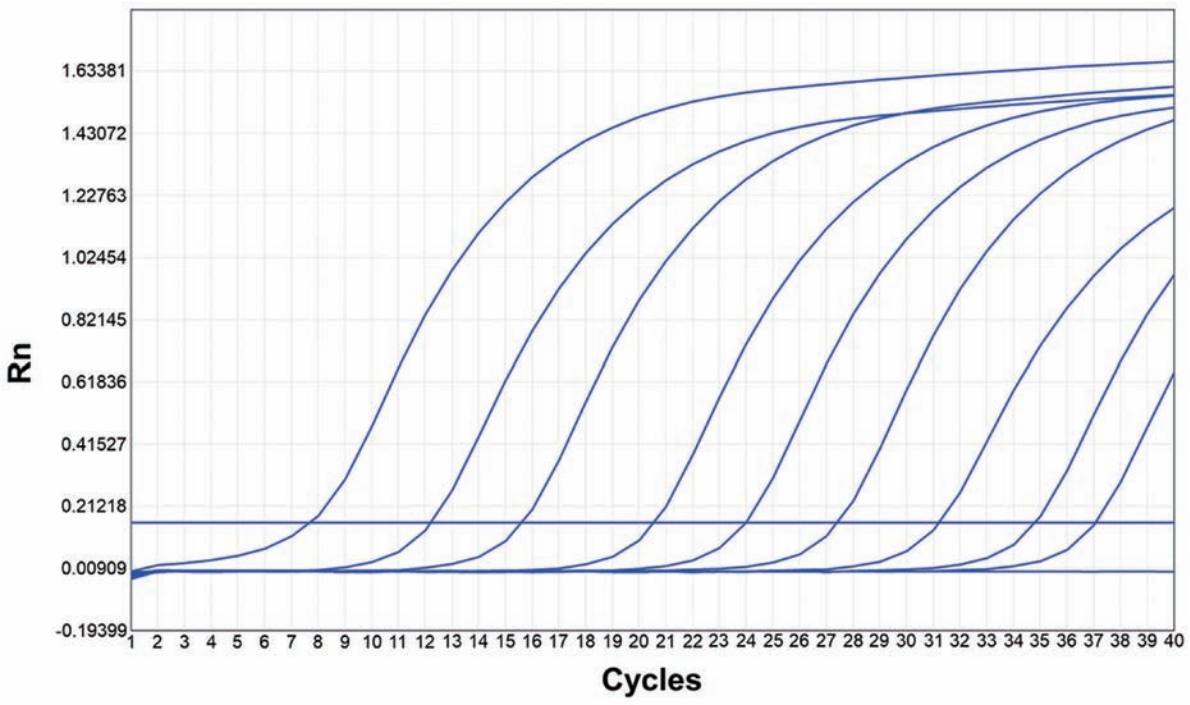

Fig. 2. Amplification curves of plasmids.

Conflict of interest

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

Author contributions

Study design (JJL, HM, YW), performance of experiments (JJL, LN), analysis and interpretation of data (JJL, GC), manuscript writing (JJL, GC), statistical analysis (JJL, GC, XML), critical revision of the manuscript (HM, YW), critical funding (HM, YW), and technical or material support (HM, LN, YW).

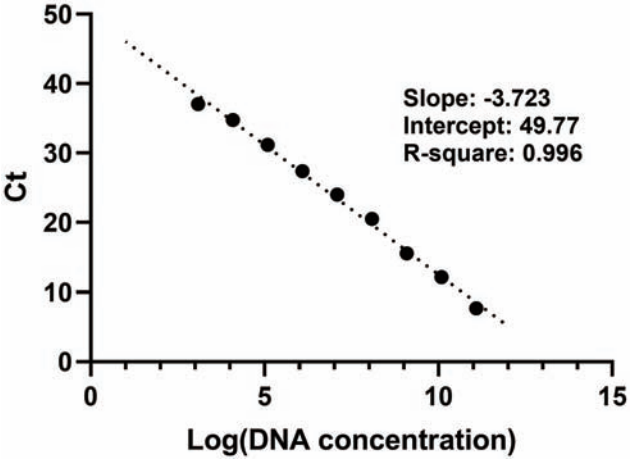

Fig. 3. Linear relationship between Ct values and log DNA concentrations.

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Journal of Clinical and Translational Hepatology **2021** vol. 9(2) | 149–159
DOI: 10.14218/JCTH.2020.00125

Original Article

Safety and Efficacy of Physical Thermal Ablation Combined Sorafenib for Hepatocellular Carcinoma: A Meta-analysis

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Received: 22 November 2020 | **Revised:** 9 February 2021 | **Accepted:** 21 February 2021 | **Published:** 8 March 2021

Abstract

Background and Aims: To compare the efficacy and safety of physical thermal ablation (PTA), including radiofrequency ablation (RFA) and microwave ablation (MWA), combined with sorafenib and physical thermal ablation alone for the control and treatment of hepatocellular carcinoma (HCC) according to the available literature. ***Methods:*** Comprehensive searches were performed on PubMed, Embase, CNKI, the Cochrane Library, China Biomedical Literature Database (known as CBM), Weipu Journal, and Wanfang Database. Meta-analysis was performed using Revman 5.3 software. ***Results:*** A total of 15 studies, consisting of 2,227 HCC patients, were selected and included in this meta-analysis. Compared with the RFA-alone group, the patients in the RFA+sorafenib group had longer 1-, 2-, and 3-year overall survival (all $p<0.05$), better overall efficacy ($p<0.0001$), longer radiofrequency interval ($p<0.001$), and lower 2-year recurrence rate ($p=0.02$). The 1-year overall survival ($p=0.003$) and overall efficacy ($p=0.002$) of the MWA+sorafenib group were also higher than those of the MWA-alone group. The incidences of adverse reactions in the RFA+sorafenib group, such as hand-foot skin reactions ($p<0.001$), diarrhea and constipation ($p=0.0001$), hypertension ($p=0.009$), and alopecia ($p<0.001$), were significantly higher than those in the RFA-alone group. ***Conclusions:*** RFA or MWA combined with sorafenib has produced a better therapeutic effect on HCC than physical thermal ablation alone; however, adverse reactions have been obvious. It is necessary to evaluate the safety of combination therapy, and pay close attention to the adverse reactions that develop in patients.

Citation of this article: Jin M, Yu Q, Liu Y, Xu W, Fu X, Ji B. Safety and efficacy of physical thermal ablation combined sorafenib for hepatocellular carcinoma: A meta-analysis. J Clin Transl Hepatol 2021;9(2):149–159. doi: 10.14218/JCTH.2020.00125.

Keywords: Physical thermal ablation; Radiofrequency ablation; Microwave ablation; Sorafenib; Hepatocellular carcinoma; Meta-analysis.

Abbreviations: CI, confidence interval; HCC, hepatocellular carcinoma; HFSR, hand-foot skin reaction; MWA, microwave ablation; OR, odds ratio; OS, overall survival; PEI, percutaneous ethanol injection; PTA, physical thermal ablation; RCT, randomized controlled trial; RFA, radiofrequency ablation; TACE, transarterial chemoembolization.

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Introduction

Hepatocellular carcinoma (HCC) is the sixth most common malignant tumor in the world. About 700,000 people die of HCC worldwide each year, with nearly half of those cases being from China.[1,2] Currently, the main treatments include liver transplantation, surgical resection, radiofrequency ablation (RFA), percutaneous ethanol injection (PEI), transarterial chemoembolization (TACE), and sorafenib.[3] Following development of medical technology and establishment of different prognosis scoring systems, like the Italian Liver Cancer tumor staging system and the Barcelona clinical liver cancer staging system, there are more therapy options for HCC patients.[4]

Surgical resection is considered to be the first-line treatment for HCC, but surgery is not always feasible due to factors such as multiple lesions, poor position, and patient status.[5] The early symptoms of liver cancer are not obvious, resulting in many patients having advanced liver cancer when they are diagnosed and missing the optimal window for surgery. The scarcity of liver sources and high costs also limit the widespread application of liver transplantation. Therefore, an effective and less invasive alternative therapy, physical thermal ablation (PTA), has been developed. PTA of the liver includes RFA and microwave ablation (MWA). Although the physical mechanisms of the two are different, they both target the tumor through imaging technology and insert the electrode into the tumor precisely. When the temperature of the tumor tissue reaches a certain level, the protein will be denatured to shrink the tumor.

A meta-analysis on the effects of RFA and hepatic resection in the treatment of liver cancer conducted by Xu *et al*.[6] showed that, compared with the hepatic resection group, the RFA group had similar 1-year overall survival (OS), lower 5-year OS, higher incidence of overall recurrence, shorter hospitalization duration and lower complication rate. Which means, compared with surgery, thermal ablation has the advantages of short duration and less complications. However, HCC patients treated with thermal ablation alone have a high recurrence rate and an unsatisfactory long-term prognosis.[7]

Sorafenib is a multi-targeted kinase inhibitor that inhibits the proliferation and differentiation of tumor cells by inhibiting the activity of B-Raf, Raf-1 and kinases in the Ras/Raf/MEK/ERK signaling pathway;[8] it can also reduce angiogenesis by inhibiting hepatocyte cytokine receptor (such as c-Kit), vascular endothelial growth factor receptors (such as the vascular endothelial growth factor receptors VEGFR-2,

VEGFR-3), platelet-derived growth factor receptors (such as the platelet-derived growth factor receptor PDGFR-β), etc.[9] A meta-analysis of 1,462 patients with unresectable HCC showed that compared with placebo, sorafenib improved disease control rate and reduced the risk of tumor progression and mortality.[10] A number of studies also pointed out that sorafenib alone or in combination with other therapies can prolong the survival of HCC patients.[11–13] However, sorafenib might delay the tissue repair after thermal ablation and adversely affect normal liver tissue. Therefore, the overall advantage of sorafenib in combination with PTA needs to be balanced, after considering its clinical efficacy effects and adverse effects.

Meta-analysis can provide a higher level of evidence for clinical decision-making by combining disaggregated data.[14] This study summarized the literature on the efficacy of PTA combined with sorafenib in the treatment of HCC, to explore the safety and efficacy of this combination therapy objectively.

Methods

Search strategy

A comprehensive literature search was conducted by two searchers on the PubMed, Embase, CNKI, Cochrane Library, China Biomedical Literature (known as CBM), Weipu Journal, and Wanfang databases on October 25–26, 2020 to identify articles published before September 2020. We collected randomized controlled trials (RCTs), controlled clinical trials, and cohort studies comparing RFA or MWA with sorafenib and PTA alone in the treatment of HCC, and reviewed the references to supplement with any missing studies. The search strategy was on the basis of the following terms: (physical thermal ablation) OR ((radiofrequency ablation OR (RFA) OR (RF ablation)) OR ((microwave ablation) OR (MWA) OR (MW ablation)) AND (sorafenib) AND ((Carcinoma, Hepatocellular) OR (HCC) OR (liver cancer) OR (liver tumor)).

Eligibility criteria

Inclusion criteria were: (1) English or Chinese language; (2) RCTs or high-quality cohort studies, quality score Jadad ≥3, Newcastle-Ottawa scale ≥5; (3) observation group treated with RFA/MWA combined with sorafenib, and control group treated with RFA/MWA alone; (4) participant Child-Pugh A/B; and (5) with data for at least one efficacy indicator (recurrence rate, survival rate, complications, radio frequency interval, etc.). Exclusion criteria were: (1) systematic review, meta-analysis, animal experiments, case reports, comments or letters; or (2) lack of required data in the results.

Quality evaluation and data extraction

Two researchers respectively scored the RCTs and non-RCTs according to the Jadad scale and the Newcastle-Ottowa scale, and independently extracted the original data according to the PICO principle (patient, intervention, comparison, and outcome), including basic information, safety indicators and effectiveness indicators.

The basic information included the first author, publication time, nationality of the patients, patient number of each group, sex ratio, age, type of study, and Child-Pugh classification. The safety indicators are the incidence of major adverse reactions, which included hand-foot skin reaction (referred to as HFSR), diarrhea and constipation, hypertension, alopecia, pyrexia, and fatigue. The effectiveness evaluation indicators included OS, recurrence rate, and overall efficacy. According to the World Health Organization solid tumor efficacy criteria, the treatment effect can be divided into four levels, namely complete remission, partial remission, the progression of the disease, and stable disease. The overall efficacy was defined as (complete remission+partial remission)/total number×100%. Different opinions on a controversial issue were solved through consultation with the third investigator.

Statistical methods

Meta-analysis and sensitivity analysis were performed using Revman 5.3 software. The categorical variables were described by odds ratio (OR) and the corresponding 95% confidence interval (CI). The continuous variables were described by mean difference and the corresponding 95% CI. The χ^2 test was used to assess heterogeneity. A fixed-effects model was applied when there was no or low heterogeneity (I^2<50%, p>0.1) and a random-effects model was applied when there was moderate or high heterogeneity (I^2≥50%, p≤0.1). The publication bias was evaluated by funnel plot analysis and Egger's test, using Stata software. A p-value of <0.05 (two-tailed) was considered statistically significant.

Results

Search results and basic information of the original literature

The process of literature screening is shown in Fig. 1. According to the criteria, this meta-analysis finally included 15 studies (3 RCTs, 5 controlled clinical trials, and 7 retrospective cohort studies).[15–29] Among these, 14 studies were high-quality and one was medium quality. A total of 2,227 patients were enrolled, of whom 1,100 were treated with PTA plus sorafenib and 1,127 were treated with PTA alone. The basic information of the studies is summarized in Table 1.

OS of HCC patients in the PTA+sorafenib group and the PTA-alone group

Seven studies, involving 1,634 individuals, reported the OS rate. The random-effects model was used because of the low grade of heterogeneity in the literature reporting OS rates at 1, 2, and 3 years OS rates (I^2=58%, 55%, and 76%, respectively). Overall, the 1- , 2- and 3-year OS rates of HCC patients in the RFA+sorafenib group were significantly higher than those of the RFA-alone group (1-year OS: OR=2.45, 95% CI: 1.25–4.79, p=0.009; 2-year OS: OR=1.87, 95% CI: 1.17–3.01, p=0.009; 3-year OS: OR=2.25, 95% CI: 1.34–4.85, p=0.004) (see Fig. 2).

MWA is another major category of physical thermal ablation, and we performed a subgroup analysis to summarize the overall survival rates of the two ablation methods. The result showed that MWA combined with sorafenib also significantly increased HCC patients' 1-year OS, with an OR of 2.74 (95% CI=1.42–5.29, p=0.009). Coupled with the results of RFA, it can be considered that HCC patients treated with PTA and sorafenib had a higher 1-year OS than those treated with PTA-alone (OR=2.43, 95% CI=1.50–3.95,

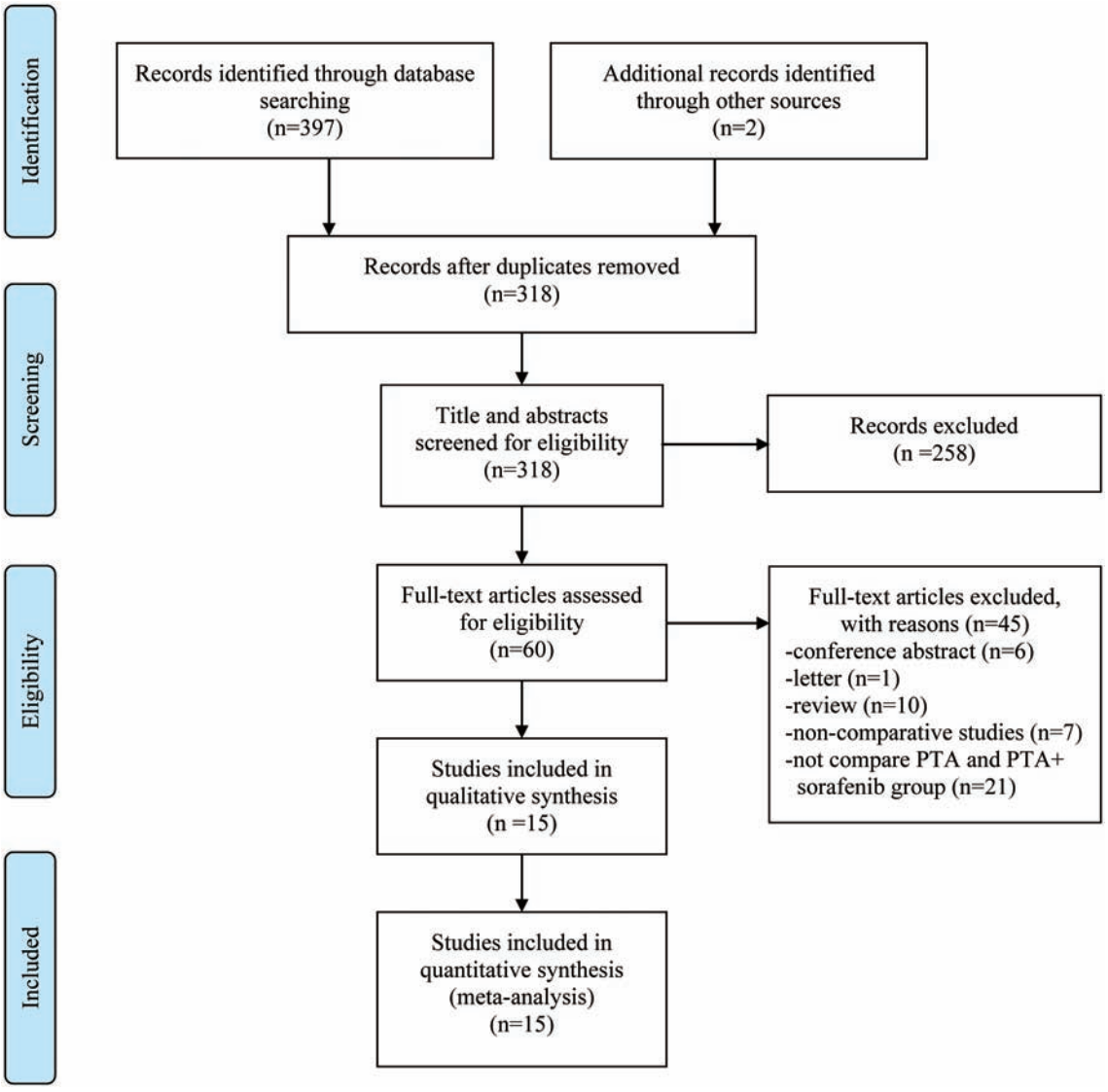

Fig. 1. Inclusion procession.

p=0.003) (see Fig. 3).

Recurrence rates of HCC patients in the RFA+sorafenib group and RFA-alone group

A total of four articles with 1,394 individuals provided information on recurrence rates. After merging them with a random-effects model, the OR of the 2-year recurrence rate was 0.40 (95% CI=0.18–0.87, p=0.02), indicating that the 2-year recurrence rate of HCC patients in the RFA+sorafenib group was lower than that of the RFA-alone group (see Fig. 4).

Overall efficacy of physical thermal ablation of HCC patients

Eight of the studies, involving 562 individuals, mentioned overall efficacy and were divided into two subgroups, according to different thermal ablation methods, four of which used RFA and three of which used MWA. A fixed-effects model was applied, as the studies were homogeneous (I^2=0%, p>0.10). Subgroup analysis showed that the overall efficacy of RFA combined with sorafenib for HCC patients was better than that of RFA alone (OR=2.72, 95% CI: 1.69–4.38, p<0.0001). The efficacy of MWA combined with sorafenib was also better than that of MWA alone (OR=2.18, 95% CI: 1.33–3.57, p=0.002). Overall, 312 patients were treated with PTA and sorafenib and 350 patients were treated with PTA alone; the total OR was 2.45 (95% CI=1.73–3.45, p<0.001), indicating that the overall efficacy of PTA combined with sorafenib was significantly better than that of PTA alone (see Fig. 5).

The radiofrequency interval of patients also indirectly reflects the effect of treatment. Three studies with 200 individuals documented the patient's radiofrequency interval, and a fixed-effects model was used since the heterogeneity test yielded results of p=0.21 and I^2=36%. The radiofrequency interval of HCC patients treated with RFA and sorafenib was longer than that of RFA alone (95% CI: 1.28–1.94, p <0.001), and the effect of combination therapy can be considered to be superior (see Supplementary Fig. 1.).

Table 1. Basic characteristics of the studies

Study	Nation	Type	No. of patients		Age in years[a]	Gender, male/female	Child-Pugh A, *n*	Quality score[b]
Bruix 2015[15]	Spain, China, Japan	RCT	RFA+so	556	58 (24–85)	451/105	541	5
			RFA	558	60 (19–83)	461/97	538	
Yu 2018[16]	China	RCT	RFA+so	23	58.19±4.34	17/6	13	3
			RFA	23	58.25±4.31	16/7	14	
Fu 2020[17]	China	RCT	RFA+so	51	57.4±3.8	34/17	32	4
			RFA	51	57.6±3.9	35/16	30	
Kan 2015[18]	China	CCT	RFA+so	30	53.7±9.6	24/6	12	6
			RFA	32	52.4±8.9	25/7	18	
Zhang 2015[19]	China	CCT	RFA+so	52	28–65 (51.2±13.4)	28/24	22	6
			RFA	68		31/37	43	
Wu 2016[20]	China	CCT	RFA+so	45	48±11	28/17	–	6
			RFA	45	50±9	30/15		
Gong 2017[21]	China	CCT	RFA+so	40	55.7±13.6	23/17	–	7
			RFA	50	53.9±12.4	28/22		
Sun 2011[22]	China	cohort	RFA+so	15	59.5 (35–80)	11/4	7	6
			RFA	15		12/3	9	
Feng 2014[23]	China	cohort	RFA+so	64	49.7±11.2	59/5	64	6
			RFA	64	50.9±10.9	59/5	64	
Fukuda 2014[24]	Japan	cohort	RFA+so	15	72.8±7.9	6/9	15	7
			RFA	30	72.1±8.0	8/22	25	
Li 2014[25]	China	cohort	RFA+so	8	53±6.8	5/3	–	5
			RFA	12	48±11.1	8/4		
Zhu 2018[26]	China	cohort	RFA+so	40	55.5±10.9	3/37	33	6
			RFA	66	54.1±10.1	5/61	48	
Hua 2012[27]	China	cohort	MWA+so	42	57.2 (38–74)	28/14	32	5
			MWA	48	54.7 (39–72)	32/16	37	
Zheng 2013[28]	China	CCT	MWA+so	44	56.2 (38–74)	30/14	34	5
			MWA	50	55.7 (39–72)	33/17	38	
Sun 2018[29]	China	cohort	MWA+so	45	48.5±7.2	31/14	41	6
			MWA	45	47.6±7.1	30/15	40	

[a]Age recorded with mean±standard deviation or median (interquartile range).

[b]Jadad score and Newcastle-Ottawa scale were used for RCTs and non-RCTs respectively.

Abbreviations: CCT, control clinical trial; MWA, microwave ablation; RCT, randomized clinical trial; RFA, radiofrequency ablation; so, sorafenib.

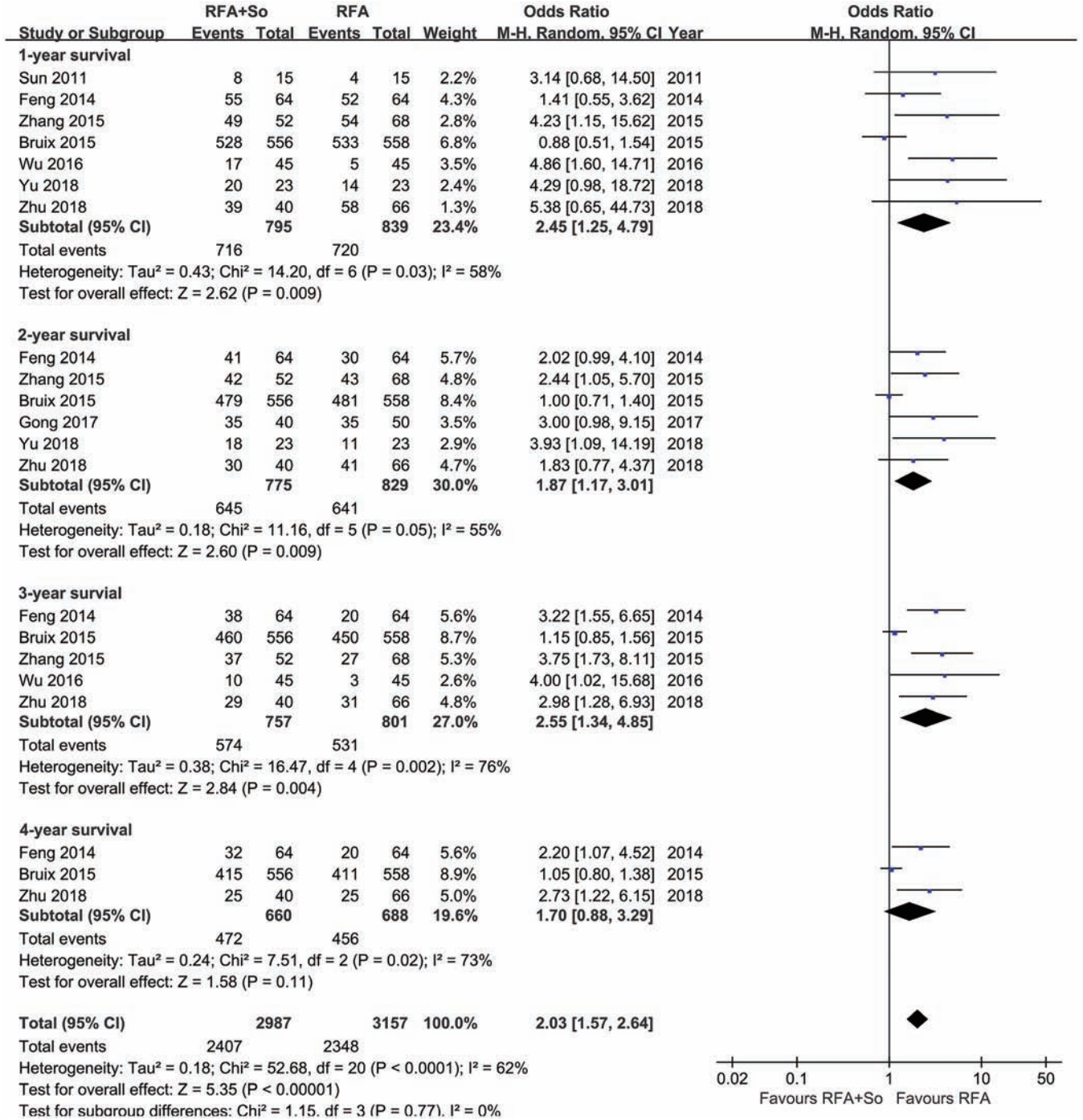

Fig. 2. OS in the RFA+sorafenib group and the RFA-alone group.

Adverse effects in the RFA+sorafenib group and the RFA-alone group

A total of nine studies, involving 1,561 individuals, reported adverse effects after treatment. The incidences of adverse reactions, such as HFSR (OR=47.57, 95% CI: 17.54–129.04, $p<0.01$), diarrhea and constipation (OR=7.01, 95% CI: 2.57–19.08, $p=0.005$), hypertension (OR=8.52, 95% CI: 1.70–42.73, $p=0.009$), and alopecia (OR=15.26, 95%CI: 9.43–24.71, $p<0.01$), in the combination therapy group were significantly higher than those in the PTA-alone group (see Fig. 6).

Sensitivity analysis and publication bias

The sensitivity analysis showed that the study conducted by Bruix *et al*.[15] significantly affected the calculated ORs of OS

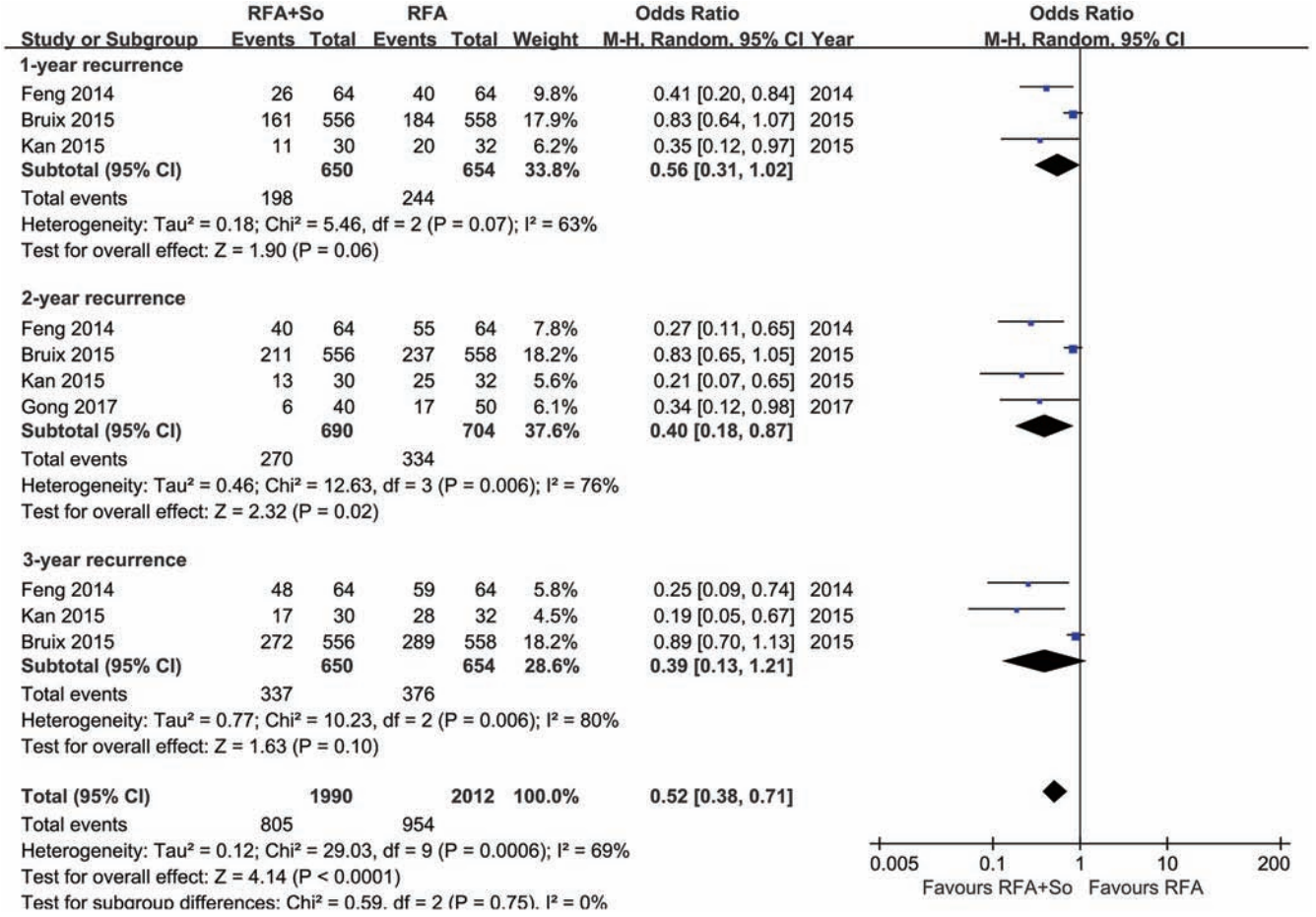

Fig. 3. Subgroup analysis of 1-year OS in the RFA and MWA treatment groups.

and recurrence rate. After excluding this trial, the I^2 value declined to 0%. The funnel plot of the 1-year OS revealed asymmetry; however, after excluding the Bruix 2015[15] study, the Egger's test results yielded p=0.107, indicating that there was no substantial publication bias (see Fig. 7). Further reading and evaluation found that this study was a high-quality RCT, recorded a number of indicators, provided results that were credible, and had application value. The reason why the results were different from others might be due to the variety of ethnicity (Spain, China, and Japan) and large sample size (n=1,114). In summary, we retained this high-quality study.

Discussion

Ablation combined with chemotherapy has been widely used in cancer treatment, such as for small cell lung cancer, advanced renal cell carcinoma, etc.[30,31] In the treatment of HCC, PTA has the advantages of little trauma and quick recovery, and can be applied as treatment of multiple times. However, the size of the lesion and the existence of heat dissipation make it difficult to ablate completely, resulting in a higher risk of local recurrence. When the diameter of the tumor is more than 3.0 cm, it is more likely to recur.[32,33] Therefore, reducing the recurrence rate of tumors after thermal ablation has become the focus of treatment improvement.

RFA+sorafenib: Higher survival rate and efficiency, longer radiofrequency interval and lower recurrence rate

Sorafenib, a kinase inhibitor, has been shown to have a synergistic effect in combination with RFA. It has the function of inhibiting angiogenesis in tumors, thereby reducing heat loss and indirectly enhancing ablation. Sorafenib itself also inhibits tumor cell proliferation and differentiation. From the perspective of evidence-based medicine, in order to explore whether the therapeutic effect of RFA combined with sorafenib is better than using RFA alone, a total of 15 studies were included in the meta-analysis, 12 of which were about RFA and included 939 patients treated with RFA plus sorafenib and 1,014 patients treated with RFA alone. We summarized the original literature and found that the RFA+sorafenib group had higher 1-, 2-, and 3-year OS and lower 2-year recurrence rate compared with the RFA-alone group; RFA combined with sorafenib also significantly extended the RF interval, which indirectly reduced the RFA-related adverse effect, and also reduced the pain and financial burden of patients.

However, the survival and recurrence indicators of the RFA+sorafenib group were not always better than the RFA-alone group. The 4-year survival rate and the 1- and 3-year recurrence rates were not significantly different between the two groups. Probably due to (1) a large-sample-size study,[15] there was no difference in the 1- and 3 recurrence

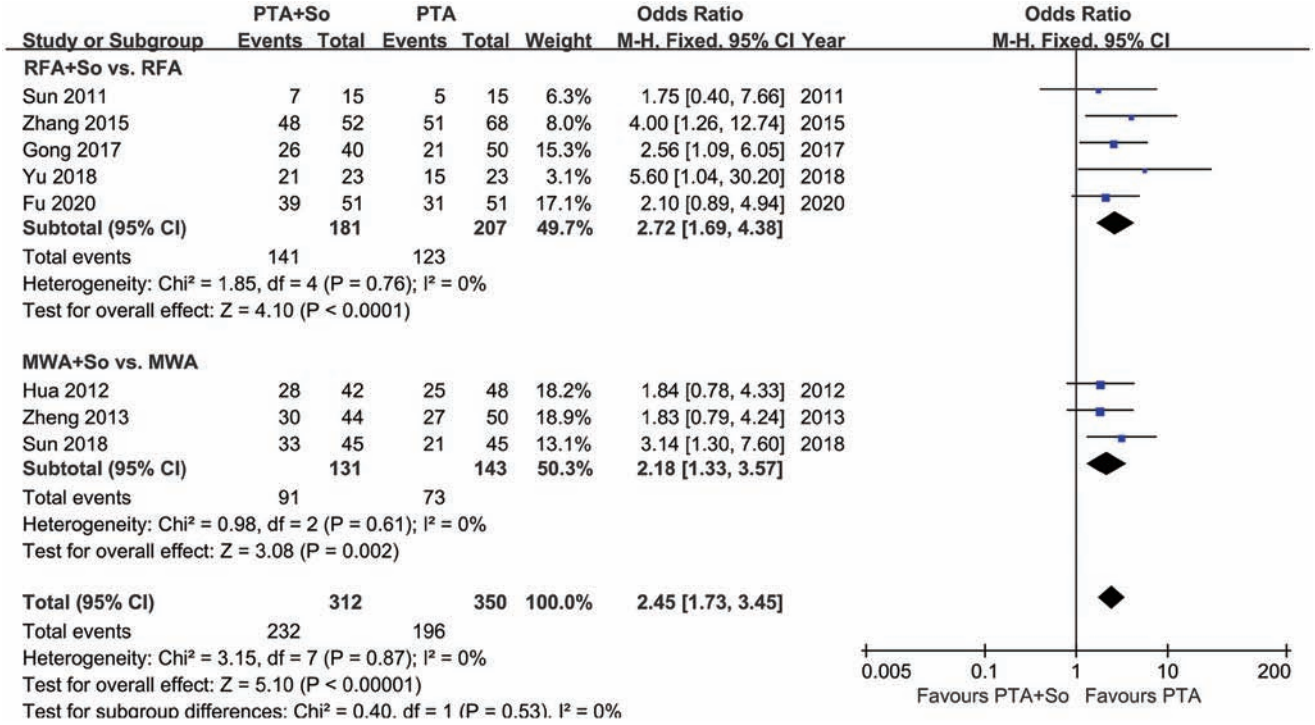

Fig. 4. Recurrence rates in the RFA+sorafenib group and the RFA-alone group.

rates between the two groups, since that study had a large weight in the meta-analysis, and (2) few studies reported the 4-year OS and, the 1- and 3-year recurrence rates and the heterogeneity was significant.

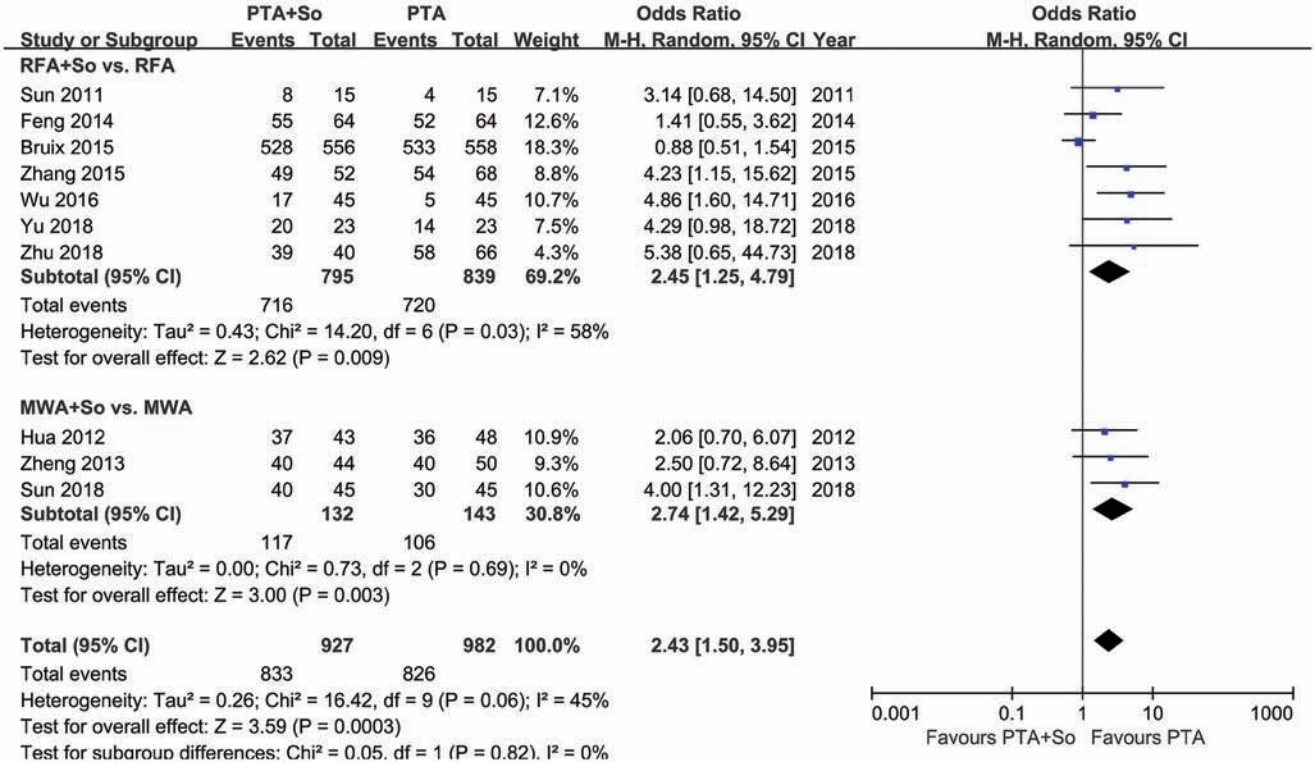

Fig. 5. Subgroup analysis of overall efficacy of RFA and MWA in HCC patients.

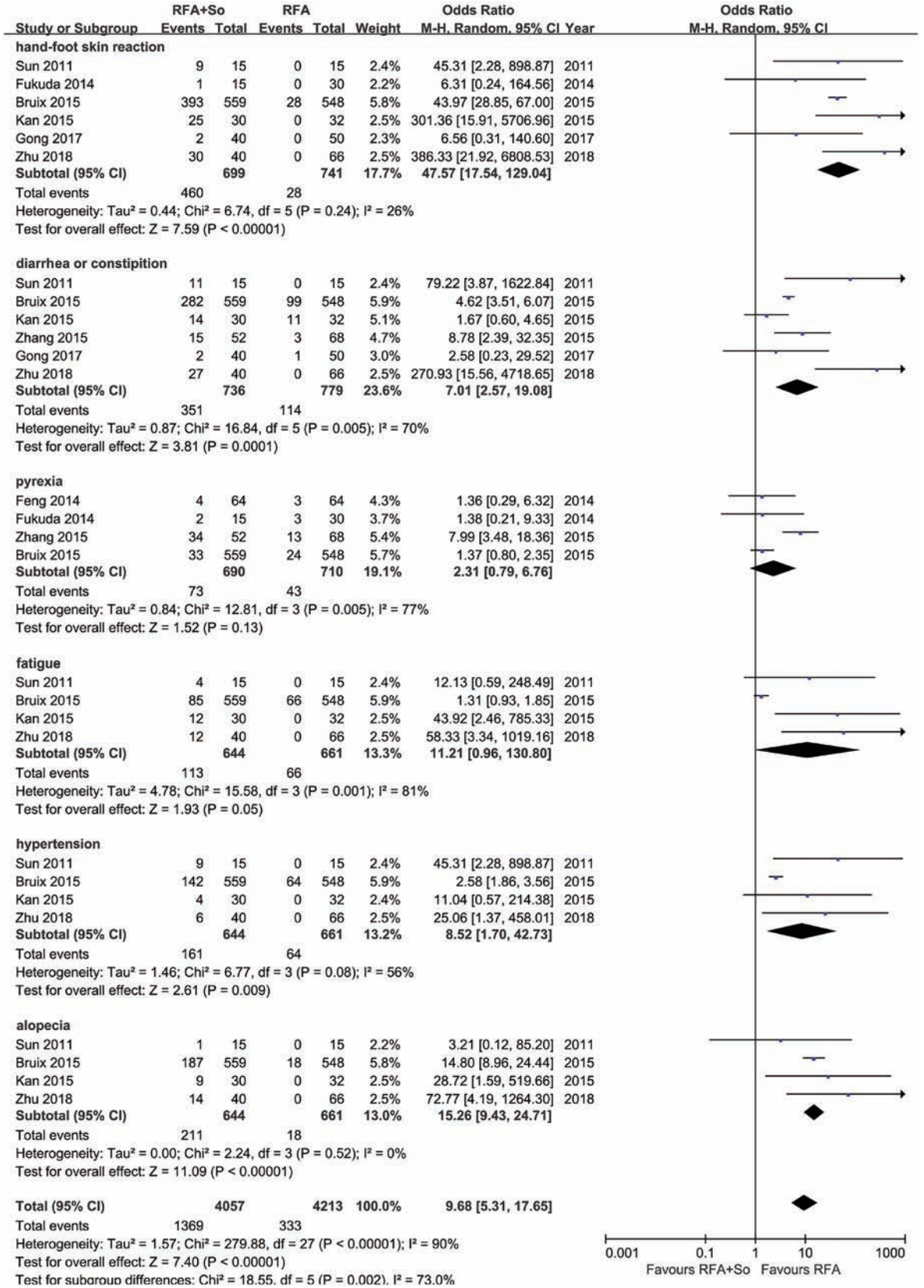

Fig. 6. Adverse effects of HCC patients in the RFA+sorafenib group and the RFA-alone group.

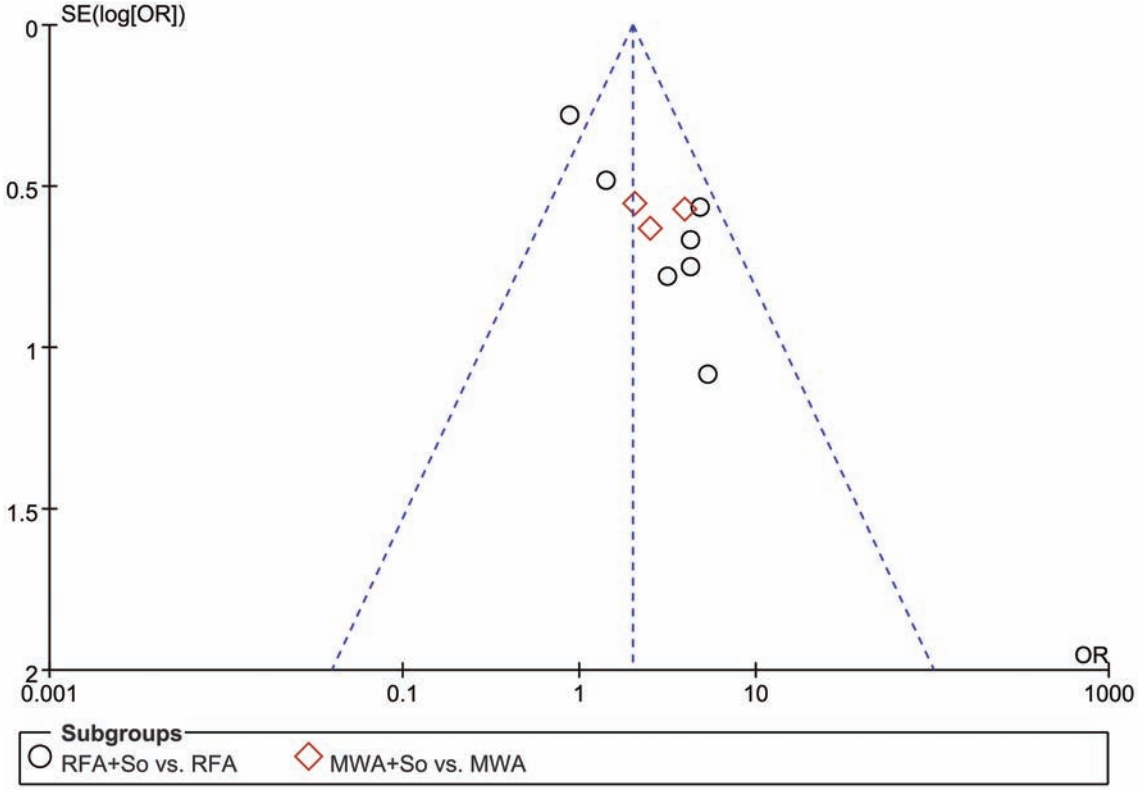

Fig. 7. Funnel plot of 1-year OS with 95% CI to assess publication bias.

Sorafenib brings significant adverse reactions

Sorafenib is a tyrosine kinase inhibitor that inhibits various receptors, such as RAF-1, VEGFR-2, and FLT-3, and has been used for first-line treatment of liver cancer, with millions of patients benefiting from it.[11] Our meta-analysis showed that combined use with sorafenib can significantly improve the effect of RFA, but the incidence of adverse reactions was significantly higher. Studies have suggested that the mechanism of HFSR may be that sorafenib can inhibit VEGF and PDGF, and damage the capillaries. When the hands and feet are subjected to direct pressure, the vessels are again mechanically damaged, thus prompting an inflammatory response and blister formation.[34] As we know, severe adverse effects may lead to the suspension of treatment and ultimately affect the patient's survival. There were also studies suggesting that diarrhea in HCC patients treated with sorafenib predicts better OS.[35,36] Reig *et al*.[37] believed that the development of dermatological adverse events within 60 days after the start of sorafenib was associated with better survival. Regardless of whether the adverse reaction can directly affect survival, it may affect the quality of life and cause a dose change or interruption of sorafenib, which may limit the anti-tumor effect. Therefore, standardized treatment and dose adjustment of sorafenib are necessary to improve the survival and life-quality of HCC patients.

RFA and MWA

Both RFA and MWA are PTA techniques. The mechanism of RFA is that the polar molecules in the tumor will run at high speed under the influence of high-voltage, generating heat to kill tumor cells. The MWA electrode emits microwaves, and the polarity of the water molecules in the tumor is changed by the voltage to form an alternating electric field to generate heat. MWA has higher thermal efficiency, faster heating speed, better heat dissipation resistance,[38] the ablation range is larger, the operation time is also shorter, and the MWA consumables are relatively inexpensive, which can reduce the economic burden on patients. Compared with RFA, the development of MWA was relatively late, first put into clinical application in China and Japan. Therefore, there were few MWA studies and limited survival index in this meta-analysis.

From the subgroup analysis of the existing literature, the total effective rate and 1-year survival rate of the combination group were higher than in the control group. There have been studies comparing the efficacy and safety of RFA and MWA, but the findings are still inconclusive. After summarizing the high-quality RCTs, this can serve as a topic of our next evaluation.

Limitations and summary

The studies selected for this meta-analysis were not all RCTs. Retrospective cohort studies have selection and recall biases, and the number of original articles was limited. In addition, the entire study cohort for this meta-analysis was incomprehensive in regards to race, and most of the research population was Chinese, with some Japanese and Spanish. The 2015 epidemiological survey report showed that nearly 27% of the world's cancer deaths are from China, and HCC is the second most common cause of cancer-related mortality in China, after lung cancer.[39] Due to hepatitis B virus infection, aflatoxin exposure, alcohol abuse and environmental pollution, China has become the country

with the highest incidence of liver cancer (about 55% of the world's full rate) and with the largest number of deaths.[40] China has a long way to go to control the incidence and mortality of liver cancer, which may be one of the important reasons why most of the research population in this meta-analysis was Chinese. Except for overall efficacy and radiofrequency interval, the heterogeneity of other indicators was remarkable. This may be due to differences in sample size, tumor size and number, patient age, and previous treatment history.

Chen *et al*.[41] have also conducted a meta-analysis of the efficacy of RFA combined with sorafenib in patients with HCC. Their results showed no significant difference in OS and recurrence rates, but only included five articles of RFA+sorafenib vs. RFA alone. In addition, their meta-analysis also included literature that did not only use RFA as a control group, which might affect the overall reliability. Our study strictly screened out 15 original studies, and our conclusions are different from theirs.

Nowadays, the ideal therapy for HCC is still being explored. A comprehensive comparative analysis of the scoring system for HCC published in the *World Journal of Hepatology* told us that an appropriate scoring system should be selected according to the patient's situation and a personalized strategy for HCC patients should be developed.[4] The characteristics and liver function of the patients determine whether the treatment is curative or only palliative, or a combination of the two, as mentioned in this study (RFA+sorafenib). Therefore, the formulation of HCC treatment strategy needs the combination of multiple disciplines, such as hepatobiliary surgery, interventional radiology, and oncology. Personalized settings and adjustments would be needed at any time, according to the patient's progression, adverse reactions and complications.

According to the current meta-analysis, PTA combined with sorafenib in the treatment of HCC is better than RFA or MWA alone. Patients who undergo the combination therapy should be closely observed for changes in skin, blood pressure, body temperature, gastrointestinal reactions, etc., to reduce the dose or discontinue the drug if necessary, and actively initiate symptomatic treatment. Although the subgroup analysis and random-effects models were applied in this study, the heterogeneity between studies may still affect the reliability of the results. The superiority of PTA plus sorafenib over PTA-alone still needs to be confirmed by more high-quality studies.

Conclusions

RFA or MWA combined with sorafenib has better efficacy than PTA alone; however, the adverse reactions are obvious. It is necessary to evaluate the safety of combination therapy and pay close attention to the adverse reactions of patients.

Funding

This work was supported by the National Natural Science Foundation of China (81802805), the Natural Science Foundation of Jilin Province (20200201490JC) and the translation-clinical joint foundation of the First Hospital of Jilin University (No.2020-ZL-04).

Conflict of interest

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

Author contributions

Study concept and design (BJ, QY), acquisition of data (YL, MJ), analysis and interpretation of data (MJ, BJ), drafting of the manuscript (MJ), critical revision of the manuscript for important intellectual content (XF, WX), administrative, technical, or material support, study supervision (QY).

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Journal of Clinical and Translational Hepatology **2021** vol. 9(2) | 160–168
DOI: 10.14218/JCTH.2020.00105

Original Article

Histone Deacetylase Inhibitors Romidepsin and Vorinostat Promote Hepatitis B Virus Replication by Inducing Cell Cycle Arrest

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Received: 11 November 2020 | **Revised:** 29 January 2021 | **Accepted:** 24 February 2021 | **Published:** 8 March 2021

Abstract

Background and Aims: Chronic hepatitis B virus (HBV) infection is a global public health challenge. HBV reactivation usually occurs in cancer patients after receiving cytotoxic chemotherapy or immunosuppressive therapies. Romidepsin (FK228) and vorinostat (SAHA) are histone deacetylase inhibitors (HDACi) approved by the Food and Drug Administration as novel antitumor agents. The aim of this study was to explore the effects and mechanisms of HDACi treatment on HBV replication. ***Methods:*** To assess these effects, human hepatoma cell lines were cultured and cell viability after FK228 or SAHA treatment was measured by the CCK-8 cell counting kit-8 assay. Then, HBV DNA and RNA were quantified by real-time PCR and Southern blotting. Furthermore, analysis by western blotting, enzyme-linked immunosorbent assay (ELISA), immunohistochemistry, and flow cytometry was performed. ***Results:*** FK228/SAHA treatment significantly promoted HBV replication and biosynthesis in both HBV-replicating cells and HBV-transgenic mouse model. Flow cytometry assay indicated that FK228/SAHA enhanced HBV replication by inducing cell cycle arrest through modulating the expression of cell cycle regulatory proteins. In addition, simultaneous inhibition of HDAC1/2 by FK228 promoted HBV replication more effectively than the broad spectrum HDAC inhibitor SAHA. ***Conclusions:*** Overall, our results demonstrate that cell cycle blockage plays an important role in FK228/SAHA-enhanced HBV replication, thus providing a potential avenue for rational use of HDACi in patients with chronic hepatitis B.

Citation of this article: Yang Y, Yan Y, Chen Z, Hu J, Wang K, Tang N, *et al*. Histone deacetylase inhibitors romidepsin and vorinostat promote hepatitis B virus replication by inducing cell cycle arrest. J Clin Transl Hepatol 2021;9(2):160–168. doi: 10.14218/JCTH.2020.00105.

Introduction

Hepatitis B virus (HBV) infection continues to be a serious public health problem worldwide. Chronic HBV infection is a major risk factor for developing cirrhosis and hepatocellular carcinoma (HCC).[1] The World Health Organization estimated that 257 million people were infected with HBV and approximately 887,000 people die from HBV/HCC complications every year.[2] HBV, the prototype virus of the *Hepadnaviridae* family that productively infects hepatocytes, contains a partially double-stranded DNA genome surrounded by an icosahedral capsid.[3] The HBV genome is only 3.2 kb long and contains four partially overlapping open reading frames, which transcribe four different lengths of mRNA, including pregenomic RNA, precore mRNA, preS/S mRNA, and X (*i.e.* HBx) mRNA.[4] HBV covalently closed circular DNA (cccDNA) serves as the transcriptional template for all viral RNAs, and accounts for HBV persistence.[5] Despite the availability of effective anti-HBV drugs, reactivation of HBV infection is a challenging issue for patients with a chronic HBV infection who undergo cytotoxic chemotherapy or immunosuppressive therapies.[6]

HBV reactivation was firstly reported in patients with hematological malignancies by Wands *et al*.[7] It is usually defined as a sudden increase in HBV DNA levels (≥10-fold relative to baseline), or an absolute increase that is more than 10^5 copies/mL in patients undergoing chemotherapy or immunosuppressive therapy.[8,9] Reactivation of HBV could lead to severe complications, such as acute liver failure or even death.[10] However, eradicative therapy for HBV is still unavailable, leading to great concerns about the potential consequences of HBV reactivation.

Histone deacetylase inhibitors (HDACi) are a family of

Keywords: HBV reactivation; Histone deacetylase inhibitor; Romidepsin; Vorinostat; Cell cycle.

Abbreviations: ALT/AST, alanine and aspartic aminotransferase; CCK-8, cell counting kit-8; CDK, cyclin-dependent kinase; C/EBPα, CCAAT/enhancer-binding protein α; EBV, Epstein-Barr virus; ELISA, enzyme-linked immunosorbent assay; FDA, Food and Drug Administration; FK228, romidepsin; HBcAg, hepatitis B core antigen; HBeAg, hepatitis B e-antigen; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; HBV cccDNA, HBV covalently closed circular DNA; HBV-Tg mice, HBV-transgenic mice; HCC, hepatocellular carcinoma; HDACi, histone deacetylase inhibitors; HIV-1, reactivates human immunodeficiency virus type 1; NTCP, solute carrier family 10 member 1; PBS, phosphate-buffered saline; qRT-PCR, real-time polymerase chain reaction; SAHA, suberoylanilide hydroxamic acid; siRNA, small interfering RNA.

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natural or synthetic small-molecule inhibitors of histone deacetylases (HDACs) which are widely applied in treating disorders such as hematopoietic malignancies and psychiatric disorders in clinical trials.[11] However, some clinical studies have indicated that virus reactivation is one of the severe complications that occur after HDACi treatment. Romidepsin (FK228), a cyclic peptide that specifically inhibits Class I HDACs, can efficiently induce the lytic cycle reactivation of Epstein-Barr virus (EBV).[12–14] Vorinostat (SAHA), a broad-spectrum HDACi,[15] reactivates human immunodeficiency virus type 1 (commonly known as HIV-1) via activation of the PI3K/Akt pathway in infected patients receiving highly active antiretroviral therapy.[16–19] However, the effects of FK228/SAHA on HBV replication are still unknown.

Herein, we investigated the role of two FDA-approved HDACi, FK228 and SAHA, in HBV replication *in vitro* and *in vivo*. Our results will provide useful information for further studies on chemotherapy-induced HBV reactivation, especially for patients undergoing HDACi treatment.

Methods

Antibodies and reagents

The antibodies used in this study were as follows: anti-hepatitis B core antigen (HBcAg) (B0586) and anti-HBsAg (NB100-62652) from Dako (Glostrup, Denmark) and Novus Biological (Littleton, CO, USA) respectively, anti-β-actin (BL005B) from Biosharp (Hefei, China). Antibodies to p21 (Cat. no. 2947T), p27 (Cat. no. 3686T) and p-cyclin-dependent kinase (CDK)2 (Cat. no. 2561S) were obtained from Cell Signaling Technology (Danvers, MA, USA). Antibodies to Rb (Cat. no. BS1310), p-Rb (Cat. no. BS4165P), cyclin A (Cat. no. BS1083), cyclin B1 (Cat. no. BS6874), cyclin E (Cat. no. BS1085), cyclin D1 (Cat. no. BS1741), CDK2 (Cat. no. BS1050), HDAC1 (Cat. no. BS5576), and HDAC2 (Cat. no. BS1162) were all from Bioworld (St. Louis Park, MN, USA).

Cell culture, transfection and viral infection

The human HCC cell lines HepG2 and HepAD38 were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (Gibco, Rockville, MD, USA), 100 U/mL penicillin and 100 μg/mL streptomycin (HyClone, Logan, UT, USA). In addition, HepAD38 cells were cultured in the presence of 500 ng/mL tetracycline to suppress HBV pregenomic RNA transcription and 500 μg/mL G418 to maintain the stably transfected HBV genome. HepG2 cells were infected with adenovirus Ad-HBV1.3 (kindly provided by Prof. Michael Nassal, University Hospital Freiburg, Freiburg, Germany) for 12 h to sustain all processes of HBV replication. All transfections were performed by using Lipofectamine™ 3000 transfection reagent (Invitrogen, Carlsbad, CA, USA).

HepG2 cells stably expressing sodium taurocholate co-transporting polypeptide, termed as HepG2-NTCP cells, were inoculated with HBV virus, as previously described.[20] Briefly, the supernatants of HepAD38 cells were collected and precipitated with 8% polyethylene glycol 8000. Then, the HepG2-NTCP cells were infected with concentrated HBV virus for 16 h in the presence of 4% polyethylene glycol 8000 and 1% DMSO.

Animal models

HBV-transgenic, termed as HBV-Tg mice, raised by the Laboratory Animal Center of Chongqing Medical University (SCXK (YU) 2017-0001), were kindly provided by Prof. Ning-shao Xia from the School of Public Health (Xiamen University, Xiamen, China). Mice (6–8 weeks-old, n=6 for each group) were intraperitoneally injected with FK228, SAHA (2.5 mg/kg, 40 mg/kg body weight, respectively) or phosphate-buffered saline (PBS; control) every other day for seven times. At the 14th-day after injection, all mice were euthanized. Then, mice serum and liver tissue specimens were collected for RT-qPCR, Southern blotting, and immunohistochemical staining. All the animal procedures were conducted in compliance with the protocols approved by the Laboratory Animal Center of Chongqing Medical University, following the national guidelines and regulations for experimental animal use and welfare of China.

Chemical inhibitors and small interfering RNAs

The HDAC1 and HDAC2 inhibitors romidepsin (FK228) and the broad-spectrum HDAC activity inhibitor vorinostat (SAHA) were purchased from Selleckchem (Houston, TX, USA). Both of the chemicals were dissolved in DMSO and stored at −20 °C. Small interfering RNAs (siRNAs) were obtained from TranSheep Bio (Shanghai, China). The siRNA sequences targeting human HDAC1, HDAC2 are listed in Supplementary Table 1. Scrambled siRNA was used as a control. Cells were transfected with specific or non-specific control siRNAs at a concentration of 20 μM by Lipofectamine™ 3000 (Invitrogen) according to the manufacturer's protocol.

Cell growth curve and cell viability assay

The proliferation capacity of HepAD38 and HepG2 cells was measured by using a cell growth curve. Cells were seeded into 96-well plates (2,000–3,000 cells/well), with three replicate wells per group. Then, the cells were incubated with various concentrations of FK228 (0, 0.5, 1, 2.5, 5, 10, 20 and 40 nM) and of SAHA (0, 0.25, 0.5, 1, 2.5, 5, 10 and 20 μM) for 120 h. Cell number was enumerated automatically every 24 h, and the growth curve was plotted. The cell viability was measured by the CCK-8 cell counting kit-8 assay (Dojindo Molecular Technologies Inc., Kumamoto, Japan). Cells were seeded into 96-well culture plates for 12 h with three replicate wells per group, then various concentrations of FK228 and of SAHA were added to the cells for 120 h. The absorbance at 450 nm was measured after the treatment with 10 μL of CCK-8 for 1 h.

Quantification of HBV DNA via RT-qPCR

RT-qPCR was performed to detect the HBV DNA copies. Cells or liver tissues were first lysed at 37 °C for 30 m with cell lysis buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA, 2% sucrose, and 1% NP-40). Then, the mixture was centrifuged at 13,000 × g for 5 m and the supernatant was treated with micrococcal nuclease (Cat. no. 70196Y; Affymetrix, Santa Clara, CA, USA) and $CaCl_2$ for 60 m at 37 °C to eliminate residual DNA. Then, EDTA was used to terminate the reaction. A 35% polyethylene glycol 8000 solution was used for precipitation and a 0.5 mg/mL proteinase K solution (Cat. no. 3115879001; Roche Diagnostics GmbH, Mannheim, Germany) was used for digestion of viral DNAs at 45 °C for 12 h. Nucleic acids were purified via phenol:chloroform:isoamyl alcohol (25:24:1) extraction three times and precipitated with ethanol. Then, the SYBR Green qPCR Master Mix (Bio-Rad, Hercules, CA, USA) was used to perform qPCR with the indicated primers (Supplementary Table 1). The pCH9/3091 plasmid (containing 1.1 copies of HBV genome) served as a

template for the standard curve.

Quantification of HBV cccDNA via RT-qPCR

Cells or liver tissues were lysed at 37 °C for 20 m with cell lysis buffer (50 mM Tris-HCl pH 8.0, 10 mM EDTA, 150 mM NaCl, 1% SDS), and then the lysate was incubated with 2.5 M KCl overnight. After centrifugation at 14,000 × *g* for 30 m, the supernatant was extracted by phenol:chloroform:isoamyl alcohol (25:24:1) three times and precipitated with isopropanol. The extraction was treated with Plasmid-Safe ATP-Dependent DNase (Epicenter, Madison, WI, USA) to remove double-stranded DNA, and then the RT-qPCR was performed with the indicated primers (Supplementary Table 1) to determine HBV cccDNA.

RNA isolation and RT-qPCR

Total RNA was extracted using TRIzol (Invitrogen) and reverse transcribed using Moloney murine leukemia virus reverse transcriptase (A3500; Promega, Madison, WI, USA), in accordance with the manufacturer's instructions. RT-qPCR was performed to quantify mRNA levels, using the SYBR Green qPCR Master Mix (Bio-Rad) and a Bio-Rad CFX Connect Real-time PCR Detection System (Bio-Rad), according to the manufacturer's instructions. HBV 3.5-kb mRNA was standardized to genomic β-actin. The primer sequences are listed in Supplementary Table 1.

Southern blotting

Southern blotting was performed as previously described.[21] Briefly, extracted DNA samples were separated via electrophoresis in 1% agarose gel. After denaturation in a solution of 0.5 M NaOH and 1.5 M NaCl, and neutralization in a solution of 1 M Tris-HCl (pH 7.4) and 1.5 M NaCl, the DNA fragments were transferred onto a nylon membrane (Cat. no. 11417240001; Roche Diagnostics GmbH). Then, the membrane was fixed via ultraviolet-crosslinking. A digoxigenin-labeled full-length HBV genome probe (Digoxigenin High Prime DNA Labeling and Detection Starter Kit; Roche Diagnostics GmbH) was used to detect HBV DNA via hybridization.

Western blotting

For SDS-PAGE and immunoblotting, cells or liver tissues were lysed in whole cell lysis buffer (Cat. no. P0013; Beyotime, Nantong, China). The protein concentration of the homogenates was measured by BCA protein assay (Cat. no. BCA02; Dingguo, Beijing, China). Then, the protein samples were boiled at 100 °C for 10 m. The boiled protein samples were subjected to gel electrophoresis and then were electrotransferred to polyvinylidene difluoride membranes (Cat. no. IPVH00010; Millipore, Billerica, MA, USA). The immunoblots were incubated at 4 °C overnight with primary antibodies. Horseradish peroxidase-coupled secondary antibodies (Abcam, Cambridge, UK) were applied on the 2nd day. At last, the blots were visualized by using Clarity Western ECL Substrate (Bio-Rad).

Detection of HBV antigen, alanine and aspartic aminotransferase (ALT/AST)

Quantification of hepatitis B surface antigen (HBsAg) and hepatitis B e-antigen (HBeAg) in culture supernatants and in mouse serum were assayed by ELISA kits (Kehua Bio-Engineering, Shanghai, China). Serum ALT/AST was measured with ELISA kits (Kanglang, Shanghai, China), according to the manufacturer's protocols.

Immunohistochemistry

After fixation in 4% paraformaldehyde for 24 h, liver tissue samples were embedded in paraffin according to standard procedures. The resultant sections were incubated with anti-HBcAg (Cat. no. B0586; Dako, Glostrup, Denmark) separately. Subsequently, the slides were incubated with secondary anti-rabbit IgG (Cat. no. ZB-2301; ZSGB-BIO, Beijing, China) and visualized using 3, 3′-diaminobenzidine (ZSGB-BIO). After rinsing, the samples were dehydrated, treated with xylene for transparency, and scanned with an Olympus BX61 microscope.

Flow cytometry analysis

Cells were synchronized by starvation with 1% fetal bovine serum for 72 h, after treatment with HDACi FK228/SAHA, or transfection with an siRNA; then, the cells were re-stimulated with 10% fetal bovine serum. The cells were then fixed with 70% alcohol at 4 °C overnight, and resuspended in PBS with propidium iodide and RNaseA for 30 m before application to a flow cytometry assay (FACS Calibur; BD Biosciences, San Jose, CA, USA).

Statistical analysis

Data were expressed as mean ± standard deviation. Data were analyzed using one-way analysis of variance for multiple comparisons and Student's *t*-test for between-group comparisons. A *p*-value less than 0.05 was considered statistically significant.

Results

HDACi directly promotes HBV replication in vitro

To investigate the effects of FK228 and SAHA on HBV replication, we first explored the optimal dose of FK228 and SAHA in the stable HBV-expressing HCC cell line HepAD38 and transient HBV-replicating cells (HepG2 cells infected with AdHBV-1.3, HepG2-HBV1.3), respectively. As shown in Supplementary Fig. 1A and 1C, cell proliferation of HepAD38 and HepG2-HBV1.3 was not affected by FK228 up to a concentration of 10 nM, or SAHA up to 5 μM. In addition, the cytotoxic effects of the two HDACi were measured by CCK-8 assay. The EC_{50} values of FK228 and SAHA were 27.10 nM and 17.61 μM in HepAD38 cells, and 29.59 nM and 18.18 μM in HepG2 cells, respectively (Supplementary Fig. 1B and 1D). Thus, HepAD38 and HepG2-HBV1.3 cells were incubated with FK228 at concentrations between 1 and 5 nM, or with SAHA between 0.5 and 2.5 μM, for 72 h to investigate their effects on HBV replication in a safe range.

We observed that FK228 and SAHA significantly stimulated HBV replication in a concentration-dependent manner (Fig. 1). Treatment with FK228 and SAHA accounted for a significant increase of HBV 3.5-kb RNA levels (Fig. 1A and Supplementary Fig. 2C), HBV cccDNA levels (Fig. 1B), and HBV DNA levels ($p<0.01$; Fig. 1C and Supplementary Fig. 2D), as determined by qPCR. Moreover, FK228/SAHA treat-

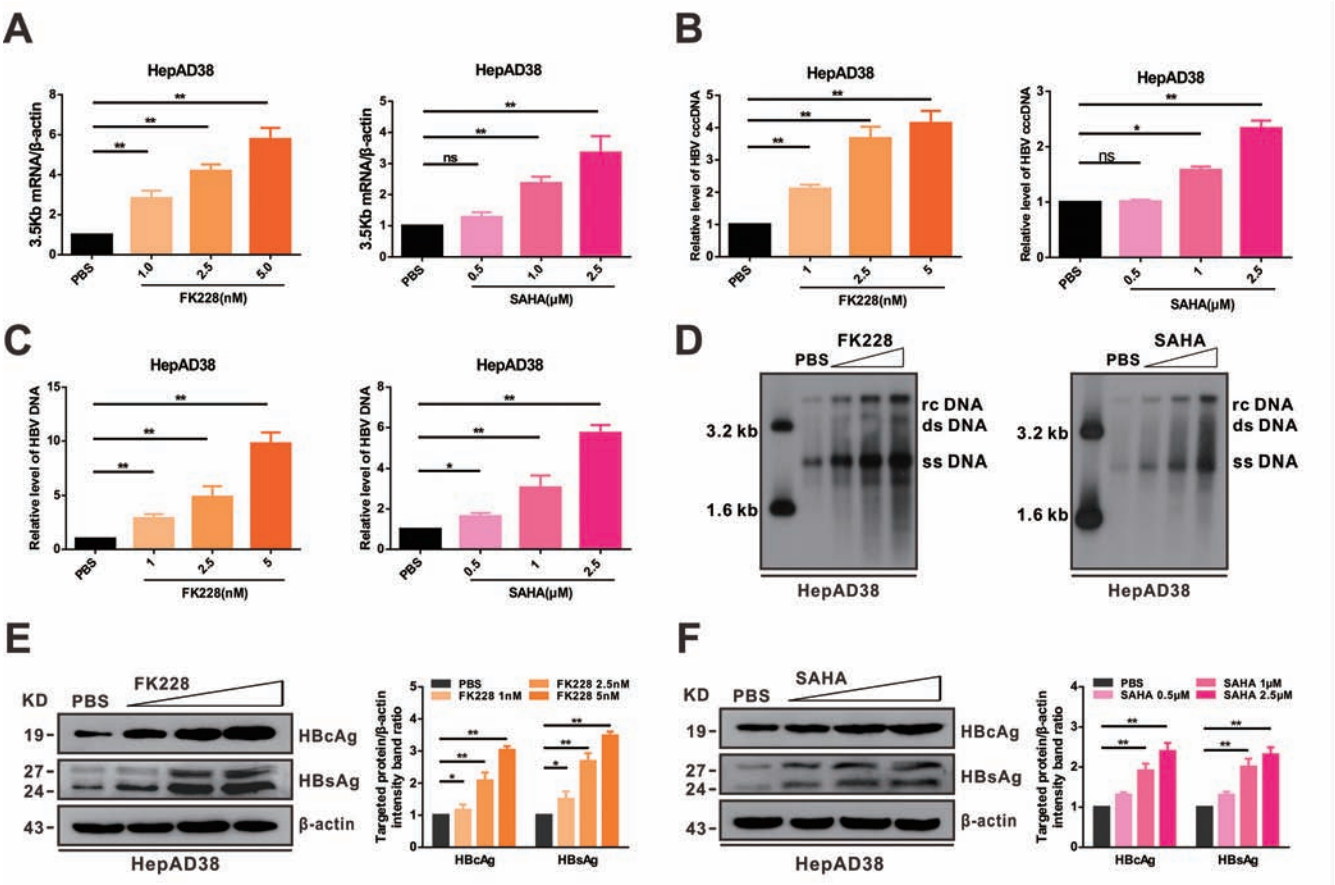

Fig. 1. HDACi enhance HBV DNA replication, viral protein production, and virion secretion. (A–C) Quantification of HBV 3.5-kb mRNA levels, intracellular HBV cccDNA levels, and HBV DNA levels by RT-qPCR assay in HepAD38 cells treated with FK228 (left) and SAHA (right). (D) Southern blotting to determine intracellular HBV replicative intermediates (RIs) treated as previously described in HepAD38 cells. rc DNA, relaxed circular DNA; ds DNA, double-stranded DNA; ss DNA, single-stranded DNA. (E–F) Western blotting to assess expression levels of HBcAg and HBsAg in HepAD38 cells treated with FK228 (left) and SAHA (right). Relative levels of HBcAg and HBsAg were measured by densitometry. Values represent the mean ± standard deviation (n=3, performed in triplicate), with statistical significance by comparison with PBS indicated by *p<0.05 and **p<0.01.

ment enhanced HBV replicative intermediates (Fig. 1D and Supplementary Fig. 2F), intracellular expression of HBsAg and HBcAg (Fig. 1E, 1F and Supplementary Fig. 2E), and HBsAg and HBeAg levels in the supernatants of culture medium (p<0.01; Supplementary Fig. 2A–B). In addition, similar results were observed in HBV-infected HepG2-NTCP cells after HDACi treatment (p<0.01; Supplementary Fig. 5A–D). Interestingly, FK228 promoted HBV replication more significantly than SAHA, with approximately a 10-fold increase in HBV DNA levels compared to a 6-fold change obtained with SAHA treatment (p<0.01). These results demonstrate that FK228 and SAHA promote HBV replication and biosynthesis in HBV-expressing hepatoma cells.

FK228/SAHA block the cell cycle of HBV-replicating cells

HDACi contribute to apoptosis and growth arrest by inducing cell cycle arrest in cancer cells. To investigate the effects of FK228 and SAHA on cell cycle distribution during HBV replication, the HepAD38, HepG2-HBV1.3 and HBV-infected HepG2-NTCP cells were cultured with 5 nM FK228 or 2.5 μM SAHA for 72 h, respectively, and flow cytometry was performed. As shown in Fig. 2A and Supplementary Fig. 3A, treatment with FK228/SAHA approximately induced a 2-fold decrease (p<0.01) in the S phase and a corresponding increase in the G1 phase in HBV-replicating cells, suggesting that FK228/SAHA treatment induced hepatocytes to stall at G1 phase and prevent cells from G1/S transition.

As mentioned earlier, FK228 is a HDAC1 and HDAC2 selective inhibitor, while SAHA is a broad-spectrum HDACi. To further elucidate the influence of HDAC1 and HDAC2 on the cell cycle in HBV-expressing hepatoma cells, siRNAs were used to lower HDAC1 and/or HDAC2 expression. As previously indicated, simultaneous silencing of HDAC1/2 significantly promoted HBV replication compared with inhibiting HDAC1 or HDAC2 alone (p<0.01; Fig. 3A–C, Supplementary Fig. 4A–B).

Additionally, we also examined the cell cycle-related protein levels after cells were treated with FK228/SAHA. The cyclin-dependent kinase inhibitors p21 and p27, which are key regulators of G1/S transition, were significantly upregulated (Fig. 2B, Supplementary Fig. 3B and Supplementary Fig. 5E) while the positive cell cycle regulators cyclin A, cyclin B1, cyclin D1, cyclin E, p-Rb, and p-CDK2 were apparently downregulated (Fig. 2C, Supplementary Fig. 3C, and Supplementary Fig. 5E), indicating that the cell cycle was arrested by FK228/SAHA treatment at G1 phase. Moreover, FK228 showed a more remarkable inhibitory effect on the cell cycle than SAHA; meanwhile, the siHDAC1+2 showed a

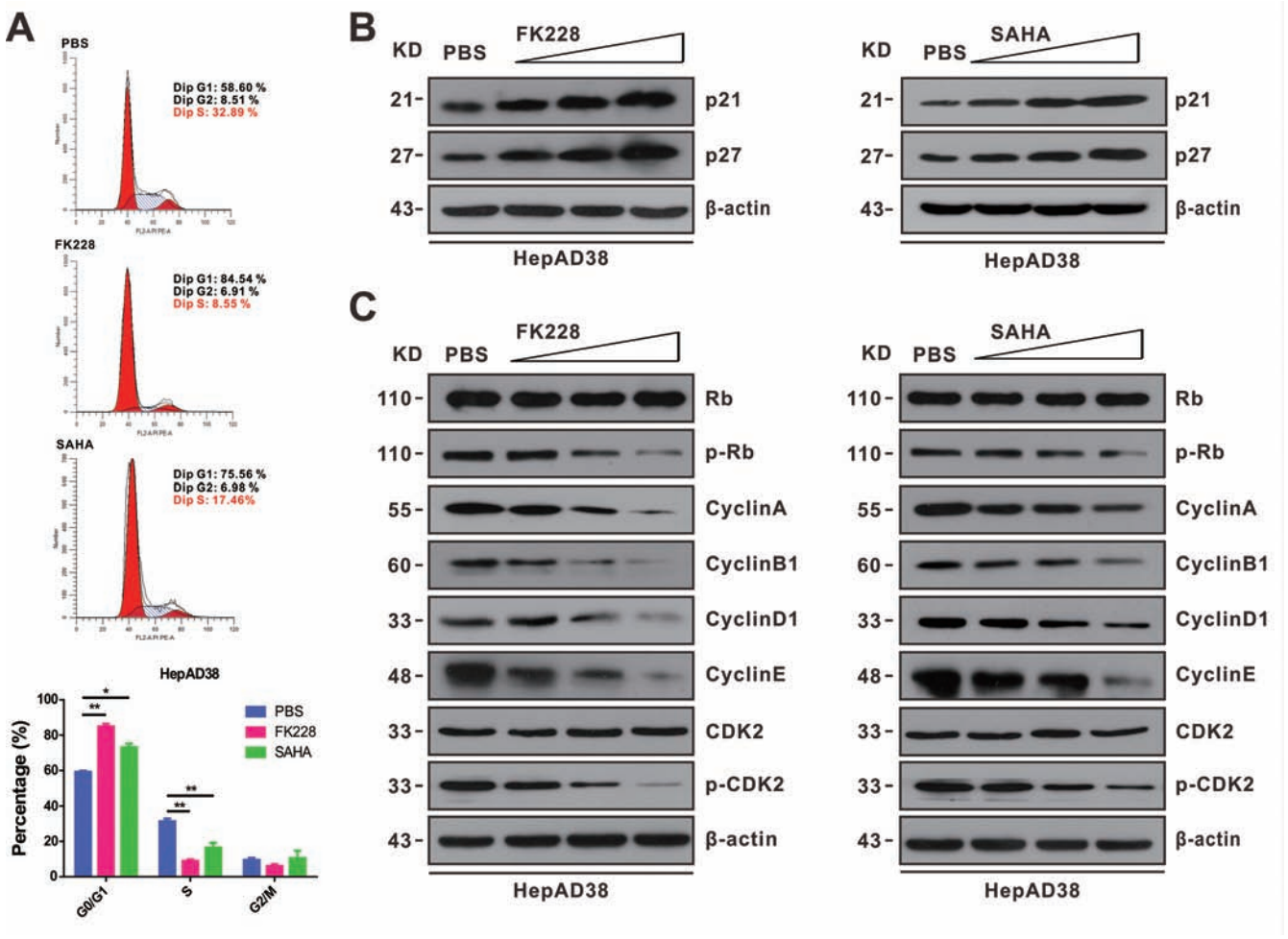

Fig. 2. Effect of HDACi on the cell cycle in HBV-expressing hepatoma cells. HepAD38 were incubated with different concentrations of FK228 (0, 1, 2.5, and 5 nM) and SAHA (0, 0.5, 1, and 2.5 μM) for 72 h. (A) Cell cycle distribution was detected by flow cytometry analysis. (B) Protein levels of CDK inhibitors p21 and p27 were detected by western blotting. (C) Protein levels of cell cycle-related proteins Rb, p-Rb, cyclin A, cyclin B1, cyclin D1, cyclin E, CDK2, and p-CDK2 were detected by western blotting. Values represent the mean ± standard deviation (n=3, performed in triplicate), with *p<0.05 and **p<0.01 vs. PBS control.

similar effect to FK228 (Fig. 3D–F and Supplementary Fig. 4C–D). Taken together, FK228/SAHA treatment, as well as transcriptional inhibition of HDAC1 and HDAC2, significantly promotes HBV replication by blocking the cell cycle at G1 phase.

FK228/SAHA promote HBV replication by inducing cell cycle arrest

Several studies have reported that HBV replication is cell cycle-dependent and highly associated with the growth status of hepatocytes.[22,23] To further explore the relationship between enhanced HBV replication and cell cycle blockage induced by HDACi, we used siRNA to knockdown CDK inhibitors p21 and p27 to promote cell cycle conversion especially G0/G1 to S phase; then, we treated cells with FK228 or SAHA and examined HBV replication. We found that p21 and p27-knockdown significantly impaired the enhanced HBV replication induced by HDACi treatment, when compared with the negative control group which was followed with HDACi (Supplementary Fig. 6A–D). Conversely, when cells were first cultured with serum-free media to induce G0/G1 arrest and subsequently treated with FK228 or SAHA, HBV replication showed a higher level than the serum-free group (Supplementary Fig. 6E).

FK228/SAHA enhance HBV replication in vivo

Finally, we examined the effects of FK228 and SAHA on HBV replication in the HBV-Tg mouse model. After the six HBV-Tg mice were treated with 2.5 mg/kg FK228, or 40 mg/kg SAHA or PBS (control) for 2 weeks, administration of FK228 and SAHA significantly increased serum levels of HBeAg and HBsAg secretion to varying degrees (p<0.05; Fig. 4A). Meanwhile, HBV 3.5-kb RNA, HBV cccDNA, and HBV DNA levels in liver tissues were also significantly upregulated by the FK228/SAHA treatment (p<0.01; Fig. 4C), consistent with results in the HBV-replication cell model. Furthermore, HBcAg levels in hepatocytes (examined by immunohistochemistry) also increased significantly after HDACi treatment (Fig. 4D).

A previous study had indicated that HBV reactivation after chemotherapy could generally induce liver injury.[24] In order to determine whether HDACi treatment could induce liver injury in HBV-Tg mice, we examined a serum inflammation marker (*i.e.* ALT/AST level) after FK228/SAHA treat-

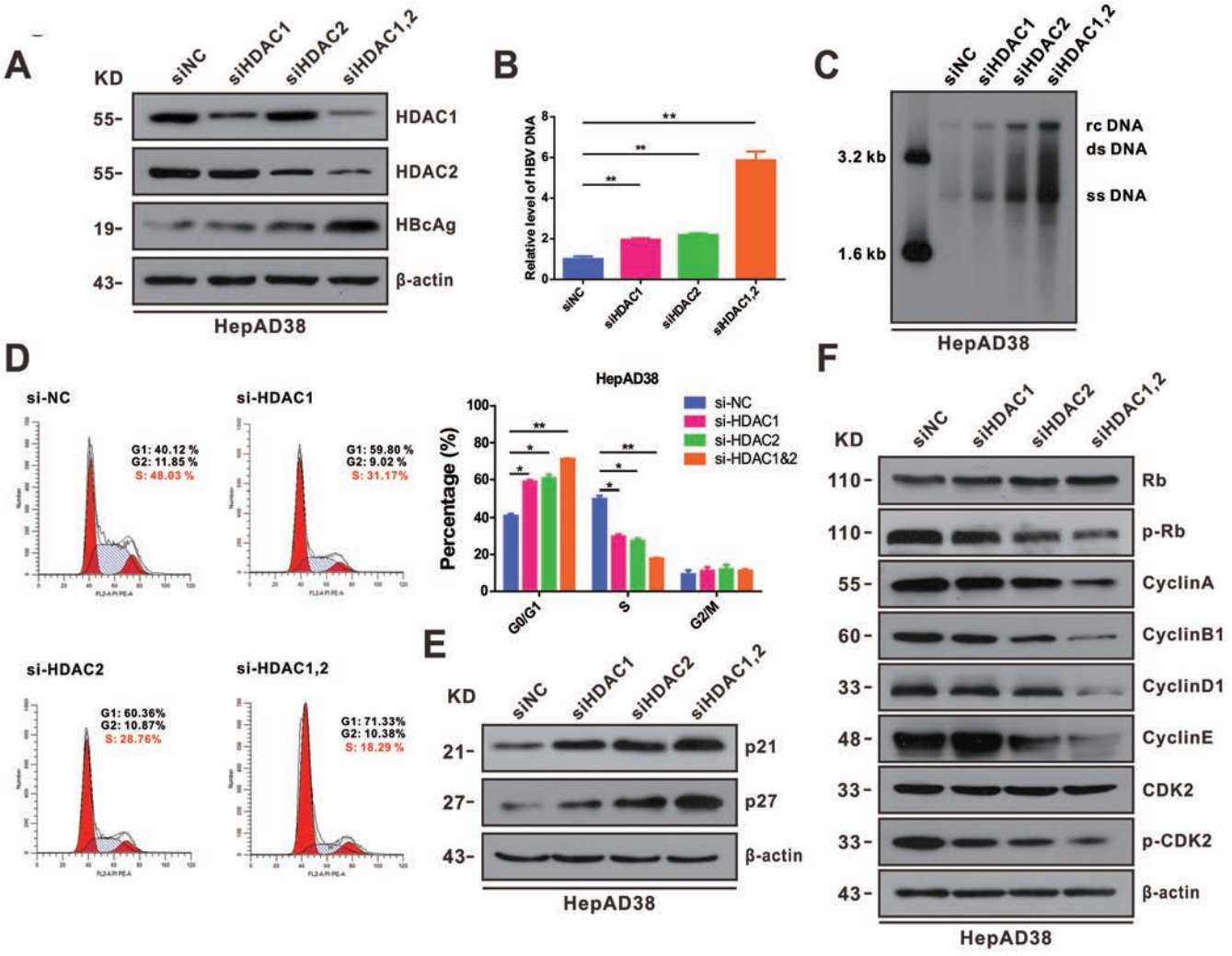

Fig. 3. Transcriptional HDAC1/2 inhibition leads to cell cycle blockage in HBV-expressing hepatoma cells. HepAD38 cells were transfected with 20 μM siRNA duplexes against HDAC1, HDAC2, or HDAC1+2 for 72 h. (A) Protein levels of HDAC1, HDAC2, and HBcAg detected by western blotting. (B) Intracellular HBV DNA level detected by RT-qPCR assay. (C) Intracellular HBV replicative intermediates (RIs) by Southern blotting. (D) Cell cycle distribution detected by flow cytometry analysis. (E) Protein levels of p21 and p27. (F) Protein levels of Rb, p-Rb, cyclin A, cyclin B1, cyclin D1, cyclin E, CDK2, and p-CDK2. Values represent the mean ± standard deviation (n=3, performed in triplicate), with *p<0.05 and **p<0.01 vs. siNC control.

ment. The ALT and AST levels were significantly elevated (p<0.05; Fig. 4B). Overall, administration of FK228/SAHA enhanced HBV replication and aggravated liver damage in the HBV-Tg mice. Additionally, FK228 showed a more significant damage effect than SAHA in the HBV mouse model.

Discussion

Previous studies have suggested that the immune system may be suppressed by systemic chemotherapy, leading to HBV reactivation. Moreover, it has been reported that chemotherapy can enhance the interactions between the promyelocytic leukemia protein and HBV core protein, which inhibit promyelocytic leukemia-associated HDAC activity, eventually leading to HBV exacerbation.[25] In fact, as early as 2009, it was reported that HDACi induce HBV reactivation and liver damage,[26] but the detailed molecular mechanisms remain to be explored to date. In this study, we investigated the effects of HDACi FK228 and SAHA on HBV reactivation. Further analysis revealed that cell cycle arrest played an important role in FK228/SAHA-induced HBV replication.

The HDAC family is composed of 18 different enzymes, which are divided into four separate classes on the basis of their homology to yeast proteins.[27] Several HDACs are involved in HBV replication. For example, Pollicino *et al*.[28] found that recruitment of HDAC1 onto the HBV cccDNA can inhibit HBV replication. Moreover, inhibition of HDAC4 by miRNA-548ah can inhibit the deacetylation of histones combining with cccDNA, therefore, enhancing the replication of cccDNA.[29] Additionally, acetylated histone H3 also participates in HBV DNA replication.[30] However, the exact roles of HDACi in HBV replication are still unknown. In this study, we demonstrated that both the selective HDAC1 and HDAC2 inhibitors romidepsin and broad-spectrum HDACi vorinostat promoted HBV replication in a concentration-dependent manner *in vitro*; FK228 had a more significant effect than SAHA. We further investigated the effects of FK228 and SAHA *in vivo*, and found that these two HDACi could also induce HBV reactivation in HBV-Tg mice.

HDACi represent a new wave of anticancer drugs due to

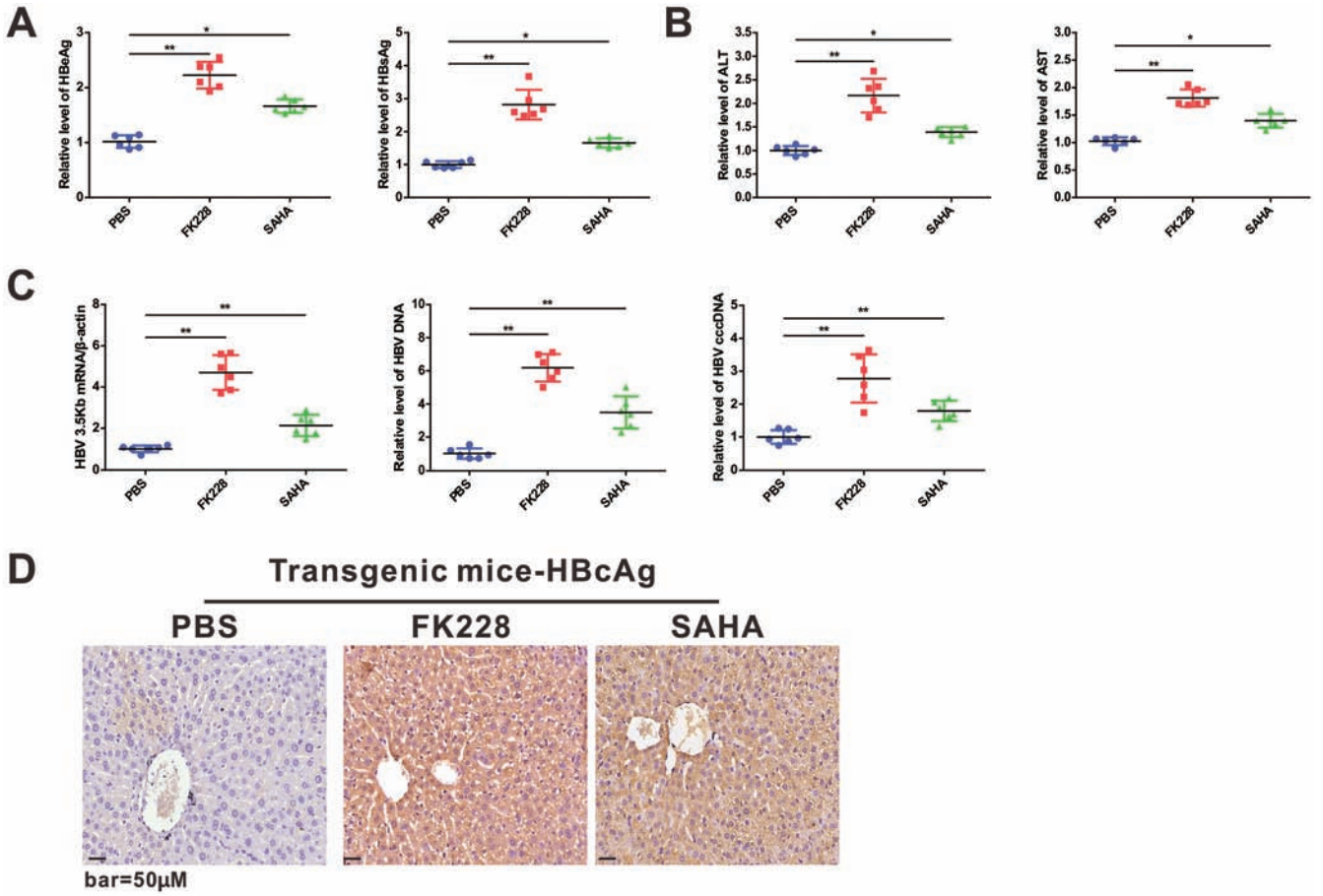

Fig. 4. Effect of HDACi in C57-HBV-Tg mice. HBV-Tg mice (6–8 weeks-old, n=6) were intraperitoneally injected with FK228, SAHA (2.5 mg/kg and 40 mg/kg body weight, respectively) or PBS (control) every other day for seven times. After the final injection, serum and liver tissue specimens were collected. (A–B) Quantification of HBeAg, HBsAg, and ALT/AST in mouse serum by ELISA. (C) Quantification of 3.5-kb mRNA, HBV DNA, and HBV cccDNA in liver tissues by RT-qPCR. (D) Immunohistochemistry analysis of HBcAg in liver tissues, scale bar: 50 μm. Values represent the mean ± standard deviation (n=3, performed in triplicate), with *p<0.05 and **p<0.01 vs. PBS control.

their biological effects, such as regulation of gene expression, cell cycle progression, and apoptosis.[31,32] Increased expression of p21/p27 and, subsequently, cell cycle arrest are common responses to HDACi treatment.[33] Consistent with a previous study, our results showed that the expression of cyclin-dependent kinase inhibitors, such as p21 and p27, were significantly increased, while cyclin D1/E and CDK2, which are required for the G1/S checkpoint complex, were significantly decreased after FK228/SAHA treatment.

In fact, several studies have indicated that HBV replication is cell cycle-dependent and highly associated with the growth status of hepatocytes.[22,23] For example, HBV replication was more active in quiescent cells but slowed down when cells started to divide,[23] the number of viral replicative intermediates was significantly increased after cells reached confluence,[34] and the chemotherapy drug vincristine could strongly stimulate HBV replication through S-phase arrest.[35] Therefore, our results strongly suggest that FK228/SAHA treatment enhances HBV replication mainly by stalling cell cycle progression at the G1 phase and preventing its transition to S phase. It is well known that viruses such as influenza A virus,[36] EBV,[37] and hepatitis C virus[38] usually utilize different strategies to deregulate cell cycle checkpoint controls, and regulate cell proliferation in order to replicate in cells and produce new progeny. HDACi-induced G1/S phase arrest might contribute to *de novo* HBV replication before cells enter into mitosis, and regulation of some transcriptional factors which are involved in cell growth and differentiation, such as E2F transcription factor 5, CCAAT/enhancer-binding protein α (C/EBPα), hepatocyte nuclear factor 4 alpha, etc.[39] Increased p21 has been shown to recruit C/EBPα to the HBV promoter after doxorubicin treatment;[40] thus, FK228/SAHA-induced elevated p21/p27 expression might stimulate HBV replication by increasing the recruitment of C/EBPα or other transcriptional factors to HBV promoters. In addition, HDACi treatment has been reported to induce the depletion of uracil DNA glycosylase, which can counteract APOBEC3-induced hypermutations.[41] As such, the enhanced host genome mutation may also, subsequently, help the virus to escape the immune system and evolve.[42] The exact mechanism needs further investigation.

Recent reports have indicated that FK228 could potently induce the lytic cycle of EBV through inhibition of HDAC1/2, which subsequently leads to G2/M phase arrest.[14] Moreover, FK228 was able to induce the EBV lytic cycle at a lower concentration and showed a more significant effect than SAHA. Our results on cell cycle distribution also verified that FK228 treatment induced cell cycle arrest at a much higher degree through simultaneous inhibition of HDAC1/2, compared to the global inhibitor SAHA. This may be because, although inhibition of HDAC1/2 increases the promoter activities of

p21 and p27,[43] there is a limitation of specific HDAC-targeted inhibition when treated with SAHA. Therefore, FK228 treatment showed a stronger promotion of HBV replication than SAHA.

In conclusion, FK228 and SAHA significantly promoted HBV replication in a dose-dependent manner in HBV-expressing HCC cell lines and the HBV-Tg mice model. Further analysis showed that cell cycle blockage played an important role in HDACi-induced HBV reactivation. Higher HBV replication levels were found after FK228 treatment when compared with SAHA, suggesting simultaneous inhibition of HDAC1/2 had a stronger effect on the cell cycle arrest.

Conclusions

In summary, we have proposed herein a possible mechanism for FK228/SAHA-mediated HBV reactivation. According to our study's findings, FK228/SAHA induce cell cycle arrest to enhance HBV replication through the inhibition of HDAC1/2. Pharmacological or transcriptional inhibition of HDAC1/2 exhibits more significant effects than broad-spectrum inhibition of HDACs by increasing of p21/p27 and decreasing cyclins and CDKs, thereby stimulating HBV replication more robustly. Further studies in HBV-infected and reactivated animal models and clinical patients are required to verify and supplement the current information.

Funding

This study was supported by the National Natural Science Foundation of China (81871653, 82072286 and 82073251), Natural Science Foundation of Chongqing (cstc2020jcyj-msxmX0159, cstc2018jcyjAX0254 and cstc2019jcyj-msxmX0587), Chongqing Medical Science Project (2018MSXM065), Technology Research Project of Chongqing Municipal Education Commission (KJQN201900449, KJZD-M202000401) and Scientific Research Innovation Project for Postgraduates in Chongqing (CYS19193 and CYB19168).

Conflict of interest

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

Author contributions

Conception and design of the research (ZZ, XL), acquisition of the data (YY, YuY, ZC, JH), analysis and interpretation of the data (YY, YuY, ZC), drafting of the manuscript (YY, XL), materials and technical support (KW, NT), critical revision of the manuscript for important intellectual content (YY, ZZ), supervision (ZZ). All authors read and approved the final version for publication.

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Journal of Clinical and Translational Hepatology **2021** vol. 9(2) | 169–179
DOI: 10.14218/JCTH.2020.00114

Original Article

Development and Validation of a Metabolic-related Prognostic Model for Hepatocellular Carcinoma

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Received: 14 November 2020 | **Revised:** 3 January 2021 | **Accepted:** 26 January 2021 | **Published:** 22 February 2021

Abstract

Background and Aims: Growing evidence suggests that metabolic-related genes have a significant impact on the occurrence and development of hepatocellular carcinoma (HCC). However, the prognostic value of metabolic-related genes for HCC has not been fully revealed. ***Methods:*** mRNA sequencing and clinical data were obtained from The Cancer Genome Atlas and the GTEx Genotype-Tissue Expression comprehensive database. Differentially expressed metabolic-related genes in tumor tissues (n=374) and normal tissues (n=160) were identified by the Wilcoxon test. Time-dependent receiver operating characteristic curve analysis, univariate multivariate Cox regression analysis and Kaplan-Meier survival analysis were used to evaluate the predictive effectiveness and independence of the prognostic model. Two independent cohorts (International Cancer Genome Consortiums and GSE14520) were applied to verify the prognostic model. ***Results:*** Our study included a total of 793 patients with HCC. We constructed a risk score consisting of five metabolic-genes (BDH1, RRM2, CYP2C9, PLA2G7, and TXNRD1). For the overall survival rate, the low-risk group had a considerably higher rate than the high-risk group. Univariate and multivariate Cox regression analyses indicated that the risk score was an independent predictor for the prognosis of HCC. ***Conclusions:*** We constructed and validated a novel prognostic model, which may provide support for the precise treatment of HCC.

Citation of this article: Huo J, Wu L, Zang Y. Development and validation of a metabolic-related prognostic model for hepatocellular carcinoma. J Clin Transl Hepatol 2021; 9(2):169–179. doi: 10.14218/JCTH.2020.00114.

Keywords: Hepatocellular carcinoma; Metabolic; Prognostic; Signature.

Abbreviations: AFP, α-fetoprotein; ALT, glutamic pyruvic transaminase; BCLC, Barcelona Clinic Liver Cancer; BDH1, 3-hydroxybutyrate dehydrogenase; CLIP, Cancer of the Liver Italian Program; DEMRGs, differentially expressed metabolic-related genes; DFS, disease-free survival; DSS, disease-specific survival; GEO, Gene Expression Ominibus; GO, Gene Ontology; GSEA, gene set enrichment analysis; GTEx, Genotype-Tissue Expression comprehensive database; HCC, hepatocellular carcinoma; HR, high-risk; ICGC, International Cancer Genome Consortium; KEGG, Kyoto Encyclopedia of Genes and Genomes; LR, low-risk; OS, overall survival; PFS, progression-free survival; ROC, receiver operating characteristic; RS, risk score; TCGA, The Cancer Genome Atlas; TME, tumor microenvironment.

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Introduction

Hepatocellular carcinoma (HCC) is the second major cause of cancer-related death in the world.[1,2] The prognosis of HCC is still not ideal, although related research has made great progress in recent years.[1,3] This is mainly related to the high heterogeneity of HCC and the high diagnostic rate of advanced HCC,[4–7] even with identical pathological types and clinical stages, patients' individual responses to the same treatment can be diversified.[8,9] It is worth noting that patients with HCC usually have a background of liver cirrhosis. Nevertheless, in clinical practice, which monitoring strategy is most effective for early tumor detection is uncertain.[10] There is a pressing need to identify reliable biomarkers for the diagnosis and prognosis of HCC, to improve the survival of HCC.

In recent years, growing evidence has shown that the metabolic pattern of the cell cancerization process has changed significantly, which involves many aspects, such as glycolysis, the citric acid cycle, and oxidative phosphorylation of amino acids metabolism, fatty acid metabolism and nucleic acid metabolism, etc. This phenomenon is known as the reprogramming of energy metabolism of tumor cells, which is crucial for tumor growth.[11,12] Some scholars have found that metabolic abnormalities are an important factor in the pathogenesis of HCC.[6,7,13] Lee *et al*.[13] noted that the gene expression levels involved in glycolysis and oxidative metabolism in HCC livers were much higher than those in normal livers, which was indeed relevant to an increased risk of liver cancer and may represent a potential target for the prevention of HCC. Gao *et al*.[6] conducted a multidimensional proteomics study of 159 hepatitis B virus-positive liver and para cancer samples from patients in China, and found that most of the liver-specific metabolic pathway proteins (such as sugar dysplasia, detoxification, ammonia and urea metabolism) in liver tumors were significantly reduced; however, the key enzymes of cholesterol metabolism (SOAT1, SOAT2, etc.) and glutamine metabolism-related proteins (GLS and GLUD2) expressed in tumors were increased significantly, suggesting that hepato-specific metabolic pathways are reprogrammed in hepatitis B virus-associated HCC. Some scholars have also proposed that metabolic changes in the tumor microenvironment (TME) can inhibit antitumor immunity (such as immune cell infiltration) by producing immunosuppressive metabolites.[14,15] However, there is still a lack of research on genes related to metabolism in predicting the prognosis of patients with liver cancer. Investigations into the metabolic genes of HCC are expected to open up new avenues for the treatment of HCC.

This research study established a risk score (RS) based upon the expression levels of five metabolism-related genes and analyzed the diverse clinicopathological features correlated with the new RS. The correlations of the RS with tumor immune cell infiltration were also evaluated.

Methods

Data collection and extraction of metabolic genes

The clinical data and mRNA expression profiles of patients with HCC were taken from The Cancer Genome Atlas (TCGA; https://portal.gdc.cancer.gov/), including 374 HCC samples and 50 normal samples; the Genotype-Tissue Expression project (GTEx; www.gtexportal.org), including 110 normal samples; the International Cancer Genome Consortium (ICGC; https://icgc.org/), including 215 patients with HCC; and the Gene Expression Omnibus (GEO; https://www.ncbi.nlm.nih.gov/geo/) (GSE14520), including 235 patients with HCC. A total of 2,752 metabolic-related genes that encoded all the known human transporters and metabolic enzymes were obtained from a previously published paper,[16] for subsequent analysis. The gene expression profiles obtained from different databases were normalized with the "combat" package in R software. The collected data were used in accordance with the data access strategies of TCGA, ICGC and GEO. All research processes and analyses were conducted in compliance with relevant regulations and guidelines. HCC clinical survival data and mRNA profile data were publicly available, and approval of the local ethics committee was not required.[17]

Identification of differentially expressed metabolic-related genes (DEMRGs)

Using the Wilcoxon method in the R package "limma" to detect differential genes related to metabolism in HCC and normal tissues, the results of log2 fold change >1 and false discovery rate <0.05 were regarded as significantly different. The "limma" and "heatmap" packages in the R software were used to form volcano and heat maps of DEMRGs.

Annotations of DEMRGs' functions and pathways

This research applied the R package "cluster profile" for DEMRGs annotation (gene ontology [GO] and Kyoto Encyclopedia of Genes and Genomes [KEGG] pathway)[18] to evaluate the underlying biological function of DEMRGs.

Identification of prognostic-related genes and construction of the prognostic model in the TCGA cohort

We used 343 patients (survival ≥1 month) from the TCGA dataset as the training cohort, to develop the prognostic model. During the building process of the prognostic model, we combined univariate Cox regression analysis, Lasso regression analysis, and multivariate Cox regression analyses. First, univariate Cox regression analysis was applied to screen DEMRGs associated with prognosis (p-value <0.001 considered significant).[19,20] Next, the least absolute shrinkage and selection operator algorithm was utilized to avoid overfitting of the prognosis-related genes. During the process of this analysis, we subsampled the dataset 1,000 times and chose the genes that were repeated >900 times. A subselection of prognosis-related genes was determined by penalty parameter tuning performed via 10-fold cross-validation. Only genes with non-zero regression coefficients were retained for subsequent multivariate Cox regression analyses[19–22] (Supplementary Fig. 1). The formula of the RS was as follows: RS = the sum of each multivariate Cox regression coefficient of mRNA multiplied by each normalized mRNA expression level. According to the median RS, patients were divided into two groups: the low-risk (LR) group and the high-risk (HR) group. Utilizing the Kaplan-Meier approach in the R-package "suvminer" produces survival curves, and the log-rank test was used to contrast discrepancies between the two groups. Using the time-dependent receiver operating characteristic (ROC) curve analysis feature in the R package, "survival ROC" aimed to evaluate the prognostic ability of the RS.

Independence validation of the prognostic model

Univariate and multivariate Cox regression analyses were applied to detect whether the RS was an independent prognostic predictor. A value of $p<0.05$ was statistically significant.

Internal validation of the prognostic model in the TCGA cohort

We divided the patients into several subgroups for internal validation according to their pathological features (including α-fetoprotein [AFP] level, vascular invasion, histological grade, AJCC-TNM stage, new tumor after initial treatment, and individual tumor status). The analysis of survival adopted the Kaplan-Meier method, and when the log-rank test detected a value of $p<0.05$, it was considered statistically significant.

External validation of the prognostic model using multiple independent cohorts

We calculated the RS of patients in the validation cohort (ICGC, GSE14520) using the same formula established by the TCGA cohort. The patients were separated into a LR group and a HR group based on the same cutoff value. Kaplan-Meier survival analysis, ROC curve analysis and univariate and multivariate Cox regression analysis were conducted as described above.

Correlation analysis between the RS and clinicopathology

We used the chi-square test to analyze the correlation between the RS and clinicopathology (including gender, age, AJCC-TNM stage, Barcelona Clinic Liver Cancer [BCLC] stage, Cancer of the Liver Italian Program [CLIP] stage, main tumor size, histologic grade, AFP, and vascular tumor cell type). A value of $p<0.05$ was statistically significant.

Correlation analysis between RS and tumor immune cell infiltration

The CIBERSORT method (using the characteristic matrix of 547 genes to express 22 types of infiltrating immune cells) was used to measure the infiltration ratio of immune cells as a number in tumor tissues, and the samples with $p<0.05$

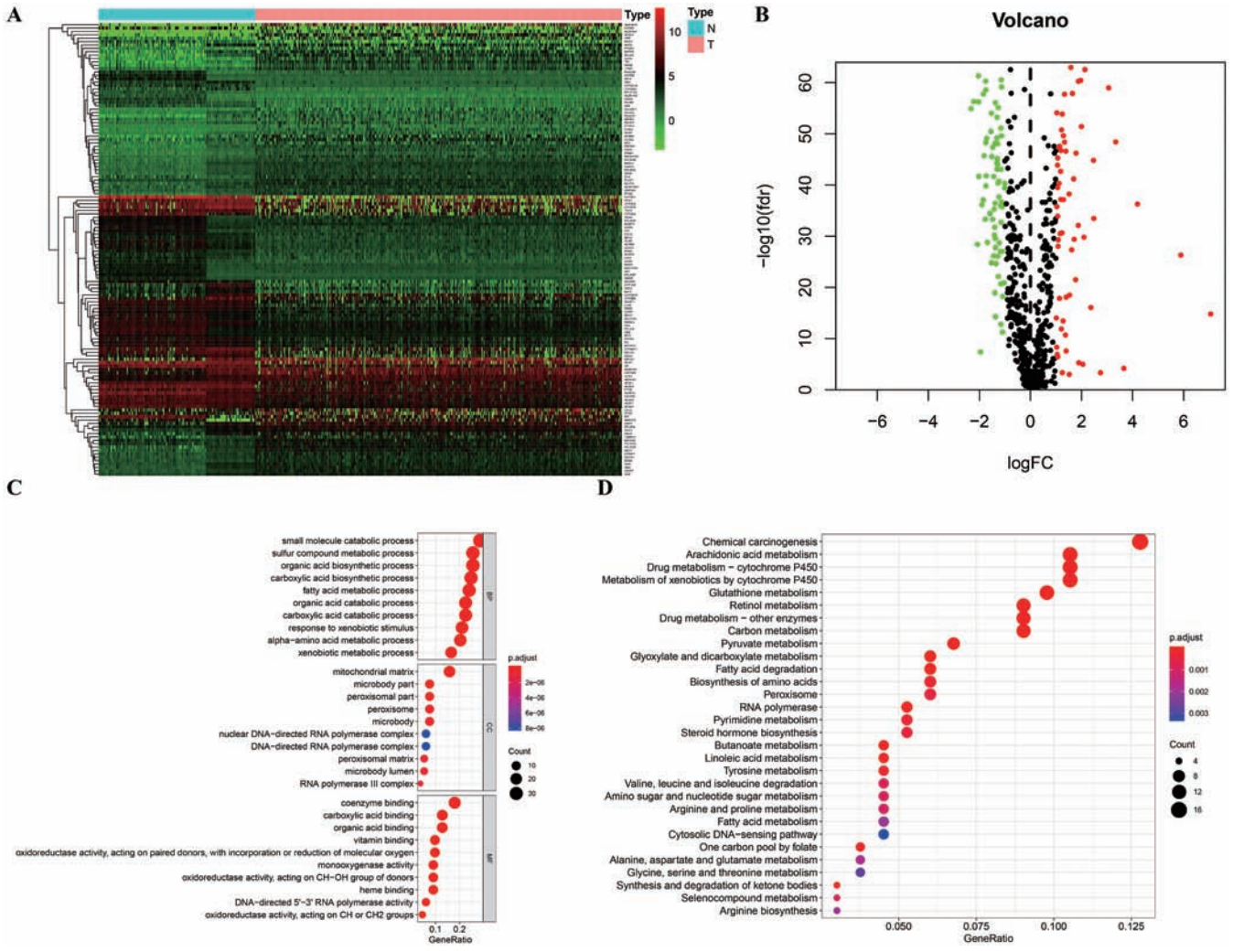

Fig. 1. Identification and functional enrichment analysis of DEMRGs. (A–B) The heatmap and volcano plot of DMRGs. (C) GO enrichment analysis of DMRGs. (D) KEGG enrichment analysis of DMRGs.

were selected for subsequent analysis.

Gene set enrichment analysis (GSEA)

To further explore the internal mechanism of the prognostic model, we conducted GSEA on the LR and HR groups of the three independent cohorts to reveal the molecular biological characteristics of the LR and HR groups.

Results

Functional enrichment analysis and survival analysis of DEMRGs

A total of 134 metabolic-related genes were differentially expressed in HCC tissues (n=374) compared with normal tissues (n=160) (Fig. 1A–B). GO enrichment analysis showed that the main functions of these differentially metabolized genes included small molecule catabolism, organic acid biosynthesis, sulfur compound metabolism, carboxylic acid biosynthesis, organic acid catabolism, fatty acid metabolism, carboxylic acid catabolism and other processes (Fig. 1C). The KEGG enrichment analysis identified these genes as being prevailingly related to chemical carcinogenesis, arachidonic acid metabolism, drug metabolism, glutathione metabolism, retinol metabolism, and carbon metabolism (Fig. 1D).

Construction of the five-metabolic gene prognostic model

To facilitate the clinical application of our prognostic model, five metabolic-related genes were identified by Lasso-penalized Cox analysis to establish a predictive model. RS=(−0.02928*BDH1 normalized expression level)+(0.04763*RRM2 normalized expression level)+(−0.0018*CYP2C9 normalized expression level)+(0.0111*PLA2G7 normalized expression level)+(0.0111*TXNRD1 normalized expression level). The median RS (0.967) of the TCGA cohort is a critical value that divides all patients with HCC into HR and LR groups. This research applied disease-specific survival (DSS), overall survival (OS), progression-free survival (PFS) and disease-

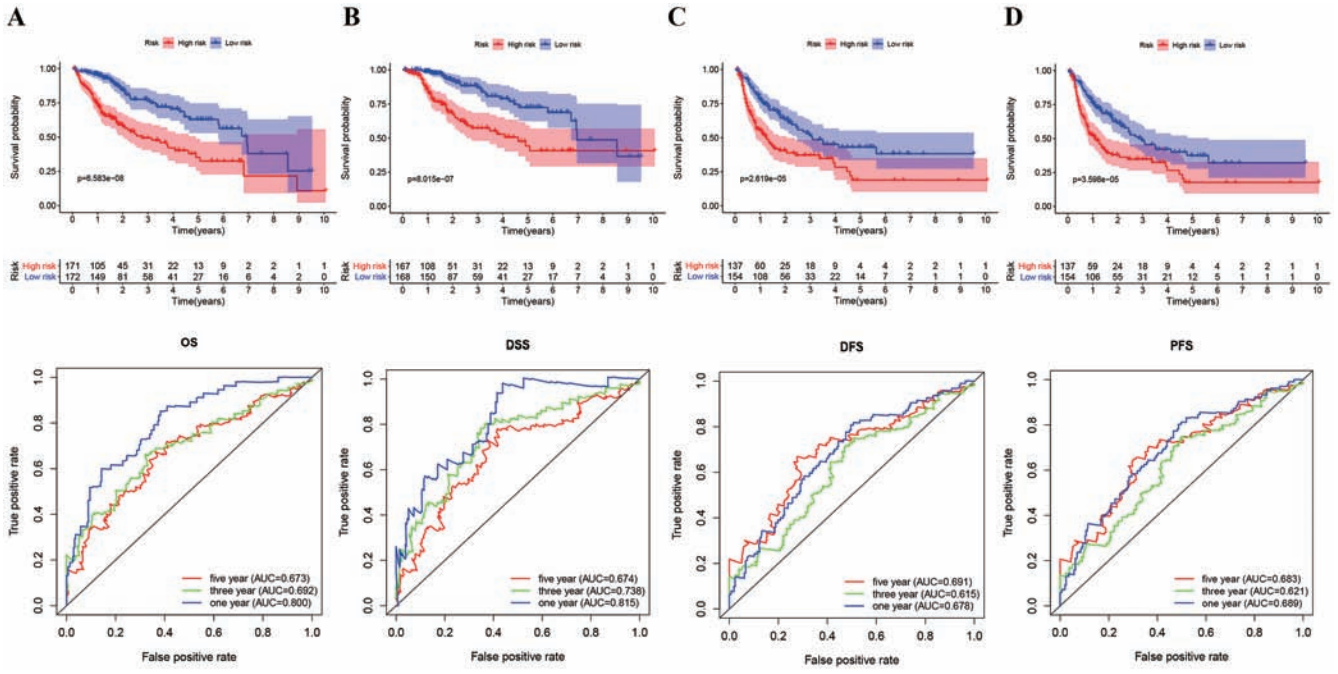

Fig. 2. Kaplan-Meier survival analysis and time-dependent ROC analysis for (A) OS, (B) DSS, (C) DFS and (D) PFS.

free survival (DFS) to compare the prognosis of patients with different risks. The Kaplan-Meier curve showed that compared with the HR group, the PFS, DFS, DSS, and OS of the LR group were remarkably higher ($p<0.001$) (Fig. 2A–D).

When assessing the performance of the prognostic model by measuring the area over time under the ROC curve, the higher the area under the curve, the better was the model performance. The areas under the curve for the 1-year, 3-year, and 5-year PFS were 0.689, 0.621, and 0.683, respectively (Fig. 2D); the areas under the curve for the 1-year, 3-year, and 5-year DFS were 0.678, 0.615, and 0.691, respectively (Fig. 2C); the areas under the curve for the 1-year, 3-year, and 5-year DSS were 0.815, 0.738, and 0.674, respectively (Fig. 2B); and, the areas under the curve for the 1-year, 3-year, and 5-year OS were 0.8, 0.692, and 0.673, respectively (Fig. 2A). The RS was an independent prognostic indicator linked to PFS, DFS, DSS and OS, as presented by univariate and multivariate Cox regression analyses (Fig. 3A–D).

Internal validation of the prognostic model in the TCGA cohort

We divided the patients into several subgroups for internal validation according to their pathological features, consistent with previous results. Compared to the LR group, the HR group patients' OS rates were notably lower (Fig. 4A–F).

External validation of the prognostic model in the ICGC and GSE14520 cohorts

Two independent datasets (ICGC, $n=215$; GSE14520, $n=235$) were used to test the prognostic value of the RS. The calculation formula of RS and the threshold value for dividing the HR and LR groups were consistent with that of the TCGA cohort. The HR patients' OS was notably lower than that of LR patients in the two independent cohorts (Fig. 5A, D). The area under the ROC curve of the 1-year, 3-year and 5-year overall survival rates of the ICGC cohort and GSE14520 cohort were 0.750, 0.734, 0.829, 0.675, 0.671, and 0.673, respectively (Fig. 5A, D). The RS could be regarded as an independent prognostic indicator by univariate and multivariate Cox regression analyses (Fig. 5C, F). Due to the lack of relevant information about the Child/model for end-stage liver disease score, we cannot directly compare the prognostic value of the Child/model for end-stage liver disease score with the RS. However, by comparing the area under the curve values of the ROC curve between the RS and the traditional TNM staging system, we found that the RS had better performance in predicting prognosis (Supplementary Fig. 2).

Correlation of the prognostic model with clinicopathological characteristics

We performed chi-square tests on three independent cohorts (TCGA, ICGC, and GSE14520) and revealed that stage, grade, vascular tumor cell type, individual neoplasm status, main tumor size, and new tumor event after initial treatment concerned the RS of patients with HCC (Supplementary Tables 1–3).

Correlation analysis between the RS and tumor immune cell infiltration

The CIBERSORT algorithm was used to further analyze the infiltration degree of immune cell subtypes (samples were screened by $p<0.05$). The results showed that, compared

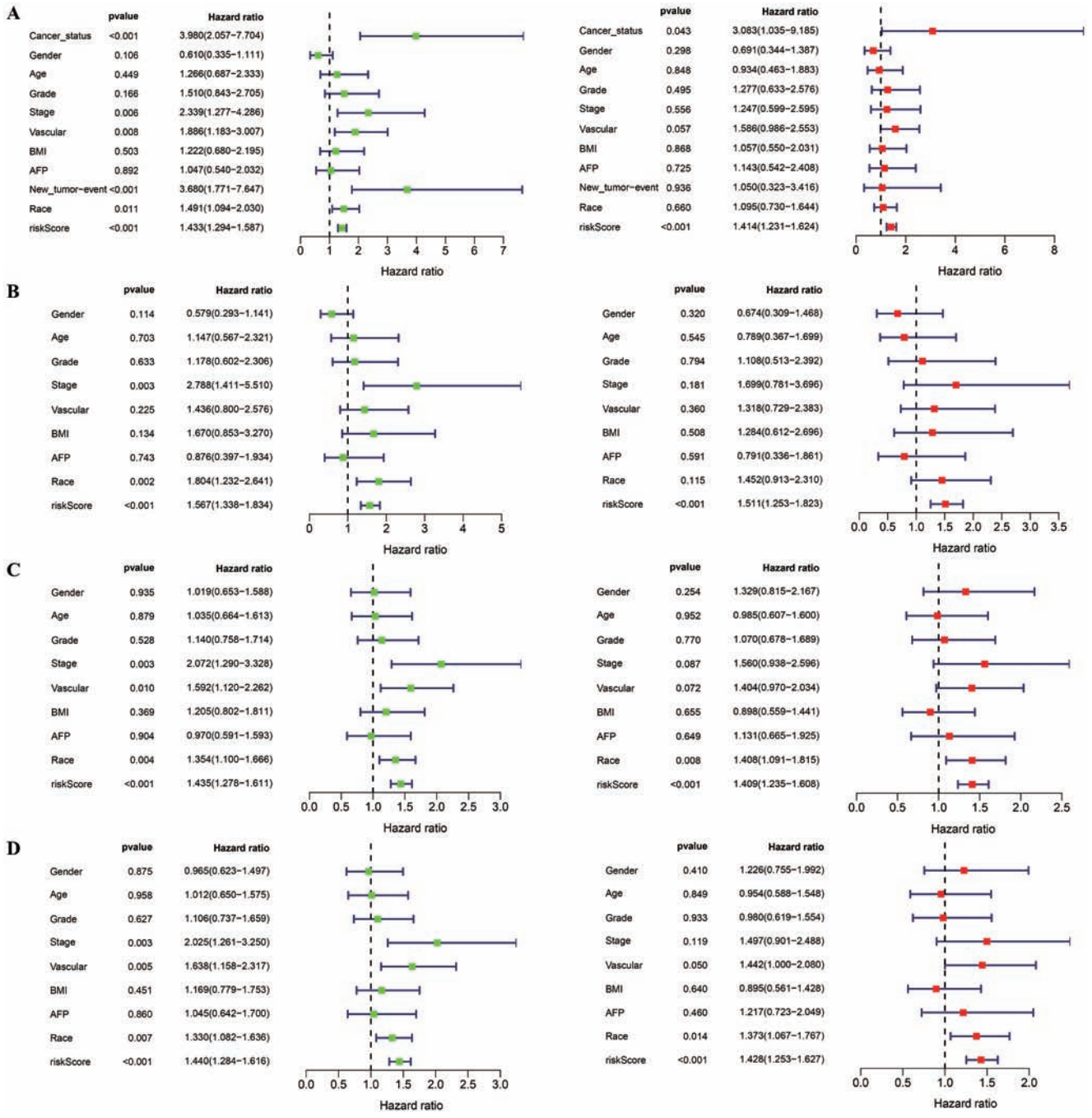

Fig. 3. Forrest plot of the univariate and multivariate regression analysis regarding (A) OS, (B) DSS, (C) DFS and (D) PFS in the TCGA cohort. Green represents univariate analysis, and red represents multivariate analysis.

with the LR group, the HR group had a markedly higher infiltration degree of M2 macrophages and a lower infiltration degree of M1 macrophages (Fig. 6A–C).

GSEA

As shown in Supplementary Fig. 3, the activity of metabolism-related pathways in the LR group was significantly stronger than that in the HR group, suggesting that we may be able to find new therapeutic strategies to improve the prognosis of HCC by targeting metabolic reprogramming of HCC.

Discussion

Because HCC usually occurs in the context of cirrhosis, it has high morbidity, mortality, recurrence and heterogeneity,[1–3] and poses a great threat to human health. With the worldwide application of next-generation gene sequencing

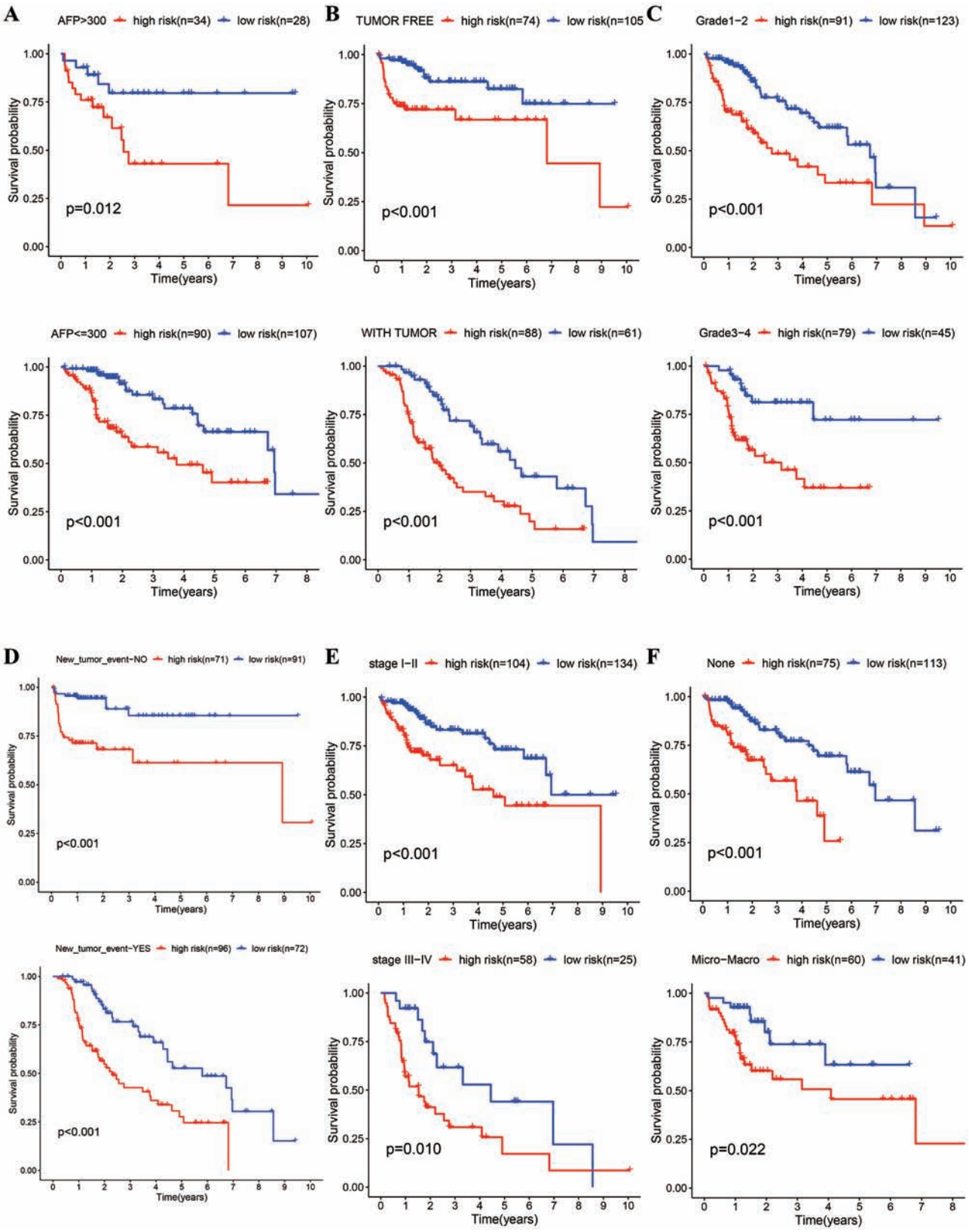

Fig. 4. Internal validation in the TCGA cohort based on clinical features. (A) AFP. (B) Tumor status. (C) Histopathological grade. (D) New tumor event after initiate treatment. (E) AJCC-TNM stage. (F) Vascular tumor cell type.

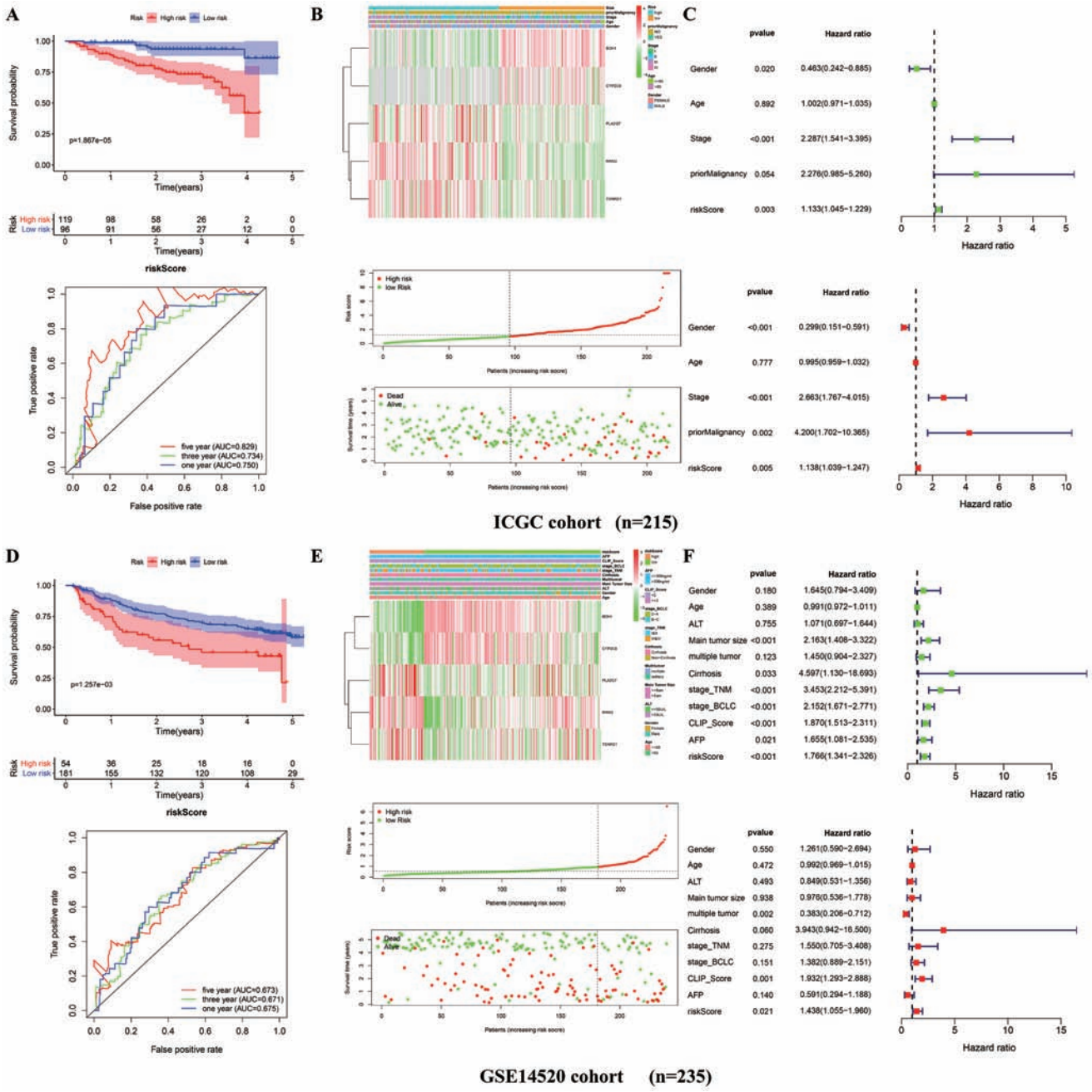

Fig. 5. External validation of the prognostic model in two independent cohorts. (A) Kaplan-Meier curve of OS and time-dependent ROC analysis in the ICGC cohort. (B) Heatmap of the five genes and the distribution of RS and the survival status of patients of the ICGC cohort. (C) Univariate and multivariate Cox regression analysis of the five-gene signature in the ICGC cohort (green represents univariate analysis, and red represents multivariate analysis). (D) Kaplan-Meier curve of OS and time-dependent ROC analysis in the GSE14520 cohort. (E) Heatmap of the five genes and the distribution of RS and the survival status of patients of the GSE14520 cohort. (F) Univariate and multivariate Cox regression analysis of the five-gene signature in the GSE14520 cohort (green represents univariate analysis, and red represents multivariate analysis).

technology, people have gradually realized that the prognosis of patients with HCC is not only dependent on the traditional clinical staging system but is also related to molecular genetic factors.[5,7,23–25] Growing evidence has shown that metabolic reprogramming exerts a huge function in the emergence and growth of HCC.[11,13] The prognostic value of some metabolic genes has been validated,[6,26] but they are still numerically inadequate. Thus, there remains a pressing need to identify more biomarkers related to the prognosis of HCC.[20]

Compared with previous studies,[18–20,26,27] this study highlighted the following aspects. First, in this study, mRNA data from TCGA, ICGC, GEO, and GTEx were integrated to study the prognostic value of metabolic-related genes in HCC. Second, we used three independent cohorts (TCGA, ICGC, and GSE14520) to construct and validate the prognostic model, making the conclusion more reliable. Third, we investigated the relationship between the prognostic

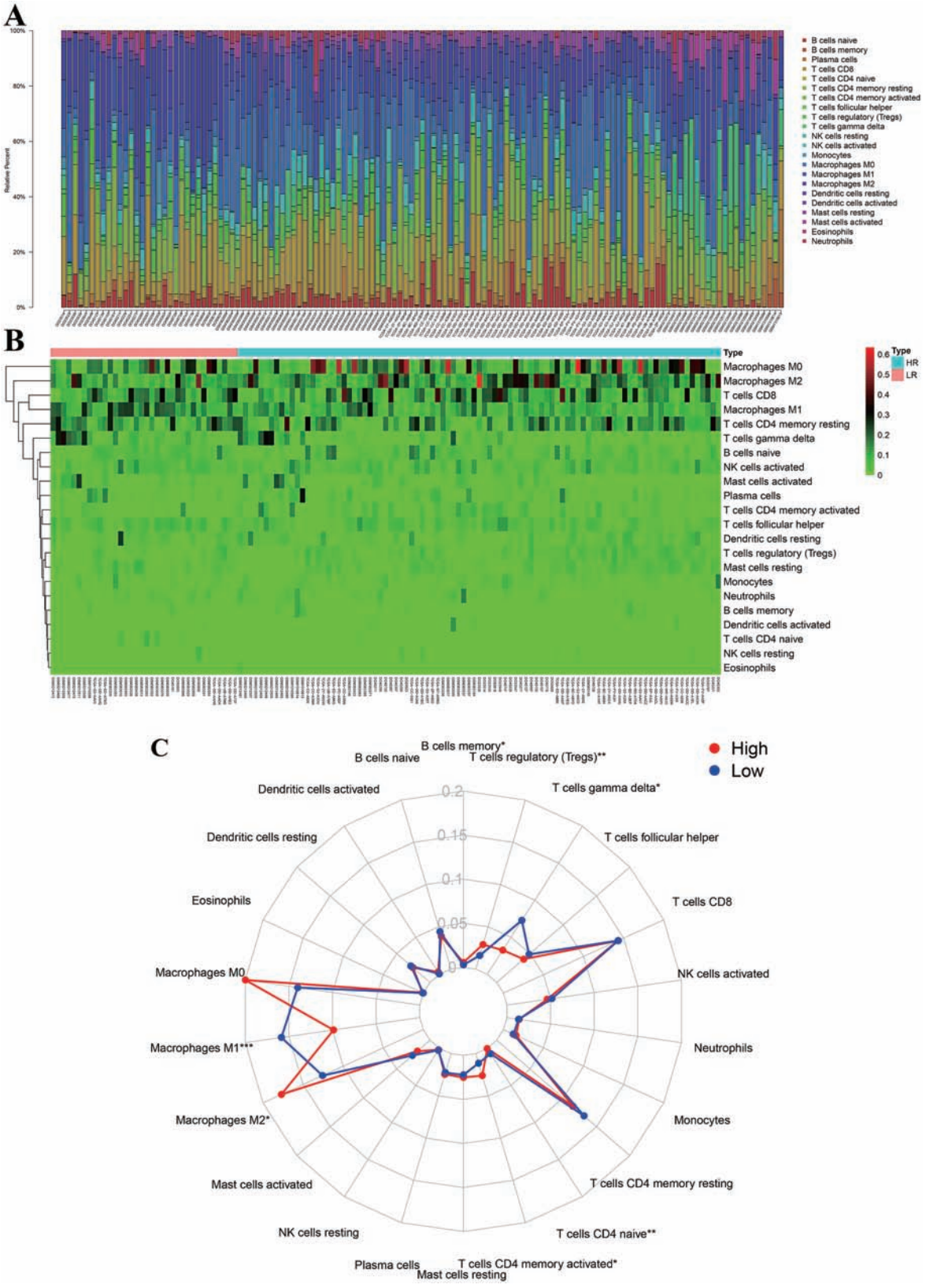

Fig. 6. Relative proportion of 22 kinds of immune cell infiltration in HR and LR patients estimated by the CIBERSORT method. (A) Barplot. (B) Heatmap. (C) Radar plot visualizing significantly different infiltration immune cells between HR and LR groups (*p*-value significant codes: 0≤ *** <0.001 ≤ ** < 0.01 ≤ * < 0.05).

model and tumor immune cell infiltration.

Patients were classified into a LR group and a HR group, based on a uniform cutoff (0.967). In both the training cohort (TCGA) and the validation cohort (ICGC and GSE14520), the OS of the HR group was significantly lower than that of the LR group. The area under the curve of the ROC curve showed that the RS had higher specificity and sensitivity for the prediction of prognosis. Clinical correlation analysis showed that patients with a HR score were found to be significantly correlated with higher tumor grade, larger main tumor size (>5 cm), older (age >65 years), vessel invasion, AFP >300 ng/mL, advanced BCLC stage (B–C), advanced CLIP stage (≥2) and advanced TNM stage (III–IV), and these results suggest the high-RS patients had a higher degree of malignancy. For patients with the same clinical features, the prognosis of the HR group patients was markedly worse than for those of the LR group, which highlights the importance of our RS establishment because it can better reflect the heterogeneity of patients compared with the traditional clinical stage.

It has been reported that abnormal metabolism of purines (such as abnormal elevation of uric acid) is related to the appearance and growth of different malignant tumors, such as colorectal cancer metastasis, non-small cell lung cancer brain metastasis, and the prognosis of pancreatic cancer, etc.,[28–33] but there have been few reports on HCC. Immune cells are the main non-tumor components in the TME, and earlier studies have suggested that the infiltration of immune cells (neutrophils, dendritic cells, macrophages, etc.) in tumors has a close relationship with unadvanced HCC prognosis.[34–39] Macrophages are the most numerous in tumor tissues and have the most significant regulatory effect on tumors. As such, they are called tumor-associated macrophages. Studies have found that M1-type macrophages can recognize tumor antigens, and phagocytose or kill tumor cells. Type II interferon (interferon-γ) is a classic inducer of M1 macrophage polarization and tumor cell killing.[36,38] M2-type macrophages inhibit the activation and proliferation of T cells and natural killer cells by producing interleukin-10, transforming growth factor-β and prostaglandin E2 (prostaglandin E-2, PGE-2), and induce immune tolerance of tumor cells, thus promoting the proliferation, invasion and metastasis of tumor cells.[36] In this study, it was found that the HR group had a higher infiltration degree of M2 macrophages and a lower degree of M1 macrophages than the LR group. These results suggested that this model could be used as an effective predictor of immune cell infiltration.

At present, obesity and metabolic diseases have become important factors that induce liver cancer.[40] The beige fat cells in the body have come under scrutiny for their ability to burn energy to prevent obesity.[41,42] Wang *et al*.[43] found that catabolic metabolism of n-hydroxybutyrate mediated by 3-hydroxybutyrate dehydrogenase (BDH1) is an important step in the formation of beige fat cells in the body. Martinez-Outschoorn *et al*.[44,45] found that BDH1 is preferentially expressed in breast tumor mesenchymal cells and that overexpression of BDH1 can promote the growth of cancer cells by generating fibroblasts to drive increased mitochondrial synthesis. Saraon *et al*.[46] also found significant upregulation of BDH1 expression in prostate cancer tissues. The current study discovered that, compared with normal tissues, BDH1 expression in tumor tissues was markedly down-regulated (Supplementary Fig. 4). Moreover, the high expression of BDH1 in tumor tissues was correlated with better OS and earlier TNM stage and a maximum tumor diameter of ≤5 cm and AFP of ≤300 ng/mL (Supplementary Figs. 5–8). Whether the mechanism is related to the previously reported promotion of beige fat cell formation *in vivo* deserves further study.

The ribonucleotide reductase m2 RRM2 has been confirmed repeatedly to have a relationship with HCC prognosis in recent years.[47–49] Kosakowska *et al*.[50] found a reduction in RRM2 remarkably suppressed HCC cell proliferation, and that RRM2 catalyzes the conversion of ribonucleoside 5′-diphosphate into a corresponding 2′-deoxyribonucleotide, and since this reaction is a rate-limiting step in DNA synthesis, RRM2 has been identified as a new target for cancer therapy.[51,52] Additionally, this study discovered that poor OS was responsible for high RRM2 expression and predicted low tumor differentiation (Supplementary Figs. 5–8).

The cytochrome P450 system of the liver plays an important role in drug metabolism. The CYP2 family is the largest family of the CYP450 enzyme family, among which CYP2C9 is one of the most important subtypes.[53] Nebert *et al*.[54] once reported that the expression of CYP can affect the production of arachidonic acid-derived molecules and change various downstream signal transduction pathways, thus causing cell cancerization. Yan *et al*.'s[55] study found that the expression level of CYP was significantly destroyed during the cancer process, while the activity of CYP was highly correlated with the expression level of the protein. The current study found that the expression level of CYP2C9 in tumor tissue was remarkably lower than that in normal tissue (Supplementary Fig. 4), and patients with high CYP2C9 expression in tumor tissues had a better prognosis (Supplementary Figs. 5–8). In addition, the reduced CYP2C9 expression level had a significant relationship with higher TNM stage and higher BCLC stage, a maximum tumor diameter of >5 cm, AFP of >300 ng/mL and vessel invasion (Supplementary Figs. 5–8). The results were similar to those of CYP4A11 in the study by Eun *et al*.[56] These findings indicate that the high CYP2C9 expression is likely to be a favorable signal for the prognosis of HCC (Supplementary Figs. 5–8). Clinically, we can consider using CYP2C9 as a therapeutic target to further improve the prognosis of HCC.

The platelet-activating factor acetylhydrolase PLA2G7 is an effective proinflammatory and anti-inflammatory molecule involved in a variety of inflammatory processes.[57] Nair *et al*.[58] found that PLA2G7 expression was significantly upregulated in fat cell precursors in obese individuals. Hou *et al*.[59] and Hoffmann *et al*.[60] identified PLA2G7 as a risk factor for cardiovascular disease. The current study found that compared with the level in normal tissues, the PLA2G7 expression level in tumors was remarkably higher (Supplementary Fig. 4), and the high expression level of PLA2G7 was significantly correlated with AFP >300 ng/mL (Supplementary Figs. 5–8), so PLA2G7 may be a new diagnostic marker for HCC.

Increasing evidence shows that oxidative stress caused by the destruction of the reduction-oxidation system is closely related to the occurrence of liver cancer.[61–63] The thioredoxin reductase 1 TXNRD1, as a member of the thioredoxin system, is essential for maintaining the balance of the redox state in cells.[62,64] This study found that TXNRD1 was significantly correlated with poor OS and higher TNM staging (Supplementary Figs. 5–8), which was similar to the report of Fu *et al*.[65] and Lee *et al*.[66] Therefore, TXNRD1 may be a biomarker with important prognostic value for HCC.

Targeted sequencing based on five metabolic genes can undoubtedly significantly reduce the cost of sequencing, but there are some limitations because our research results are mainly based on the description of the phenomenon, and we need to explore its mechanism through experiments. HCC is a complex disease caused by multiple mechanisms, not just metabolic disorders. Although we made full use of data resources, the lack of some clinical data will cause inevitable limitations, for example, the adjuvant treatment methods patients receive, such as chemotherapy, targeted therapy, and immunotherapy, comorbidities of patients and whether patients have underlying cirrhosis, because these

factors have a significant impact on the clinical outcome. This study was retrospective and needs to be improved upon and verified in future multicenter prospective studies.

Conclusions

This research established and verified a reliable prognostic model for HCC patients. The five metabolic genes in the model may be promising targets for the precise treatment of HCC. Therefore, it is likely to have influential potential for clinical practice in the near future.

Funding

None to declare.

Conflict of interest

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

Author contributions

Designed this study (JH, LW), performed the data analyses and wrote the manuscript (JH), revision of the manuscript (JH, LW, YZ), reviewed the final version of the manuscript (JH, LW, YZ).

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Journal of Clinical and Translational Hepatology **2021** vol. 9(2) | 180–186
DOI: 10.14218/JCTH.2020.00108

Original Article

UGT1A1-related Bilirubin Encephalopathy/Kernicterus in Adults

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Received: 12 November 2020 | **Revised:** 15 January 2021 | **Accepted:** 4 February 2021 | **Published:** 11 March 2021

Abstract

Background and Aims: Bilirubin encephalopathy/kernicterus is very rare in adults. This study is aimed to investigate the clinical manifestations and genetic features of two patients with *UGT1A1*-related kernicterus. ***Methods:*** Sanger sequencing analysis was performed to identify *UGT1A1* gene mutations in the patients and their families. Bioinformatics analysis was used to predict the potential functional effects of novel missense mutations. Clinical manifestations and biochemical parameters were collected and analyzed. ***Results:*** Two patients with Crigler-Najjar syndrome type II (CNS2) developed kernicterus in adulthood. Sanger sequencing identified a compound heterozygous mutation in the *UGT1A1* gene in patient 1, which was inherited from his mother (G71R) and his father (c.-3279T>G; S191F). Patient 2 carried three heterozygous mutations, namely G71R, R209W and M391K; among which, the M391K mutation has not been reported before. Multiple prediction software showed that the M391K mutation was pathogenic. Symptoms were relieved in the two patients after phenobarbital and artificial liver support treatment. Patient 1 also underwent liver transplantation. ***Conclusions:*** Adults with CNS2 are at risk for kernicterus. Phenobarbital treatment is beneficial for maintaining bilirubin levels and preventing kernicterus.

Citation of this article: Bai J, Li L, Liu H, Liu S, Bai L, Song W, *et al*. *UGT1A1*-related bilirubin encephalopathy/kernicterus in adults. J Clin Transl Hepatol 2021;9(2):180–186. doi: 10.14218/JCTH.2020.00108.

Keywords: Kernicterus; *UGT1A1*; Crigler-Najjar syndrome type II; Phenobarbital.

Abbreviations: ACLF, acute-on-chronic liver failure; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BAMR, bilirubin-albumin molar ratio; CNS2, Crigler-Najjar syndrome type II; CT, computed tomography; DPMAS, double plasma molecular adsorption system combined with plasma exchange; GS, Gilbert syndrome; Ig, immunoglobulin; TB, total bilirubin; UCB, unconjugated bilirubin; UGT1A1, uridine diphosphate glucuronosyl transferase 1A1.

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Introduction

Bilirubin encephalopathy/kernicterus is an uncommon disabling neurologic disease caused by the toxicity of unconjugated bilirubin (UCB) to the basal ganglia and various brain stem nuclei.[1] Neonatal jaundice is quite common, affecting 60–80% of newborns, as a whole.[2] However, severe hyperbilirubinemia (>20 mg/dL), which may potentially lead to kernicterus and neurodevelopmental complications, is very rare, accounting for less than 2% of newborns.[3] The incidence of kernicterus is about 0.2 to 2.7 cases per 100,000 live births.[1] Common risk factors may include preterm delivery, hemolytic disease [glucose-6-phosphate dehydrogenase deficiency and ABO hemolysis], perinatal infection and exclusive breastfeeding.[1,4]

For adults, elevated UCB caused by hemolytic disease and inherited non-hemolytic unconjugated hyperbilirubinemia (bilirubin glucuronidation defects) is relatively common. Bilirubin glucuronidation is regulated by the uridine diphosphate glucuronosyl transferase 1A1 (UGT1A1) enzyme. It is encoded by the *UGT1A1* gene, which is located on chromosome 2 (2q37), and covers a promoter, enhancers, and five exons. According to the severity of UGT1A1 enzyme deficiency, inherited unconjugated hyperbilirubinemia can be classified into Crigler-Najjar syndrome type I (CNS1), Crigler-Najjar syndrome type II (CNS2), and Gilbert syndrome (GS).[5,6] CNS1 is the most severe form, determined by a complete lack of bilirubin glucuronidation, and patients exhibit a toxic level of hyperbilirubinemia (≥340 μmol/L) shortly after birth.[7]

CNS1 patients usually suffer from bilirubin encephalopathy, and are prone to death within the first 2 years of their lives.[8,9] At present, orthotopic liver transplantation is the only radical treatment.[10,11] CNS2 is characterized with not very high bilirubin (from 103 to <340 μmol/L)[12] and the bilirubin glucuronidation is less than 10% of normal level but not completely eliminated.[13] Although the phenotype of CNS2 is less severe, patients with CNS2 remain vulnerable to brain injury throughout life, especially in the setting of concurrent diseases, after injury, or during surgery.[14] Poddar *et al*.[15] reported a case of kernicterus in a CNS2 child due to a dramatic increase in UCB caused by hemolysis. GS is a mild hyperbilirubinemia (from normal level up to 80–100 μmol/L) that occurs in 5–10% of the population,[12] with approximately 70% reduction in bilirubin glucuronidation.[16] GS is considered as a benign condition without neurological damage and treatment requirement.

At present, there are few reports of kernicterus in adults.

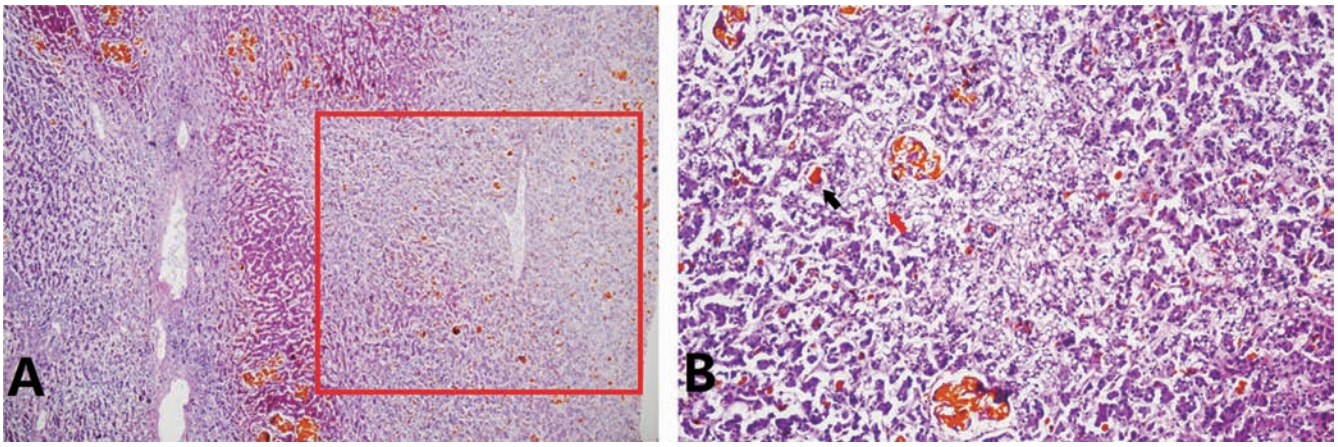

Fig. 1. Liver histopathology of patient 1 revealed massive and submassive necrosis, cholestasis and steatosis. (A) Hematoxylin-eosin stain, 40 x. (B) Hematoxylin-eosin stain, 200 x. Massive and submassive necrosis, cholestasis and steatosis are indicated by the red box, and black and red arrows, respectively.

Here, we aimed to report two CNS2 adults with *UGT1A1* mutations who developed kernicterus.

Methods

Subjects and sample collection

The patients and all family members received careful clinical examinations and laboratory assessments by experienced physicians in Beijing You'an Hospital, Capital Medical University. Fasting blood samples were collected from all participants; clinical manifestations and biochemical parameters were collected and analyzed.

This study was approved by the Ethics Committee of Beijing You'an Hospital, Capital Medical University, and a written informed consent form was obtained from all participants.

DNA extraction and screening for the mutations in UGT1A1

Genomic DNA was extracted from whole blood using a QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany), according to the manufacturer's protocol. The promoter, all five exons, exon-intron boundaries, and a region in the distal promoter (the phenobarbital response enhancer module, PBREM) of *UGT1A1* were amplified by PCR technology, then purified through agarose gel electrophoresis and sequenced using a 3730XL sequencer (Applied Biosystems Inc., Foster City, CA, USA). Finally, Sanger sequencing data were compared and analyzed by SeqMan software (DNASTAR, Madison, WI, USA).

Bioinformatics analyses

Potential functional effects of novel missense mutations were predicted by PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2/index.shtml), SIFT (http://sift.jcvi.org/), PROVEAN (http://provean.jcvi.org/index.php), MutationTaster (http://mutationtaster.org/), FATHMM (http://fathmm.biocompute.org.uk/), InterVar (http://wintervar.wglab.org/), and MutPred2 (http://mutpred.mutdb.org/). The grade of conservation of the mutant nucleotides was determined by PhastCons and PhyloP.

Results

Patient 1

The patient was a 32-year-old man with a 30-year history of jaundice. In May 2020, he lost his appetite after drinking (about 500 mL beer, 20 g ethanol). He took Chinese medicine (unknown pharmaceutical ingredients) for a week, but the symptoms were not alleviated. Then, he developed dizziness, headache, and mild neuropsychological disorder. Liver function results were abnormal, with decreased albumin (23.8 g/L) and increased aspartate aminotransferase (commonly referred to as AST; 148U/L), alanine aminotransferase (commonly referred to as ALT; 54 U/L), total bilirubin (TB, 411.8 μmol/L), indirect bilirubin (266.3 μmol/L) and direct bilirubin (145.5 μmol/L). Blood routine test showed a high proportion of neutrophils (81.5%) and a decrease in hemoglobin (109 g/L). His prothrombin time activity was 42% and the Coombs test was negative. Blood ammonia (16 μg/dL), fasting plasma lipids, autoantibodies profile, anti-neutrophil cytoplasmic antibody, and immunoglobulins (Igs) including IgG, IgA, IgM, and IgE were normal. The viral hepatitis markers were negative. Computed tomography (referred to as CT) and magnetic resonance imaging showed hepatosplenomegaly (Supplementary Fig. 1). Brain CT scans were normal (Supplementary Fig. 1). The patient was diagnosed with acute-on-chronic liver failure (ACLF), CNS2 and kernicterus, and received oral phenobarbital, albumin infusion, and anti-infection therapy.

One week after admission, the patient suddenly manifested hematemesis and showed restlessness, then lost consciousness. TB, indirect bilirubin, and blood ammonia levels increased to 532.3 μmol/L, 379.1 μmol/L, and 123 μg/dL, respectively. Gastroscopy revealed cardiac mucosal laceration syndrome (Mallory-Weiss syndrome). The patient was treated with hemostasis, sedation, intramuscular injection of phenobarbital, and artificial liver support treatment of double plasma molecular adsorption system combined with plasma exchange (DPMAS) and plasma exchange. Later, the patient's consciousness gradually recovered and he underwent liver transplantation. Histopathology of the removed liver showed massive and submassive hepatocyte necrosis, cholestasis, and steatosis (Fig. 1). The patient is now in a relatively stable state. Table 1 and Fig. 2 show his blood test results.

Sanger sequencing of the *UGT1A1* gene identified a compound heterozygous mutation in this patient, which was in-

Table 1. Biochemical characteristics of patient 1

Characteristics	Reference	Days after admission										
		1	3	8[a]	13[b]	15[c]	16[c]	17[c]	18	24	25[c]	26
TB (μmol/L)	5–21	411.8	513.6	532.3	616.4	570.3	353.8	453.3	417.8	524	561.5	378.5
IB (μmol/L)	<7	266.3	370.6	379.1	450.1	427.7	274.9	374.3	352.7	428.6	490.4	330.4
DB (μmol/L)	–	145.5	143	153.2	166.3	142.6	78.9	79	65.1	95.4	71.1	48.1
ALB (g/L)	40–55	23.8	27.8	27.4	39.4	35.9	35.7	46.2	43.3	41.3	42.2	43.2
BAMR	–	1.1	1.2	1.3	1.0	1.0	0.7	0.6	0.6	0.8	0.9	0.6
ALT (U/L)	9–50	54	60	62	64	130	81	79	75	108	93	75
AST (U/L)	15–40	148	152	147	164	366	207	206	184	207	191	150
GGT (U/L)	10–60		114		99							
ALP (U/L)	45–125		474		412							
TBA (μmol/L)	<10		175.3		122.8							
WBC ($\times 10^9$/L)	3.5–9.5	6.68	5.54	8.26	7.47	7.02	7.2	6.87	4.21	6.25	4.08	7.69
N%	40–75	81.5	71.1	80.1	83.2	75.8	72.8	72.7	83.4	75.9	71.6	90.1
HGB (g/L)	130–175	109	112	105	80	82	78	85	80	65	62	68
PLT ($\times 10^9$/L)	125–350	145	138	128	85	87	81	90	89	85	70	75
Amon (μg/dL)	19–54	16	25	123	79	20	8	25	48	59		

[a]The patient's neuropsychiatric symptoms deteriorated after hematemesis.

[b]The patient underwent plasma exchange.

[c]The patient underwent DPMAS and plasma exchange.

Abbreviations: %N, proportion of neutrophils; ALB, albumin; ALP, alkaline phosphatase; Amon, blood ammonia; BAMR, Bilirubin-albumin molar ratio; DB, direct bilirubin; GGT, γ-glutamyl transferase; HGB, hemoglobin; IB, indirect bilirubin; PLT, platelets; TBA, total serum bile acid; WBC, white blood cell.

herited from his mother (c.211G>A, p.G71R) and his father (c.-3279T>G; c.572C>T, p.S191F) (Fig. 3 and Supplementary Fig. 2).

Interestingly, the patient's elder sister also showed jaundice since childhood. Her TB was about 400 μmol/L. She was diagnosed with CNS2 but had never developed kernicterus. In addition, the patient's father had mild unconjugated hyperbilirubinemia (TB of 32.1 μmol/L, indirect bilirubin of 23.2 μmol/L) and was diagnosed with GS. They were both also found to carry the same compound heterozygous mutation as the patient (Fig. 3).

Patient 2

The patient was a 35-year-old man. He had been suffer-

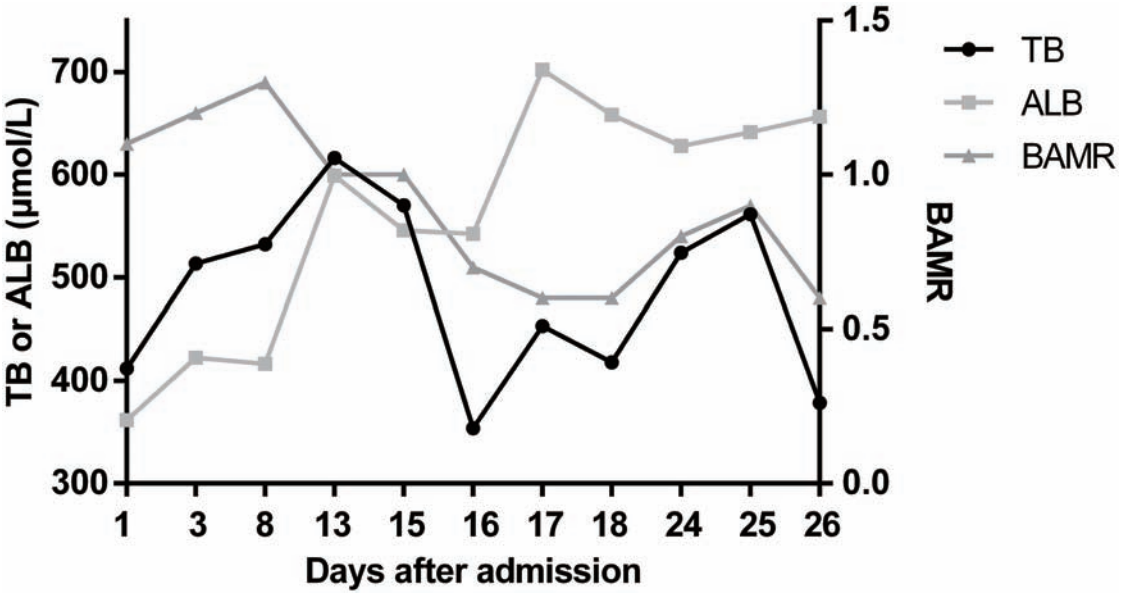

Fig. 2. BAMR in patient 1 after admission.

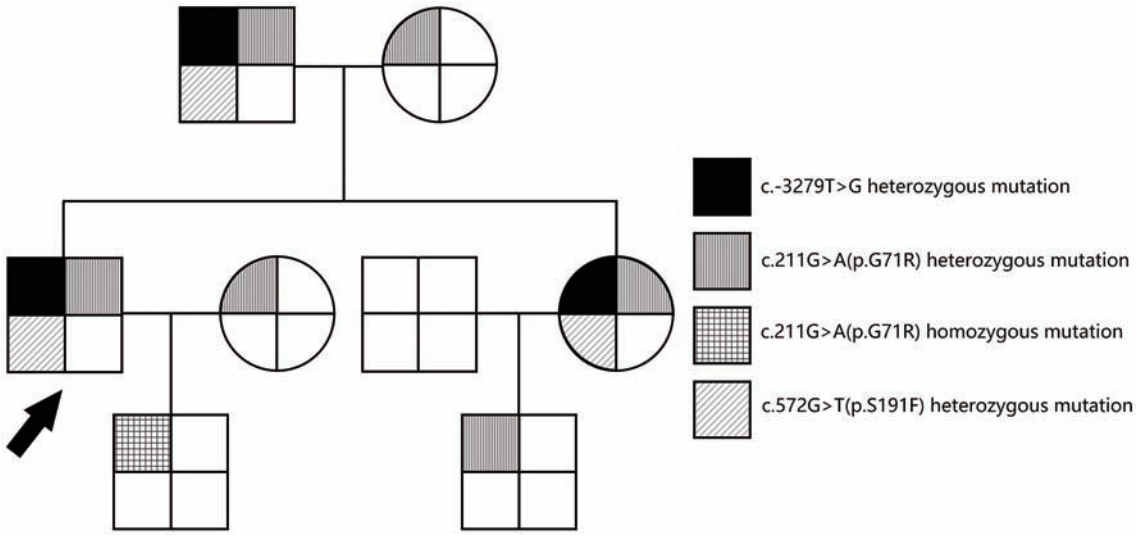

Fig. 3. Family pedigree of patient 1. The arrow indicates patient 1.

ing from jaundice since birth, with TB ranging from 70–80 μmol/L. In adulthood, his TB levels were about 300 μmol/L. At the age of 31, he developed right upper abdominal pain and severe jaundice. His TB level had been found to have increased to above 500 μmol/L. The patient was diagnosed with gallbladder stones, cholecystitis and CNS2. After treatment with antibiotics and oral phenobarbital, the patient's pain was relieved and TB was reduced to 300 μmol/L. Later, the patient experienced repeated abdominal pain, fever, and severe jaundice. In November 2019, he became lethargic and unresponsive after a fever. Then, he developed limb convulsions and urinary incontinence. The patient was admitted on November 15, 2019.

The results of liver function showed decreased albumin (21.5 g/L) and increased AST (222 U/L), ALT (57 U/L), TB (417.4 μmol/L), and direct bilirubin (195 μmol/L). The proportion of neutrophils (88.1%) and blood ammonia (112 μg/dL) were elevated. The Coombs test was negative. The fasting plasma lipids, autoantibodies profile, and anti-neutrophil cytoplasmic antibody were normal. Cerebrospinal fluid results did not suggest central nervous system infection. Abdominal ultrasonography showed gallstones, cholecystitis, and splenomegaly. Brain CT scans were normal.

Sanger sequencing of the *UGT1A1* gene identified three heterozygous mutations, namely G71R (c.211G>A), R209W (c. 625C>T), and M391K (c.1172T>A) (Supplementary Fig. 3). The M391K mutation has not been reported before. Seven software programs were used to predict the pathogenicity of the mutation. As shown in Supplementary Table 1, all software programs showed that the M391K mutation was pathogenic or damaging. The PhastCons score of the mutation was 1, and the corresponding PhyloP value was 2.307, suggesting the high conservation of this amino acid.

The patient was treated with antibiotics, sedation, intramuscular injection of phenobarbital, and artificial liver support treatment with DPMAS and plasma exchange. The patient's neurological status gradually returned to normal. Table 2 and Fig. 4 show the results of his blood investigations.

Discussion

High concentrations of UCB can cause nervous system damage, known as bilirubin encephalopathy or kernicterus. In general, kernicterus is found in infants and young children, especially those who are premature and/or have hemolysis.[1,17] On one hand, the blood-brain barrier of newborns and children is immature. On the other hand, the albumin/bilirubin-binding capacity and tissue-binding capacity vary significantly among newborns, and these values are particularly low for premature babies.[18] Kernicterus in adults is a rare condition. To date, there are only two published cases.[19,20] In one case, the disorder was associated with liver failure.[19] The other case was an adult with CNS2, who developed kernicterus after laparoscopic surgery.[20] This report describes two new cases of adult kernicterus associated with *UGT1A1* mutations (CNS2).

CNS2 is usually caused by missense mutations in the *UGT1A1* gene, which reduces enzyme activity but does not eliminate it.[21,22] Most patients with CNS2 have homozygous missense mutations or compound heterozygous mutations.[23] This explains the milder phenotype and inducibility of the residual enzyme activity by phenobarbital administration. Variants c.211G>A (p.G71R) and c.1456T>G (p.Y486D) are the most frequently reported mutation sites in Asian CNS2 patients.[24–26] In our patients, *UGT1A1* sequencing analysis was performed. Patient 1 is a compound heterozygote with mutations c.-3279T>G, G71R, and S191F. His father and sister share the same *UGT1A1* genotype as him, and were diagnosed with GS and CNS2, respectively. But none of them suffered from kernicterus. The above evidence suggests that the same genotype may result in different phenotypes and clinical manifestations. A possible explanation is that a multifactorial etiology including hormonal, environmental, and genetic factors contributes to the development of inherited diseases. Besides, liver histology suggested massive and submassive hepatocytes necrosis and cholestasis, consistent with ACLF and CNS2, respectively.[27,28] Histopathology also showed steatosis, but the related mechanism is not clear. Before the onset of ACLF, Patient 1 drank about 20 g ethanol (500 mL beer) and then took traditional Chinese medicine for 1 week. Considering the relatively small intake of ethanol and the complex composition of Chinese medicine (although the composition is unknown), we speculated that Chinese medicine was the main cause of his acute liver injury, and eventually lead to his ACLF on the basis of Crigler-Najjar syndrome.

Patient 2 is heterozygous for the mutations G71R, R209W and M391K. The mutations G71R, S191F, and R209W are

Table 2. Biochemical characteristics of patient 2

Characteristics	Reference	Days after admission							
		1	4	5[a]	7[b]	8[b]	9	12[b]	13
TB (μmol/L)	5–21	417.4	527.7	547.3	448.9	432.5	342.1	486.2	257.1
IB (μmol/L)	<7	222.4	320	360	322.8	322.9	260.4	361.8	217.5
DB (μmol/L)	–	195	207.7	187.3	126.1	109.6	81.7	124.4	39.6
ALB (g/L)	40–55	21.5	31.7	32.8	32.1	35	29.6	33.4	26.7
BAMR	–	1.3	1.1	1.1	0.9	0.8	0.8	1.0	0.6
ALT (U/L)	9–50	57	58	59	58	63	71	88	63
AST (U/L)	15–40	222	249	233	171	160	171	191	169
GGT (U/L)	10–60		78	76	73	74		112	
ALP (U/L)	45–125		513	460	327	282		334	
TBA (μmol/L)	<10		159.1	148.9	137.4	136.6		133.8	
WBC (×10^9/L)	3.5–9.5	12.77	5.56	6.22	8.35	9.46	6.24	10.6	
N%	40–75	88.1	76.6	79.9	79.8	84.7	79	86.7	
HGB (g/L)	130–175	116	108	105	102	102	93	93	
PLT (×10^9/L)	125–350	237	161	176	176	188	156	173	
Amon (μg/dL)	19–54	41	107	116	81	74	108	75	89

[a]The patient underwent DPMAS and plasma exchange.

[b]The patient underwent plasma exchange.

Abbreviations: %N, proportion of neutrophils; ALB, albumin; ALP, alkaline phosphatase; Amon, blood ammonia; BAMR, Bilirubin-albumin molar ratio; DB, direct bilirubin; GGT, γ-glutamyl transferase; HGB, hemoglobin; IB, indirect bilirubin; PLT, platelets; TBA, total serum bile acid; WBC, white blood cell.

located in exon 1 of the *UGT1A1* gene, and have been shown to be associated with CNS2 (moderate hyperbilirubinemia).[29] The mutation c.-3279T>G is located in the phenobarbital response enhancer module, which is related to GS (mild hyperbilirubinemia).[30] The M391K mutation has not been reported yet. All prediction software indicated that the mutation is pathogenic. PhastCons and PhyloP showed a high degree of amino acid conservation, suggesting that the mutation has a great impact on amino acids and the protein. According to the variant classification criteria of the American College of Medical Genetics and Genomics and the Association for Molecular Pathology,[31] the novel M391K mutation is pathogenic to CNS2 in this patient. The effect on the UGT1A1 enzyme's activity needs to be further verified through cell experimentation. In summary, the mutation site, number, and genotype of *UGT1A1* are related to bilirubin level.

The pathogenesis of kernicterus has not been fully elu-

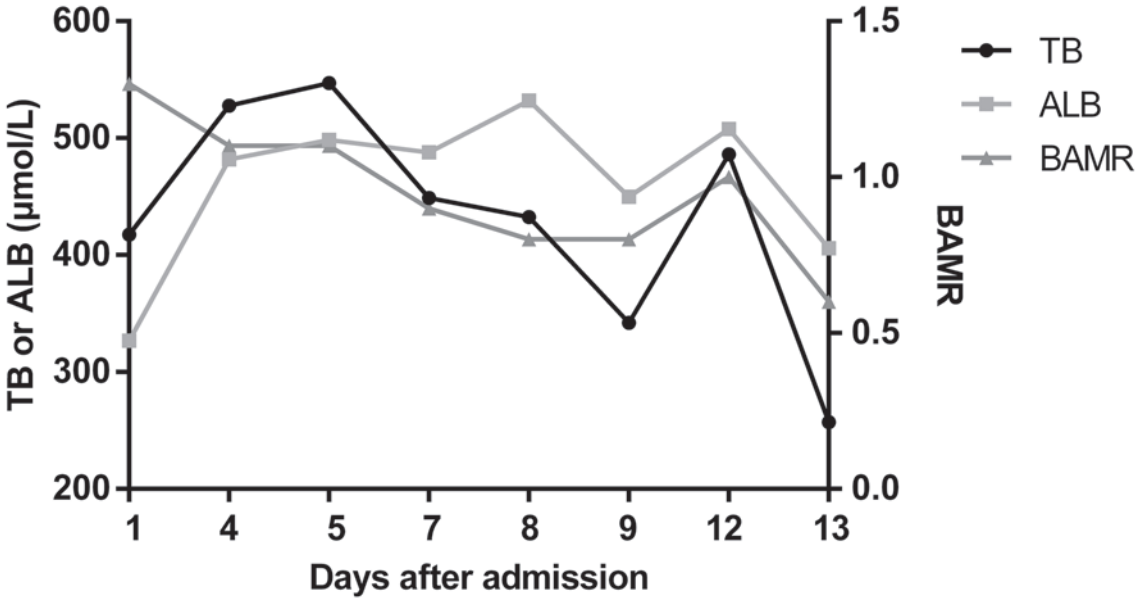

Fig. 4. BAMR in patient 2 after admission.

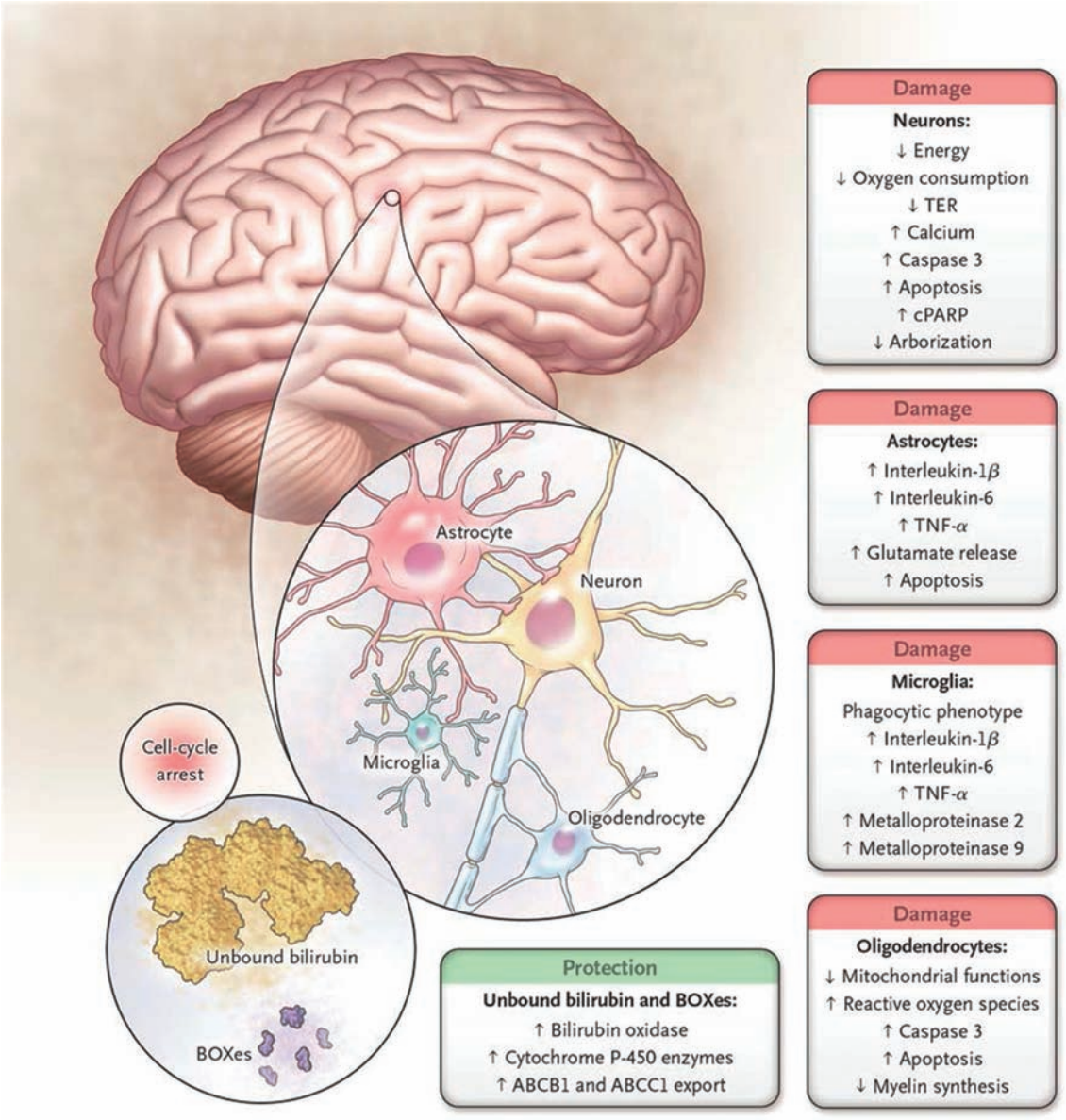

Fig. 5. Cell types and metabolic processes affected by bilirubin in the central nervous system. The main effects of bilirubin on neurons are decreased oxygen consumption and increased release of calcium and caspase 3, resulting in apoptosis. There is also decreased dendritic and axonal arborization. A similar pattern is observed in oligodendrocytes with increased apoptosis, impairment of the redox state (oxidative stress), and reduced synthesis of myelin. Microglia react to toxic injury associated with bilirubin by increased release of proinflammatory cytokines and metalloproteinase activity as cells manifest a phagocytic phenotype. A similar proinflammatory pattern is observed in astrocytes, with enhanced release of glutamate and apoptosis. At the same time, cells may reduce the intracellular concentration of bilirubin either by extruding the pigment through the ATP-binding cassette transporters or by increasing the formation of the less toxic products through bilirubin oxidation products (BOXes) and/or cytochrome P-450 enzymes (1a1 and 1a2, in particular). These responses are protective, whereas all others result in cell damage; this suggests that once the intracellular concentration of bilirubin exceeds a toxic threshold (still to be defined), the polymorphic metabolic cascade leading to neurotoxicity ensues. (From Watchko JF, Tiribelli C. Bilirubin-induced neurologic damage-mechanism and management approaches. N Engl J Med 2013; 369: 2025). Abbreviations: cPARP, cleaved poly (adenosine diphosphate-ribose) polymerase; TER, transcellular resistance.

cidated but the main reason is excessive bilirubin production (i.e. hemolysis) and/or insufficient liver glucuronidation, leading to higher levels of free unbound bilirubin. The potential mechanism of UCB neurotoxicity is shown in Fig. 5.[18] In our two patients, CNS2 reduced bilirubin glucuronidation. Infection, fever and liver injury cause a decrease in serum albumin levels. Therefore, free unbound bilirubin increased. In infants, a bilirubin-albumin molar ratio (referred to as BAMR) value >0.8 is considered dangerous because bilirubin/albumin binding is unpredictable at these levels.[17] However, for adults, the BAMR value predicting kernicterus is still unclear. In our two patients, bilirubin concentrations increased to 616 μmol/L and 547 μmol/L, respectively, with BAMR value >0.8 in both. Then, kernicterus occurred. Therefore, for patients with CNS2, it is important to avoid particularly high bilirubin levels and maintain normal serum albumin levels.

Infection, liver damage, and hemolytic disease should also be avoided. It should be noted that CNS2 patients respond to phenobarbital. To prevent severe hyperbilirubine-

mia and kernicterus, phenobarbital therapy should be adhered to. If the patient develops kernicterus, treatments that eliminate the cause, or those such as albumin supplementation and artificial liver support treatment, are effective. Due to severe necrosis and insufficient regeneration of hepatocytes in patient 1, the bilirubin level still slightly fluctuated in the case of artificial liver support treatment. At the same time, although the patient recovered consciousness after albumin supplementation and artificial liver support treatment, increased bilirubin due to CNS2 may lead to recurrence of kernicterus. At present, liver transplantation is the only treatment option that completely replaces UGT1A1 function and normalizes serum bilirubin levels.

In conclusion, although very rare, adults with CNS2 are at risk of kernicterus. Phenobarbital administration helps maintain bilirubin levels and prevent kernicterus.

Funding

This study was supported by Beijing Municipal Science and Technology Project (No. Z171100002217070), National Key R&D Program of China (No. 2017YFA0103000), National Science and Technology Key Project on "Major Infectious Diseases such as HIV/AIDS, Viral Hepatitis Prevention and Treatment" (Nos. 2012ZX10002004-006, 2017ZX10203201-005, 2017ZX10201201, 2017ZX10202203-006-001 and 2017ZX 10302201-004-002), "Beijing Municipal Administration of Hospitals" Ascent Plan (No. DFL20151601), Beijing Municipal Administration of Hospitals Clinical Medicine Development of Special Funding Support (Nos. ZYLX201806 and ZYLX202125), Digestive Medical Coordinated Development Center of Beijing Municipal Administration of Hospitals (No. XXZ0503), You An Fund for Liver Diseases and AIDS (No. YNKTTS201801189), Beijing Municipal Natural Science Foundation (No. 7202068).

Conflict of interest

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

Author contributions

Conception and design (JB, LL, SZ, ZD), patient recruitment (JB, LL), data collection (HL, SL, LB, WS), data analysis (JB, LL, HL, WS), writing the draft (JB, LL), revising and polishing the manuscript (JB, LL, YC, SZ, ZD). All authors read and approved the final manuscript.

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Journal of Clinical and Translational Hepatology **2021** vol. 9(2) | 187–193
DOI: 10.14218/JCTH.2020.00127

Original Article

Predictive Model of Ursodeoxycholic Acid Treatment Response in Primary Biliary Cholangitis

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Received: 24 November 2020 | **Revised:** 21 January 2021 | **Accepted:** 13 February 2021 | **Published:** 4 March 2021

Abstract

Background and Aims: Although ursodeoxycholic acid (UDCA) treatment in primary biliary cholangitis is effective in many patients, there are still many people who respond poorly to it. Identifying and intervening these patients early is important. Therefore, exploring the risk factors and proposing a predictor index to predict the UDCA treatment nonresponse earlier among primary biliary cholangitis patients were the aims of this research. ***Methods:*** A total of 135 primary biliary cholangitis patients treated with UDCA (13–15 mg/kg/d) were enrolled in this retrospective study. The response to treatment was evaluated based on Paris I criteria. The univariate and logistic multivariate regression analyses were adopted to determine the independent risk factors and propose a predictor index. Receiver operating characteristic curve was used to evaluate the predictive ability of the predictor index. ***Results:*** Total bilirubin, albumin, globulin, immunoglobin M, and aspartate aminotransferase-to-platelet ratio index were the five independent risk factors associating with early biochemical nonresponse to UDCA treatment. Based on these factors, we established a predictor index with the predictive value being 0.886 (sensitivity: 82.80%, specificity: 84.40%). ***Conclusions:*** We developed a predictor index that had an accurate prediction of the early biochemical nonresponse to UDCA treatment, which is expected to provide valuable information for the high-risk group before treatment begins.

Citation of this article: Shu Y, Song Y, Bai T, Pan X, Shang H, Yang L, *et al*. Predictive model of ursodeoxycholic acid treatment response in primary biliary cholangitis. J Clin Transl Hepatol 2021;9(2):187–193. doi: 10.14218/JCTH.2020.00127.

Introduction

Primary biliary cholangitis (PBC) is a kind of autoimmune liver disease marked by destruction of the small bile duct, rising alkaline phosphatase levels and positivity for anti-mitochondrial antibody (AMA) in serum, especially for the AMA-M2 form.[1] The current standard treatment of PBC is ursodeoxycholic acid (UDCA), 13–15 mg/kg/day, which can significantly improve the clinical manifestation, serum profile and histology.[2–4] However, the response to UDCA treatment in some patients is unsatisfactory, which may result in poor prognosis.[5,6]

In recent years, with the increasing prevalence of PBC (39.2 per 100,000),[7] the cases in China have risen up to 19.1 cases per 100,000.[8] Furthermore, because of the uncertainty of UDCA treatment response in some patients, identifying patients at high-risk of poor response to UDCA before the start of treatment and starting the second-line treatment early will help to control disease progression. Therefore, prediction of UDCA treatment nonresponse in PBC is drawing more and more attention.

Several studies have been conducted to identify inadequate response to UDCA.[5,6,9–13] These studies, which are based on 1- or 2-year treatment data, have effectively predicted the long-term outcome but they have not identified the patients earlier. To make up for this deficiency, some criteria have been conducted based upon admission data,[14–16] but the findings still need validation. Given that the risk factors that are associated with early biochemical nonresponse have been subject to misidentification, the aims of our study were to accurately identify the independent risk factors of the early biochemical nonresponse and propose a relatively accurate predictor index for insufficient early biochemical response to UDCA treatment before treatment begins, ultimately providing more evidence of relevant aspects in PBC patients.

Methods

Study design

In total, 241 PBC patients, at admission and in the outpatient setting from January 2010 to July 2018, were identified through search of the electronic medical record system. The 135 patients who met the research needs were enrolled in this retrospective study. The patients were regularly treated with UDCA upon diagnosis. The baseline data were obtained when the patients were first diagnosed with PBC. The follow-up data were obtained at the 1-year regular UDCA treatment appointment (the 1-year follow-up endpoint).

The study was approved by the Ethics Committee of Tongji Medical College, HUST. This Ethics Committee was constituted and still functions in accordance with the Inter-

Keywords: Primary biliary cholangitis; Therapy; Risk factors; Statistical model.
Abbreviations: AMA, anti-mitochondrial antibody; APRI, aspartate aminotransferase- to-platelet ratio index; AUC, area under the ROC curve; ESR, erythrocyte sedimentation rate; FIB-4, fibrosis index based on the four factors; Ig, immunoglobin; PBC, primary biliary cholangitis; ROC, receiver operating characteristic; UDCA, ursodeoxycholic acid; ULN, upper limit of normal.
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national Conference on Harmonization-Good Clinical Practice, the Good Clinical Practice in China, and the Declaration of Helsinki. The study is also registered on the Chinese Clinical Trial Registry Platform (http://www.chictr.org.cn/), as ChiCTR1800019712.

Diagnostic criteria

According to the American Association for the Study of Liver Disease and the European Association for the Study of the Liver,[9,17] a patient meeting any two of following three criteria was diagnosed with PBC: (1) titer of AMA-M2 ≥1:40; (2) alkaline phosphatase elevation of unknown causes (≥1.5 times normal) for 6 months; (3) and liver biopsy findings of non-suppurative cholangitis, interlobular bile duct injury, or bile duct granuloma.[9,17] Positive or weak detection of AMA-M2 was noted when the titer was ≥1:40, according to the equipment system setting.

Inclusion and exclusion criteria

The inclusion criteria were as follows: (1) patients diagnosed with PBC; and (2) PBC patients treated with UDCA (13–15 mg/kg/d) regularly following diagnosis. The excluded criteria were as follows: (1) patients complicated with other kinds of hepatitis; (2) patients complicated with liver cancer; (3) pregnant or lactational women; (4) patients who died during this hospitalization; (5) patients with incomplete baseline data; and (6) patients with follow-up less than 1 year.

Data collection

The clinical, laboratory and pathological data were collected from Wuhan Union Hospital and included measures of leukocytes, hemoglobin, platelets, prothrombin time, fibrinogen, alanine aminotransferase, aspartate aminotransferase, total bilirubin, alkaline phosphatase, γ-glutamyl transpeptidase, albumin, globulin, erythrocyte sedimentation rate (referred to as ESR), immunoglobin (Ig) and hepatic-related autoimmune antibodies, as well as findings from liver pathology. Aspartate aminotransferase-to-platelet ratio index (ARP1)[10] and fibrosis index based on the four factors (FIB-4),[11] the two noninvasive liver fibrosis indexes, were analyzed as part of the baseline data. The liver specimens were assessed blindly by two experienced hepatopathologists.

Response to UDCA

The Paris I criteria proposed by Corpechot *et al*.[12] in 2008 was adopted in this study to evaluate the response to UDCA treatment. Early biochemical response was defined as the patients' indexes having met the requirements of the Paris I criteria after a 1-year period of UDCA treatment, in which the level of alkaline phosphatase was ≤3 the upper limit of normal (referred to as ULN), the level of aspartate aminotransferase was ≤2 ULN, and the level of total bilirubin was ≤1 mg/dL.[12] Whereas, the early biochemical nonresponse was defined as the patients' indexes not having met the requirements mentioned above.

Statistical analysis

SPSS software v23.0 (IBM Corp., Armonk, NY, USA) was employed for the data processing. Continuous variables were expressed as median (interquartile range) because of skewed distribution. Categorical variables were described in terms of numbers and percentages. The cut-off value of continuous variables were determined by receiver operating characteristic (ROC) curve, using MedCalc statistical software. Univariate analysis was conducted by χ^2 test or Fisher's exact test, while multivariate analysis was conducted by forward logistic regression analysis based on maximum likelihood estimation, predictor index was obtained by logistic analysis and the ROC curve was measured to evaluate prediction value. Statistical significance was signified by p-value <0.05.

Results

Baseline and follow-up data of PBC patients

In total, 122 females and 13 males (totaling 135 patients) were enrolled, and the gender ratio of female to male was 9.4:1. The median age of the total 135 patients was 51 (range, 45–58) years-old. The liver biopsy had been conducted for 52 (38.5%) of the patients because of the need for diagnosis. The destruction of small bile duct was apparent in all of the patients upon histological examination, with 40 (76.9%) being at stages I and II. Meanwhile, interface hepatitis was apparent in 36 (69.2%) of the patients upon histological examination, but 34 (94.5%) were only at the mild or moderate stages (Table 1).

The follow-up time for this entire group was 1 year. After 1-year of the UDCA treatment, 77 (57%) patients had achieved early biochemical response, whereas 58 (43%) patients had not. The alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase, γ-glutamyl transpeptidase and total bilirubin levels from follow-up were significantly lower than those of baseline (p<0.05), which showed the therapeutic effect of UDCA in our PBC patients (Table 1).

ROC curve and univariate analysis of risk factors

In continuous indexes, the cut-off value for sorting the patients with early biochemical response from those with nonresponse was determined by ROC curve. The indexes which might influence the biochemical response (p<0.05) (Table 2) and the categorical variables were evaluated by univariate analysis. Hemoglobin, prothrombin time, aspartate aminotransferase, total bilirubin, alkaline phosphatase, albumin, globulin, IgG, IgM, IgA, APRI, and FIB-4 were identified as factors that might influence the early biochemical response (p<0.05) (Table 3).

Multivariate analysis of the risk factors and development of the predictor index

Multivariate logistic regression analysis was employed to determine the independent risk factors. The analysis included all the factors that were statistically significant in the univariate analysis. Collinearity diagnostics was employed and the multi-collinearity of the indexes of those factors were excluded. After adjusting for sex and age, the forward logistic regression analysis based on maximum likelihood estimation indicated that total bilirubin ≥1.98 mg/dL, albumin ≤35.30 g/L, globulin ≥33.00 g/L, IgM ≥3.10 g/L and APRI ≥1.63 were independent risk factors of early biochemical nonresponse in PBC patients, with the

Table 1. Characteristics of the PBC patients in our study cohort

Variables	Total patients	Response patients	Nonresponse patients
Gender, male (%)	13 (9.6%)	9 (11.7%)	4 (6.9%)
Age, years	51 (45, 58)	53 (45, 58)	50.5 (44, 56)
Leukocyte, 4–10*10^9/L	4.64 (3.49, 5.84)	4.809 (3.53, 5.67)	4.245 (2.89, 6.15)
Hemoglobin, male: 120–160 g/L; female: 110–150 g/L	111 (99, 122)	116 (106, 125)	102.5 (85,115)
Platelet, 100–300*10^9/L	150 (95, 240)	158 (106.5, 243.5)	138.5 (78.0, 235.5)
Prothrombin time, 11–16 s	12.7 (12.0, 13.5)	12.5 (12.0, 13.2)	12.8 (12.0, 15.0)
Fibrinogen, 2–4 g/L	3.10 (2.57, 3.63)	3.10 (2.71, 3.60)	3.15 (2.49, 3.69)
Alanine aminotransferase, 5–35 U/L	67.0 (41.0, 115.0)	67.0 (39.5, 115.5)	70.5 (45.5,108.8)
Aspartate aminotransferase, 8–40 U/L	86.0 (52.0, 129.0)	63.0 (45.0, 99.5)	113.0 (74.8, 159.3)
Total bilirubin, 0.1–1 mg/dL	1.44(0.82, 2.59)	16.95 (10.93, 27.60)	41.75(25.05, 87.55)
Alkaline phosphatase, 40–150 U/L	318.0 (209.0, 537.0)	264.0 (181.5, 484.0)	387.5(250.5,638.3)
γ-glutamyl transpeptidase, 7–32 U/L	346.0 (160.0, 612.3)	325.0 (156.8, 568.5)	380.5 (177.3, 670.3)
Albumin, 35–55 g/L	37.8 (33.2, 41.3)	39.7 (36.6, 42.2)	35.1 (30.9, 39.6)
Globulin, 20–30 g/L	33.7 (28.1, 38.9)	31.8 (27.0, 36.6)	36.5 (31.1, 42.2)
ESR, male <15 mm/h; female <20 mm/h	33.0 (17.8, 67.5)	25.0 (16.0, 67.0)	46.0 (27.0, 73.0)
IgG, 7.51–15.60 g/L	16.89 (12.60, 21.00)	14.65 (11.93,18.73)	18.89 (15.90, 25.20)
IgM, 0.460–3.040 g/L	4.24 (2.94, 5.49)	3.61 (2.33, 5.47)	4.68 (3.94, 5.66)
IgA, 0.82–4.53 g/L	2.65 (1.92, 3.79)	2.47 (1.87, 3.37)	3.46 (2.21, 4.55)
Complement 3, 0.790–1.520 g/L	1.03 (0.79, 1.23)	1.00 (0.81, 1.16)	1.09 (0.67, 1.33)
Complement 4, 0.160–0.380 g/L	0.18 (0.14, 0.22)	0.18 (0.14, 0.21)	0.17 (0.13, 0.22)
APRI	1.25 (0.81, 2.48)	1.05 (0.70, 1.70)	1.98 (1.04, 3.54)
FIB-4	3.49 (1.97, 6.09)	2.57 (1.70, 4.65)	4.38 (2.47, 8.02)
ANA, *n* (%)	119 (89.5%)	67 (89.3%)	52 (89.7%)
ASMA, *n* (%)	4 (3.00%)	2 (2.67%)	2 (3.45%)
AMA-M2, *n* (%)	111 (83.5%)	60 (80.0%)	51 (87.9%)
Anti-sp100 antibody, *n* (%)	8 (25.00%)	4 (17.39%)	4 (44.44%)
Anti-gp210 antibody, *n* (%)	16 (50.00%)	11 (47.83%)	5 (55.56%)
Anti-3E-BPO antibody	25 (78.10%)	17 (73.91%)	8 (88.89%)
Interface hepatitis, *n* (%)	36 (69.2%)	27 (71.1%)	9 (64.3%)
Cholangitis (Ludwig)			
I–II	40 (76.9%)	32 (82.1%)	8 (61.5%)
III–IV	12 (23.1%)	7 (17.9%)	5 (38.5%)
Alanine aminotransferase T12	30.0 (21.0, 47.5)	25.0 (19.0, 33.3)	49.0 (30.0, 83.0)
Aspartate aminotransferase T12	42.0 (29.5, 77.5)	33.0 (27.0, 41.3)	83.0 (54.0, 114.0)
Total bilirubin T12	1.02 (0.75, 2.03)	13.85 (11.40, 17.00)	42.00 (26.80, 74.20)
Alkaline phosphatase T12	174.0 (108.5, 264.5)	119.5 (86.8, 183.5)	277.0 (201.0, 386.0)
γ-glutamyl transpeptidase T12	129.0 (62.0, 308.0)	99.0 (40.0, 195.8)	234.0 (104.0, 454.0)
Albumin T12	39.0 (34.1, 43.0)	41.9 (38.0, 44.0)	35.0 (27.3,38.8)
Globulin T12	33.0 (28.4, 38.6)	32.2 (28.6, 35.5)	35.1 (28.1, 43.0)

Abbreviations: ANA, anti-nuclear antibody; anti-3E-BPO antibody, anti-BCOADC-E2PDC-E2OGDC-E2 antibody; ASMA, anti-smooth muscle antibody; dsDNA antibody, double stranded DNA antibody.

area under the ROC curve (AUC) values for each being 0.804, 0.704, 0.676, 0.640 and 0.711, respectively (Tables 2 and 4).

In assigning the independent risk factors that were men-

Table 2. ROC curve of continuous variations of baseline data

Variables	AUC	Cut-off value	*p*-value
Age, years	0.554	51.00	0.286
Leukocyte, 4−10*10^9/L	0.520	4.50	0.7
Hemoglobin, male: 120−160 g/L; female: 110−150 g/L	0.710	108.00	<0.001***
Platelet, 100−300*10^9/L	0.570	104.00	0.173
Prothrombin time, 11−16 s	0.605	13.90	0.042*
Fibrinogen, 2−4 g/L	0.524	2.80	0.651
Alanine aminotransferase, 5−35 U/L	0.545	53.00	0.362
Aspartate aminotransferase, 8−40 U/L	0.732	107.00	<0.001***
Total bilirubin, 0.1−1 mg/dL	0.804	1.98	<0.001***
Alkaline phosphatase, 40−150 U/L	0.668	317.00	<0.001***
γ-glutamyl transpeptidase, 7−32 U/L	0.554	440.00	0.285
Albumin, 35−55 g/L	0.704	35.30	<0.001***
Globulin, 20−30 g/L	0.676	33.00	<0.001***
ESR, male <15 mm/h; female <20 mm/h	0.622	23.00	0.062
IgG, 7.51−15.60 g/L	0.710	15.20	<0.001***
IgM, 0.460−3.040 g/L	0.640	3.10	0.013*
IgA, 0.82−4.53 g/L	0.646	3.30	0.01*
Complement 3, 0.790−1.520 g/L	0.527	1.31	0.656
Complement 4, 0.160−0.380 g/L	0.509	0.18	0.887
APRI	0.711	1.63	<0.001***
FIB-4	0.686	3.33	<0.001***

*p<0.05, ***p<0.001.

Table 3. Results of univariate analysis of risk factor between the response group and nonresponse group

Variables	Response group	Non-response group	Statistics	*p* value
Hemoglobin >108.00 g/L, *n* (%)	55 (71.4%)	20 (34.5%)	18.29	<0.001***
Prothrombin time >13.90 s, *n* (%)	6 (7.8%)	20 (34.5%)	15.16	<0.001***
Aspartate aminotransferase >107.00 U/L, *n* (%)	13 (16.9%)	31 (53.4%)	20.13	<0.001***
Total bilirubin >1.98 mg/dL, *n* (%)	10 (13.0%)	38 (65.5%)	39.84	<0.001***
Alkaline phosphatase >317.00 U/L, *n* (%)	29 (37.7%)	39 (67.2%)	11.58	0.001**
Albumin <35.30 g/L, *n* (%)	11 (14.3%)	30 (51.8%)	21.93	<0.001***
Globulin >33.00g/L, *n* (%)	29 (37.7%)	41 (70.7%)	14.45	0.001**
IgG >15.20 g/L, *n* (%)	29 (37.7%)	35 (60.3%)	6.83	0.009**
IgM >3.10 g/L, *n* (%)	40 (51.9%)	41 (70.7%)	4.84	0.028*
IgA >3.32 g/L, *n* (%)	17 (22.1%)	24 (41.4%)	5.83	0.016*
APRI >1.63, *n* (%)	20 (26.0%)	36 (62.1%)	17.76	<0.001***
FIB-4 >3.33, *n* (%)	29 (37.7%)	41 (70.7%)	14.45	<0.001***
AMA-M2, *n* (%)	60 (80.0%)	51 (87.9%)	1.49	0.222
Anti-sp100 antibody, *n* (%)	4 (17.4%)	4 (44.4%)		0.176
Anti-gp210 antibody, *n* (%)	11 (33.3%)	5 (55.6%)		1
Anti-3E-BPO antibody, *n* (%)	17 (73.9%)	8 (88.9%)		0.64
Interface hepatitis	27 (71.1%)	9 (64.3%)	0.017	0.896
Cholangitis (Ludwig)				
III−IV, *n* (%)	7 (17.9%)	5 (38.5%)	1.30	0.254

*p<0.05, **p<0.01, ***p<0.001.

Table 4. Result of logistics regression analysis of risk factor between response group and nonresponse group

Variables	Assignment	Multivariate analysis				
		B	S.E.	Wald	Exp(B) (95%CI)	Sig.
X1 (total bilirubin)	>1.98 mg/dL=1; <1.98 mg/dL=0	2.456	0.539	20.794	11.66 (40.56, 33.49)	<0.001***
X2 (globulin)	>33.00 g/L=1; <33.00 g/L=0	1.156	0.516	5.023	3.18 (1.16, 8.73)	0.025*
X3 (IgM)	>3.10 g/L=1; <3.10 g/L=0	1.217	0.561	4.695	3.38 (1.12, 10.15)	0.03*
X4 (APRI)	>1.63=1; <1.63=0	1.217	0.489	6.2	3.38 (1.30, 8.80)	0.013*
X5 (albumin)	<35.30 g/L=1; >35.30 g/L=0	1.533	0.573	7.162	4.63 (1.51, 14.23)	0.007**
Constant		−3.548	0.646	30.183	0.03	<0.001***

*p<0.05, **p<0.01, ***p<0.001.

tioned above (Table 4), the logistic equation was established and the predictor index was formed (Table 4). The ROC curve was adopted to evaluate the prediction value of the predictor index, and the obtained value of the AUC was 0.886, which was better than that obtained for any of the single independent risk factors, with the cut-off value being 0.3102 (Fig. 1). Transferring the logistic equation, the predictor index was obtained.

The logistic equation was:

$$\text{logit}(p) = -3.548+2.456*X1+1.156*X2 +1.217*X3+1.217*X4+1.533*X5$$

$$\text{predictor index} = 1/e^{(-3.548+2.456*X1 +1.156*X2+1.217*X3+1.217*X4+1.533*X5)}$$

Discussion

In this study, we adopted univariate analysis, logistic multivariate regression analysis and ROC curve analysis to identify the risk factors of UDCA nonresponse and propose a predictor index to predict treatment response of PBC patients to UDCA. We observed that total bilirubin, albumin, globulin, IgM, and APRI were independent risk factors and the cut-off value of the predictor index was 0.3102, with the AUC being 0.886, indicating good predictive value.

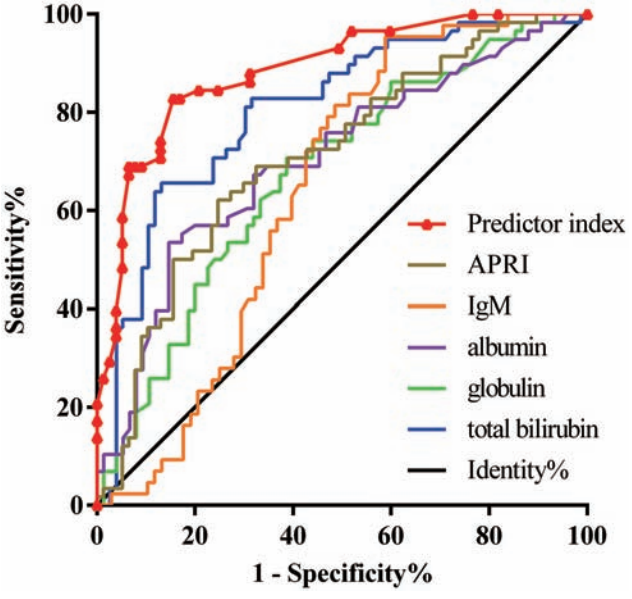

Fig. 1. ROC curve of the five independent risk factors and the predictor index established by the five variables. The AUCs of total bilirubin, globulin, IgM, albumin and APRI were 0.804, 0.67, 0.640, 0.704 and 0.711, respectively. The AUC of the predictor index was 0.886.

UDCA is widely recommended as the first-line treatment for PBC, and the disease can be effectively delayed if the patient responds well to the UDCA. But, unfortunately, there are still some patients whose response is poor to this treatment. To evaluate the treatment response to UDCA, a number of criteria have been published, such as Barcelona, Paris-I/II, GLOBE score, UK-PBC score and so on, which are all based on data from 1 or 2 years of UDCA treatment.[5,6,12,13,18–20] Among the published criteria which identified the treatment response of UDCA, Paris I has been the widely used.[1] The GLOBE score and UK-PBC score were proposed recently and are considered to be better than the Paris I criteria but they both still need further validation.[21] Compared to the Barcelona, Rochester, Rotterdam, Ehime and Toronto criteria, the Paris I criteria has a relatively better predictive value and has been validated by several large studies.[21–23] In our study, we compared the criteria and found the response rate in Paris I was close to the Guideline.[1] With the intent of providing a supplement of Paris I, we tried to use Paris II criteria to decide on the biochemical response of early-stage patients, but the response rate was no different from that of the Paris I criteria. Considering the situation above and the Paris I criteria being recommended by the Chinese Guideline,[24] so we chose Paris I to determine the early biochemical response.

Consistent with previous studies, total bilirubin, albumin, and APRI were found to be associated with biochemical nonresponse to UDCA treatment.[9,25,26] The elevation of total bilirubin level associated with the adverse outcome of PBC patients has been confirmed by many other surveys.[9–11,27-30] The elevation of total bilirubin might reflect progression of PBC.[9] Therefore, there is no doubt that bilirubin is one of the risk factors of early biochemical nonresponse. Albumin, as a protective factor, has already been reported as associated with the adverse long-term outcome in PBC.[13,18,31] As is known, albumin is synthesized by the liver; hence, the decline of albumin reflects the decline of hepatic function, which represents the severity of the disease. APRI is a non-invasive measurement of liver fibrosis in chronic hepatitis of C, and is calculated by Wai's formula.[10] The previous studies showed that APRI could act as a non-invasive diagnostic tool for hepatitis C virus-related liver fibrosis[32] and are associated with Ludwig's stages of PBC.[33] Moreover, the APRI was supposed to be able to predict UDCA treatment response.[25]

A key difference between our and other studies is that the globulin and IgM were identified as independent risk factors in ours and each was determined to significantly influence the UDCA response (odds ratio of 3.38); most of the previous studies did not include the Igs in their analyses. IgM is the one of the established biomarkers of PBC.[34] Moreover, increase of globulin is related to liver inflammation and

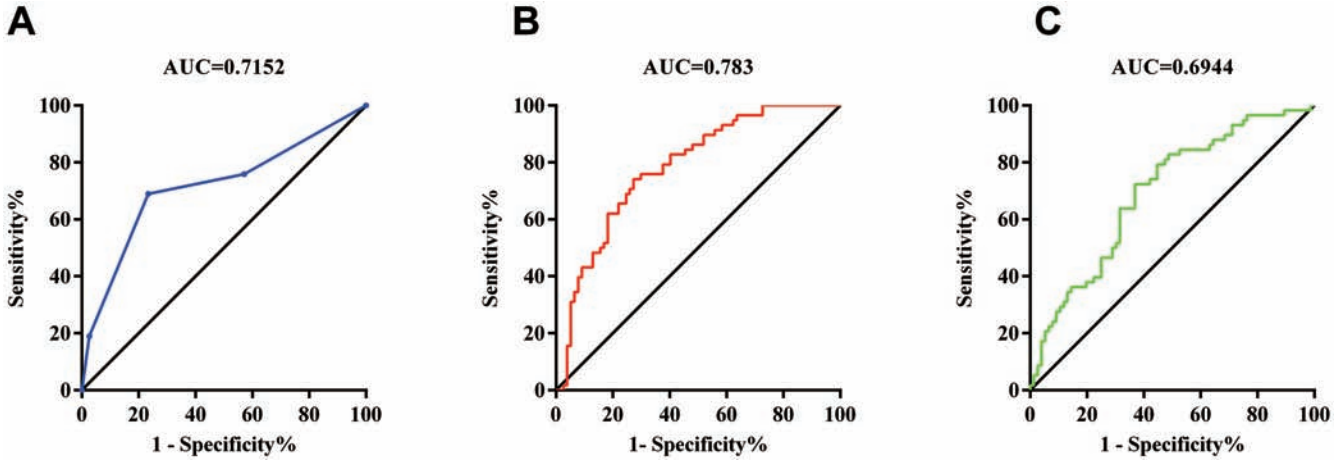

Fig. 2. Predictive value of the proposed criteria in our cohort. (A) The model proposed by Chen *et al*.[15] (B) The model proposed by Tian *et al*.[16] (C) The model proposed by Carbone *et al*.[14]

fibrosis in chronic hepatitis patients, including those with autoimmune liver disease.[35] Therefore, we supposed that the elevations of globulin and IgM were predictive for complicated conditions of patients or a longer diagnostic delay of such patients, linking them to early biochemical nonresponse.

What interested us most was that alkaline phosphatase was not included in the last predictor index. In previous studies, alkaline phosphatase was included in many published models established from the data of European and North American patients.[9,13,18] When involving Chinese patients, we found that alkaline phosphatase was not included in some groups for predicting early biochemical response[15] or long-term outcome.[30] The reasons that might account for this phenomenon are small sample size, different populations of PBC patients and the different natural histories of Chinese and European or North American patients.

The model established by the five risk factors mentioned above had a relatively high predictive ability, with AUC being 0.886 (sensitivity: 82.80%, specificity: 84.40%). Compared to the previous studies that established the predictive model,[14–16] our study has some key distinctions. First of all, the independent risk factors that formed the predictor index were different. The risk factors in our study were total bilirubin, albumin, globulin, IgM, and APRI. Among them, the IgM and globulin were first discovered by us, both of which showed great influence on the response to UDCA (odds ratio for them was 3.38) (Table 4). Second, we screened more factors that were probably associated with the response to UDCA, including complement 3, complement 4, IgA, IgM, IgG, ESR, ANA, ASMA, AMA-M2, interface hepatitis and so on, among which the IgM showed significant relevance to the UDCA treatment response. Furthermore, we tested the predictive value of the model proposed by previous studies[14–16] in our cohort. It turned out that the predictive ability of them was relatively low (Fig. 2).

This retrospective study established a relatively accurate predictor index for the response of PBC patients to UDCA treatment, but there might be some limitations. Mainly, our sample size was small, so there might exist selection bias and we did not have validation data. Because of the short-term follow-up, we also could not identify the predictive value for long-term outcomes.

In conclusion, we found that total bilirubin, albumin, globulin, IgM, and APRI were independent risk factors of early biochemical nonresponse in PBC patients after 1-year of UDCA treatment. The predictive value of the predictor index established based on those five variables was excellent, and it is expected to contribute to the future recognition of high-risk patients before the start of treatment and provide important information for the physician.

Funding

This work was supported by the National Natural Science Foundation of China (grant number 81770582).

Conflict of interest

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

Author contributions

Conception and design of the study (JY, FD), data collection and analysis (YYS, YHS, TB, HTS), review of patient charts and data interpretation (XLP, LY), statistical analysis (YYS, YHS), and manuscript preparation (YYS, FD, JY).

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Journal of Clinical and Translational Hepatology **2021** vol. 9(2) | 194–202
DOI: 10.14218/JCTH.2020.00151

Original Article

Associations of Hydroxysteroid 17-beta Dehydrogenase 13 Variants with Liver Histology in Chinese Patients with Metabolic-associated Fatty Liver Disease

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Received: 5 December 2020 | **Revised:** 24 January 2021 | **Accepted:** 7 February 2021 | **Published:** 22 February 2021

Abstract

Background and Aims: In Europeans, variants in the *hydroxysteroid 17-beta dehydrogenase 13* (*HSD17B13*) gene impact liver histology in metabolic-associated fatty liver disease (MAFLD). The impact of these variants in ethnic Chinese is unknown. The aim of this study was to investigate the potential associations in Chinese patients. ***Methods:*** In total, 427 Han Chinese with biopsy-confirmed MAFLD were enrolled. Two single nucleotide polymorphisms in *HSD17B13* were genotyped: rs72613567 and rs6531975. Logistic regression was used to test the association between the single nucleotide polymorphisms and liver histology. ***Results:*** In our cohort, the minor allele TA of the rs72613567 variant was related to an increased risk of fibrosis [odds ratio (OR): 2.93 (1.20–7.17), p=0.019 for the additive model; OR: 3.32 (1.39–7.91), p=0.007 for the recessive model], representing an inverse association as compared to the results from European cohorts. In contrast, we observed a protective effect on fibrosis for the minor A allele carriers of the *HSD17B13* rs6531975 variant [OR: 0.48 (0.24–0.98), p=0.043 for the additive model; OR: 0.62 (0.40–0.94), p=0.025 for the dominant model]. *HSD17B13* variants were only associated with fibrosis but no other histological features. Furthermore, *HSD17B13* rs6531975 modulated the effect of *PNPLA3* rs738409 on hepatic steatosis. ***Conclusions:*** *HSD17B13* rs72613567 is a risk variant for fibrosis in a Han Chinese MAFLD population but with a different direction for allelic association to that seen in Europeans. These data exemplify the need for studying diverse populations in genetic studies in order to fine map genome-wide association studies signals.

Citation of this article: Liu WY, Eslam M, Zheng KI, Ma HL, Rios RS, Lv MZ, *et al*. Associations of hydroxysteroid 17-beta dehydrogenase 13 variants with liver histology in Chinese patients with metabolic-associated fatty liver disease. J Clin Transl Hepatol 2021;9(2):194–202. doi: 10.14218/JCTH.2020.00151.

Keywords: Metabolic-associated fatty liver disease (MAFLD); Nonalcoholic fatty liver disease (NAFLD); Hydroxysteroid 17-beta dehydrogenase 13 (HSD17B13); Single nucleotide polymorphism (SNP).

Abbreviations: BMI, body mass index; CI, confidence interval; GWAS, genome-wide association studies; HOMA, homoeostasis model assessment; *HSD17B13*, *hydroxysteroid 17-beta dehydrogenase 13*; IFNL3, interferon lambda-3; IR, insulin resistance; MAF, minor allele frequency; MAFL, metabolic-associated fatty liver; MAFLD, metabolic-associated fatty liver disease; *MICA*, *MHC class I polypeptide-related chain A*; *NCAN*, *neurocan*; OR, odds ratio; *PNPLA3*, *patatin-like phospholipase domain containing protein 3*; SNP, single nucleotide polymorphism; TLL1, tolloid-like 1; TLR3, toll-like receptor 3.

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Introduction

Metabolic-associated fatty liver disease (MAFLD) is recognized as a leading cause of liver-related morbidity and mortality.[1,2] In China, the MAFLD burden is increasing, with prevalence rising from 18% to 29% in the last decade.[3] MAFLD comprises a spectrum of disease, ranging from simple steatosis or metabolic-associated fatty liver (MAFL) to the presence of steatohepatitis with varying degrees of

fibrosis and cirrhosis.[4] MAFLD arises from "multiple hits", with genes acting as important modifiers of the clinical phenotype.[5] Our understanding of the underpinnings of MAFLD has been enhanced by numerous genetic association studies, and all of the polymorphisms identified to date explain only 10–20% of disease heritability.[6,7]

It is broadly acknowledged that there is overrepresentation of subjects of European ancestry in human genetics research, with ~79% of all genome-wide association studies (GWAS) participants being of European descent. This overrepresentation hinders a complete understanding of the human genetic architecture. Moreover, it can also have a negative impact, including prediction accuracies between 1.6-4.9-fold lower for other ethnicities than Europeans.[8] Hence, increasing the representation of diverse populations and studying other ethnicities has become a research priority.

Several variants in the *hydroxysteroid 17-beta dehydrogenase 13* (*HSD17B13*) gene encoding a hepatic lipid droplet protein have been identified to impact the histological features of MAFLD. However, the impact of *HSD17B13* gene variants on MAFLD histology among those of Chinese ancestry is unknown. Notably, allele frequencies, haplotype patterns and the effect size of polymorphisms vary considerably across populations and ethnicities.[6] As *HSD17B13* has been proposed as a therapeutic target for MAFLD, it is pivotal to explore whether the effect of this variant observed in Caucasian populations extends to other populations, as also to the effect size.

It is known that the genetic association of variants in *HSD17B13* with the histological features of MAFLD is complex, with different potentially causative single nucleotide polymorphisms (SNPs) and various SNPs associated with different phenotypic patterns. For example, alleles of rs6834314 and rs72613567 associate with decreased injury and with increased hepatic fat.[9] However, there are other studies that show no association of rs72613567 with steatosis.[10,11] Non-coding SNPs (e.g., rs6531975) not in linkage disequilibrium with rs72613567 have also been associated with decreased hepatic fat.[9] Adding to this complexity, a recent study of 487 patients suggested that those harboring the 'protective' TA-allele of rs72613567 have a numerically increased risk for mortality, liver-related death and hepatic decompensation.[12] Likewise, while some reports have suggested that there is a potential interaction between *HSD17B13* and variants in the *patatin-like phospholipase domain containing protein 3* (*PNPLA3*) gene in MAFLD, subsequent reports have cited a failure to discern an association.[13,14]

Given these controversies, the aims of this study were 1) to explore the role of variants in the *HSD17B13* gene in a cohort of Han Chinese with biopsy-confirmed MAFLD, 2) to clarify the role of the variants on the various morphological features of MAFLD, and 3) to discern if there is any interaction between the variants and variants in *PNPLA3*.

Methods

Study population

We recruited 427 consecutive Han Chinese patients with biopsy-confirmed MAFLD from the PERSONS cohort (2017.01–2019.05). The definition of MAFLD was based on the criteria proposed by an international expert panel.[15] The study cohort included patients from a previously published study as well as additional subjects.[16] To ascertain the effects of the *HSD17B13* variant on liver disease solely due to MAFLD, patients with other causes of liver disease (including alcohol use disorder or viral hepatitis) were excluded. Briefly, all consecutive patients, aged ≥18, with biopsy-proven MAFLD, and without alternative causes of liver disease were recruited to the study.

The study protocol was approved by the ethics committee of the First Affiliated Hospital of Wenzhou Medical University (2016-246, 1 December 2016) and registered in the Chinese Clinical Trial Registry (ChiCTR-EOC-17013562). Written informed consent was obtained from each subject before their participation in the study. Patient identifiers were anonymized and replaced by the health examination number.

Clinical and biochemical data

Clinical and biochemical data were collected from all patients within 24 hours of liver biopsy. Body mass index (BMI) was calculated as weight (kg) divided by the square of height (m). Insulin resistance (IR) was estimated according by the homoeostasis model assessment (commonly referred to as HOMA).[17] Diagnosis of diabetes was based on criteria of the American Diabetes Association.[18]

Assessment of liver histology

Liver biopsies were performed using a 16-gauge needle under ultrasound guidance. The histology was reviewed by a single liver pathologist (X.D. Wang) who was blinded to the clinical and biochemical data. Histologic scoring was based on the Activity Score.[19] Steatohepatitis was diagnosed as a score ≥4 and a score of at least one for each feature of steatosis, ballooning, and lobular inflammation. Severe steatosis, severe ballooning and severe lobular inflammation were defined if their scores were ≥2.

Genetic analysis

Genotyping for the *HSD17B13* (rs72613567 and rs6531975) and *PNPLA3* (rs738409) variants were performed using the MassARRAY (Agena Biosciences, San Diego, CA, USA) or *Taq*Man assay (Bio-Rad, Hercules, CA, USA) platforms, according to the manufacturer's protocol. For the purpose of genotyping, each sample used approximately 20 ng of genomic DNA. Locus-specific PCR and detection primers were designed using Assay Design Suite v3.1.

Statistical analysis

Statistical analyses were performed using R software (v3.5.2; R Foundation for Statistical Computing, Vienna, Austria) and SPSS 19.0 (SPSS Inc., Armonk, NY, USA). Continuous variables were expressed as mean±standard deviation and compared using the one-way analysis of variance test. Categorical variables were expressed as frequency (%) and compared using the chi-square test. The Hardy-Weinberg equilibrium was assessed using the chi-square test. Multivariate logistic regression models were undertaken to test the association between the aforementioned SNPs and liver histology features. A p-value <0.05 was considered to be statistically significant.

Results

Patient characteristics

The study comprised 427 consecutive biopsy-confirmed MAFLD patients; their clinical, biochemical, and histological features are depicted in Supplementary Table 1. The average age was 41 years, with 73.8% being male. About 287

Table 1. Baseline characteristics of biopsy-confirmed MAFLD patients according to rs72613567 genotypes

	T/T (*n*=198)	T/TA (*n*=176)	TA/TA (*n*=45)	*p*-value
Age in years	40.2±11.9	41.4±11.5	43.1±14.8	0.299
Male sex, %	150 (75.8%)	126 (71.6%)	33 (73.3%)	0.657
Diabetes, %	63 (31.8%)	54 (30.7%)	18 (40.0%)	0.484
Hypertension, %	74 (37.4%)	59 (33.5%)	22 (48.9%)	0.161
Waist circumference in cm	92.2±9.0	90.6±8.7	91.7±6.8	0.212
BMI in kg/m^2	27.0±3.5	26.5±3.3	26.3±2.9	0.255
HOMA-IR score	5.3±8.4	5.1±6.6	6.5±7.5	0.541
Platelet count as 10^9/L	242.2±61.0	246.7±56.2	253.1±84.6	0.520
Hemoglobin A1c, %	6.0±1.3	6.2±1.5	6.3±1.5	0.427
Fasting glucose in mmol/L	5.7±1.5	5.5±1.2	6.2±2.4	0.012
Total cholesterol in mmol/L	5.2±1.3	4.9±1.1	5.0±1.0	0.100
Triglycerides in mmol/L	2.4±1.7	2.0±1.1	2.3±1.3	0.044
HDL-cholesterol in mmol/L	1.0±0.2	1.0±0.2	1.1±0.4	0.019
LDL-cholesterol in mmol/L	3.1±1.0	3.0±0.9	2.9±0.8	0.331
Albumin in g/L	46.4±4.2	46.4±3.4	46.2±3.6	0.957
ALT in U/L	83.4±79.9	67.9±56.9	70.6±46.6	0.079
AST in U/L	50.4±35.7	45.2±35.0	40.8±20.6	0.139
GGT in U/L	75.8±83.7	68.7±108.9	84.6±98.2	0.567
Creatinine in μmol/L	67.1±14.3	66.1±12.9	70.6±17.4	0.159
Uric acid in μmol/L	395.7±102.9	385.8±108.1	398.2±120.3	0.615
PNPLA3 rs738409				0.256
C/C	56 (28.7%)	51 (29.7%)	16 (35.6%)	
C/G	101 (51.8%)	73 (42.4%)	19 (42.2%)	
G/G	38 (19.5%)	48 (27.9%)	10 (22.2%)	

Categorical values are shown as *n* (%). Continuous variables are shown as mean±standard deviation.
Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; HDL, high-density lipoprotein; LDL, low-density lipoprotein.

(67.2%) had fibrosis (≥F1), 226 (52.9%) had severe steatosis (S2-S3), 157 (36.8%) had severe ballooning (B2) and 84 (19.7%) had severe inflammation (A2-A3).

Genotype distribution, Hardy-Weinberg equilibrium calculations

Two SNPs in *HSD17B13* were genotyped: rs72613567 and rs6531975. The genotype distributions of rs72613567 and rs6531975 in *HSD17B13* were in Hardy-Weinberg equilibrium (all, $p>0.05$). The minor allele frequency (MAF) for rs72613567 and rs6531975 was 0.32 and 0.30 in our cohort, respectively. Each of these MAFs is close to the MAF in general East Asian population in the 1000 Genomes Project.[20] The overall genotype distribution of rs72613567 T/T, T/TA and TA/TA was 47.3%, 42.0% and 10.7%, while the distribution of rs6531975 G/G, G/A and A/A was 49.8%, 40.5% and 9.8%, respectively.

Clinical and laboratory characteristics stratified by HSD17B13 variants

The baseline characteristics of study participants according to rs72613567 genotypes is presented in Table 1. There were significant differences in levels of fasting glucose, triglycerides and high-density lipoprotein cholesterol among rs72613567 genotypes (all, $p<0.05$). Table 2 shows the baseline characteristics of study participants according to rs6531975 genotypes. No significant differences were observed among the rs6531975 genotypes.

HSD17B13 variants and hepatic steatosis

The proportion of severe steatosis in rs72613567 T/T, T/TA and TA/TA was 103 (52.0%), 91 (51.7%)and 27 (60.0%) respectively, while the proportion of severe steatosis in rs6531975 G/G, G/A and A/A was 113 (54.1%), 84 (49.4%) and 24 (58.5%) respectively (Table 3). No association between *HSD17B13* variants and severe steatosis was observed in multivariate logistic regression model (Table 4).

HSD17B13 variants and hepatocyte ballooning and lobular inflammation

The proportion of severe ballooning in rs72613567 T/T, T/TA and TA/TA was 73 (36.9%), 58 (33.0%)and 21 (46.7%)

Table 2. Baseline characteristics of biopsy-confirmed MAFLD patients according to rs6531975 genotypes

	G/G (*n*=209)	G/A (*n*=170)	A/A (*n*=41)	*p*-value
Age in years	41.8±12.3	40.6±11.2	38.9±13.8	0.300
Male sex, %	160 (76.6%)	122 (71.8%)	27 (65.9%)	0.287
Diabetes, %	61 (29.2%)	60 (35.3%)	12 (29.3%)	0.420
Hypertension, %	74 (35.4%)	67 (39.4%)	14 (34.1%)	0.672
Waist circumference in cm	91.6±7.9	91.2±9.3	90.8±9.8	0.824
BMI in kg/m^2	26.5±3.1	26.8±3.6	26.7±3.5	0.690
HOMA-IR score	5.8±8.0	5.2±8.8	4.3±3.5	0.472
Platelet count as 10^9/L	246.0±62.3	243.9±60.9	257.4±65.1	0.457
Hemoglobin A1c, %	6.1±1.4	6.1±1.4	5.9±1.3	0.537
Fasting glucose in mmol/L	5.7±1.6	5.7±1.5	5.4±1.1	0.440
Total cholesterol in mmol/L	5.0±1.1	5.1±1.1	5.3±1.6	0.324
Triglycerides in mmol/L	2.2±1.4	2.4±1.6	2.1±1.0	0.284
HDL-cholesterol in mmol/L	1.0±0.2	1.0±0.2	1.0±0.2	0.665
LDL-cholesterol in mmol/L	3.0±0.9	3.0±0.9	3.4±1.2	0.061
Albumin in g/L	46.1±3.6	46.5±4.3	46.7±3.1	0.412
ALT in U/L	70.3±53.4	81.2±93.1	84.3±73.5	0.275
AST in U/L	44.1±30.1	50.2±40.8	51.0±35.7	0.193
GGT in U/L	72.6±103.3	76.7±96.9	60.9±41.7	0.636
Creatinine in µmol/L	68.0±13.0	66.4±15.2	63.5±13.7	0.137
Uric acid in µmol/L	390.8±100.9	391.6±112.9	412.2±115.7	0.489
PNPLA3 rs738409				0.684
C/C	62 (30.1%)	48 (29.1%)	14 (34.1%)	
C/G	93 (45.1%)	83 (50.3%)	16 (39.0%)	
G/G	51 (24.8%)	34 (20.6%)	11 (26.8%)	

Categorical values are shown as *n* (%). Continuous variables are shown as mean±standard deviation.

respectively, while the proportion of severe ballooning in rs6531975 G/G, G/A and A/A was 79 (37.8%), 63 (37.1%) and 11 (26.8%) respectively. The proportion of severe inflammation in rs72613567 T/T, T/TA and TA/TA was 35 (17.7%), 35 (19.9%) and 12 (26.7%) respectively, while the proportion of severe inflammation in rs6531975 G/G, G/A and A/A was 40 (19.1%), 35 (20.6%) and 8 (19.5%) respectively (Table 3). Both severe ballooning and inflammation were unrelated to *HSD17B13* variants in multivariate analysis (Table 4).

HSD17B13 variants and fibrosis

The prevalence of having fibrosis in rs72613567 T/T, T/TA and TA/TA was 135 (68.2%), 111 (63.1%) and 38 (84.4%) respectively. A higher prevalence of fibrosis was observed in patients with the TA/TA genotype in rs72613567 ($p<0.05$) (Table 3). In rs6531975 genotypes, the prevalence of having fibrosis in G/G, G/A and A/A was 150 (71.8%), 109 (64.1%) and 23 (56.1%) respectively. The A allele carriers of rs6531975 showed a nonsignificant trend for a reduced prevalence of having fibrosis ($p=0.082$) (Table 3).

To further understand the association between *HSD17B13* variants and liver histology in Chinese patients with MAFLD, multivariate logistic regression modeling was undertaken. As shown in Table 4, rs72613567 TA/TA increased the risk of fibrosis with an odds ratio (OR) of 2.93 [TA/TA vs. T/T, 95% confidence interval (CI): 1.20–7.17, $p=0.019$] for the additive model and an OR of 3.32 (TA/TA vs. T/T+T/TA, 95% CI: 1.39–7.91, $p=0.007$) for the recessive model after adjusting for age, sex, BMI, presence of diabetes, fasting glucose, triglycerides and high-density lipoprotein cholesterol. In contrast, the rs6531975 A allele appeared to have a protective impact on fibrosis, with an OR of 0.48 (A/A vs. G/G, 95% CI: 0.24–0.98, $p=0.043$) for the additive model and an OR of 0.62 (G/A+A/A vs. G/G, 95% CI: 0.40–0.94, $p=0.025$) for the dominant model after adjusting for age, sex, BMI and presence of diabetes.

Interaction of PNPLA3 and HSD17B13 variants

Next, we conducted interaction analysis for *HSD17B13* (rs72613567 and rs6531975) and *PNPLA3* (rs738409) variants for their impact on liver histology. For fibrosis, no interaction effects were observed between the two genes. In contrast, there was an interaction between rs6531975 and rs738409 with regard to hepatic steatosis (Fig. 1). For the rs738409 risk allele carriers (CG+GG), the proportion of severe steatosis was lower in patients with the rs6531975 A allele (G/A+A/A) compared to those with rs6531975 G/G (Fig. 1A). Using the latter as reference, the rs6531975 A allele (G/A+A/A) attenuated the risk effect of the rs738409

Table 3. Liver histology features of biopsy-confirmed MAFLD patients according to *HSD17B13* genotypes

	HSD17B13 rs72613567					*HSD17B13* rs6531975		
	T/T (*n*=198)	T/TA (*n*=176)	TA/TA (*n*=45)	*p*-value	G/G (*n*=209)	G/A (*n*=170)	A/A (*n*=41)	*p*-value
Steatosis, *n* (%)				0.586				0.484
Mild steatosis: <2	95 (48.0%)	85 (48.3%)	18 (40.0%)		96 (45.9%)	86 (50.6%)	17 (41.5%)	
Severe steatosis: ≥2	103 (52.0%)	91 (51.7%)	27 (60.0%)		113 (54.1%)	84 (49.4%)	24 (58.5%)	
Hepatocyte ballooning, *n* (%)				0.226				0.401
Mild ballooning: <2	125 (63.1%)	118 (67.0%)	24 (53.3%)		130 (62.2%)	107 (62.9%)	30 (73.2%)	
Severe ballooning: =2	73 (36.9%)	58 (33.0%)	21 (46.7%)		79 (37.8%)	63 (37.1%)	11 (26.8%)	
Lobular inflammation, *n* (%)				0.386				0.939
Mild inflammation: <2	163 (82.3%)	141 (80.1%)	33 (73.3%)		169 (80.9%)	135 (79.4%)	33 (80.5%)	
Severe inflammation: ≥2	35 (17.7%)	35 (19.9%)	12 (26.7%)		40 (19.1%)	35 (20.6%)	8 (19.5%)	
Presence of fibrosis, *n* (%)	135 (68.2%)	111 (63.1%)	38 (84.4%)	0.023	150 (71.8%)	109 (64.1%)	23 (56.1%)	0.082

Table 4. Association between *HSD17B13* variants and liver histology features in Chinese MAFLD patients

SNP	Severe steatosis			Severe ballooning			Severe inflammation			Presence of fibrosis		
	OR	95% CI	*p*	OR	95% CI	*p*	OR	95% CI	*p*	OR	95% CI	*p*
HSD17B13 rs72613567[†]												
Additive model												
T/T	ref.	–	–	ref.	–	–	ref.	–	–	ref.	–	–
T/TA	1.24	0.78–1.96	0.368	0.93	0.60–1.44	0.737	1.24	0.72–2.16	0.437	0.77	0.49–1.20	0.252
TA/TA	1.62	0.77–3.42	0.203	1.37	0.69–2.72	0.368	1.99	0.89–4.43	0.092	2.93	1.20–7.17	0.019
Dominant model												
T/T	ref.	–	–	ref.	–	–	ref.	–	–	ref.	–	–
T/TA+TA/TA	1.30	0.84–2.02	0.234	1.01	0.67–1.52	0.973	1.38	0.83–2.31	0.216	0.96	0.63–1.48	0.867
Recessive model												
T/T+T/TA	ref.	–	–	ref.	–	–	ref.	–	–	ref.	–	–
TA/TA	1.46	0.72–2.98	0.292	1.42	0.74–2.73	0.295	1.80	0.85–3.83	0.127	3.32	1.39–7.91	0.007
HSD17B13 rs6531975[‡]												
Additive model												
G/G	ref.	–	–	ref.	–	–	ref.	–	–	ref.	–	–
G/A	0.69	0.44–1.08	0.104	0.95	0.62–1.45	0.802	0.94	0.56–1.60	0.830	0.65	0.42–1.02	0.063
A/A	0.91	0.43–1.94	0.809	0.59	0.28–1.24	0.164	0.84	0.35–2.00	0.690	0.48	0.24–0.98	0.043
Dominant model												
G/G	ref.	–	–	ref.	–	–	ref.	–	–	ref.	–	–
G/A+A/A	0.73	0.48–1.11	0.138	0.87	0.58–1.30	0.496	0.92	0.56–1.52	0.751	0.62	0.40–0.94	0.025
Recessive model												
G/G+G/A	ref.	–	–	ref.	–	–	ref.	–	–	ref.	–	–
A/A	1.08	0.52–2.23	0.833	0.60	0.29–1.24	0.170	0.86	0.37–1.98	0.726	0.59	0.30–1.16	0.123

[†]OR and 95% CI obtained by binary logistic regression analysis adjusted for age, sex, BMI, presence of diabetes, fasting glucose, triglycerides and HDL-cholesterol.

[‡]OR and 95% CI obtained by binary logistic regression analysis adjusted for age, sex, BMI, presence of diabetes. ref., reference.

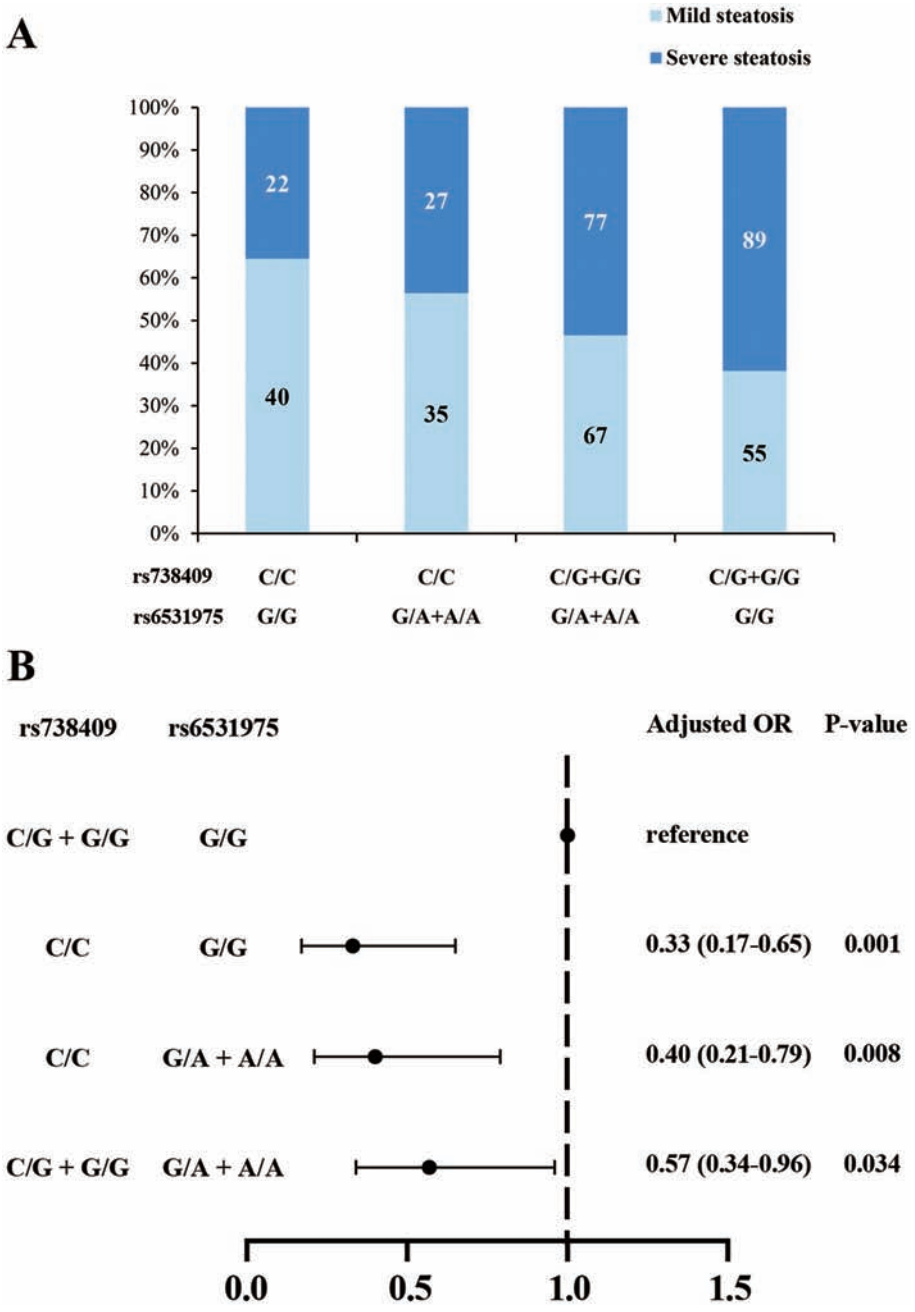

Fig. 1. Interaction of *HSD17B13* rs6531975 and *PNPLA3* rs738409 on liver steatosis. (A) Prevalence of mild steatosis and severe steatosis according to rs6531975 and rs738409 genotypes. (B) Interaction effect of rs6531975 and rs738409 on steatosis after adjusting for age, sex, BMI and presence of diabetes. Patients with the rs6531975 A allele (G/A+A/A) attenuated the risk effect of the rs738409 G allele (C/G+G/G) on steatosis, with an OR of 0.57 (95% CI: 0.34-0.96, p=0.034).

G allele (C/G+G/G) on steatosis, with an OR of 0.57 (95% CI: 0.34–0.96, p=0.034) after adjusting for age, sex, BMI and presence of diabetes (Fig. 1B). The interaction between rs72613567 and rs738409 on liver steatosis was also performed (Fig. 2); however, no effect was observed.

Discussion

We characterized the impact of *HSD17B13* gene variants on histological features in a cohort of Han Chinese with MAFLD. This study has three key findings. First, we confirmed the *HSD17B13* region as a susceptibility locus for MAFLD-related fibrosis but extended these findings toward the identification of an inverse allelic direction of association as compared to that reported in Europeans. Second, the *HSD17B13* variants are only associated with fibrosis and not any other histological feature. Third, the *HSD17B13* variants modulate the effect of *PNPLA3* rs738409 on hepatic steatosis but no other histological features.

The association between *HSD17B13* variants and liver histological features seems to be complex, with multiple

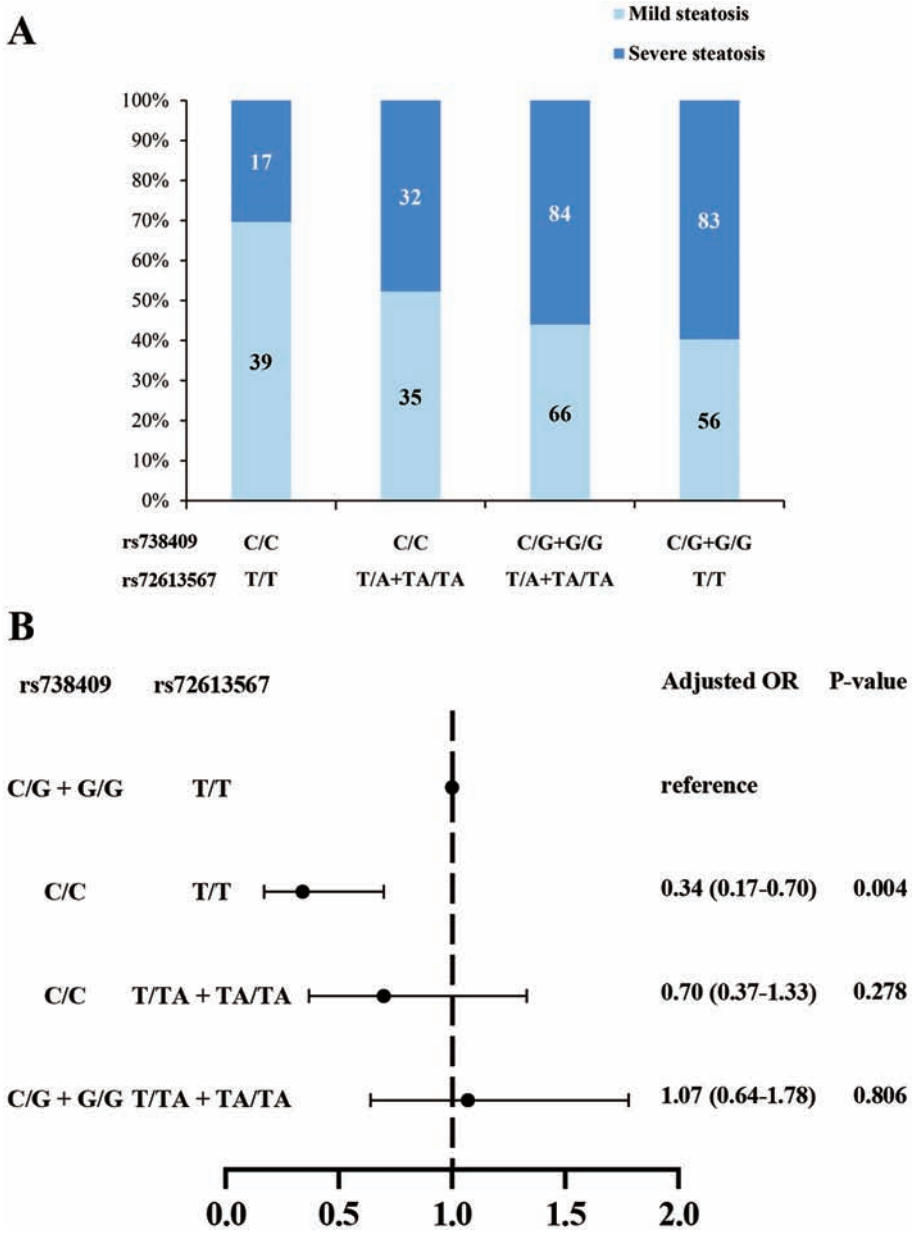

Fig. 2. Interaction of *HSD17B13* rs72613567 and *PNPLA3* rs738409 on liver steatosis. (A) Prevalence of mild steatosis and severe steatosis according to rs72613567 and rs738409 genotypes. (B) Interaction effect of rs72613567 and rs738409 on steatosis after adjusting for age, sex, BMI and presence of diabetes. No interaction effect was observed between rs72613567 and rs738409.

suggested functional variants. Notably, in our cohort, the minor allele TA of the rs72613567 variant was related to an increased risk of fibrosis, representing an inverse association as compared to the results in European cohorts. Hence, if there is a shared causal variant across European and Chinese populations, it is unlikely to be rs72613567. In this regard, we observed a protective effect in the minor A allele carriers of the *HSD17B13* rs6531975 variant, but this is not in strong linkage disequilibrium with rs72613567. Thus, further fine-mapping studies in Han Chinese populations and comparison to other populations would be helpful to identify shared causal variants across different ethnicities.

The differential effect size and allele direction of variants discovered by GWAS between ethnicities is not uncommon. In one Chinese MAFLD cohort, researchers found that the *neurocan* (known as *NCAN*) rs2228603 T variant associated with a higher level of high-density lipoprotein,[21] while it was positively related to liver steatosis in the USA population.[22] Similarly, toll-like receptor 3 (known as *TLR3*) rs3775290[23,24] and interferon lambda-3 (known as *IFNL3*) rs12979860[25,26] variants in Chinese hepatocellular carcinoma populations showed opposite effects to those in non-Asian populations. Inconsistent results have also been observed in other Asian populations, such as among Japanese. For example, tolloid-like 1 (known as *TLL1*) rs17047200[27] and *MHC class I polypeptide-related chain A* (known as *MICA*) rs2596542[28] variants were suggested to have protective impacts on fibrosis and hepatocellular carcinoma in

Caucasians. The associations were inverse to those of a Japanese cohort.[29,30] Besides, there are several MAFLD-related SNPs in Europeans for which there has been no association in Chinese populations.[31–33] Along the same line, lower genetic prediction accuracies (between 1.6-4.9-fold lower) were observed in other ethnicities compared to Europeans.[8] Hence, increasing the representation of diverse populations and studying other ethnicities has recently become a research priority to enhance understanding of the human genetic architecture and its translational implications.

The ethnic differences in the characteristics of patients with MAFLD might also contribute to the observed differences in the genetic findings. There is growing evidence, for example, that the MAFLD disease course in Asian populations is different to that in Caucasians. As an example, for the same BMI, there is a higher prevalence of MAFLD in Asians. Published reports also indicate that lean MAFLD accounts for 36.9% of cases in China,[3] but only 17.3% of the total disease burden in the USA.[34] Differences in metabolic adaptation have been reported between lean and non-lean MAFLD patients, suggesting that lean fatty liver disease likely has a distinct pathophysiology.[35]

Another intriguing aspect of this study is the lack of association found between *HSD17B13* variants and other histological features. To date, the nature of the association between the rs72613567 allelic variant and the histological features of MAFLD, particularly steatosis, is unclear. Abul-Husn and colleagues[10] suggested a lack of association between the rs72613567 TA variant and steatosis in human liver, consistent with the study of Pirola *et al*.[11] However, a study by Ma *et al*.[9] found a significant association with hepatic steatosis. Similarly, in animal and *in vitro* studies, inconsistent results have been reported for an effect of *HSD17B13* on hepatic lipid accumulation. Abul-Husn *et al*.[10] showed no differences in lipid accumulation according *HSD17B13* isoforms. Similarly Ma *et al*.[9] reported that *HSD17B13* overexpression or knockout in HepG2 cells did not affect lipid content. On the other hand, Marion *et al*.[36] noted hepatic steatosis in *HSD17B13* knockout mice, whilst Su *et al*.[37] observed steatosis in mice that overexpressed *HSD17B13*. Collectively, these results imply that *HSD17B13* variants could have a direct impact on fibrosis rather than effects on steatosis. These findings may be associated with retinol metabolism, since retinol plays a crucial role in the activation and transformation of hepatic stellate cells to matrix secreting myofibroblasts and the development of hepatic fibrosis.[38] Since *HSD17B13* participates in the rate limiting step of retinol metabolism,[9] the mutant in *HSD17B13* might conceivably influence the process of fibrosis.

The interaction between *HSD17B13* and *PNPLA3* variants in MAFLD is also a subject of controversy.[14,39] In this work, we noted an interaction between these variants with regard to steatosis, but not with other histological features. As *HSD17B13* has been suggested as a potential therapeutic target for MAFLD and considering the growing concerns about the failure of phase 2 and 3 clinical trials in this disease[40,41] that was at least partially attributed to clinical heterogeneity, our study highlights the importance of first understanding the functional basis of the various proposed genomic and other targets before therapeutic development.[40,42] Collectively, our data support such an approach. The data from *HSD17B13*-knockout mice, in fact, suggest that HSD17B13 triggers steatosis and inflammation,[36] which is opposite to what has been reported in humans.

The present study has limitations. First, the sample size is modest. In case the observed opposite finding is due to the sample size, we performed a post-hoc power analysis. The power calculated for the model was 72%. It is close to but less than 80%. Considering the low proportion of the rs72613567 TA variant in the general population, we think it is acceptable. In addition, lack of a validation cohort from populations in other parts of China or those of Chinese ancestry living outside mainland China is another limitation.

In conclusion, the *HSD17B13* rs72613567 variant appears to be a risk variant for hepatic fibrosis in a Han Chinese MAFLD population, with a different direction for allelic association to that seen in Europeans.

Funding

This work was supported by grants from the National Natural Science Foundation of China (82070588), High Level Creative Talents from Department of Public Health in Zhejiang Province (S2032102600032) and Project of New Century 551 Talent Nurturing in Wenzhou. GT was supported in part by grants from the University School of Medicine of Verona (Verona, Italy). CDB was supported in part by the Southampton NIHR Biomedical Research Centre (IS-BRC-20004), UK. ME and JG were supported by the Robert W. Storr Bequest to the Sydney Medical Foundation, University of Sydney (Sydney, Australia) and the National Health and Medical Research Council of Australia (NHMRC) Program (APP1053206, APP1149976) and Project (APP1107178 and APP1108422) grants.

Conflict of interest

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

Author contributions

Study concept and design (WYL, ME, JG, MHZ), acquisition of data (HLM, LJT, GL, PWZ), pathology analysis (XDW), drafting of the manuscript (WYL, ME, JG, KIZ, RSR), critical revision of the manuscript (ME, JG, GT, CDB), statistical analysis (WYL, ME, MZL), study supervision (JG, MHZ), guarantor of the article (MHZ).

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Journal of Clinical and Translational Hepatology **2021** vol. 9(2) | 203–209
DOI: 10.14218/JCTH.2020.00163

Original Article

Association of LDLR rs1433099 with the Risk of NAFLD and CVD in Chinese Han Population

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Received: 13 December 2020 | **Revised:** 1 February 2021 | **Accepted:** 7 February 2021 | **Published:** 11 March 2021

Abstract

Background and Aims: Recent genome-wide association studies have shown that low-density lipoprotein receptor (LDLR) rs1433099 polymorphism is associated with cardiovascular disease (CVD) risk in many countries. However, the association of LDLR rs1433099 with CVD in China has not been reported yet. There are no studies on LDLR rs1433099 and non-alcoholic fatty liver disease (NAFLD) as well. The purpose of this study was to investigate whether LDLR rs1433099 is related to CVD or NAFLD in the Chinese population. ***Methods:*** LDLR rs1433099 polymorphism was genotyped in 507 individuals, including 140 healthy controls, 79 NAFLD patients, 185 CVD patients, and 103 patients with NAFLD combined with CVD. The expression of LDLR was tested by the sequence detection system, and clinical parameters were assessed by biochemical tests and physical examination. ***Results:*** The genotype distribution of LDLR rs1433099 was not statistically different among the NAFLD group, the CVD group, the combined group, and the healthy control group ($p>0.05$). There was no significant correlation of LDLR rs1433099 genotypic distribution or allele frequency and the risk of NAFLD, CVD or NAFLD combined with CVD ($p>0.05$). In the CVD group, T allele carriers had higher alkaline phosphatase and gamma-glutamyl transpeptidase than non-carriers ($p<0.05$). ***Conclusions:*** Our study demonstrated that the LDLR rs1433099 polymorphism is not a risk factor of NAFLD. The LDLR rs1433099 polymorphism may increase the risk of CVD through a mechanism involving alkaline phosphatase and gamma-glutamyl transpeptidase.

Citation of this article: Han Y, Zhang Y, Liu S, Chen G, Cao L, Xin Y. Association of LDLR rs1433099 with the risk of NAFLD and CVD in Chinese Han population. J Clin Transl Hepatol 2021;9(2):203–209. doi: 10.14218/JCTH.2020.00163.

Keywords: LDLR rs1433099; C44857T; CVD; NAFLD; Gene polymorphism.

Abbreviations: ALP, alkaline phosphatase; ALT, alanine transaminase; AST, aspartate aminotransferase; BMI, body mass index; CVD, cardiovascular disease; FBG, fasting blood glucose; GGT, gamma-glutamyl transpeptidase; HDL-C, high-density lipoprotein cholesterol; LDL, low-density lipoprotein; LDL-C, low-density lipoprotein cholesterol; LDLR, low-density lipoprotein receptor; NAFLD, non-alcoholic fatty liver disease; PCR, polymerase chain reaction; TC, total cholesterol; TG, triglyceride.

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Introduction

Non-alcoholic fatty liver disease (NAFLD) is becoming the most common chronic liver disease. The prevalence of NAFLD is constantly increasing, rising from 15% in 2005 to 25% in 2018.[1] Approximately 27% of adults in Asia suffer from NAFLD, the rate of which is even higher in the Middle East and South America, with an estimated prevalence of 32% and 31% respectively.[1] It was estimated in 2016 that the annual burden of NAFLD-related cases was $103 billion in the USA and 35 billion in four European countries per year.[2]

NAFLD consists of a broad spectrum of fatty liver disease, ranging from simple fatty infiltration in >5% of hepatocytes (steatosis), fatty infiltration plus inflammation, fibrosis, and ultimately cirrhosis, ending with liver failure and hepatocellular carcinoma.[3] NAFLD is in close relationship with type 2 diabetes mellitus, obesity, and metabolic syndrome.[4] Younossi *et al.*[1] estimated that of all patients that have developed non-alcoholic steatohepatitis in the USA, 82% are obese, 48% have type 2 diabetes mellitus, 82% get hyperlipidemia, 76% are diagnosed with metabolic syndrome, and 70% suffer from hypertension. Biopsy remains the gold-standard for assessing the progression of NAFLD, but its side effects keep many patients away, especially in the early stage of fatty liver disease. The most commonly used biomarker of chronic liver disease to evaluate the function of the liver is alanine transaminase (ALT), while it has a low specificity.[5]

With the development of the genome-wide association studies, many gene loci modulating metabolism have been demonstrated to influence the risk of diseases.[6] *PNPLA3* and *TM6SF2* were of the first genes to be related to NAFLD.[7,8] The low-density lipoprotein receptor (LDLR) is a widely distributed transmembrane glycoprotein regulating cholesterol homeostasis. Cells can internalize lipoprotein ligands, including chylomicrons, low-density lipoprotein (LDL), intermediate-density lipoprotein, or very-LDL mediated by LDL, facilitating cholesterol utilization.[9] The gene for LDLR is located at 19p13.1–13.3 and spans 45 kb, including 18 exons and 17 introns.[10] Early studies showed that mutations in LDLR can cause familial hypercholesterolemia, an autosomal dominant

disorder characterized by severe hypercholesterolemia.[11]

C44857T (rs1433099) is a single nucleotide polymorphism within the 5′ region of the 3′ untranslated region of LDLR.[12] Polisecki *et al*.[13] found that carriers of the T allele at the C44857T locus had significantly lower levels of LDL-C, suggesting it as a decisive pathogenic factor of NAFLD and cardiovascular disease (CVD). However, the relationship between LDLR rs1433099 and NAFLD is still unknown. It remains unclear whether LDLR rs1433099 affects CVD risk in the Chinese population. Our study aimed to investigate whether LDLR rs1433099 is associated with NAFLD or CVD in the Chinese population.

Methods

Subjects

This study was permitted by the hospital ethical committee of Qingdao Municipal Hospital (Qingdao, China), following the principles of the Declaration of Helsinki and its appendices.[14] The study selected 367 in-patients of Qingdao Municipal Hospital from January 2018 to September 2019, including 79 NAFLD patients (NAFLD group), 185 CVD patients (CVD group), and 103 patients with NAFLD combined with CVD (NAFLD combined with CVD group, the combined group). NAFLD patients were selected from the Department of Gastroenterology; CVD patients and NAFLD combined with CVD patients were selected from the Department of Cardiology. At the same time, 140 healthy controls were selected from the Health Examination Center of Qingdao Municipal Hospital. All individuals were unrelated, ethnically Chinese Han adults.

The diagnosis of NAFLD met the standards of the "Guidelines for Prevention and Treatment of Non-alcoholic Fatty Liver Disease"[15] and was confirmed by β-ultrasonography. CVD was diagnosed by coronary angiography of the coronary artery or its branches. Excluded were patients with alcoholic hepatitis, viral or autoimmune hepatitis, drug-induced hepatitis, acute fatty liver of pregnancy, and other causes of liver disease, as well as aortic dissection, atrial fibrillation, rheumatic immune disease, cardiomyopathy, aortic arteritis, etc. that may cause secondary CVD. Healthy controls were confirmed by biochemical indicators combined with findings from ultrasound examination.

Specimen and data collection

After 12 hours of fasting, 8 mL of venous blood was collected routinely into two EDTA anti-coagulant tubes (designated as A and B respectively), with 4 mL in each. Tube A was used to detect biochemical indexes, including ALT, aspartate aminotransferase (AST), fasting blood glucose (FBG), triglycerides (TG), total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), alkaline phosphatase (ALP) and gamma-glutamyl transpeptidase (GGT). Tube B was centrifuged and stored at −80 °C for genotyping. The basic information, such as name, sex, and age of the subjects, was gathered by a standard questionnaire. Height and body mass was measured with professional instruments, and the body mass index (BMI) was calculated.

Genotyping

Genomic DNA was isolated from peripheral blood using a purification kit (Bio Miao Biological Technology, Beijing, China). The rs1433099 polymorphism of LDLR was detected by polymerase chain reaction (PCR) using the following primers designed and synthesized for LDLR polymorphism: 5′-ACGTTGGATGAATGATGCCACTTCCCAGAG-3′, 5′-ACGTTGGATGAAGGTAACCGGGTGTCTCAG-3′. PCR amplification was performed under the following conditions: 5 m at 94 °C, then 45 cycles before denaturation at 94 °C for 20 s, annealing at 56 °C for 30 s, and elongation for 1 m at 72 °C. For direct DNA sequencing, the ABI Prism sequence detection system ABI veriti-384 (Foster City, CA, USA) was applied for the assay of LDLR genotypes. The average genotype call rate was above 95% and the genotype concordance rate of blind replicates was above 99%.

Statistical analysis

Statistical analysis was carried out using SPSS Statistics software, version 24.0 (IBM Corp., Armonk, NY, USA). When comparing the general clinical data among the four groups, the count data were compared by the χ^2 test. The measurement data, not in accordance with normality and homogeneity of variance by Kolmogorov-Smirnov test, were expressed as median (quartile), compared by Kruskal-Wallis H test. For indexes with statistical differences by rank-sum test, the results were corrected by Bonferroni correction. The χ^2 test was used to analyze whether the distribution of LDLR rs1433099 genotypes conformed to the law of Hardy-Weinberg genetic equilibrium to avoid the lack of population representativeness. The χ^2 test was used to analyze the differences of LDLR rs1433099 genotype distribution and allele frequency among the four groups. Logistic regression analysis was performed to analyze the relationship between polymorphism and disease risk. Student's *t*-test, Kruskal-Wallis test, and rank-sum test were used to evaluate the association of LDLR rs1433099 genotypic distribution with clinical characteristics. A *p*-value of <0.05 was considered statistically significant.

Results

Clinical characteristics of the individuals

We investigated 507 individuals in total. Table 1 shows the clinical characteristics and serum lipid levels of the subjects as well as comparisons of groups in sex (χ^2 test) and other clinical parameters (Kruskal-Wallis test). NAFLD vs. control group: BMI, ALT, TG, GGT, and FBG of the NAFLD group are higher than those of the control group, while age is lower than that of the control group ($|Z|=3.053$~17.418, $p<0.05$). CVD vs. control group: age, ALT, TC, GGT, ALP, and FBG of the CVD group are higher than those of the control group, while TG, HDL-C, and LDL-C are lower than those of the control group ($|Z|=2.828$~10.768, $p<0.05$). Combined group vs. control group: age, BMI, ALT, TC, GGT, ALP, and FBG of the combined group are higher than those of the control group, while TG, HDL-C, and LDL-C of the combined group are lower than those of the control group ($|Z|=3.065$~11.713, $p<0.05$). Combined group vs. NAFLD group: age, ALP, and FBG of the combined group are higher than those of the NAFLD group, while TG, HDL-C, and LDL-C are lower than those of the NAFLD group ($|Z|=3.685$–9.803, $p<0.05$).

LDLR rs1433099 genotypes and allele distribution

The genotype distribution of the LDLR rs1433099 corre-

Table 1. Clinical characteristics of individuals in the four study groups

	Control, *n*=140	NAFLD, *n*=79	CVD, *n*=185	Combined, *n*=103	p_1*	p_2*	p_3*	p_4*
Sex, M/F	60/80	57/22	120/65	67/36	<0.001	<0.001	0.001	0.308
Age, years	52.5 (42–59)	43 (39–45)	67 (60–76)	62 (56–67)	<0.001	<0.001	<0.001	<0.001
BMI, kg/m^2	23.71 (21.26–25.95)	26.26 (23.44–28.09)	24.54 (22.52–26.72)	25.26 (23.78–26.77)	<0.001	0.214	<0.001	1.000
ALT, U/L	17.79 (12.51–23.68)	25.82 (21.71–32.33)	21.71 (15.04–32.02)	22.63 (15.64–32.78)	<0.001	0.001	0.004	0.098
AST, U/L	20.75 (17.71–24.84)	21.19 (18.31–24.05)	21.84 (17.09–33.68)	22.09 (16.83–32.46)	–	–	–	–
TG, mmol/L	5.11 (4.60–5.72)	5.99 (5.34–6.17)	4.47 (3.78–5.37)	4.27 (3.77–5.55)	<0.001	<0.001	<0.001	<0.001
TC, mmol/L	1.12 (0.87–1.57)	1.03 (1.03–1.59)	1.33 (0.96–1.83)	1.39 (0.94–2.17)	0.937	0.028	0.013	1.000
HDL-C, mmol/L	1.29 (1.05–1.50)	1.21 (1.08–1.35)	1.01 (0.85–1.16)	1.05 (0.88–1.19)	1.000	<0.001	<0.001	<0.001
LDL-C, mmol/L	3.06 (2.65–3.62)	3.27 (2.88–3.59)	2.66 (2.08–3.33)	2.53 (2.12–3.40)	0.853	<0.001	0.002	<0.001
GGT, U/L	19.90 (14.96–30.26)	30.63 (21.21–49.62)	27.19 (19.14–44.29)	26.43 (18.88–38.98)	<0.001	<0.001	0.002	0.292
ALP, U/L	70.26 (58.89–83.81)	66.17 (55.65–81.47)	82.90 (64.86–107.72)	81.09 (73.51–98.01)	1.000	<0.001	<0.001	<0.001
FBG, mmol/L	4.51 (4.00–5.15)	4.83 (4.70–4.96)	5.23 (4.55–6.75)	5.48 (4.81–6.47)	0.014	<0.001	<0.001	0.001

P_1, NAFLD vs. control; P_2, CVD vs. control; P_3, Combined vs. control; P_4, Combined vs. NAFLD.
*All *p*-values are Bonferroni corrected.
Abbreviations: NAFLD, non-alcoholic fatty liver disease; CVD, cardiovascular disease; BMI, body mass index; ALT, alanine transaminase; AST, aspartate aminotransferase; TG, triglyceride; TC, total cholesterol; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; GGT, gamma-glutamyl transpeptidase; ALP, alkaline phosphatase; FBG, fasting blood glucose.

sponds to the Hardy-Weinberg equilibrium in NAFLD, CVD, combined and control groups ($p_{Control}$=0.681, p_{NAFLD}=0.986, p_{CVD}=0.796, $p_{Combined}$=0.723). The distribution of LDLR rs1433099 genotypes is shown in Table 2, and there is no significant difference between patients with the NAFLD group, the CVD group, the combined group, and the healthy controls (p>0.05).

Association of LDLR rs1433099 genotypic distribution and allele frequency with the risk of NAFLD and CVD

Table 3 shows the unconditional logistic regression model analysis for genotypes and alleles of rs1433099. There is no significant correlation between LDLR rs1433099 genotypic distribution or allele frequency for the risk of NAFLD, CVD or NAFLD combined with CVD (p>0.05). We observed no significant difference after adjustment for age, sex, and BMI (p>0.05).

Association of LDLR rs1433099 genotypic distribution with clinical characteristics

Table 4 shows clinical characteristics in LDLR rs1433099 T carriers and non-carriers. Statistical analysis indicates no significant difference between the T-carriers and non-carriers among all subjects (p>0.05). Further analysis among the three genotypes suggests no statistical difference as well (Table 5; p>0.05). Analysis of clinical characteristics of LDLR rs1433099 in each group shows that T-carriers have higher ALP and GGT than non-carriers in the CVD group (Table 6; p<0.05).

Discussion

LDLR is a cell-surface receptor that removes excessive LDL from plasma and maintains the circulating cholesterol level.[16] LDLR is closely related to metabolic syndrome.[17] In the whole population, 0.2–0.5% of people have heterozygous mutations in LDLR.[18]

Recently, international experts reached a consensus recommending a change in name from NAFLD to metabolic (dysfunction)-associated fatty liver disease,[19] emphasizing it as a consequence of metabolic syndrome. Lipotoxicity is the initial factor in NAFLD development. Former studies showed that LDLR rs1433099 mutation can induce dyslipidemia,[12,13,20] suggesting that it may influence the risk of NAFLD. We explored the relationship between LDLR rs1433099 and NAFLD for the first time. But this research shows no association for LDLR rs1433099 polymorphism with the incidence of NAFLD.

LDLR also has a close relationship with the development of atherosclerosis.[21,22] Abnormal LDLR alleles in the human manifest as familial hypercholesterolemia, with dramatically increased risk of CVD.[9,18] The severity of atherosclerosis is in correlation with the level and activity of liver LDLR.[23]

Previously, Anand *et al*.[24] conducted the INTERHEART case-control study, which included 8,795 individuals of European, South Asian, Arab, Iranian, and Nepalese origin. The investigators found that LDLR rs1433099 is associated with a lower apolipoprotein B/A1 ratio, an indicator proportional to the narrowness of coronary artery (p=0.0022). No direct correlation between LDLR rs1433099 and myocardial infarction was found. Takeuchi *et al*.[20] investigated the relationship of LDLR rs1433099 and the risk of CVD in Japan from 12,066 individuals. Their study indicated a strong as-

Table 2. Correlation of the rs1433099 polymorphism in the LDLR gene with NAFLD and CVD

		Control, *n*=140	NAFLD, *n*=79	CVD, *n*=185	Combined, *n*=103
Genotypes	CC	70 (50.00%)	43 (54.43%)	96 (51.89%)	62 (60.19%)
	CT	62 (44.29%)	30 (37.97%)	78 (42.16%)	33 (32.04%)
	TT	8 (5.71%)	6 (7.59%)	11 (5.95%)	8 (7.77%)
Alleles	C	202 (72.14%)	116 (73.42%)	270 (72.97%)	157 (76.21%)
	T	78 (27.86%)	42 (26.58%)	100 (27.03%)	49 (23.79%)

Abbreviations: NAFLD, non-alcoholic fatty liver disease; CVD, cardiovascular disease.

Table 3. LDLR rs1433099 genotypes, alleles, and risk of NAFLD and CVD

	NAFLD vs. control		CVD vs. control		Combined vs. control		Combined vs. NAFLD	
	OR (95% CI)	*p**	OR (95% CI)	*p**	OR (95% CI)	*p**	OR (95% CI)	*p**
Genotypes								
CC	1	0.529	1	0.735	1	0.116	1	0.436
CT+TT	0.84 (0.48–1.46)		0.93 (0.60–1.44)		0.66 (0.40–1.11)		0.79 (0.44–1.43)	
Allele								
C	1	0.774	1	0.814	1	0.313	1	0.542
T	0.94 (0.60–1.46)		0.96 (0.68–1.36)		0.81 (0.53–1.22)		0.862 (0.535–1.389)	
Adjusted Genotypes								
CC	1	0.273	1	0.450	1	0.198	1	0.358
CT+TT	0.68 (0.34–1.35)		0.80 (0.45–1.42)		0.67 (0.37–1.23)		0.43 (0.07–2.64)	
Allele								
C	1	0.722	1	0.340	1	0.344	1	0.444
T	0.91 (0.53–1.55)		0.81 (0.52–1.26)		0.79 (0.49–1.28)		0.571 (0.136–2.401)	

*All *p*-values are Bonferroni corrected.
Abbreviations: NAFLD, non-alcoholic fatty liver disease; CVD, cardiovascular disease; OR, odds ratio; CI, confidence interval.

sociation of LDLR rs1433099 genotype with the risk of CVD ($p=2.1\times10^{-7}$). However, clinical parameters between our study and the Japanese study did not show a significant difference. The inconsistent results between this study and the previous study may be contributed by the smaller sample size and differences in region and ethnicity.

Our data indicate that LDLR rs1433099 T-carriers have higher ALP and GGT than non-carriers in the CVD group.

Table 4. Clinical characteristics of LDLR rs1433099 T carriers and non-carriers

	CC, *n*=271	CT+TT, *n*=236	*p*
Sex, M/F	154/117	150/86	0.123
Age, years	57.84±14.02	57.44±13.68	0.743
BMI, kg/m^2	24.97 (22.53–27.14)	24.67 (22.86–26.47)	0.385
ALT, U/L	21.71 (15.49–30.21)	20.92 (14.80–31.92)	0.952
AST, U/L	21.19 (18.01–25.83)	22.02 (18.17–27.59)	0.375
TG, mmol/L	4.99±1.18	4.87±1.20	0.252
TC, mmol/L	1.30 (0.96–1.86)	1.26 (0.90–1.76)	0.434
HDL-C, mmol/L	1.10 (0.94–1.31)	1.12 (0.94–1.29)	0.962
LDL-C, mmol/L	2.90 (2.33–3.45)	2.94 (2.28–3.46)	0.683
GGT, U/L	25.21 (17.97–38.14)	26.74 (18.50–42.04)	0.526
ALP, U/L	74.59 (61.30–91.52)	79.38 (64.15–97.37)	0.052
FBG, mmol/L	4.91 (4.55–5.72)	4.97 (4.49–5.98)	0.530

Abbreviations: BMI, body mass index; ALT, alanine transaminase; AST, aspartate aminotransferase; TG, triglyceride; TC, total cholesterol; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; GGT, gamma-glutamyl transpeptidase; ALP, alkaline phosphatase; FBG, fasting blood glucose.

Table 5. Analysis of clinical characteristics for LDLR rs1433099 C/T genotypes

	CC, *n*=271	CT, *n*=203	TT, *n*=33	*p*
Sex, M/F	154/117	127/76	23/10	0.225
Age, years	59 (46–67)	59 (46–66)	60 (51–68)	0.556
BMI, kg/m^2	24.87±3.35	24.70±3.24	24.85±2.95	0.850
ALT, U/L	21.71 (15.49–30.21)	20.88 (15.21–32.33)	21.67 (13.28–30.21)	0.947
AST, U/L	21.19 (18.01–25.83)	22.04 (18.15–28.09)	21.19 (17.95–25.43)	0.653
TG, mmol/L	4.99±1.18	4.88±1.19	4.78±1.30	0.466
TC, mmol/L	1.30 (0.96–1.86)	1.25 (0.90–1.79)	1.41 (0.92–1.67)	0.601
HDL-C, mmol/L	1.10 (0.94–1.31)	1.12 (0.95–1.29)	1.01 (0.88–1.32)	0.678
LDL-C, mmol/L	2.90 (2.33–3.45)	2.96 (2.29–3.45)	2.93 (2.22–3.64)	0.918
GGT, U/L	25.21 (17.97–38.14)	26.53 (18.49–42.97)	29.46 (18.51–38.91)	0.818
ALP, U/L	74.59 (61.30–91.52)	80.28 (63.92–98.34)	78.06 (65.18–92.93)	0.148
FBG, mmol/L	4.91 (4.55–5.72)	4.95 (4.47–5.84)	5.00 (4.63–6.51)	0.552

Abbreviations: BMI, body mass index; ALT, alanine transaminase; AST, aspartate aminotransferase; TG, triglyceride; TC, total cholesterol; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; GGT, gamma-glutamyl transpeptidase; ALP, alkaline phosphatase; FBG, fasting blood glucose.

Numerous studies have revealed the association of CVD with ALP and GGT. A cross-sectional research study including 5,995 individuals found that elevated serum ALP is correlated with peripheral arterial disease.[25] A Korean study including 3,091 participants indicated an independently positive relationship of ALP with carotid-femoral pulse wave velocity, a surrogate marker for arterial stiffness.[26] Many studies have shown that GGT is correlated with traditional risk factors for CVD, such as TC, LDL-C, glucose, insulin, BMI, etc.[27–32] Further studies showed that higher GGT may increase cardiovascular mortality,[33–38] and is an independent predictor for future cardiovascular mortality.[39,40] Even within the normal range, higher GGT is associated with CVD risk factors,[41–44] suggesting GGT as a superior marker for predicting CVD risk.[45] Kunutsor *et al*.[46] performed a meta-analysis including 20 GGT-related studies and 4 ALP-related studies, and found that baseline levels of ALP and GGT are each positively related to CVD risk. Recently, a dose-response meta-analysis including 23 studies with 1,067,922 participants revealed a direct relationship between ALP and GGT levels and the risk of CVD mortality.[47] While the specific mechanisms remain unclear for the moment, increasing evidence has demonstrated that ALP and GGT can promote CVD by facilitating oxidative stress and vascular calcification.[48,49]

Table 6. Analysis of clinical characteristics in LDLR rs1433099 T carriers and non-carriers of each group

	$p_{Control}$*	p_{NAFLD}*	p_{CVD}*
Age, years	0.608	0.847	0.532
BMI, kg/m^2	0.512	0.456	0.701
ALT, U/L	0.376	0.276	0.524
AST, U/L	0.497	0.267	0.349
TG, mmol/L	0.563	0.477	0.157
TC, mmol/L	0.522	0.701	0.957
HDL-C, mmol/L	0.582	0.619	0.595
LDL-C, mmol/L	0.170	0.400	0.392
GGT, U/L	0.179	0.929	0.002#
ALP, U/L	0.181	0.370	0.024#
FBG, mmol/L	0.488	0.228	0.312

*Significance of each group comparing CT+TT and CC individuals.

#T carriers have higher ALP and GGT in the CVD group.

Abbreviations: NAFLD, non-alcoholic fatty liver disease; CVD, cardiovascular disease; BMI, body mass index; ALT, alanine transaminase; AST, aspartate aminotransferase; TG, triglyceride; TC, total cholesterol; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; GGT, gamma-glutamyl transpeptidase; ALP, alkaline phosphatase; FBG, fasting blood glucose.

We can conclude that LDLR rs1433099 polymorphism may increase the risk of CVD through ALP and GGT, from this first related research study in China; moreover, these findings are consistent with previous findings.[20,24]

Our study has the following limitations. First, selection bias may exist since subjects comprised only a small sample size of patients in Qingdao district. Second, this study was confined to Chinese Han population in northern China, possibly with racial and geographical bias. Third, this study did not grade the severity of NAFLD patients. Further studies with more subjects should be conducted to illustrate the relationship of LDLR rs1433099 polymorphism with the risk of NAFLD in other ethnicities.

Conclusions

In conclusion, this study addressed that there was no association between LDLR rs1433099 polymorphism and incidence of NAFLD, for the first time. The LDLR rs1433099 T allele was found to significantly affect serum ALP and GGT in the CVD group. We can assume that LDLR rs1433099 polymorphism may influence the risk of CVD by ALP and GGT. The variant may be a risk factor in the early stage. Further studies on a large-scale population of subjects and of different ethnicity are needed to estimate the impact of LDLR rs1433099 on CVD and NAFLD patients. Further research on the role of LDLR rs1433099 in CVD might help to enhance the application of future therapeutic strategies and interventions.

Acknowledgments

We thank Dr. Zhenzhen Zhao for the assistance in this study.

Funding

This study was supported by grants from the National Natural Science Foundation of China (No. 31770837).

Conflict of interest

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

Author contributions

Design and data interpretation (YNX, YH, YSZ), manuscript writing (YH, SSL), critical revision of the manuscript (YSZ, SSL, GXC, LLC). All authors reviewed and commented on the manuscript and approved the final version.

Data sharing statement

All data generated or analyzed in this study are available from the corresponding author for the reasonable request.

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Journal of Clinical and Translational Hepatology **2021** vol. 9(2) | 210–219
DOI: 10.14218/JCTH.2020.00121

Original Article

Clinical Validation of Global Coagulation Tests to Guide Blood Component Transfusions in Cirrhosis and ACLF

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Received: 16 November 2020 | **Revised:** 6 January 2021 | **Accepted:** 20 January 2021 | **Published:** 18 February 2021

Abstract

Background and Aims: Patients with cirrhosis and acute-on-chronic liver failure (ACLF) may have bleeding complications and need for invasive procedures. Point-of-care (POC) coagulation tests like thromboelastography (TEG) and Sonoclot may be better for guiding patient management than the standard coagulation tests (SCTs), like prothrombin time, platelet count and international normalized ratio. ***Methods:*** We prospectively compared and validated the POC tests and SCTs in 70 persons with ACLF and 72 persons with decompensated cirrhosis who had clinical bleeding and checked for episodes of re-bleeding and transfusion requirements. We assessed pre-procedure requirement of blood components when correction was done based on an SCT or POC strategy. ***Results:*** Episodes of bleeding were seen in 45% and 28% of ACLF and cirrhosis patient, respectively (p=0.036), with the major site of bleeding being gastrointestinal (31% and 16%, respectively). Platelet counts correlated with TEG-maximum amplitude in cirrhosis (p=0.045) and prothrombin time correlated positively with TEG-reaction (R) time (p=0.032), TEG-Clot kinetics (K) time (p=0.042), Son-activated clotting time (p=0.038) and negatively with clot rate (p=0.043) in ACLF, making these correctable target variables in POC transfusion algorithms. Of 223 procedures, transfusion of fresh frozen plasma and platelet concentrate was reduced by 25% (p=0.035) and 20.8% (p=0.045) by using a POC strategy in 76 patients. Correction of deranged Son-activated clotting time and TEG-reaction time was noted in 68% and 72% after 24 h of fresh frozen plasma transfusion in ACLF and 85% and 80% in cirrhosis, respectively. ***Conclusions:*** Our study clinically validates that POC tests can better detect coagulation defects and transfusion thresholds in ACLF and cirrhosis, whereas use of conventional tests appear to be less suitable in patients with clinical bleeding. ***Trial Registration:*** NCT04332484.

Citation of this article: Premkumar M, Mehtani R, Divyaveer S, Kajal K, Kulkarni AV, Ahmed S, *et al*. Clinical validation of global coagulation tests to guide blood component transfusions in cirrhosis and ACLF. J Clin Transl Hepatol 2021;9(2): 210–219. doi: 10.14218/JCTH.2020.00121.

Introduction

Standard coagulation tests (SCTs) like prothrombin time (PT), international normalized ratio (INR), activated partial thromboplastin time (aPTT) and platelet count have been traditionally used to assess the hemostatic system in patients with cirrhosis of liver and acute-on-chronic liver failure (ACLF). However, these may not be accurate in cirrhosis, as there is deficiency of both pro-coagulants and anti-coagulants. Point-of-care (POC) viscoelastic coagulation devices, including those for thromboelastography (TEG) and the rotational thromboelastometer (ROTEM) and Sonoclot, are now being used increasingly for perioperative monitoring and for guiding blood component transfusion in patients with ACLF, variceal bleeding, or those undergoing liver transplantation. Fisher *et al*.[1] showed lower thrombin generation potential in ACLF compared to cirrhosis with acute decompensation. Studies in ACLF patients have also shown that viscoelastic tests may guide coagulation factor replacement effectively.[2,3] We previously described the dynamic changes in specific coagulation factors, like Factor VIII, von-Willebrand factor (vWF), protein C and antithrombin III, in addition to standard coagulation tests (SCTs) and TEG in relation to presence of sepsis/ SIRS and bleeding events in ACLF.[4]

Algorithms for management of peri-transplant coagu-

Keywords: Coagulation; Cirrhosis; ACLF; Validation; Sonoclot; Thromboelastography.

Abbreviations: ACLF, acute-on-chronic liver failure; ACT, activated clotting time; aPTT, activated partial thromboplastin time; ATIII, antithrombin III; CI, confidence interval; CLIF-SOFA, chronic liver failure-sequential organ failure assessment; CR, clot rate; CTP, Child-Turcotte-Pugh score; FFP, fresh frozen plasma; GCT, global coagulation tests; GI, gastrointestinal; HR, hazard ratio; INR, international normalized ratio; K, clot kinetics; Lys30 and Lys 60, lysis at 30 or 60 minutes, respectively; MA, maximum amplitude; MELD, model for end-stage liver disease; MELD-Na, model for end-stage liver disease-sodium; PAI, plasminogen activator inhibitor; R, reaction time; ROTEM, rotational thromboelastometry; SCT, standard coagulation test; SIRS, systemic inflammatory syndrome; TEG, thromboelastography; TPA, tissue plasminogen activator; TRALI, transfusion-related acute lung injury; TACO, transfusion-associated circulatory overload; vWF, von Willebrand factor.

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lopathy and blood component transfusion have been described.[5] These instruments are based on similar principles and provide a graphical output depicting various facets of coagulation, like platelet function, clot initiation, stability, and lysis. However, it is still not clear whether TEG and Sonoclot provide identical information. In this study, we aimed to clinically validate and compare the results obtained from TEG and Sonoclot instruments, and whether these correlated with SCTs in ACLF and cirrhosis.

Methods

This was a prospective observational study conducted at the Department of Hepatology, Postgraduate Institute of Medical Education and Research, Chandigarh, India between January 2018 and February 2020. Consecutive patients with ACLF (n=70) and cirrhosis (n=72) of any etiology, aged between 18 and 65 years of either sex were recruited. The exclusion criteria were blood or blood component transfusion in the last 2 weeks, human immunodeficiency virus infection, anti-platelet, anticoagulant or anti-fibrinolytic therapy, dialysis, pregnancy, active malignancy in the last 5 years, chronic heart failure, chronic pulmonary or end-stage renal disease. All patients were enrolled after providing written informed consent, and the protocol was performed in accordance with the Declaration of Helsinki. Ethical clearance was obtained from the Postgraduate Institute of Medical Education and Research Institutional Ethics Committee (IEC/NK 5412/Study/586, dated 13/8/2019). The trial was registered at NCT04332484, available at https://clinicaltrials.gov/ct2/show/NCT04332484. All authors had access to study data and approved the final manuscript. The primary objective was to clinically validate the results of TEG and Sonoclot instruments in patients with cirrhosis and ACLF, and whether a POC- versus SCT-based strategy resulted in different transfusion volumes of platelet concentrate and fresh frozen plasma (FFP).

Definitions

ACLF was defined as a syndrome that defines a subgroup of cirrhotic patients who develop organ failure with or without an identifiable precipitating event and have increased mortality rates in concordance with criteria reported in the CANONIC study.[6]

Systemic inflammatory response syndrome and sepsis (life-threatening organ dysfunction caused by a dysregulated host response to infection) was defined as per the Third International Consensus Definitions for Sepsis and Septic Shock (Sepsis-3).[7] The presence of sepsis was assessed by various cultures, detection of C-reactive protein and procalcitonin, or findings of new infiltrates on chest radiographs. Major bleeding was defined according to the International Society on Thrombosis and Hemostasis[8] as fatal bleeding, symptomatic bleeding in a critical area/organ (intracranial, intraspinal, intraocular, retroperitoneal, intra-articular or pericardial or intramuscular with compartment syndrome) and/or causing a fall in hemoglobin by ≥2 g/dL or requiring transfusion of ≥2 U of packed red cells. Assay validation was defined as documented control of the test performance according to predefined criteria relating to precision, linearity, accuracy, robustness, or measurement limits. Clinical validation of an assay requires the assessment of relevance of the test to clinical practice. Key considerations include comparabilityof results with previous results and evaluating effects of factors that may be encountered in clinical practice (e.g., variations in patient characteristics).[9,10] Follow-up of enrolled patients for episodes of bleeding, sepsis events, procedures and transfusions were carried out until 28 days or death.

Procedure risk in cirrhosis and ACLF

Procedure-related bleeding is common in cirrhosis patients but estimates of incidence vary widely. Intagliata *et al*.[11] classified the risk of invasive procedures in liver disease with examples of high risk (cardiac, thoracic, intracranial surgery, etc.), intermediate risk (lumbar puncture, percutaneous or trans-jugular liver biopsy, trans-arterial embolization procedures, etc.) and low risk (paracentesis, thoracentesis, central line, etc.).

Transfusion strategy

Royston and von Kier developed the use of TEG in 60 patients undergoing complex cardiac surgery and compared the actual use of blood and blood components during cardiac surgery.[12] Spalding *et al*.[13] and Görlinger *et al*.[14] devised algorithms based on thromboelastometry according to the same principle. We have modified the concept for our patients with liver disease to predict the requirement of FFP and platelets using a TEG-based algorithm (Table 1).

Standard medical therapy

Standard medical therapy included nutritional intervention, antibiotics, albumin infusion, diuretics, and vitamin supplements. Administration of blood components was limited to patients with active bleeding, and prophylactic transfusions were not performed. Prognostic scores like the model for end-stage liver disease (MELD) and chronic liver failure-sequential organ failure assessment (CLIF-SOFA) were used for assessment of severity of ACLF.[15,16]

Outcome measures

The main objective of the project was to evaluate the relationships of individual variables of the POC tests (activated clotting time (ACT), reaction time (R) and clot kinetics (K) time for clotting initiation) with the corresponding elements of serial SCTs (PT and aPTT). Platelet count and fibrinogen level was compared with clot rate (CR), maximum amplitude (MA), and α angle of the POC tests (Supplementary Table 1). We evaluated the ability of viscoelastic tests to predict spontaneous or procedure-related bleeding. We also studied the change in global coagulation tests (GCT) and SCT parameters over time and assessed correction with intervention. Since coagulation failure is associated with bleeding and sepsis, we also assessed the association of GCT variables, clinical events, and mortality in this large prospective cohort of patients. We also assessed the current practice of prophylactic FFP and platelet transfusion on the perceived risk of procedure-related bleeding and assessed transfusion requirements using an SCT correction or POC-based strategy. Lastly, we assessed whether any derangement in the SCT or GCT values at baseline could predict mortality in this large prospective cohort.

Sample size

Sample size was estimated using the G*Power program. Assuming incidence of coagulation defect in cirrhosis to be 10% and in ACLF to be increased by additional 20%,[17] a total sample size of 60 patients would be required with an effect size of 0.5, alpha of 0.05, and power of 0.85. Therefore,

Table 1. Prophylactic transfusion thresholds for patients administered FFP or platelets based on perceived risk of interventions

	Low risk procedures, *n*=167		High risk procedures, *n*=56		All procedures, *n*=223	
Pre-procedure INR	FFP given, *n* (%)	Number of procedures	FFP given, *n* (%)	Number of procedures	FFP given, *n* (%)	Number of procedures
≤1.5	0 (0)	75	0 (0)	25	0 (0)	100
1.6–1.7	2 (6.89)	29	4 (12.9)	20	6 (10)	49
1.8–2.0	8 (27.58)	18	24 (77.4)	8	32 (53.33)	26
>2.0	19 (65.51)	45	3 (9.67)	3	22 (36.67)	48
Not checked	0	0	0	0	0	0
Total	29	167	31	56	60	223
Pre-procedure platelet count as $\times 10^9$/L	Platelets given, *n* (%)	Number of procedures	Platelets given, *n* (%)	Number of procedures	Platelets given, *n* (%)	Number of procedures
<50	28 (96.55)	75	20 (80)	25	48 (90.5)	100
50–69	1 (3.45)	29	3 (12)	20	4 (7.54)	49
70–99	0 (0)	18	1 (4)	8	1 (1.88)	26
≥100	0 (0)	45	1 (4)	3	0	48
Not checked	0 (0)	0	0 (0)	0	0	0
Total	29 (100)	167	25 (100)	56	53	223

70 patients each with ACLF and cirrhosis were recruited to account for 10% attrition.

Assessment of coagulation parameters

Blood samples for assessment of complete blood count, PT, INR, aPTT, D-dimer and fibrinogen (Supplementary materials) and the viscoelastic tests of TEG and Sonoclot were collected at presentation and 72 h later. The need for repeat testing was to assess correction of the coagulation defect after the first intervention. In case of clinically evident bleeding, POC tests were repeated to check for changes. No patient received drugs that could potentially alter coagulation results, such as anticoagulants or antiplatelet agents. The standard antibiotic started was ceftriaxone or piperacillin-tazobactam. All coagulation tests were performed by the same technician and all Sonoclot and TEG tracings were interpreted by a single investigator (MP). The operating principles of TEG and Sonoclot analyzer are described in the Supplementary Table 1, with nomenclature of different TEG/Sonoclot variables.

Statement of ethics

All patients were enrolled after providing written informed consent, and the protocol was performed in accordance with the Declaration of Helsinki. Ethical clearance was obtained from the Postgraduate Institute of Medical Education and Research Institutional Ethics Committee (IEC/NK 5412/Study/586, dated 13/8/2019).

Statistical methods

Descriptive statistics were presented as mean ± standard deviation and median with inter-quartile range. Comparative analysis was done by Student's *t*-test/Mann-Whitney *U* test for continuous and Chi-square/Fisher's exact test for qualitative variables. Repeated measures analysis of variance (ANOVA) was used for analysis of changes in continuous variables by time. If necessary, logarithmic, or rank transformation was performed to obtain a good model fit. Linear correlations were evaluated by Pearson's coefficient of correlation. A p-value of <0.05 was considered statistically significant. Logistic-regression analysis was performed for predictors of 28-day mortality. Adjusted hazard ratios (HRs) and 95% confidence intervals (CIs) were computed to estimate the association of each predictor to clinical event/death. Statistical analysis was performed using SPSS for Windows, version 16.0 (SPSS Inc. Chicago, IL, USA).

Results

One hundred sixty-five eligible patients with liver disease were screened; of them, 23 were excluded (Fig. 1). Finally, 72 patients with decompensated cirrhosis and 70 with ACLF were enrolled. Patient demographics, clinical and laboratory data are presented in Table 2. Results of the POC tests at two time points are shown in Supplementary Table 2. Of the analyzed SCTs, repeated 72 h apart, only platelet count ($p=0.042$) and fibrinogen ($p=0.037$) values differed significantly with time and none correlated with clinical bleeding. There were significant changes for TEG variables R, K, alpha (clot rate) and MA (clot strength) at the two time points, indicating the dynamic nature of the coagulation milieu. For Sonoclot, only Son-ACT values differed significantly with time, especially in those with clinical bleeding.

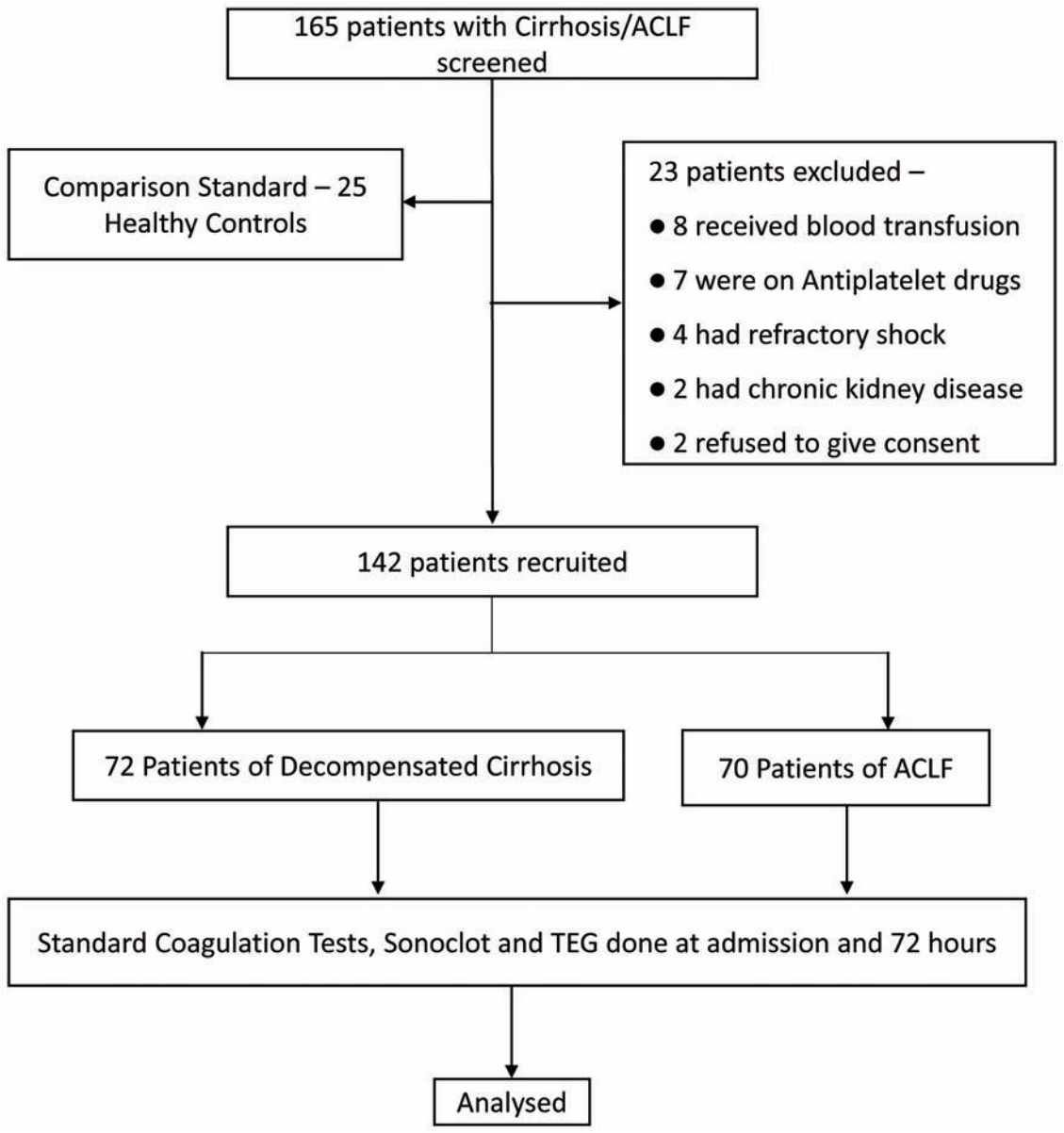

Fig. 1. Study participant enrollment flowchart.

Correlations of POC tests and SCTs

We performed multiple correlations between individual coagulation variables between POCs and SCTs to test plausibility based on the stage of coagulation (Table 3). Fig. 2A shows the TEG curve, with the stages of coagulation and the SCTs and TEG variables associated with defects at each step of clotting. Even though both TEG-alpha (r=0.76, p=0.048) and -K time (r=−0.65, p=0.050) were correlated to fibrinogen, in ACLF, only TEG-K was correlated to prediction of clinically evident bleeding (r=0.73, p=0.040). TEG-alpha and Sonoclot CR were the only variables that correlated with fibrinogen at all time points in ACLF.

Platelet counts correlated with TEG-MA in cirrhosis (p=0.045) but not in ACLF. PT correlated positively with TEG-R (p=0.032), TEG-K time (p=0.042) and Son-ACT (p=0.038), and negatively with CR (p=0.043) in ACLF but not in cirrhosis, making it useful as a correctable target variable in POC transfusion algorithms. The only correlations with aPTT were with Son-ACT (p=0.027) and CR (p=0.043) in ACLF. Fig. 2B shows the Sonoclot signature and the corresponding SCTs used to detect the defect at each stage of coagulation. The TEG-R (p=0.032) and TEG-K (p=0.040) times correlated with Son-ACT in ACLF but, in cirrhosis, only the K time correlated with ACT (p=0.052). Overall, the TEG-MA, CR and platelet function did not show a direct correlation with each other, even when done simultaneously.

Clinical presentation of bleeding and thromboses in ACLF and cirrhosis

Bleeding episodes were seen in 45% and 28% of patients in ACLF and cirrhosis, respectively (p=0.036). The most com-

Table 2. Baseline characteristics of the ACLF patients and cirrhosis patients

Parameters	Cirrhosis, *n*=72, mean ± SD	ACLF, *n*=70, mean ± SD	*P* value
Age in years	46.7±12.2	41.5±12.5	0.053
Males, *n* (%)	65 (90.6%)	62 (89.7%)	0.870
Etiology	Ethanol-related (44, 61%) NASH (16, 22%) Chronic hepatitis C (10, 13.8%) AIH (2, 2.7%)	Ethanol-related (48, 68.5%) NASH (14.20%) Chronic hepatitis B (6, 8.5%) Chronic hepatitis C (2, 2.8%)	
Acute insult	–	Ethanol (48, 68.5%) Acute viral hepatitis (12, 17.4%) DILI (8,11.4%)	
Duration of jaundice in days	20.5±6.5	16.1±8.3	0.041
Duration of ascites in days	10.4±4.7	8.2±2.5	0.045
Time between onset of disease to presentation to hospital in days	17.9±4.5	19.7±7.1	0.079
Total duration of stay in hospital in days	8.4±5.4	20.7±12.6	0.033
MELD-Na score	11.6±3.3	24.8±4.9	0.026
CLIF-SOFA score	–	8.6±2.1	–
APACHE II score	9.08±3.1	8.6±2.2	0.452
Age in years	46.7±12.2	41.5±12.5	0.053
Males, *n* (%)	65 (90.6%)	62 (89.7%)	0.870
BMI in kg/m^2	20.1±2.4	22.5±4.0	0.494
Hemoglobin in g/dL	10.9±2.1	10.5±0.31	0.177
Total leucocyte count as ×10^9/L	10.1±2.3	16.4±2.2	0.067
Platelet count as ×10^9/L*	130.3 (78.6–155.4)	153.6 (93.8–168.9)	0.034
Total bilirubin in mg/dL*	3.6 (2.5–7.8)	11.7 (5.6–18.5)	0.040
AST in IU/L*	36.0 (32–55)	120 (60–145)	0.050
ALT in IU/L*	30.5 (25–65)	95 (70–130)	0.024
Serum albumin in g/dL	2.9±0.5	2.6±0.7	0.319
S ferritin in ng/mL	237.3±94.5	514.5±167.3	0.036
Prothrombin time in s	18.5±7.5	23.5±7.5	
INR	1.56±0.8	2.32±0.5	0.039
S fibrinogen in mg/dL	145.8±45.9	144.2±25.9	0.180

*Values of these parameters are in median (interquartile range).

Abbreviations: AIH, autoimmune hepatitis; AST, aspartate transaminase; ALT, alanine transaminase; APACHE, acute physiology and chronic health evaluation; BMI, body mass index; DILI, drug-induced liver injury; NASH, non-alcoholic steatohepatitis; S, serum; SD, standard deviation.

Table 3. Correlations between SCTs and POCs

Conventional tests		ACLF		Cirrhosis	
		***r* value**	***p* value**	***r* value**	***p* value**
Platelet count	TEG-MA	0.64	0.073	0.76	0.045
	Sonoclot platelet function	0.73	0.052	0.75	0.06
PT	TEG-R time	0.81	0.032	0.66	0.083
	TEG-K time	0.79	0.042	0.72	0.057
	Son-ACT	0.8	0.038	0.67	0.076
	CR	−0.65	0.043	−0.54	0.058
aPTT	TEG-R time	0.76	0.078	0.72	0.057
	Son-ACT	0.81	0.027	0.61	0.076
	CR	−0.7	0.043	−0.55	0.058
Fibrinogen	TEG-alpha	0.76	0.048	0.72	0.076
	TEG-K time	−0.65	0.050	−0.70	0.037
	TEG-MA	0.6	0.069	0.58	0.089
	CR	−0.65	0.043	−0.54	0.058
Viscoelastic tests					
TEG-R time	Son-ACT	0.81	0.032	0.66	0.083
TEG-K time	Son-ACT	0.76	0.04	0.72	0.052
TEG-MA	Sonoclot platelet function	0.67	0.076	0.66	0.085
TEG-MA	CR	0.78	0.031	0.61	0.076
TEG-alpha	CR	0.72	0.067	0.62	0.071
TEG-MA	Platelet function	0.6	0.072	0.59	0.073

mon sites of minor bleeding were cutaneous ecchymoses (45% and 18%) and epistaxis (7% and 2.3%). The most common site of major bleeding in ACLF and cirrhosis was gastrointestinal (GI), at 31% and 16%, respectively. The GI bleeding was variceal in 44.4% and 40.6% or diffuse portal hypertensive gastropathy related in 55.5% and 59.3% in ACLF and cirrhosis, respectively. Post-variceal ligation ulcer bleeds (5.4%) were rare but severe, with a high re-bleeding rate and mortality. Spontaneous or iatrogenic hematomas (6.5%) and hemoperitoneum (3.4%) were the other bleeding sites. Thrombosis was observed in four patients with cirrhosis (one deep vein thrombosis, two portal vein, thrombosis, one superior mesenteric vein thrombosis). Clotting of central lines was noted in 14 subjects, but none was attrib-

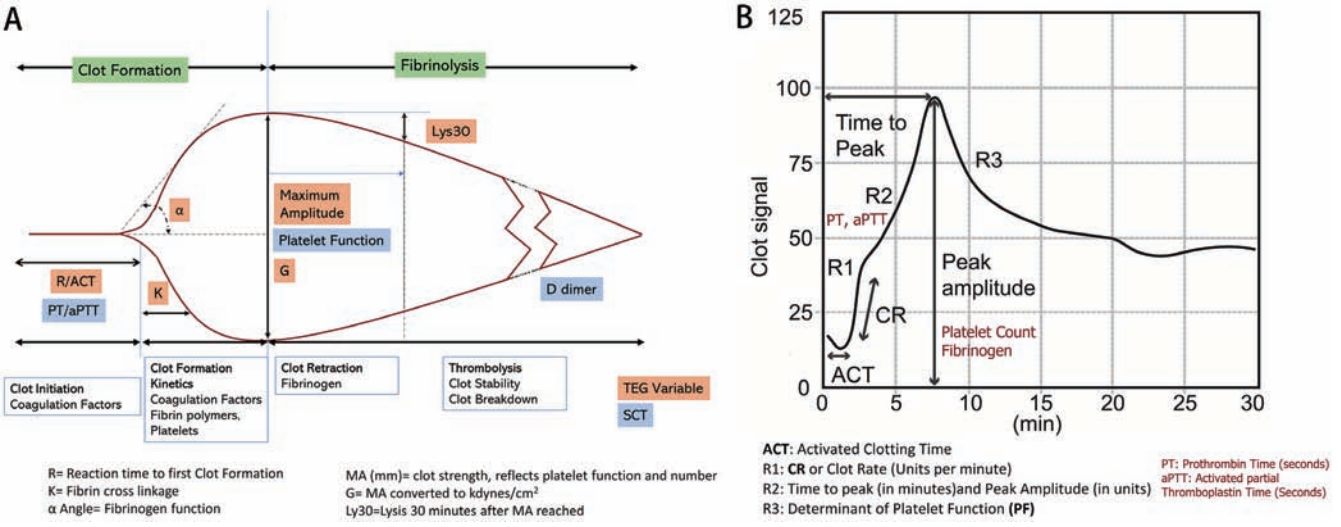

Fig. 2. Correlations of POC tests and SCTs. (A) TEG trace showing the stages of coagulation with corresponding standard coagulation tests. (B) Sonoclot signature showing the phases of clot formation and retraction, with corresponding standard coagulation tests.

Table 4. TEG-based transfusion algorithm at our center based on the method by Royston and von Kier[12]

TEG variable	Implication	Therapy
R>14 and <21 mm	↓ Clotting factors	Two FFPs
R>21 and <28 mm	↓↓ Clotting factors	Four FFPs
R>28 mm	↓↓↓ Clotting factors	Six to eight FFPs
MA <48 mm	↓↓ Platelet number/function	One SDP or four RDPCs
MA <40 mm	↓↓↓ Platelet number/function	Two SDPs or six to eight RDPCs
Lys30 >7.5%	Increased lysis	Tranexamic acid

Abbreviations: RDPC, random donor platelet concentrate; SDP, single-donor platelet.

utable to a hypercoagulable TEG/Sonoclot.

Bleeding, sepsis, and coagulation failure

Baseline SCTs were similar in patients with and without bleeding episodes. Also, the viscoelastic test values in cirrhosis did not differ much in patients who presented with a bleed and in those who did not. However, deranged TEG-R time at baseline was a predictor of bleeding [hazard ratio (HR) of 1.8; 95% confidence interval (CI) of 1.5–4.9, p=0.040) in ACLF. Overall, TEG-K time >9 m (HR of 1.3; 95% CI of 1.1–4.9, p=0.039), lysis >10% (HR of 1.9; 95% CI of 1.2–2.9, p=0.040) and MA<18mm (HR of 1.2; 95% CI of 1.1–3.5, p=0.034) predicted a major bleeding event. About 34% and 50% of patients with cirrhosis and ACLF respectively had sepsis at presentation. The sites of sepsis were spontaneous bacterial peritonitis (43%), pneumonia (24%), urinary tract infection (8%), and skin and soft tissue infection (3%). The presence of sepsis at baseline was associated with increased risk of a bleeding event in cirrhosis (HR of 1.2; 95% CI of 1.1–4.9, p=0.052) and ACLF (HR of 1.8; 95% CI of 1.5–9.1 p=0.040). Prolongation of the Son-ACT was observed in ACLF with sepsis at presentation (HR of 1.3; 95% CI of 1.1–7.6, p=0.043) when compared to ACLF without sepsis. There were no significant differences in CR, peak amplitude, or time to peak amplitude, with obvious trends related to sepsis. Supplementary Table 2 shows that in the absence of a new bleeding, thrombosis, or sepsis event, the GCT remained the same. Therefore, the GCT is a POC individualized test, should be compared in an individual patient for change with respect to baseline, and no absolute cut-offs can be given for the cirrhosis or ACLF population.

Transfusion thresholds for coagulation correction

Packed red cells were transfused when hemoglobin concentration was <7g/dL in a setting of clinical bleeding. We assessed the requirement for blood components pre-procedure when correction of a coagulation defect was done using a strategy based on SCTs versus POC. The correction was done based on SCTs, POCs or both, as per the discretion of the treating clinician. Table 4 shows the TEG-based transfusion algorithm followed at our center.[12,14] A total of 223 procedures were included, which were divided into low-risk and high-risk (Table 1). Patients with major bleeding were transfused with red cell concentrates (median of 2.3; 2–6 U), platelet concentrates (2.4; 0–4 U) and FFP (3.5; 3–6 U) at the discretion of the clinician in consultation with surgical teams. An INR≤1.5 and platelet count≥70×10^9/L did not merit prophylactic transfusion in low-risk procedures and INR≥1.8 and platelet count ≤50×10^9/L were all transfused. However, compliance with the POC algorithm was erratic. Of the 223 procedures, only 76 were treated as per the POC strategy. Median transfusion of FFP and platelet concentrate was reduced by 25% and 20.8% by using a POC strategy in cirrhosis. Only two patients developed transfusion-related acute lung injury (referred to as TRALI) after platelet transfusion in ACLF. Since the volume of products was minimized, we did not observe any transfusion related circulatory overload (referred to as TACO).

Eighteen patients received tranexamic acid post-endoscopy. Tranexamic acid was given when clot lysis was >5% in patients with bleeding. Correction of deranged Son-ACT and TEG-R time after 24 h was noted in 68% and 72% after 24 h of FFP transfusion in ACLF and 85% and 80% in cirrhosis, respectively.

Predictors of all-cause mortality in ACLF

None of the patients with cirrhosis died. Of the 14 (19.4%) deaths among the ACLF patients, causes of 28-day mortality included sepsis (57.1%), progressive liver failure (28.5%), and refractory variceal bleeding (14.3%). On univariate analysis, baseline model for end-stage liver disease-sodium (MELD-Na) >26 (HR of 6.7; 95% CI of 2.1–10.3, p=0.027), INR>2.6 (HR of 2.3; 95% CI of 1.8–8.5, p=0.010), CLIF-SOFA score >10.5 (HR 2.4; 95% CI of 1.4–6.1, p=0.038), clot lysis on TEG>10% (HR of 2.2; 95% CI of 1.9–3.4, p=0.033), and Sonoclot CR<15 (HR of 2.8; 95% CI of 1.1–4.5, p=0.044) predicted mortality in ACLF. However, on multivariate analysis, only MELD-Na (HR of 2.2; p=0.041), INR (HR of 1.9; p=0.039) and CLIF-SOFA score (HR of 1.2; p=0.040) predicted mortality (Table 5). Cox proportional HR for TEG and Sonoclot parameters served as predictors of mortality at 28 days in ACLF across two models with adjustment for age and baseline MELD. Derangement in four or five TEG parameters independently predicted mortality (Table 6).

Discussion

POC viscoelastic tests demonstrate specific functional coagulation defects that can direct blood component transfusion therapy in ACLF/cirrhosis, with clinical validation of individual parameters. This study clinically validates the use of two POC tests when done sequentially in cirrhosis and ACLF to estimate hypo- or hypercoagulability of any given patient. Secondly, our data suggest that the POC tests can be used to prevent unnecessary prophylactic transfusions if they demonstrate preserved global coagulation. Conventional practice of using target PT or platelet count correction for pre-procedure prophylaxis (i.e., correction of platelet counts or INR to an absolute value) has no evidence to support it. Our study demonstrates that the main utility of POCs is that they detect the defect in a stage of the coagulation and clot retraction process and, therefore, give a better actionable target for components like cryoprecipitate, platelets, FFP or antifibrinolytic drugs, like tranexamic

Table 5. Predictors of mortality in ACLF based on coagulation tests and severity scores

Variable	Cut-off at baseline	Univariate analysis			Multivariate analysis		
		HR	95% CI	*p* value	HR	95% CI	*p* value
MELD-Na	>26	6.7	2.1–10.3	0.027	2.2	1.2–7.1	0.041
INR	>2.6	2.3	1.8–8.5	0.010	1.9	1.2–4.3	0.039
CLIF-SOFA score	>10.5	2.4	1.4–6.1	0.038	1.2	1.1–5.5	0.040
Clot lysis, TEG	>10%	2.2	1.9–3.4	0.033			
CR	< 15	2.8	1.1–4.5	0.044			

acid. There was minimal comparability in the POC test and SCT variables, like INR or platelet count, suggesting that we need to re-evaluate the practice of pre-procedure correction of coagulation defects, and dose of blood components in patients with bleeding and ACLF.

Use of POCs for patients with bleeding

A POC test should be sensitive enough to indicate alterations in hemostasis in a timely fashion, with data at the time of bleeding being most relevant. We demonstrated correction of Son-ACT and TEG-R time in 68% and 72% after 24 h of FFP transfusion in ACLF and 85% and 80% in cirrhosis, respectively. Initial parameters like R and K times and alpha angle in TEG and ACT in Sonoclot can guide the use of FFP. These parameters are recorded in about 10–15 m and use of platelets (MA or platelet function) can be assessed in 30–40 m and data for use of fibrinolytics (lysis) is available in 30–60 m. These data suggest that the POC tests could better guide the transfusion protocol for bleeding patients and prophylactic transfusions and a corrected POC might be a better target than a corrected SCT, like INR or platelet count, which did not change much in serial tests.

Appraisal of POC tests: TEG and Sonoclot

Previous studies demonstrating interchangeability between TEG and thromboelastometry have been reported.[18–20] We suggest comparing individual parameters of these tests is futile even if they correspond to the same step in the coagulation cascade, as they are based on different estimation techniques (Tables 3 and 4). The same test, when used sequentially provides better information regarding adequacy of coagulation correction. Correction of ACT or R and K times after transfusion or resolution of sepsis is of greater significance than an individual parameter's numerical value. Structural differences in these POC tests, as highlighted in the supplementary materials, may explain divergent results obtained from the two instruments used in our study and in those by other authors.[21–24] TEG and Sonoclot may be equally useful to quantify changes in fibrinogen, consistent with previous studies.[18,25]

Comparison of POC tests and SCTs

Although we tried to find correlations between individual components of the POCs with corresponding SCTs in the coagulation cascade, we found little association. No correlations were found between INR and the TEG variable R or Sonoclot ACT, even though they all measure the time to the first fibrin formation. However, TEG-R time, TEG-K time and Son-ACT correlated with PT in ACLF. The reason for this discrepancy might be the different activators used, kaolin for TEG and tissue factor for INR. Sonoclot variables ACT and CR correlated significantly with aPTT. This is expected, as aPTT is usually prolonged in hypo-coagulable states, where a low CR can be found. Comparison of magnitude of the coagulation defect between two patients using numerical values of the component results is also fallacious. Rather, serial comparison of dynamic POC changes in the same patient makes clini-

Table 6. Cox proportional HR models for TEG and Sonoclot parameters as predictors of mortality at 28 days in ACLF across two models with different levels of adjustment

	Multivariate HR (95% CI)			
	Model 1[†]	*P* value	Model 2[‡]	*P* value
Number of TEG parameters deranged				
*None/1 parameter	1.0 (reference)		1.0 (reference)	
2	1.2 (0.9–3.98)	0.096	–	–
3	2.1 (2.2–4.3)	0.043	–	–
4	2.7 (1.9–6.3)	0.031	1.26 (1.38–2.17)	0.044
5	2.2 (1.8–7.4)	0.018	1.22 (1.32–1.54)	0.040
Number of Sonoclot parameters deranged				
*None/1 parameter	1.0 (reference)		1.0 (reference)	
2	1.1 (0.6–1.98)	0.080	–	–
3	1.3 (1.2–4.3)	0.070	–	–

[†]Model 1adjusted for age.

[‡]Model 2, adjusted as Model 1, and baseline MELD.

cal sense. Although viscoelastic tests are not perfect, they are the best currently available tests for determination of coagulation status. They certainly perform better objectively in patients with clinical bleeding than the SCTs like aPTT, PT, fibrinogen, platelet count, and INR. Since we have demonstrated a reduction in transfusions and reduced the number of additional coagulation tests, replacing it with a POC strategy, our costs have reduced, and transfusion-related adverse events like TRALI and TACO have been minimized. No POC tests showed correlations with platelet counts at the same time points. Thus, the use of MA/platelet function in POC tests is more clinically relevant than platelet count.

Our study was not designed to investigate clinical outcomes in relation to POC tests, which would require a much larger patient population with more frequent measurement of variables. The main limitation was difficulty performing the POC tests at the exact time of bleeding. The way to overcome this limitation will be to adopt use of GCTs in our Intensive Care Units in the same way that it is currently being used in the transplantation operating rooms. At our center, we have adopted a viscoelastic test as the standard algorithm, gradually replacing the use of platelet count or INR to guide blood transfusion for pre-procedure prophylaxis for invasive procedures or for coagulopathy-related GI re-bleeding. Our results are consistent with previous studies that separately evaluated TEG and Sonoclot in cardiac surgery.[26,27] Although transfusion of FFP and platelet concentrate in our Intensive Care Unit was reduced by 25% and 20.8% by using a POC strategy in cirrhosis, the agreement for the raised transfusion threshold for primary prophylaxis prior to invasive procedures was difficult, as interventional radiologists and surgeons remained skeptical. Ideally, GCTs should be repeatedly only when a new bleeding or clotting event occurs, or correction of coagulopathy is required (as is clear from the results of our study). Secondly, since individual baseline TEG and Sonoclot parameters did not correlate with survival, there is no need to repeat a TEG or Sonoclot assessment, unless an elective procedure is contemplated or there is a bleeding diathesis or thrombosis event. A GCT is a useful indicator of the coagulation defect in a patient who is bleeding and will indicate the appropriate correction. It should not be used as a predictor of bleeding in an individual case but should be done as a reliable test to define the need for blood products in a patient with bleeding or new thrombosis.

MELD-Na, INR, CLIF-SOFA score, and derangement of four or five TEG variables predicted mortality. Though a greater number of abnormal parameters on a GCT may predict increased likelihood of mortality, these are dynamic tests, and a single cut-off does not carry any relevance as a predictor of mortality.[28]

Until better standardization in liver disease is possible and knowledge about factors affecting POCs is increased, SCTs and POCs will remain complimentary for assessment of hemostasis.[29] Our study supports the use of POC tests to guide clinicians in the choice of blood products, contributing to better transfusion management in ACLF and cirrhosis. However, more studies are needed for safe POC algorithms in patients with bleeding for correction of the coagulation defect. If these GCTs replace use of ancillary tests like fibrinogen, D-dimer and aPTT and remain complementary to platelet count and INR, then TEG and Sonoclot are more cost effective than a SCT strategy. This is because a single POC test gives the ACT or R and K times (indicating coagulation factor deficiency, and guising FFP), the CR or MA which guide use of platelets or cryoprecipitate and the fibrinolys is guiding the use of tranexamic acid.

Conclusions

In conclusion, TEG and Sonoclot can be used to detect hemostatic defects and correction targets in ACLF and cirrhosis, whereas use of conventional tests like INR appears to be less suitable, at least in patients with clinical bleeding. It is not possible to match SCTs and variables from POC tests, even though they apparently match the same stage of coagulation. POC tests vary with time and can be normalized after correction of sepsis or use of blood components, unlike SCTs. Variables from TEG and Sonoclot provide more actionable targets at the bedside than SCTs, including correction of platelet function and clot lysis. By reducing additional tests, and reducing transfusion of blood components, the POC strategy is safer and more cost effective than an SCT transfusion strategy. Further studies are necessary to establish adequate reference values for patients with active bleeding and to standardize these assays in ACLF to achieve reliable results.

Funding

The study was funded by PGIMER institutional intramural grant (No RGC/6701/2019).

Conflict of interest

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

Author contributions

Design of the study (RM, MP, KK), diagnostic procedures and data collection (MP, RD, AD), TEG performance (KK), Sonoclot performance (Harpreet K, Harman K), statistical analysis and manuscript preparation (SD, AVK, MP). All the authors have read and approved the manuscript and had access to the study data.

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Journal of Clinical and Translational Hepatology **2021** vol. 9(2) | 220–226
DOI: 10.14218/JCTH.2020.00037

Review Article

Hepatocellular Carcinoma: Downstaging to Liver Transplantation as Curative Therapy

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Received: 30 April 2020 | **Revised:** 4 October 2020 | **Accepted:** 4 March 2021 | **Published:** 22 March 2021

Abstract

Hepatocellular carcinoma (HCC) ranks among the leading cancer-related causes of morbidity and mortality worldwide. Downstaging of HCC has prevailed as a key method to curative therapy for patients who present with unresectable HCC outside of the listing criteria for liver transplantation (LT). Even though LT paves the way to lifesaving curative therapy for HCC, perpetually severe organ shortage limits its broader application. Debate over the optimal protocol and assessment of response to downstaging treatment has fueled immense research activity and is pushing the boundaries of LT candidate selection criteria. The implicit obligation of refining downstaging protocol is to ensure the maximization of the transplant survival benefit by taking into account the waitlist life expectancy. In the following review, we critically discuss strategies to best optimize downstaging HCC to LT on the basis of existing literature.

Citation of this article: Frankul L, Frenette C. Hepatocellular carcinoma: Downstaging to liver transplantation as curative therapy. J Clin Transl Hepatol 2021;9(2):220–226. doi: 10.14218/JCTH.2020.00037.

Introduction

Hepatocellular carcinoma (HCC) is the most prevalent primary liver malignancy. It is the sixth most common neoplasm and fourth cause of cancer-related mortality globally.[1,2] As the incidence of HCC is projected to increase in the USA as nonalcoholic fatty liver disease continues to increase exponentially and alcohol and hepatitis C remain public health issues, HCC has emerged as a leading indication for liver transplantation (LT).[3–5]

LT offers a successful therapy for early-stage HCC patients because it simultaneously removes the lesion(s) and the preneoplastic liver.[6] Early records of post-LT outcome delineated high recurrence rates and were plagued with dismal patient survival.[7,8] Apart from tumor measurements, factors influencing recurrence include vascular invasion, histologic differentiation, previous response to local-regional therapy (LRT) and serum marker levels.[9–12]

The primary aims of establishing criteria for LT are to select candidates with good post-LT prognoses and to exclude patients whose disease conditions are suitable for other therapies, such as resection or systemic therapy. The Milan criteria (MC) (a single nodule ≤5 cm, 2–3 nodules ≤3 cm), proposed in 1996, emerged as an international benchmark to select patients with HCC for LT. According to MC, post-LT 5-year survival in HCC is >70% with a recurrence rate <10–15%.[13–15] The American Association for the Study of Liver Disease (commonly known as AASLD) and Guidelines of the European Association for the Study of the Liver (commonly known as EASL) recommend LT for HCC patients within MC but unsuitable for resection.[16,17]

However, debate in the past two decades has revolved around the dichotomous nature of MC. The stringent MC precludes access to LT for patients with larger or more numerous tumors who potentially have acceptable post-LT outcomes but who otherwise are not candidates for curative therapy. A plethora of studies have evaluated the liberalization from conventional criteria for HCC LT.[18–24] An alternative form of expansion relates to LT of candidates whose tumor burden exceeds MC without utilizing pre-LT treatment, while another form is linked to using treatment to successfully "downstage" tumor burden to within standard LT listing criteria based on radiographic assessment and markers of tumor biology. The current article reviews the framework for the downstaging of HCC and sheds light on recent updates in the field of prognosticators of post-LT outcomes.

Expanded selection criteria

Several expanded criteria for HCC beyond MC have been proposed (Table 1).[13–15,19–24] It is important to preface that most of the earlier studies predominantly relied on tumor morphological characteristics, which undermined their power in establishing ideal cutoffs. Additionally, prospective study design constructs a stronger evidential foundation for expanded criteria than does retrospective study proposals,

Keywords: Downstaging; Milan criteria; Hepatocellular carcinoma; Liver transplantation.

Abbreviations: 18F-FDG, 18F-fluorodeoxyglucose; AASLD, American Association for the Study of Liver Disease; AFP, alpha-fetoprotein; AFP-L3, lens culinaris agglutinin-reactive alpha-fetoprotein; CT, computed tomography; CTLA-4, cytotoxic T-lymphocyte-associated antigen 4; DCP, des-γ-carboxy prothrombin; EASL, European Association for the Study of the Liver; ETC, extended Toronto criteria; HCC, hepatocellular carcinoma; LT, liver transplantation; LRT, local-regional therapy; MC, Milan criteria; mRECIST, modified response evaluation criteria in solid tumors; MWA, microwave ablation; OLT, orthotopic liver transplantation; OPTN, Organ Procurement and Transplantation Network; PD-1/PDL-1, programmed cell death protein 1/programmed death ligand 1; PET, positron emission tomography; RFA, radiofrequency ablation; SBRT, stereotactic body radiotherapy; TACE, transarterial chemoembolization; UCSF, University of California in San Francisco; UNOS, United Network for Organ Sharing; VEGF, vascular endothelial growth factor; Y-90, Yttrium-90.

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Table 1. Details of different criteria for LT in HCC

Selection system	Assessment	Criteria	Years of follow-up	Survival, %	Recurrence rate, %
MC[13–15]	Radiology	Tumor size of ≤5 cm; up to three separate lesions, none larger than 3 cm; no evidence of gross vascular invasion; and no regional nodal or distant metastases	4	>70 (OS)	<10–15
UCSF criteria[19]	Radiology	Single tumor ≤6.5 cm or two to three lesions, none exceeding 4.5 cm with total tumor diameter ≤8 cm	5	80.9 (RFS)	9.1
Total tumor volume and AFP criteria[21]	Radiology	Total tumor volume ≤115 cm^3 and AFP ≤400 ng/mL, without macrovascular invasion	4	74.6 (OS)	9.4
Up-to-seven criteria[20]	Pathology	Size of largest HCC plus number of HCCs ≤7	5	71.2 (OS)	9.1
ETC[24]	Radiology	Any size or number of tumors, provided no extrahepatic spread, vascular invasion, or poor differentiation on pre-LT biopsy	5	68 (OS)	25.6
Hangzhou criteria[22]	Pathology	Total tumor diameter ≤8 cm or a total tumor diameter >8 cm, with a histopathologic grade I or II and a preoperative AFP ≤400 ng/mL	5	70.7 (OS)	N/A
Kyoto criteria[23]	Radiology	Tumor number ≤10; all ≤5 cm; and serum DCP ≤400 mAU /mL	5	65 (OS)	30

OS, overall survival; RFS, recurrence-free survival.

by eliminating confounding variables and carefully selecting participants.[25,26]

In 2001, Yao *et al*.[18] retrospectively analyzed LT recipients and propounded a modestly expanded criteria for orthotopic liver transplantation (referred to herein as OLT) on the grounds of explant histological characteristics. The exploratory study set forth the University of California in San Francisco (UCSF) criteria: (1) single lesion ≤6.5 cm; or (2) ≤3 lesions, with the largest ≤4.5 cm and total sum of diameters ≤8 cm. In a follow-up study, Yao *et al*.[19] prospectively validated the UCSF criteria for OLT based on pretransplant imaging and outlined post-OLT tumor recurrence and survival. The 5-year patient survival without recurrence was 81% and the recurrence-free probability exceeded 90% for patients meeting the UCSF criteria, which were similar to the patients fulfilling the MC.

Mazzaferro *et al*.[20] examined the feasibility of "up-to-seven criteria" (the sum of the size of the largest nodule and the number of nodules ≤7 without microvascular invasion) derived from explant pathology collected from 36 centers worldwide. Notably, the 71.2% 5-year OS rate achieved among patients beyond MC but within the "up-to-seven" criteria was associated with the absence of microvascular invasion, a variable difficult to ascertain pre-LT. It is noteworthy that upper tumor size and number limits beyond MC may increase the likelihood of microvascular invasion.[27]

In a prospective validation attempt to extend MC, Toso *et al*.[21] presented data in which LT candidate selection depended on a composite of the total tumor volume (≤115 cm^3) and alpha-fetoprotein (AFP) ≤400 ng/mL without macrovascular invasion or extrahepatic disease. Even though post-LT survival and recurrence were comparable to patients meeting MC, the waitlist drop-out rates posed a disadvantage. In China, the Hangzhou criteria also accounted for AFP levels in their protocol for selecting HCC patients for LT. Specifically, the Hangzhou criteria integrated total tumor diameter ≤8 cm or a total tumor diameter >8 cm, with a histopathologic grade I or II and preoperative AFP ≤400 ng/mL.[22]

A research group from Kyoto University proposed the Kyoto criteria that involved HCC tumor number ≤10, each tumor diameter no larger than 5 cm, and serum des-γ-carboxy prothrombin (referred to herein as DCP) ≤400 mAU /mL. The group's recent intention to treat analysis resulted in a 5-year OS rate and recurrence rate of 82% and 7%, respectively.[23]

Researchers at the University of Toronto endeavored to validate their extended Toronto criteria (commonly known as ETC), which relied on poor tumor differentiation, elevated AFP and cancer-related symptoms to select HCC candidates for LT, rather than the conventional measurements of tumor size and number at presentation. Although the 5-year OS of 68% for patients transplanted according to ETC was not statistically inferior to patients within MC amongst the prospective cohort of patients followed, tumor recurrence post-LT was higher for patients who exceeded MC but satisfied the ETC.[24]

Nonetheless, MC remains the gold standard for HCC patient selection and prognostic evaluation in LT.[28] The adoption of extended selection criteria generates the dilemma of a sharp rise in HCC patients on the LT waitlist with unknown regional repercussions on non-HCC patients waiting for LT, while persistent shortages of donor organs highlight the fundamental challenge of maintaining equity in liver transplant allocation.

Dynamism of serum markers

The multifactorial nature of HCC necessitates the integration of prognostic markers to assess tumor biological features and vascular invasion during the transplant evaluation process. No longer a contentious tool in candidate selection, AFP is widely used to distinguish the subset of LT candidates with a reasonable life expectancy after LT.[29–32] Many liver transplant centers globally incorporate AFP into their listing criteria, with differences in cutoffs. Therefore, the optimal serum AFP level cutoff as an exclusion criterion for LT in pretransplant HCC patients has garnered conspicuous research focus. In a detailed analysis of national United Network for Organ Sharing (UNOS) data, the subset of patients outside

the MC with low serum AFP levels (0–15 ng/mL) displayed improved post-LT survival.[30] The high end of AFP level cut-off ranges from 400 ng/mL to 1,000 ng/mL.[33–35] Mounting evidence reveals that AFP >1,000 ng/mL manifested in HCC patients either within or outside MC portends reduced post-LT survival and considerable risk for HCC recurrence.[34,36,37]

There is a paucity of data on the predictive value of other serum markers for post-transplant mortality and HCC recurrence. In the absence of a universal AFP cutoff point, some members of the liver transplant community have investigated DCP, lens culinaris agglutinin-reactive alpha-fetoprotein (AFP-L3) and/or the ratio of AFP-L3 to total AFP (AFP-L3%) as adjuncts within patient selection algorithms.[38–40] Moreover, the elevated neutrophil-lymphocyte ratio, an index of systemic inflammation, has been pursued as a maker of propensity to recurrence and unfavorable prognosis in parallel with AFP.[41] External validation is needed prior to amending organ allocation strategies to embrace these promising serum markers.

LRT: Bridging and downstaging

LRT plays a pivotal role in the therapeutic management of HCC patients. Forms of LRT encompass a wide range of modalities that include transarterial chemoembolization (TACE), radiofrequency ablation (RFA), microwave ablation (commonly known as MWA), radioembolization, stereotactic body radiotherapy (commonly known as SBRT) and/or hepatic resection.[42,43]

LRT is frequently employed as a bridge to transplant in patients listed for LT within the Organ Procurement and Transplantation Network (commonly known as OPTN) T2 (Milan) criteria to prevent dropout from the waiting list by inducing tumor necrosis and deterring tumor progression.[44,45] The rationale for bridging therapy lies in noncomparative studies reporting waitlist dropout rates as low as 8.7% at 6 months and between 22.9% at 12 months. By comparison, reported waitlist dropout rates are as high as 25% at 6 months and 38% at 12 months without the use of LRT.[44,46–48] The possible beneficial effect of bridging therapy for HCC patients' waitlist times of <6 months remains poorly characterized.[28] Despite the liability for selection bias and lack of randomized control trials, European guidelines recommend LRT to reduce the risk of pre-LT drop-out in regions of anticipated wait times longer than 6 months.[17] It is imperative to consider the risk of hepatic decompensation in advance of undergoing LRT. Furthermore, the variability in organ availability and hence vastly differing median waiting times across geographic regions culminate in a conditional recommendation for bridging therapy. Consequently, studies exploring LT waitlist dropout and post-LT outcomes founded on pre-transplantation treatment response radiologically evaluated by modified Response Evaluation Criteria in Solid Tumors (commonly known as mRECIST) are more logistically plausible to conduct than randomized controlled trials to elucidate the net effects of bridging LRT.[49,50] Such future studies will also lend insight into how the development of new lesion(s) notwithstanding partial or complete response of the target lesion(s) affects outcomes. With the changes in UNOS model for end-stage liver disease score exception criteria now mandating a 6-month delay before exception points can be obtained, LRT has become standard of care in patients with HCC awaiting liver transplant. These changes inevitably cause a prolonged wait time that reinforces the usefulness of LRT. In a multivariate analysis of the UNOS database, Halazun *et al*.[5] demonstrated that a waiting time of less than 4 to 6 months adversely impacts post-LT survival. Transplantation of patients with aggressive tumors in areas without a mandatory observational period can theoretically occur prematurely before tumor biologic behavior is assessed, thereby causing poor outcomes with aggressive recurrence. Accordingly, a minimal observation period aids in better candidate selection and possibly leads to lower risks of post-LT HCC recurrence.[51,52]

Tumor "downstaging" is a process that applies LRT to decrease tumor size and number in patients first deemed outside of the locally predefined criteria, commonly MC, for LT.[53] First recommended in 1997, tumor downstaging provides a viable alternative approach to expanding MC limits to select a subgroup of patients whose LT candidacy would otherwise be disregarded.[54–56] Sustained response to LRT can function as a measure of favorable tumor biology, whereas unresponsive and proliferative tumor burden after LRT yields worse post-LT outcomes.[45,57–60] The latest AASLD guidelines suggest that patients beyond the MC (T3) should be considered for LT after successful downstaging to MC.[16] Due to non-standardized downstaging protocol with precisely defined upper tumor limits across geographic regions, UNOS adopted the UCSF inclusion criteria for downstaging (single nodule ≤8 cm, 2–3 nodules each ≤5 cm, or 4–5 nodules each ≤3 cm with sum of the maximal tumor diameters ≤8 cm) as USA policy in 2017.[58,61] The notion of placing restrictions to enter downstaging is predicated on concerns over fairness and appropriate prioritization in liver allocation for all indications.

The first analysis of the UNOS database of 3,276 patients within MC and 422 patients within UNOS downstaging criteria, who underwent LT from 2012 to 2015, confirmed the validity of UNOS downstaging criteria by showing similar 3-year post-LT survival between HCC patients always meeting MC and patients whose initial tumor burden met the UNOS downstaging criteria and were then downstaged to LT.[62] Given the study's dependence on pre-LT data submitted to UNOS by LT centers, reporting biases pertaining to radiographic response to LRT are plausible. For example, underestimation of tumor size, whether intentional or unintentional, can inflate the proportion of patients in the downstaging group with explant tumor burden beyond MC.[63] Nevertheless, the findings that AFP ≥100 ng/mL at LT and short wait regions (median wait time of 2.6 months) or mid wait regions (median wait time of 6.5 months) were predictors for impaired post-LT survival in the downstaging groups support the need to incorporate AFP and expected wait times into tumor downstaging models.[62]

Efficacy of downstaging modalities

Currently, there are sparse data to draw conclusions on the optimal form of LRT for downstaging. Reported efficacies of common downstaging techniques defined as the successful anatomical reduction of tumor burden to within MC are highly variable.[64–67] A systematic review by Parikh *et al*.[65] revealed an overall downstaging success rate of 48%, with a post-LT HCC recurrence rate of 16%. The discrepancies in success of downstaging are attributed to various factors, such as initial tumor burden, choice of LRT utilized, LT program's downstaging procedures, and lack of a standardized time interval to determine radiographic response to LRT. The type of LRT performed for each patient is contingent upon the location of tumor, underlying liver function, performance status of patient, as well as local expertise in each treatment modality. In this systematic review, there was no significant difference comparing TACE and transarterial radioembolization, but the highest success rates were in patients that underwent multimodal therapy. There was not a significant difference in downstaging success rates in patients with more or less advanced liver disease, although other studies have reported lower success in patients with

Child's C cirrhosis.[65] Overall, the studies are variable in terms of success of downstaging, but overall it can be expected that approximately half of patients that are attempted to be downstaged will actually undergo LT.

Hepatic resection is the preferred curative treatment for patients with small localized tumors and well compensated liver disease and is an option for downstaging.[68] Comprehensive pathological examination of resected specimens may facilitate the identification of patients with histological features of poor prognosis, for instance macrovascular invasion gone unobserved.[69] This significantly influences subsequent treatment choices during postoperative surveillance of tumor recurrence patterns. Although large lesion size is not an absolute contraindication to hepatic resection, portal hypertension and end-stage liver disease are major risk factors for postsurgical complications and death.[70] There is a subset of patients who require resection in conjunction with LRT to complete downstaging. However, surgical resection has been reported in a minority of studies as a downstaging modality so no statement can be made about its efficacy.

TACE is the most frequently used palliative treatment technique in downstaging protocols, particularly for multifocal HCC.[43] The reported downstaging success rates with TACE (23.7–90%) are inconsistent and should be interpreted with caution.[64] Since the TACE mechanism of action targets the hepatic arterial supply, its efficacy depends on responsive HCCs with good blood supply and uptake. While TACE is not advised to be performed in the presence of portal vein thrombosis, transarterial radioembolization with Yttrium-90 (Y-90) beads is a safe alternative downstaging therapy.[71,72] Per available data there is no statistically significant difference between success rates of TACE and radioembolization for downstaging.[65] It is important to note the risk of inaccurate staging when relying on imaging results to gauge radiological response to TACE or radioembolization in terms of tumor size and viability. For example, tumor response to Y-90 typically evolves gradually and may require 3–6 months to exhibit an adequate response on triphasic computed tomography (commonly known as CT) or magnetic resonance imaging.[73] Therefore, timely intervals between treatment sessions and imaging are crucial to reduce confounding by image interpretation.

RFA confers its curative effects through thermal energy to achieve complete necrosis at a success rate of up to 90% in tumors of ≤3.0 cm in diameter.[74] The rare complication of tumor seeding and risk of bleed with superficially located tumors are a few limitations within RFA's safety profile.[42] RFA is contraindicated near large vessels because of the heat sink effect, whereas MWA is a safe therapeutic option.[70,75] SBRT, an extracorporeal technique, administers high doses of radiation to the target tumor. Published data investigating SBRT for downstaging are scant, but it appears to be a safe LRT for patients with decompensated liver function, especially in tumors near the major bile ducts.[76,77]

No evidence appears to render the superiority of one downstaging modality over another. The heterogeneity in the quality of data on the downstaging effectiveness of LRTs warrants large, multicenter, prospective cohort studies enriched with multidisciplinary tumor board referrals and standardized data reporting criteria in regions of differing waitlist times.

Systemic therapy and immunotherapy in advanced HCC

The goal of treatment is to maximize survival while prolonging the highest quality of life. Hence, it is paramount to assess the strength of scientific data for the selection of an appropriate treatment approach in HCC patients with advanced disease. When liver-directed therapy fails to successfully downstage patients into MC, HCC patients often transition into systemic therapy. Sorafenib is an oral tyrosine kinase inhibitor, whose anti-vascular endothelial growth factor (i.e. VEGF) receptor properties are proven to improve survival in advanced HCC patients, with a median survival of 10.7 months compared to a median survival of 7.9 months in placebo controls.[78,79] In the scenario of sorafenib's failure as a first-line systemic therapy, regorafenib, followed by cabozantinib, demonstrates a comparable survival benefit as second-line systemic therapy.[80,81] Recently, in an open-label, phase III, multicenter, non-inferiority trial, lenvatinib, another oral multikinase inhibitor, displayed clinically meaningful improvement in objective response rate, progression-free survival, and time to progression compared to sorafenib in unresectable and treatment-naive HCC. However, the median survival was not statistically significantly between 13.6 months for lenvatinib and 12.3 months for sorafenib, (hazard ratio of 0.92, 95% confidence interval of 0.79–1.06).[82] Newly, the REACH-2 phase III trial established the efficacy of ramucirumab, a monoclonal antibody that antagonizes VEGF receptor 2, in sorafenib-refractory patients with high AFP (of at least 400 ng/mL).[83] Notwithstanding the emergence of systemic therapies, it is pertinent to mention that the role of the systemic therapies remains under study in the tumor downstaging to transplant setting. In a pilot, single-center, randomized controlled trial, the safety and adverse event profile of sorafenib plus Y-90 was compared to Y-90 alone in HCC patients as a bridge to LT. Data from the study's limited sample size suggests the combination of sorafenib plus Y-90 in patients awaiting LT was linked with more peri-transplant biliary complications and a trend of higher acute cellular rejection rates.[84] Given the lack of robust data, further studies are required to investigate and elucidate the utility of tyrosine kinase inhibitors or other systemic therapies in the pre-LT patient population, with regards to both efficacy and safety in the transplant setting. Tyrosine kinase inhibitors are known to inhibit wound healing, and patients who undergo liver transplant while being treated with tyrosine kinase inhibitors may be at risk for higher complications.

In cases of unresponsiveness or unfitness to receive tyrosine kinase inhibitors, negative regulators of T cell immune function, such as programmed cell death protein 1/programmed death ligand 1 (i.e. PD-1/PDL-1) or cytotoxic T-lymphocyte-associated antigen 4 (i.e. CTLA-4), have been identified as potential therapeutic targets.[85] Two PD-1 checkpoint inhibitors, nivolumab and pembrolizumab, are promising immunotherapies for advanced HCC as second-line therapies.[86,87] These two immunotherapies remain under Food and Drug Administration conditional approval, based on phase II data. There is also a recent approval of atezolizumab in combination with bevacizumab for treatment of advanced HCC, as well as the combination of PD-1 with CTLA-4 immunotherapy (nivolumab and ipilimumab). The lack of safety data with immunotherapy prior to transplant warrants further investigation. There is little to no data in the literature on the effects of immunotherapy in the liver transplant setting, with regards to the possibility of hyperacute or acute rejection after treatment.

AFP response to LRT

In the context of downstaging, the degree of a decrease in AFP in response to LRT is a valuable indicator of tumor biological aggressiveness. Policy implemented in the USA requires patients with AFP >1,000 ng/mL to exhibit a reduction in AFP to <500 ng/mL with LRT before proceeding with LT, in an effort to preserve comparable 5-year survival rates

between HCC and non-HCC LT recipients.[61,88] Recently, Mehta *et al*.[89] endeavored to retrospectively validate the effects of this USA national policy using the UNOS database. In a multivariable analysis, a reduction in AFP from >1,000 ng/mL to 101–499 ng/mL was correlated with a greater than 2-fold reduction in post-LT death and close to a 3-fold reduction in HCC recurrence. The French AFP model identified a stricter AFP cutoff of ≤100 ng/mL for the subgroup of patients outside the MC as a predictor of nearly 70% 5-year overall survival rates and a low risk of recurrence.[36] Interestingly, increasing AFP slope as low as 7.5 ng/mL/month and as high as 15 ng/mL/month in spite of LRT is associated with unfortunate outcomes in patients awaiting LT.[90] While the implications of an AFP slope may seem irrelevant in world regions without a minimum 6 months waiting time, an observation period is essential for the "ablate and wait" strategy.[91] Thus, the lack of durable response to LRT measured by AFP captures a supplementary exclusion criterion for LT.

18F-fluorodeoxyglucose (18F-FDG) positron emission tomography (PET)

Another potential diagnostic tool for patients being downstaged is 18F-FDG-PET imaging. Increased 18F-FDG accumulation of HCC consistently reflects tumor aggressiveness and is connected to undesirable post-LT survival.[92,93] Poorly differentiated HCC expresses high 18F-FDG metabolism with a lesion-to-liver uptake ratio of more than 2.[94] Despite its high sensitivity for detecting extrahepatic metastases, 18F-FDG-PET is not a widespread routine imaging modality due to the absence of cost-effectiveness analyses and prospective validation studies in regions with scarce donor resources.[95] Ultimately, 18F-FDG-PET scans can help determine appropriate treatment options for 18F-FDG-PET-positive patients beyond MC by clarifying aggressiveness of disease.

Conclusions

In light of growing societal demands for LT, tumor downstaging surfaces at the heart of efforts to optimize the LT selection scheme. The premise of downstaging is to allow the opportunity of LT to a larger portion of HCC patients without affecting the transplant survival benefit. A multitude of robust data emphasize that the sole reliance on radiologic tumor size and number is a relatively crude method to gauge the complexity of HCC cases. Meanwhile, limited organ supply and waitlist life expectancy stress the value of surrogates for refined patient selection. AFP and novel biomarkers, LRT approaches, radiographic and AFP response to LRT, in combination with 18F-FDG-PET scans could be utilized as predictors of post-LT outcomes in a multifaceted LT evaluation process. Forthcoming longitudinal multicenter, well-designed studies are necessary to identify and prospectively validate reliable selection parameters. Overall, regional disparities in LT wait times and program-specific practices, like live donor LT, dictate patient eligibility for downstaging and individualized treatment decisions per recommendation and thorough follow-up by the program's multidisciplinary team involving, but not exclusively, radiologists, hepatologists, surgeons, pathologists, and oncologists. Given the complexity of this disease, it is difficult to determine one particular downstaging method that is most successful, as each patient needs to be evaluated on an individual basis for which pre-LT treatment they can tolerate and will best downstage them to within transplant criteria. In general, careful patient selection combined with aggressive locoregional therapy appears to have the best outcomes in long-term.

Funding

None to declare.

Conflict of interest

Catherine Frenette reports the following conflicts of interest: Speakers Bureau for Bayer, Bristol Meyers Squibb, Eisai, Exelixis, Genentech; Advisory Board/Consultancy for Bayer, Eisai, Exelixis, Genentech; Research Support from Bayer, Merck, and Exelixis. The other author has no conflict of interests related to this publication.

Author contributions

Writing and editing this manuscript (LF, CF).

Data sharing statement

All data are available upon request.

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Journal of Clinical and Translational Hepatology **2021** vol. 9(2) | 227–238
DOI: 10.14218/JCTH.2020.00131

Review Article

Gut Microbiota in Metabolic-associated Fatty Liver Disease and in Other Chronic Metabolic Diseases

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Received: 26 November 2020 | **Revised:** 5 February 2021 | **Accepted:** 22 February 2021 | **Published:** 8 March 2021

Abstract

The gut microbiome plays a key role in the health-disease balance in the human body. Although its composition is unique for each person and tends to remain stable throughout lifetime, it has been shown that certain bacterial patterns may be determining factors in the onset of certain chronic metabolic diseases, such as type 2 diabetes mellitus (T2DM), obesity, metabolic-associated fatty liver disease (MAFLD), and metabolic syndrome. The gut-liver axis embodies the close relationship between the gut and the liver; disturbance of the normal gut microbiota, also known as dysbiosis, may lead to a cascade of mechanisms that modify the epithelial properties and facilitate bacterial translocation. Regulation of gut microbiota is fundamental to maintaining gut integrity, as well as the bile acids composition. In the present review, we summarize the current knowledge regarding the microbiota, bile acids composition and their association with MAFLD, obesity, T2DM and metabolic syndrome.

Citation of this article: Hernández-Ceballos W, Cordova-Gallardo J, Mendez-Sanchez N. Gut microbiota in metabolic-associated fatty liver disease and in other chronic metabolic diseases. J Clin Transl Hepatol 2021;9(2):227–238. doi: 10.14218/JCTH.2020.00131.

Introduction

The prevalence of metabolic chronic diseases is increasing around the world, mainly due to the increased incidence of obesity and type 2 diabetes mellitus (T2DM). The global prevalence of T2DM is 8.8%, which translates into approximately 422 million afflicted people worldwide; the USA alone has a T2DM prevalence of 25% among its seniors and an alarming amount of obesity, with 604 million adults and 108 million children being obese.[1] Metabolic-associated fatty liver disease (MAFLD) affects 25% of the global population,[2–9] representing the principal cause of chronic liver disease.[10–12]

This phenomenon can be associated with the modern lifestyle across industrialized countries that favors a sedentary life and high-calorie diets, thus promoting obesity and other chronic comorbidities. This situation constitutes a serious public health problem.[6,13–15]

The pathophysiology of these metabolic diseases is very complex and multiple factors seem to play a role in their development and progression; however, the gut microbiota is an emerging topic, as over the past few decades it has been demonstrated to play a critical role in their development. Signals generated by dietary intake and environmental factors disturb the composition of the microbiota, altering intestinal barrier homeostasis as well as the bile acid (BA) composition, and activating inflammatory pathways.[16]

Furthermore, it has been demonstrated that diet not only determines calorie intake but also impacts composition of the BA pool and gut microbiota, along with the intestinal barrier homeostasis.[17]

Throughout this paper, we will discuss the role of gut microbiota and BAs composition in metabolic diseases such as T2DM, obesity and MAFLD.

Epidemiology and risk factors of MAFLD

Elements such as ethnicity and gender have been described as determinants of the susceptibility and predictors of the severity and progression of MAFLD. Hispanics are the most susceptible to liver damage, followed by African Americans. Countries with the highest prevalence are USA, Belize, Barbados, and Mexico, with 30% of adults and 10% of adolescents having a MAFLD diagnosis, equaling to more than 80 million MAFLD patients.[6] The highest prevalence of MAFLD is between the ages of 50 to 70 years.[13] Men are at higher risk than women are; although, after menopause, this protective effect is lost. Life-style related factors, like little physical activity and a high-fat diet, are also important due to the close relationship of MAFLD with other chronic meta-

Keywords: Metabolic-associated fatty liver disease; Metabolic-associated steatohepatitis; Gut microbiota; Gut-liver axis; Dysbiosis; Bile acids.

Abbreviations: APC, antigen presenting cells; BA, bile acids; MAFLD, metabolic associated fatty liver disease; T2DM, type 2 diabetes mellitus; GWAS, genome wide association studies; TJPs, tight junction proteins; AJPs, adherent junction proteins; DC, dendritic cells; PBA, primary bile acids; SBA, secondary bile acids; FXR, farnesoid X receptor; TGR5, takeda G protein coupled receptor; FMT, fecal microbiota transfer; SCFAs, short chan fatty acids; IR, insulin resistance; HCC, hepatocellular carcinoma; SIBO, small intestine bacterial overgrowth; LPS, lipopolysaccharide.

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bolic diseases like T2DM, obesity and metabolic syndrome.[6]

Genetics and MAFLD

Genetics have always been an important determinant and risk factor for the development of numerous diseases. Several studies have strongly suggested the existence of a hereditary component of MAFLD.[18,19]

Multiple polymorphisms have been associated with MAFLD, among the most studied are those involving the *patatin-like phospholipase domain containing 3* (*PNPLA3*), *TM6SF2*, *LYPLAL1*, *GCKR* and *MBOAT7* genes.[20] *PNPLA3* has been consistently identified across different genome-wide association studies (commonly known as GWAS). This gene is located on chromosome 22 and encodes up to 81 amino acids related to the synthesis of the enzyme adipose triglyceride lipase, which contributes to the degradation of triacylglycerol in adipose tissue.[18] The variant I148M (rs738409) substitutes an isoleucine with a methionine, reducing the capability of the enzyme to catalyze triacylglycerol in lipid droplets as they are gathered in the adipose tissue, leading to the accumulation of lipids within the liver; thus, there is a close relationship with the development of MAFLD.[18,21,22] This polymorphism has also been associated with the severity of inflammation and fibrosis progression[21,23,24] and, worryingly, some data have also indicated that it might increase the risk of MAFLD-associated hepatocellular carcinoma.[18]

Other polymorphisms that have been studied in MAFLD patients involve *TM6SF2* (rs58542926) and *MBOAT7* (rs641738). Their association with the severity of hepatic steatosis has been demonstrated across studies, but a recent study suggested that they are not associated with the severity of hepatic fibrosis.[25]

On the other hand, the *HSD17B13 17-beta hydroxysteroid dehydrogenase 13* polymorphism rs6834314 (located in chromosome 14 and expressed mainly in the liver) has been described to have a protective effect on MAFLD. Although it does not appear to influence hepatocyte lipid accumulation, it might protect against hepatic fibrosis by modulating retinol dehydrogenase activity.[26–28] This polymorphism was found to be associated with a protective effect in MAFLD patients, limiting progression from hepatic steatosis to metabolic steatohepatitis and fibrosis progression,[27] and thereby reducing MAFLD chronic liver disease progression.[28]

Genetics and gut microbiota

It is believed that the gut microbiota composition is influenced, at least in part, by genetics, as it has been found that family members share similar microbial signatures.[29] Moreover, studies made in twins found that the gut microbiota was more similar among the monozygotic twins rather than the dizygotic twins.[30] The idea of a genetic influence on the microbiome and its dysbiosis is not far from the findings obtained from studies of other metabolic disorders; unfortunately, no studies have been made yet to characterize the specific genes and processes underlying this specific interaction.[29]

Intestinal barrier homeostasis

The intestinal barrier is constituted of several components that assure its homeostasis and prevent the translocation of pathogen and inflammatory factors, protecting the liver in this gut-liver axis. The main components of the intestinal barrier are the mucosal epithelium, tight junction proteins (TJPs) and the immune cellular barrier.

Mucosal epithelial barrier

Mucus represents the first anatomical barrier between the epithelial cells and the intestinal microbiota. The glycoprotein-rich mucus layer also serves as a source of nutrition and growth for some bacterial species. When fiber intake is deprived, the thickness of the mucus layer is decreased,[31] which may lead to intestinal inflammation and a reduction in the physical barrier between the microbiota and the epithelial cells.[32]

The mucus layer also represents a source of immunoglobulins, mainly IgA, and antimicrobial peptides, that hinder the translocation of "good" bacteria. The mucus layer composition and thickness have to be balanced enough to prevent commensal microbial washout due to BAs and peristaltic movements, and to conserve the integrity of the intestinal barrier.[33] Therefore, diet is very important to preserve the mucosal barrier integrity, protecting against intestinal pathogen translocation and inflammation.[33,34]

TJ proteins "leaky gut"

Among the intestinal TJs are those that hold the epithelial cells together, maintaining the integrity of the intestinal barrier. Such TJs are the ZO-1 (zonula occludens-1), occludin, claudin-1 and claudin-4. It has been shown in experimental studies in rodents that a fructose-enriched diet decreases the concentration of the TJs, as well as the concentration of adherent junction proteins, leading to an increase of bacterial endotoxin levels and contributing to the leaky gut condition.[35] In other studies, it has also been described that, in MAFLD over-nourished patients (high-fat or fructose-enriched diet), the concentration of ZO-1 and occludin is decreased, favoring bacterial translocation to the circulation, and supporting increase lipopolysaccharide (LPS) levels in the blood (known as metabolic endotoxemia). When the LPS is detected by Toll-like receptors in the liver, the characteristic low-grade inflammation associated with hepatic steatosis and fibrosis is triggered.[36]

Correlations between increased intestinal permeability and other alterations (like obesity, insulin resistance, and elevated lipid profile) have been demonstrated.[37] As described above, MAFLD patients have an increased gut permeability, and even though bacterial translocation is a relatively physiological process and the liver can regulate the bacterial concentrations through their elimination via Kupffer cells, the significant increase of harmful bacterial substances promote the production of chemokines, inflammatory cytokines, vasoactive factors, and reactive oxygen species, which lead to hepatocyte apoptosis and activation as well as proliferation of stellate cells and development of fibrosis through TGFβ.[38,39]

Immune cells barrier

Several immune cells contribute to reinforcement of the intestinal barrier. These include lymphocytes (T cells [CD4+ Th17, CD4+T regulatory]), natural killer T cells, dendritic cells, and mononuclear phagocytes.[16] The intestinal immune barrier itself is composed of intraepithelial lymphocytes that include a diversity of T cells, mainly CD4+ and T regulatory cells.[40] Natural killer T cells are also important components for the recognition and discrimination between foreign and self-antigens, when activated by antigen presenting cells triggering immune responses. They can also be activated by pro-inflammatory cytokines.[41]

Dendritic cells are another important cellular component

Table 1. Comparative view of the healthy gut bacterial composition and its alterations during disease

	Phylum	Species
Healthy/ Normal	Firmicutes, Bacteroidetes, Actinobacteria, Proteobacteria, Fusobacteria, Verrucombia	*Lactobacillus, Clostridium, Enterococcus, Ruminococcus, Bacteroides, Prevotella*
Obesity	Firmicutes (↑) Bacteroidetes (↓)	*Akkermansia muciniphila* (↓)/Tuminococcaceae, Rikenellaceae, Mollicutes (↑)
Diabetes	Firmicutes (↑) Bacteroidetes (↓)	*Roseburia, Eubacterium hallii, Faecalibacterium prausnitzii* (↓)/Lactobacillus gasseri, Streptococcus mutans, E. coli (↑)
MAFLD	Firmicutes (↑), Actinomycetes (↑), Proteobacteria (↑), Bacteroidetes (↓), Fusobacteria (↓), Lentisphaerae (↓), Proteobacteria(↓), Thermus(↓), Verrucomicrobia (↓)	*Prevotella, Porphyromonas, Veillonella* (↑)

of the innate immune system that constitute the intestinal barrier. They induce T cell activation, produce interleukin (IL)-23 and induce a T helper immune response.[42] Dendritic cells also express TJs and have the capacity to open epithelial tight junctions, playing an important role in preserving epithelial barrier integrity.[43]

Role of BAs in intestinal microbiota

BA composition and receptors also play a key role in the gut microbiota composition as well as in signaling pathways that regulate metabolic syndrome. They also seem to be part of the progression of hepatic fibrosis in patients with MAFLD.

BA composition

BAs are essential for fat-nutrients absorption and are classified as primary (PBA) and secondary (SBA). They are synthetized in the liver from cholesterol and transformed from PBA to SBA by the gut microbiota. They can be either conjugated or deconjugated. The BA signaling pathways and BA pool are controlled by the gut microbiota through different reactions (dihydroxylation and deconjugation).[17] It has been described that SBAs increase the risk of metabolic diseases. Their composition is importantly determined by gut microbiota and dysbiosis disrupts the PBA/SBA ratio.[44] Furthermore, BAs may also alter the gut microbiome by inhibiting bacterial development and altering the microbiome composition, by acting as a detergent according to the hydroxyl groups and amino acid portion, exerting this deleterious effect on the Bacteroidetes phyla especially.[17,44] BAs represent a crucial partaker in liver-microbiota communication, and their composition and concentration seem to have a positive correlation with metabolism and hepatic fibrosis.[45,46]

The composition of BAs is important in many metabolic diseases, it has been described that patients with T2DM present a change from PBA to SBA with an increase in deoxycholic acid levels. BAs are metabolic integrators that act through signaling pathways that regulate the expression of certain metabolic genes; hence, a tweak in BA signaling might promote and aggravate metabolic syndrome.[17]

BA receptors and their effect on metabolic diseases

The BA composition is very important in MAFLD development. They play a key role in the modulation of metabolic pathways and in the balance of gut microbiome by acting as signaling molecules to diverse intestinal and liver receptors, such as farnesoid X receptor (FXR), vitamin D receptor and the TGR5 Takeda G protein-coupled receptor, and modulating immune responses in the gut.[47–49] Activation of the hepatic receptors also varies depending on the type of BAs, with FXR being triggered preferably by PBAs and TGR5 by SBAs. These receptors modulate several metabolic characteristics, such as glucose tolerance, insulin sensitivity, fatty acid β-oxidation, energy expenditure, very low-density lipoprotein clearance, as well as hepatic inflammation.[17,44]

Microbial distribution in healthy subjects

Each individual has a unique gut microbiome, mainly shaped early in life by many factors, like the type of birth, milk feeding, weaning period or antibiotic use. The different bacteria species that inhabit the gut have a specific interaction with the host, regulating nutrient metabolism and immunomodulation, and protecting against pathogens or maintaining the gut mucosal barrier. Those specific variations within individuals are crucial in the predisposition to health or disease.[50]

Even though the gut microbiota tends to be stable throughout adulthood, it may vary due to exercise, lifestyle, dietary habits, pharmaceutical consumption or even metabolic or mental disorders, like stress or depression. An understanding of the healthy composition of the gut microbiota might be helpful in developing interventions that restore or maintain its equilibrium.[50,51]

Taxonomically, bacteria are classified into phyla, classes, order, families, genera, and species.

The gut microbiota is mainly composed of Firmicutes, Bacteroidetes, Actinobacteria, Proteobacteria, Fusobacteria and Verrucomicrobia, with the first two accounting for up to 95% of the entire pool. The Firmicutes phylum contains over 200 different genera (Lactobacillus, Clostridium, Enterococcus, Ruminococcus, etc.), but Clostridium is the most representative; meanwhile, Bacteroidetes' predominant genera are Bacteroides and Prevotella.[50]

Clinical implications

Gastrointestinal microbiome in obesity, T2DM, MAFLD and alcohol consumption

Several studies have analyzed the composition and role of the gut microbiome in different states of disease and have found correlations with metabolic pathologies, such as obesity, T2DM, atherosclerosis, MAFLD, irritable bowel syndrome, even some cutaneous problems, like atopic dermatitis, and psoriasis, among others (Table 1).[9,50–64]

Obesity: Most of the evidence that links changes in the

gut microbiome with obesity was obtained from animal studies. Mouse and human microbiota have close similarities, mostly due to the shared characteristic predominance of Firmicutes and Bacteroidetes.[14]

Studies of genetically obese mice revealed a lower abundance of Bacteroidetes, whereas the Firmicutes showed higher composition for species such as *Tuminococcaceae* and *Rikenellaceae*, with *Mollicutes* being the most common. Recent studies have demonstrated that *Akkermansia muciniphila*, a mucin-degrading bacterium that lives in the mucus layer, is decreased in genetically and induced obese mice.[53]

Available human studies have shown that obese individuals have several specific variations in the gut microbiome; the most evident was a decreased microbial diversity. Healthy individuals with high bacterial richness have been associated with abundant microbial species, such as *Faecalibacterium*, *Bifidobacterium*, *Lactobacillus* and *Akkermansia*; those with low bacterial richness were dominant in *Bacteroides* and *Ruminococcus*.[53]

Interestingly, the lack of microbial diversity enhances calorie harvesting, which is mainly associated with the increase in *Firmicutes* species that provide the capacity of metabolizing polysaccharides that would normally be excreted;[52] consequently, this leads to more adiposity, systemic inflammation and a tendency towards insulin resistance and dyslipidemia. It is worth mentioning that diet is a substantial point in this microbial richness; indeed, studies have found partial restoration of the gut microbiome in obese individuals with energy-restricted diets.[65]

Furthermore, studies have shown that the gut microbiome may influence weight gain by affecting host gene expression and modification of metabolic or inflammation pathways; therapeutic methods that regulate this interaction could be useful in the future.[66]

Still other studies have shown a correlation between the gut microbiome and the metabolic state through fecal microbiota transfer from obese mice to lean ones, which leads to increased fat mass, as well as the inverse situation, by transferring fecal matter from bypass operated mice to non-operated lean mice, with a final observation of reduced fat mass.[67]

The characteristic low-grade chronic inflammation in obesity can also be partly related to the alteration of the gut microbiome, evidence indicates that gut microbes exacerbate adipose tissue inflammation via increased gut permeability and increased circulating LPS.[68]

Emerging evidence based on animal studies has shown that short chain fatty acids (SCFAs), like butyrate, propionate and acetate, have an important role in obesity (apart from their normal signaling functions between the gut microbiome and the host metabolism), by increasing *de novo* lipogenesis in the liver and general lipid accumulation; however, despite human studies finding higher levels of SCFAs in obese individuals, the exact relationship with the pathophysiology remains to be clarified by more specific research.[60] Hypotheses suggest that the effects SCFAs have in obesity involve intestinal anaerobic bacteria produced by fermentation of indigestible polysaccharides; these contribute almost 200 kcal to the human body, thus increasing lipogenesis and accumulation in adipose tissue. However, other lines of evidence show that SCFAs might be beneficial for cardio metabolic health.[69]

T2DM: Evidence shows that obese people with insulin resistance have an altered gut microbiota composition characterized by an increase in the Firmicutes:Bacteroidetes ratio compared to that in healthy people.[54] However, related studies of diagnosed T2DM patients showed that this ratio is instead decreased, further accompanied by a reduction of other functional bacteria, like Bifidobacterium.[55,56] A lower abundance of butyrate-producing microbes, such as *Roseburia intestinalis* and *F. prausnitzii*, and increased *Lactobacillus* species (*L. gasseri*, *S. mutans*) has also been reported in T2DM patients.[57]

A modification in the Bacteroidetes:Firmicutes ratio has been associated with a higher expression of microbial genes that encode carbohydrate metabolism-related enzymes. This altered fermentation profile may lead to an increased capacity to harvest energy from the diet and subsequent establishment of poor energy homeostasis, which leads to hyperglycemia (as well as hyperlipidemia).[54] It is worth mentioning that the excess of adipose tissue is also a cause of insulin resistance, due to the increased production of adipokines, like resistin; on the other hand, the harmful effects of hyperglycemia are widely known, going from nephropathies, neuropathies, retinopathy to cardiovascular disease. Other effects of gut dysbiosis in diabetes are the enrichment in membrane transport of sugars, decreased butyrate synthesis, and an exaggerated oxidative stress response.[54,70]

MAFLD: Few studies have analyzed the composition of the gut microbiome in MAFLD patients. Unfortunately, the results have not been homogeneous, probably because of differences in sample sizes, variations within countries, and the unavoidable individual properties of each gut microbiota. Nevertheless, the fact that all studies showed the presence of dysbiosis confirms its role in the disease. Overall, at the phylum level, and with aid of animal studies, we can suggest that the general diversity is reduced, with increased Firmicutes, Actinomycetes and reduced Bacteroidetes, Fusobacteria, Leptosphaeria, Proteobacteria, Thermus and Verrucomicrobia.[58–60] Studies have obtained contradictory results for Actinobacteria and Bacteroidetes. Additionally, research comparing MAFLD patients and controls has revealed that certain bacteria species, such as *Prevotella* and *Porphyromonas*, are increased.[61] In patients with metabolic steatohepatitis, *Proteobacteria* species are consistently enriched; in cirrhosis, oral bacteria, such as *Prevotella* or *Veillonella*, can be observed in the distal colon. Studies that analyzed the microbial profile of cirrhotic patients with hepatocellular carcinoma (HCC) found increased fecal counts of *Escherichia coli*, as well as other Gram-negative bacteria, such as *Atopobium*, *Collinsella*, *Eggerthella* and *Coriobacterium*; others that studied the microbiota of only HCC-diagnosed patients found that *Lactobacillus* spp., *Bifidobacterium* spp., and *Enterococcus* spp. were reduced, with an additional increased concentration of H2S- and CH3SH-producing bacteria: *Fusobacterium*, F*ilifactor*, *Eubacterium*, *Parvimonas* and *Treponema*.[61,62]

Finally, it is important to mention that some bacterial signatures can overlap between MAFLD and other metabolic diseases, due to their close relationship.

Recent studies with mice have shown that the gut microbiome's state within itself may predispose to the development of MAFLD. In an experimental study, mice were fed for 16 weeks with a fat-rich diet and classified into two groups: "responders" with liver damage, and those that did not. Ultimately, transplant of the fecal microbiota from these groups into new microbe-free mice showed the mice that received "responder" microbiota were lean but had a propensity towards steatosis and insulin resistance.[58]

Pathologies like dysbiosis and small intestine bacterial overgrowth are closely correlated with MAFLD. The excess of gut bacteria leads to an increased lipid permeability and intake. These bacteria also tend to be translocated to the circulation, where they activate inflammatory pathways via the recognition of LPS. Ultimately, the Toll-like receptor 4 expression increases, production of IL-8 increases and insulin signaling decreases, leading to an increase in the influx of free fatty acids and a vicious cycle of lipotoxicity.[54]

MAFLD is closely related with obesity and insulin resistance, mainly because of the toxic effects the lipid excess causes in the liver tissue. The pathophysiology of lipotox-

icity in the liver revolves around three main points: low-grade inflammation, autoimmunity, and oxidative stress. It occurs when the hepatic capacity to store or export lipids is exceeded by the fatty acid intake (from peripheral tissues or by *de novo* synthesis). The imbalance of gut microbiota (dysbiosis), as already mentioned, leads to inflammation; this process activates the Kupffer cells and prompts recruitment of other leukocytes to the tissue, initiating a cascade of proinflammatory cytokines and chemotactic factors, which provoke autoimmunity. Stellate cells are also activated by the cytokines and initiate an overproduction of extracellular matrix, which consequently supports progressive fibrosis.[71] If these fibrotic and inflammatory processes are not regulated, steatosis can quickly progress into metabolic steatohepatitis.

The crucial point that determines the progression of liver steatosis into metabolic steatohepatitis resides within the production of fibrotic factors such as tumor necrosis factor (TNF)-α, Fas ligand and TGFβ, being mainly regulated by activation of the recurrent nuclear factor kappa-B (NF-κB) in hepatocytes (once again, the consequence of sustained inflammation). It is also important to highlight the importance of Kupffer cell activation and lymphocyte recruitment, because apart from the effects mentioned before, leukocyte presence in the liver tissue is a classic histopathological sign of metabolic steatohepatitis.[72]

Increasing evidence suggests that the gut-liver axis can take part in the onset of hepatocellular carcinoma, particularly due to the dysbiosis-induced endotoxemia. For example, gut microbial metabolites that act as antigens, like lipoteichoic acids in the case of Gram-positive bacteria or LPS in Gram-negative bacteria, can induce synthesis of the prostaglandin E2 PGE2 by hepatic stellate cells, which reduces the antitumoral activity of CD8+ lymphocytes.[61] Another pathway that promotes hepatic carcinogenesis is the Toll-like receptor 4 recognition of pathogen-associated molecular patterns; this generates a cascade that activates NF-κB, which promotes the synthesis and release of inflammatory cytokines (IL-1, IL-6, TNF-α), thereby perpetuating liver inflammation. Nevertheless, the key point is that NF-κB may also induce antiapoptotic genes (TRAF-1 and TRAF-2), having important carcinogenic consequences. Toll-like receptor 4 is also expressed in hepatic stellate cells, where it is involved in the regulation of hepatocytogen epiregulin, an epidermoid growth factor with a potent mitogen effect on liver cells (Fig. 1).[63]

Lean non-alcoholic fatty liver disease continues to be an outstanding and interesting topic to discuss. In a study performed by Chen *et al*.,[64] lean healthy subjects were found to have different microbiota and BA composition compared to lean non-alcoholic fatty liver disease patients, which have increased BA levels and bacteria species involved with BA metabolism, such as those in the *Clostridium* genus, the *Ruminococcaceae* family, and the *Dorea* genus, and a reduction of protective bacteria, like *Marvinbryantia* and *Christensellenaceae* R7 group. The authors discussed that lean non-alcoholic fatty liver disease patients have a better metabolic profile, with less insulin resistance and dyslipidemia compared to non-lean MAFLD patients, suggesting a different pathophysiology. In another study, Eslam *et al*.[9] also demonstrated that non-obese non-alcoholic fatty liver disease patients have an increase in BA levels and FXR that confers a metabolic adaptation; nevertheless, the progression of non-alcoholic fatty liver disease in lean patients continues to be a matter of debate.[9]

Alcohol consumption: Mice models have demonstrated that 2 weeks of alcohol consumption is enough to induce an evident increment in gut permeability, endotoxemia and hepatic injuries. Ethanol, similar to what happens with an inadequate hypercaloric diet, has detrimental effects on the gut epithelial integrity by altering the stability of TJs. It also has been observed that it induces mucus erosion and ulcerations, mainly by modifying glycosylation of the mucus. In addition, alcohol may also induce changes in the microbiota composition (dysbiosis), an increased predominance of *Proteobacteria*, and a decrease in *Firmicute* species like *Faecalibacterium prausnitzii* which are important for the reinforcement of intercellular gut connections through the production of butyric acid. Other bacterial products, like SCFAs, can be altered after alcohol consumption, leading to the further destabilization of the intestinal barrier integrity. Furthermore, ethanol also has an influence on BAs, as it reduces the expression of FXR and stimulates CYP7A1 hepatic activity, leading to a higher BA pool; this prevents the growth of beneficial bacterial species and promotes stellate cell activation.[73]

Treatment of MAFLD

Treatment of MAFLD with probiotics

Current interventions for MAFLD management are focused on drug administration in order to control lipid levels, diabetes and TNF-α production, while others try to encourage dietary and lifestyle modifications, despite poor patient compliance.[74] However, in the past few years, as the knowledge about gut-liver relationship has grown, several efforts have been directed towards the development of new strategies using this information. Two approaches to modulate gut dysbiosis have been established: 1) the untargeted methods, that use diet, probiotics, and antibiotics; and 2) microbiota-targeted therapy, which specifically aims at certain bacteria and host metabolites. Throughout this section we will discuss the findings related to beneficial effects of probiotic administration on the onset/treatment of MAFLD.

Probiotics are defined by the World Health Organization as a "live microorganism that—when administered in adequate amounts—confers a health benefit on the host," not to be confused with prebiotics, which are compounds in food that induce the growth or activity of microorganisms. Probiotics must be able to survive and transit the gut, as well as be able to grow and multiply in order to benefit the host.[75] Several probiotics, like *Streptococcus*, *Bifidobacterium* and *Lactobacillus* have been commercialized as fermented dairy products due to beneficial effects on the survivability of the gut epithelium and the promotion of anti-inflammatory cytokines, as well as interaction with the immune system.[74] The expected effects of these probiotics are reversion of adverse gut microbiota growth and its consequences related to the constant inflammation through the recognition of LPS, production of ethanol, alteration of the BA metabolism, SCFAs metabolism, cellular stress, and so forth; ultimately, the desired outcome is returning the microbiota to a healthy state.

Multiple animal-based studies have shown significant therapeutic effects in fatty liver mice models. Administration of probiotics could prevent the onset of liver steatosis and improve steatohepatitis and fibrosis. The mechanisms behind these protective effects are the reduction of hepatic lipid accumulation, less endotoxemia, oxidative stress and activation of anti-inflammatory pathways through the modulation of NF-κB and TNF production, as well as the regulation of collagen production.[72,75,76] For example, a study conducted by Xin *et al*.[75] showed prevention of the onset of hepatic steatosis and cellular apoptosis in mice fed with a high-fat diet through the administration of the probiotic *Lactobacillus johnsonii* BS15; the end result was an improvement in hepatic inflammation and oxidative stress. A more recent study by Liang *et al*.[77] gave a compound of probiotics to a group of mice fed with a high-fat diet and

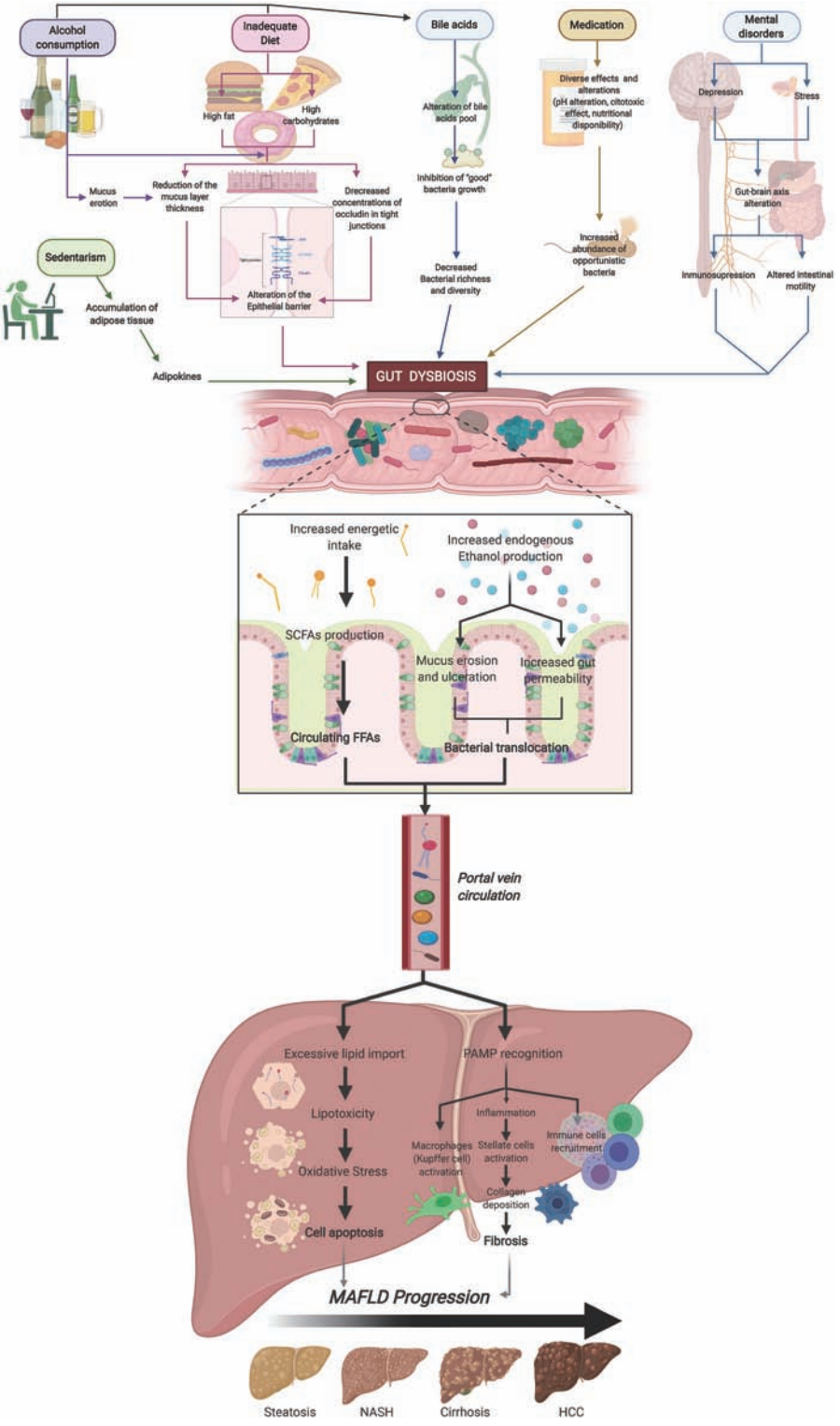

Fig. 1. Factors that promote gut dysbiosis and its effect on MAFLD. Factors such as alcohol consumption, sedentarism, inadequate diet, medication and mental disorders may lead, through diverse mechanisms, to the onset of gut dysbiosis. Alteration of the normal gut bacteria conformation enhances the energetic intake through SCFA production; this excessive energy is then converted into FFAs through anabolism in the enterocytes. At the same time, ethanol-producing bacteria increase the endogenous levels of this metabolite, which then induces mucus erosion and increases gut permeability, leading to bacterial translocation. The transport of bacteria, related antigens, and FFAs to the liver through the portal vein generates lipotoxicity in the hepatocytes, with inflammation occurring because of PAMP recognition, and ultimately immune cell recruitment. All the mechanisms mentioned favor cell apoptosis and fibrosis, and hence MAFLD severity progression. Abbreviations: FFA, free fatty acids; NASH, nonalcoholic steatohepatitis; PAMPs, pathogen-associated molecular patterns.

also showed an improvement in gut dysbiosis and a reduction of the hepatic lipid deposition. VSL#3 is a multi-strain probiotic that contains eight different species (*Lactobacillus plantarum, Lactobacillus delbrueckii, Lactobacillus casei, Lactobacillus acidophilus, Bifidobacterium breve, Bifidobacterium longum, Bifidobacterium infantis* and *Streptococcus thermophilus*), and is the most studied therapy in animals and humans. In mouse-based studies, its effects have included the modulation of NF-κB and TNF, and antifibrotic effects through TGF-β modification.[75,77]

Despite these promising results, it is important to bear in mind that animal models have their limitations. The mice used have been germ-free and even though their intestinal microbiota resembles that of the human, they are not the same. Nonetheless, the findings point to the potential benefits of pharmacologic intervention with probiotics.[76]

In terms of clinical studies, few have been conducted to explore the role of probiotics as a treatment therapy for any of the MAFLD stages, mostly due to the novelty of these discoveries.[78] Among the currently available studies, the results have been measured by biochemical parameters or through hepatic histology.

Human studies have shown, through double-blind trials, that the administration of some *Lactobacillus* species, like *rhamnosus* and *acidophilus*, in 20 obese children and 30 adults with diagnosed MAFLD, respectively, influence the reduction of hypertransaminasemia.[78–83] Other studies, using administration of other species of *Lactobacillus* (*bulgaricus*, *plantarum*), also found improvement in aspartate aminotransferase, as well as the reduction of total cholesterol and low density lipoprotein cholesterol.[84,85]

Other randomized trials have identified better effects on the disease through the administration of combined probiotics. Administration to adolescents of a capsule *containing Lactobacillus acidophilus*, *Bifidobacterium lactis*, *Bifidobacterium bifidum* and *Lactobacillus rhamnosus* showed a reduction in alanine aminotransferase, lipid profile and hepatic fat content compared to a placebo group after 12 weeks.[79] A meta-analysis conducted by Ma *et al*.[85] highlighted the beneficial impacts of probiotic therapy with *Lactobacillus*, *Bifidobacterium*, and *Streptococcus*, by reducing hepatic fat content, cholesterol, and alanine aminotransferase levels. The widely studied multi-strain probiotic VSL#3 has also been demonstrated to protect the intestine in humans by enhancing the barrier integrity, dampening endotoxemia and reducing oxidative stress, thereby leading to an improvement in chronic liver diseases.[66,86] A 24-week trial conducted by Bakhshimoghaddam *et al*.[13,87] studied 102 MAFLD patients divided into the following three groups: one control, and two intervention groups with intake of either 300 g of symbiotic yogurt or conventional yogurt. The authors concluded, after ultrasonography, that the MAFLD scores in those that consumed the symbiotic had decreased aspartate aminotransferase, alanine aminotransferase, and steatosis compared to the other groups. Some other studies have found a decrease in fibrosis levels after treatment with probiotics, apart from the results already mentioned.[88,89]

Unfortunately, and despite their effectiveness in other stages of MAFLD, studies on the effect of probiotics on cirrhosis have been controversial. Few studies have analyzed the use of probiotics as therapy for HCC; nonetheless, the ones available have presented encouraging data through positive effects. It has been observed that they favor liver function recovery and reduce complications in patients who undergo hepatic resection.[90] Drugs like norfloxacin and rifaximin, the latter being capable of inducing overgrowth of beneficial bacteria such as *Bifidobacterium*, *Faecalibacterium*, and *Lactobacillus*, have favored an increase in the survival of patients with cirrhosis and HCC, as well as prevented associated complications, like hepatic encephalopathy, portal hypertension, and spontaneous bacterial peritonitis.[63]

A meta-analysis conducted by Pan *et al*.[60] compared the mechanisms of action of a wide variety of probiotics used in MAFLD treatment and found that the most predominant was the reduction of inflammatory factors (C-reactive protein and TNF-α). Other less determinant findings were the regulation of NF-κB and a reduction of serum liver enzymes (alanine aminotransferase, gamma-glutamyltranspeptidase, and aspartate aminotransferase) and fibrotic factors (TGF-β).

Probiotics may also have antagonistic actions against specific microorganisms, reducing the number and effects, while others ensure the intestine has an adequate pH by releasing products like butyric acid, lactic acid, and propionic acid. They can also enhance immunity by activating macrophages, antibody effectiveness and even competitively against other pathogens for nutrients and growth factors (Fig. 2).[91]

As a side note, we consider it important to mention that probiotics have a wide variety of beneficial effects apart from direct gut microbiota regulation and reduction of carcinogenesis; for example, benefits have been found on mental health, mainly related with the regulation of depression through the increase of serotonin production (Table 2).[91]

Treatment of MAFLD with prebiotics

As already defined, prebiotics are substrates that are metabolized by the microbiota and promote the growth of beneficial bacteria. Oligofructose is a mixture of indigestible fermentable dietary fiber, which has been demonstrated to reduce liver oxidative stress and inflammation as well as to improve the intestinal barrier integrity.[66] Lactulose, another prebiotic, has shown great ability to promote the growth of *Bifidobacteria* and *Lactobacillus*, as well as to exert protective effects against endotoxemia by reducing Gram-negative bacteria, thereby reducing the circulating LPS levels, inflammation and liver damage.

Several other beneficial metabolic effects have been attributed to prebiotics; for example, they can reduce *de novo* lipogenesis, improve blood glucose control and control weight gain. Although prebiotic administration had been demonstrated as an effective therapy for restoring the normal gut microbiota, it may need to be provided in combination with other interventions in order to fully improve MAFLD.[92]

Treatment of MAFLD diet and exercise therapies

As discussed earlier, the onset and severity of MAFLD, obesity, insulin resistance and other chronic metabolic diseases are closely correlated with the lifestyle of the afflicted individual. The following paragraphs provide a summary of the related evidence and proposed therapies for the two pivotal elements of a lifestyle-focused treatment: diet and exercise.

Both clinical and basic research have produced robust evidence that physical exercise has a beneficial effect on MAFLD, by reducing hepatic fat content through the activation of various metabolic pathways that improve the systemic sensibility to insulin and degradation of fatty acids and glucose. Ultimately, these processes prevent excessive fatty acid influx to the liver and mitochondrial and hepatocellular damage from cellular stress. In terms of treatment regimen, many have shown effects on liver fat content, but there is no evidence as to prioritizing one over the others; rather, the selection of a training method should be based on the preferences, capability, and likelihood of continuation

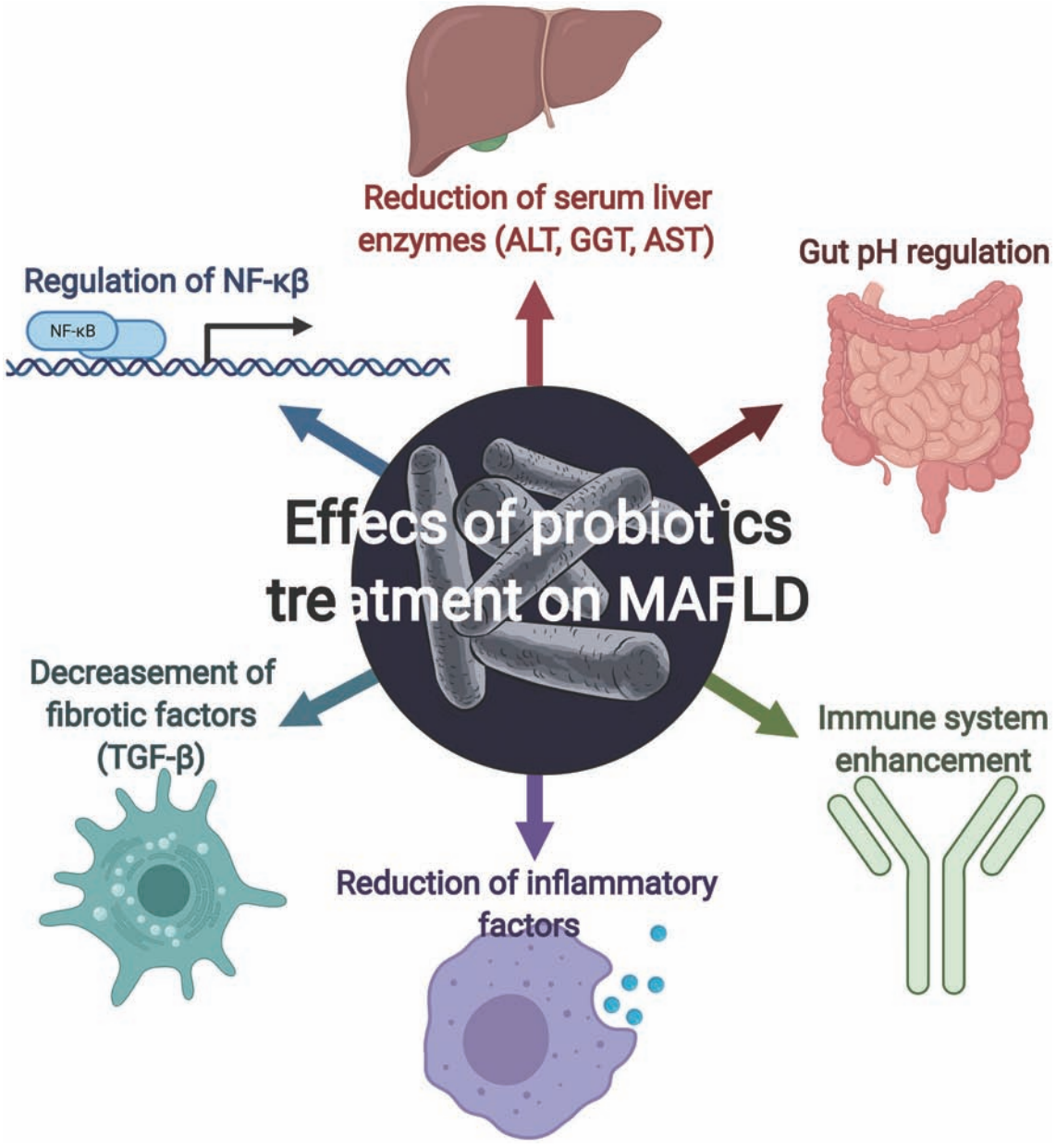

Fig. 2. Effects of probiotic treatment on MAFLD. Several trials have demonstrated that probiotic administration has beneficial effects on MAFLD patients; the most relevant are regulation of NF-κB, gut pH regulation, decrease of fibrotic factors (such as TGF-β), reduction of serum liver enzymes, and enhancement of the immune system.

of each individual patient. Two regimens worth mentioning are aerobic exercise which, even if done at low intensity and volume, has a beneficial effect on the reduction of hepatic fat content, and resistance training, which could be an alternative that provides the same improvements and results for patients who are unable to follow the aerobic regimen. It is worth mentioning that reduction of the hepatic fat content can be achieved even without an overall weight loss.[93]

On the other hand are the dietary interventions. Even though they are very controversial in terms of the determination of the most optimal regimen, they remain as a key factor in the evolution and improvement of MAFLD. In general, the recent literature reports that diets based on antioxidant intake and reduction of fatty processed foods have a better impact on metabolic health. A famous model that follows these recommendations is the Mediterranean diet, characterized by the consumption of plant-based foods and fish, and reduced meat and dairy products.[94] Future dietary approaches might include a fasting regimen (every other day fasting regimen) as experiments in mouse models have demonstrated that it selectively stimulates beige fat development within white adipose tissue, through modification of the gut microbiota composition, which drastically ameliorates obesity, insulin resistance and hepatic steatosis. Although the underlying mechanisms are poorly understood, the participation of microbial fermentation products, such as lactate and acetate, and the upregulation of the monocarboxylate transporter 1 expression in beige cells are

Table 2. Main results and discoveries of MAFLD treatment with probiotics

	Results and discoveries
Animal trials (mice)	Reduction of hepatic lipid accumulation, less endotoxemia, oxidative stress and activation of anti-inflammatory pathways Modulation of NF-κB, TNF and fibrotic factors
Hepatic steatosis (single strain probiotics)	Reduction of hypertransaminasemia Reduction of aspartate aminotransferase, low density lipoprotein, and total cholesterol levels
Hepatic steatosis (multi-strain probiotics)	Reduction of alanine aminotransferase and aspartate aminotransferase levels Less hepatic fat content Reduction of cholesterol levels Enhancement of the gut barrier
Hepatocellular carcinoma	Increased liver function recovery and reduced complications after hepatic resection

some of the main proposed protagonists.[95]

Vitamin supplementation approaches have also been suggested as treatment for MAFLD, specifically the administration of vitamin D. This is not only because vitamin D, in particular, is a molecule with notorious anti-fibrotic, anti-inflammatory, and insulin-sensitizing properties, but also because epidemiological research has found a relationship between hypovitaminosis D and the progress of liver fibrosis. Even though several pathophysiological pathways link MAFLD with vitamin D, the results from trials are still controversial and require further work; so far, available evidence supports that certain populations of MAFLD patients may benefit from vitamin D supplementation, such as those with shorter disease duration and mild to moderate liver damage.[96]

Treatment of MAFLD with fecal microbial transplantation

This technique involves transferring functional microbiomes from the feces of healthy individuals to the gastrointestinal tract of patients with MAFLD. Studies in mouse models treated with fecal transplant from lean or obese individuals have shown the consequence of induction of a microbiota signature similar to that of the donor; thus, obese mice that received microbiota of lean donors responded with a significant reduction in the adiposity and an increased insulin sensitivity, and vice versa, with lean mice that received microbiota from obese donors. Other studies applying 6-week to 8-week fecal transplant therapies to high-fat diet-induced non-alcoholic steatohepatitis mouse models, conducted to corroborate the effects, also found that the intervention increases the abundance of beneficial bacteria, alleviates endotoxemia, and reduces the severity of hepatic damage.[97] This therapy is not only viable for MAFLD patients, but also for other metabolic diseases, as studies have demonstrated its therapeutic effects on T2DM, ulcerative colitis and metabolic syndrome, associated with healthy microbiota, improved insulin sensibility and normalized blood lipid levels.[95] Despite these promising findings, further clinical trials in humans are required to fully confirm the benefits of this procedure, as there are still many unanswered questions, like what is the best way to implant the fecal matter, what is the risk of infection, and what are the long-term therapeutic effects.[97]

Conclusions

The tendency towards a sedentary lifestyle is turning out to be severely detrimental to the population's metabolic state; the growing burden of chronic diseases is not the only consequence, as it also affects the quality of life of millions of people and adds economic burdens worldwide. The gut microbiome is an important determinant of health state and tendency towards disease, and even though it is unique in each person, recent studies have found certain patterns that tend to be constant throughout life, as is seen in healthy people with a predominance of the Firmicutes and Bacteroidetes phyla.

Alterations of the normal composition of the microbiome (gut dysbiosis) should be treated as a priority research topic, due to their close relationship with the onset or severity of other pathologies, such as T2DM, obesity and MAFLD, through mechanisms that provoke systemic inflammation, metabolism alteration, infiltration of lipids to non-adipose tissue, and promotion of fibrosis, among others. Apart from drug administration, probiotic supplementation may be a safe and low-cost approach to improve the disease state

in patients, especially with multiple-strain probiotics, which have shown ability to reduce inflammatory factors (C-reactive protein and TNF-α), regulation of NF-κB, a decrease of serum liver enzymes (alanine aminotransferase, gamma-glutamyltranspeptidase, and aspartate aminotransferase) and fibrotic factors (TGF-β).

It is important that countries implement measures to control this pandemic of metabolic diseases. In general, people must have a clear understanding of the consequences they have on health and know that specific lifestyle changes can make them exponentially healthier.

Future directions

There is still much research left to be done before we fully understand the interactions between the host and the gut microbiota, the mechanisms of action of some specific bacteria strains and, ultimately, effects on health and disease. For example, the identification of ethanol-producing bacteria responsible for the increase of this endogenous metabolite would be a great step in the right direction. Another area of opportunity is to widen the knowledge about the effects of SCFAs in the pathogenesis of MAFLD. Trials that study the role of gut dysbiosis on diabetes represent a field that remains largely unexplored.

We consider it is also important to carry out epidemiological studies that analyze the prevalence of gut dysbiosis and its correlated chronic metabolic diseases. The information obtained is expected to help in decision-making and the implementation of public health measures.

Funding

None to declare.

Conflict of interest

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

Author contributions

Conceptualization and writing of the manuscript (WHC, JCG, NMS).

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Journal of Clinical and Translational Hepatology **2021** vol. 9(2) | 239–246
DOI: 10.14218/JCTH.2020.00126

Review Article

Progress in the Clinical Features and Pathogenesis of Abnormal Liver Enzymes in Coronavirus Disease 2019

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Received: 24 November 2020 | **Revised:** 15 February 2021 | **Accepted:** 28 March 2021 | **Published:** 25 April 2021

Abstract

With the rapid development of research on coronavirus disease 2019 (COVID-19), more and more attention has been drawn to its damage to extrapulmonary organs. There are increasing lines of evidence showing that liver injury is closely related to the severity of COVID-19, which may have an adverse impact on the progression and prognosis of the patients. What is more, severe acute respiratory syndrome coronavirus-2 infection, cytokine storm, ischemia/hypoxia reperfusion injury, aggravation of the primary liver disease and drug-induced liver injury may all contribute to the hepatic damage in COVID-19 patients; although, the drug-induced liver injury, especially idiosyncratic drug-induced liver injury, requires further causality confirmation by the updated Roussel Uclaf Causality Assessment Method published in 2016. Up to now, there is no specific regimen for COVID-19, and COVID-19-related liver injury is mainly controlled by symptomatic and supportive treatment. Here, we review the clinical features of abnormal liver enzymes in COVID-19 and pathogenesis of COVID-19-related liver injury based on the current evidence, which may provide help for clinicians and researchers in exploring the pathogenesis and developing treatment strategies.

Citation of this article: Wu H, Liu S, Luo H, Chen M. Progress in the clinical features and pathogenesis of abnormal liver enzymes in coronavirus disease 2019. J Clin Transl Hepatol 2021;9(2):239–246. doi: 10.14218/JCTH.2020.00126.

Keywords: Coronavirus disease 2019; Abnormal liver enzymes; Liver injury; Idiosyncratic drug-induced liver injury; Roussel Uclaf Causality Assessment Method; Clinical characteristics.

Abbreviations: ACE2, angiotensin-converting enzyme 2; ACLF, acute-on-chronic liver failure; ADE, antibody-dependent enhancement; ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; CK, creatine kinase; CLD, chronic liver disease; COVID-19, coronavirus disease 2019; CRP, C-reactive protein; CT, computed tomography; DILI, drug-induced liver injury; ESR, erythrocyte sedimentation rate; GGT, gamma-glutamyl transferase; HBV, hepatitis B virus; HBVr, hepatitis B virus reactivation; HH, hypoxic hepatitis; ICU, intensive care unit; iDILI, idiosyncratic drug-induced liver injury; INFs, interferons; IL, interleukin; LDH, lactate dehydrogenase; MAFLD, metabolic-associated fatty liver disease; PCT, procalcitonin; RNA, ribonucleic acid; RUCAM, Roussel Uclaf Causality Assessment Method; SARS-CoV, severe acute respiratory syndrome coronavirus; SARS-CoV-2, severe acute respiratory syndrome coronavirus-2; SF, serum ferritin; TBIL, total bilirubin; TNF-α, tumor necrosis factor-α; ULN, upper limit of normal.

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Introduction

Coronavirus disease 2019 (COVID-19) is an acute infectious respiratory disease caused by severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2), which is currently in the global pandemic stage. According to statistics, the average duration from onset of symptoms to hospital discharge of COVID-19 patients is about 24.7 days, with a crude case fatality ratio of 3.67%, which poses a severe threat to human public health.[1] Although COVID-19 mainly causes respiratory failure, an increasing number of extrapulmonary organ dysfunction cases have been reported, especially in critically ill patients.[2] About 14–53% of patients have presented elevated levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) at varying degrees during COVID-19,[3] suggesting that liver injury may be a common extrapulmonary manifestation of COVID-19. In this work, we reviewed the current research progress of abnormal liver enzymes in COVID-19, which may deepen our understanding of its characteristics and pathogenesis, ultimately providing help for future studies.

Definitions of COVID-19-related liver injury

At present, there is no unified standard for the definition of COVID-19-related liver injury. The China Digestion Association and the Chinese Society of Hepatology published a standard depending on the upper limit of normal (ULN) of liver enzymes. In this protocol, COVID-19-related liver injury is defined as a significant abnormality in liver biochemical test during the occurrence, development and treatment of COVID-19, namely ALT or AST >3 ULN, or total bilirubin (TBIL) >2 ULN, regardless of whether there has been any underlying liver disease in the past.[4] The patterns of liver injury in COVID-19 were classified as three types:[5] hepatocellular, if ALT and/or AST >3 ULN; cholestatic, if alkaline phosphatase (ALP) or gamma-glutamyl transferase (GGT) >2 ULN; and mixed type, if patients have both laboratory abnormalities. Some studies have used other standard definitions of ALT cut-offs. In these studies, liver injury was categorized into the following three grades based on ALT values: mild, if the ALT value was between 1 and 2 ULN; moderate, if between 2 and 5 ULN; and severe, if >5 ULN.[6]

ALT is a more specific marker for liver injury than AST, due to the more predominant extra-hepatic sources of AST, rendering it less liver-specific.[6,7] However, the serum activity of AST may be a more sensitive indicator of liver injury in conditions such as acute hepatic ischemia, alcohol-related liver disease, and some cases of autoimmune hepatitis.[7–10]

It is still unknown how SARS-CoV-2 impacts the liver. Liver biochemical abnormalities in COVID-19 can be caused by acute hepatic ischemia, hypoxia, aggravation of the primary liver disease, and drug-induced liver injury (DILI). Thus, the definition of liver injury by ALT cut-offs is incomplete or rigorous due to the variable patterns of liver enzyme abnormalities. Actually, it is recommended that the best discriminant values for recognizing acute liver injury are 200 U/L for AST and 300 U/L for ALT.[11] Nevertheless, due to the multifactorial reasons for abnormal liver enzymes in the clinical settings, the definition of COVID-19-related liver injury needs to be further explored.

Demographic and clinical characteristics of COVID-19 with abnormal liver enzymes

Demographic characteristics

For demographic characteristics, recent research studies have shown that male sex and increased age are associated with abnormal liver enzymes in COVID-19.

In the initial studies characterizing COVID-19 patients, male sex was one of the common factors associated with abnormal liver enzymes.[5,12,13] In a study, the ratio of males to females was 4:1 for patients with liver injury.[14] Li *et al*.[15] found that male sex correlated with elevated ALT and AST levels (p=0.027 and 0.036, respectively). Notably, male sex has been reported to associate with hospital admission.[16] Previous research found an increased male susceptibility to severe acute respiratory syndrome coronavirus (SARS-CoV) mediated by differences in estrogen receptor signalling,[17] so it is worth further exploring whether sex-specific differences also exist in SARS-CoV-2 infection. Furthermore, sex has been shown to influence ALT activity,[18] as men tend to have a higher serum ALT activity than women.[7] This may explain the higher percentage of males in COVID-19-related liver injury.

Further studies showed that age, male sex and body mass index (commonly known as BMI) were predictors of peak hospitalization in patients with abnormal liver enzymes >5 ULN.[5,19] However, in another study, although advanced age was associated with poor prognosis, multivariate analysis found that younger age was one of the most effective predictors of severe liver injury (ALT >5 ULN).[6] This contradiction may be explained by the overactive immune and inflammatory response to COVID-19 in young patients.

Additionally, it has been reported that the prevalence of abnormal liver enzymes in the USA-based cohorts is considerably higher than that in Chinese cohorts.[20,21] The prevalence of ALT and AST elevations in Chinese cohorts ranged between 4–33% and 4–53% respectively, while the elevated admission ALT and AST were up to 39.0% and 58.4% in a large New York, USA cohort.[20–22] Higher BMI and metabolic-associated fatty liver disease (MAFLD) prevalence in the USA may explain some of the discrepancies with Chinese cohorts.[20,21,23]

Laboratory examination characteristics

Increased ALT and AST levels were the most common abnormality found among the liver enzymes in COVID-19. Most patients presented mild to moderate elevation, rarely with more than 10 ULN abnormalities. The increase of GGT and TBIL were relatively common, while ALP level was usually not increased. In addition, inflammatory markers, such as C-reactive protein (CRP), procalcitonin (PCT), serum ferritin (SF) and interleukin (IL)-6, have tended to show an increase that is more obvious in COVID-19-related liver injury patients.[5,12,14,20,24–26]

A study that included 417 COVID-19 cases showed that a total of 318 patients had abnormal liver enzymes, of which more than 90% showed mild (<2 ULN) at admission; patients with elevated ALT, AST, GGT, and TBIL levels exceeding 3 ULN during hospitalization accounted for 10.4%, 5.7%, 11.6%, and 2.8%, while ALP level did not increase significantly.[5] Further studies determined the dynamic changes of the patterns of liver enzymes in COVID-19-related liver injury.[12,14,20] The study by Lei *et al*.[12] showed that in severe patients, AST was significantly elevated first at admission, followed by ALT elevation, and the fluctuation of TBIL and ALP levels was relatively slight. Further analysis found that the above elevated biochemical indexes were significantly associated with adverse outcomes of COVID-19, among which AST was associated with the highest mortality risk.[12] Similar results were shown by other studies,[14,20,26] and correlation analysis found that AST highly correlated with ALT throughout the illness course.

Notably, abnormal liver enzymes could also associate with systemic involvement of COVID-19. The study conducted by Bloom *et al*.[20] showed that AST had mild to moderate correlation with creatine kinase (CK), lactate dehydrogenase (LDH), CRP, erythrocyte sedimentation rate (ESR), and SF. Piano *et al*.[26] also found that patients with abnormal liver enzymes had a more severe systemic inflammation, suggesting collateral hepatic damage from systemic inflammation driven by COVID-19. Of note, some studies proposed that elevated AST in COVID-19 presented more like a cytotoxicity response rather than liver damage, which may potentially involve the muscles.[27,28] Other studies found that AST was strongly correlated with ALT throughout the illness course and to a less extent with CK, reasonably suggesting that liver injury was the predominant source of aminotransferase elevation.[12,14,20,26]

Imaging changes

Xie *et al*.[29] used computed tomography (CT) scores to analyze the relationship between chest CT manifestations and liver injury in patients with COVID-19, and the results showed that the CT scores in the liver injury group were significantly higher than those in the non-liver injury group (p<0.05); the incidence of liver injury in patients with CT scores <5, 5–15 and >15 were 13.3%, 36.4% and 77.8%, respectively; further analysis showed that CT score was an independent predictor of liver injury. Another study found that patients with abnormal liver enzymes had more frequent bilateral consolidation at chest X-ray than those without (44% vs. 32%; p=0.006).[26] Pulmonary imaging findings could accurately reflect the progression of COVID-19. Although the causal relationship between pulmonary CT findings and liver injury was not clear, CT score, as an independent predictor of liver injury, suggested that liver injury may be associated with disease severity. Therefore, for patients with severe pulmonary imaging manifestations, liver function should also be monitored when treating respiratory diseases.

Furthermore, Bhayana *et al*.[30] found that bowel abnormalities and cholestasis were common findings on abdominal imaging of patients with COVID-19. Among right upper quadrant ultrasounds, 87% (32 out of 37) were performed due to liver laboratory findings in intensive care unit (ICU) patients, and 54% (20 out of 37) demonstrated a dilated sludge-filled gallbladder which suggested cholestasis. Although the basic disease status of patients with cholestasis had not been analyzed, this research showed that not only the abnormal liver enzymes but also the cholestasis

could be present in COVID-19 patients. Indeed, based on limited evidence, the prevalence of cholestatic type has ranged from 24% to 29.25% in COVID-19 patients with abnormal liver enzymes,[5,26] and whether these patients have radiologically detectable cholestasis is worth further exploration. Notably, cholestasis could lead to severe liver damage if not effectively controlled, so it is necessary to provide timely and effective treatment for patients with cholestasis.

Pathological findings

At present, reports of the liver histopathological features of COVID-19 patients are limited. Previous studies have found that moderate microvesicular steatosis and mild periportal lymphocytic inflammation were the most frequent pathological findings in the liver of COVID-19 patients, indicating that either SARS-CoV-2 infection or DILI could cause the injury.[5,31–34] Elsoukkary *et al*.[35] observed peculiar basophilic structures of unknown origin in sinusoidal endothelium in 36% of COVID-19 patients. Notably, SARS-CoV-2 RNA has been detected in liver tissue by polymerase chain reaction.[32,34] Lagana *et al*.[36] analyzed the hepatic findings in autopsy specimens from 40 patients who died of COVID-19 complications. The results showed that macrovesicular steatosis was the most common finding (75%), followed by lobular necroinflammation (50%), portal inflammation (50%) and cholestasis (38%). Furthermore, a recent study showed that a large number of apoptotic hepatocytes and prominent binuclear hepatocytes were found in liver autopsies of two COVID-19 patients.[37] Although no obvious viral inclusion bodies were found, transmission electron microscopy examination revealed that hepatocyte cytoplasm contained a large number of typical coronavirus particles with spike structure.[37] In addition, obvious virus invasion manifestations, such as mitochondrial swelling, endoplasmic reticulum dilatation and impaired cell membrane, were also found, suggesting that SARS-CoV-2 can directly infect hepatocytes and lead to liver injury.[37]

Association between abnormal liver enzymes and clinical outcome of COVID-19

At present, a large number of studies have shown that liver biochemical abnormalities are more prevalent in severe patients. Guan *et al*.[38] collected 1,099 COVID-19 cases from 552 hospitals in China, and found that the incidence of elevated AST, ALT, and TBIL levels in severe patients were significantly higher than those in non-severe patients (AST: 39.4% vs. 18.2%; ALT: 28.1% vs. 19.8%; TBIL: 13.3% vs. 9.9%;). Among 82 hospitalized patients who died of COVID-19 in Wuhan, 78% had liver injury, and further analysis showed that AST (p=0.002) and ALT (p=0.037) were significantly correlated with the time from initial symptom onset to death.[39] Furthermore, a study involving 1,590 COVID-19 cases showed that patients with elevated AST, ALT and TBIL levels at admission had case fatality rate of 68.6%, 54.3% and 39% respectively, suggesting that abnormal liver enzymes may be related to poor prognosis.[40] In another large cohort study, the clinical course of 145 patients with severe liver injury (ALT >5 ULN) was worse, including higher rates of ICU admission (69%), intubation (65%), renal replacement therapy (33%), and mortality (42%).[6] Therefore, the severity of COVID-19 is closely related to liver injury, which may predict adverse clinical outcomes.

Recently, with the increase of clinical data, the relationship between liver injury and the clinical outcomes of COVID-19 is being explored further. Ponziani *et al*.[41] found that baseline of liver enzyme abnormality was associated with increased risk of ICU admission (odds ratio: 2.19 [95% confidence interval: 1.24–3.89], p=0.007) but not with mortality (odds ratio: 0.84 [95% confidence interval: 0.49–1.41], p=0.51), and it tended to normalize over time. However, more studies have shown that liver enzyme abnormality is an independent predictor of poor prognosis for COVID-19 patients.[26,42,43] Yip *et al*.[42] found that the incidence of elevated ALT/AST levels and acute liver injury were significantly higher in COVID-19 patients who developed adverse clinical outcomes (including ICU admission, use of invasive mechanical ventilation and/or death) than in those who did not (ALT/AST elevation: 70.9% vs. 19.1%, p<0.001; acute liver injury: 14.5% vs. 0.9%, p<0.001); multivariate analysis showed that ALT/AST elevation and acute liver injury were independently associated with adverse clinical outcomes. Similarly, the study by Piano *et al*.[26] showed that liver enzyme abnormality was an independent predictor of transfer to ICU or death. Therefore, according to current evidence, it is necessary to regularly monitor the liver function of COVID-19 patients.

COVID-19-related liver injury in special populations

Pediatric patients with COVID-19: Severe COVID-19 is not common among children. The Chinese Center for Disease Control and Prevention survey of 72,314 cases of COVID-19 confirmed that the proportion of children under 10 years of age was less than 1%, most children presented with mild symptoms or asymptomatic infection.[44] A study of 171 children with SARS-CoV-2 infection admitted to Wuhan Children's Hospital showed that the incidence of elevated ALT and AST were 12.1% and 14.6%.[45] Wang *et al*.[46] analyzed 31 cases of SARS-CoV-2 infection in children from six provinces in northern China and found that 22.2% of them had elevated transaminases, with the peak values of ALT and AST levels being 68 U/L and 67 U/L, respectively. Due to the rare occurrence of liver enzyme abnormalities in children with COVID-19, the American Association for the Study of Liver Diseases warns that COVID-19 children with elevated AST or ALT should be fully evaluated for potential liver disease and other infections.[47] Notably, Cui *et al*.[48] reported a female infant case presented with pneumonia, liver injury and heart damage after infection with SARS-CoV-2, suggesting that children with COVID-19 may also suffer from multiple organ damage and rapid disease changes.

COVID-19 patients with chronic liver disease (CLD): CLD poses a major public health burden. Patients with CLD, especially those with advanced liver diseases, such as cirrhosis and liver cancer, may be more susceptible to SARS-CoV-2 infection due to dysregulated innate and acquired immunity.[49,50] Thus, the effects of different basic liver conditions on COVID-19-related liver injury need to be meticulously evaluated.[3] According to existing reports, the proportion of CLD patients was low in COVID-19 cases. A large-scale meta-analysis showed that the pooled prevalence of underlying CLD among COVID-19 patients and critically ill patients were 3.6% and 3.9%, respectively.[51] Similar to the clinical features of ordinary COVID-19 patients, the liver injury in COVID-19 patients with CLD is also correlated with the severity of disease and poor prognosis. A study involving 105 SARS-CoV-2 and chronic hepatitis B virus (HBV) co-infected patients showed that abnormal liver enzymes of those patients were relatively common at admission and significantly elevated during hospitalization, of which 14 patients (13.33%) developed liver injury, being more common in men (p=0.001); patients with liver injury had a higher proportion of severe cases, complications rate,

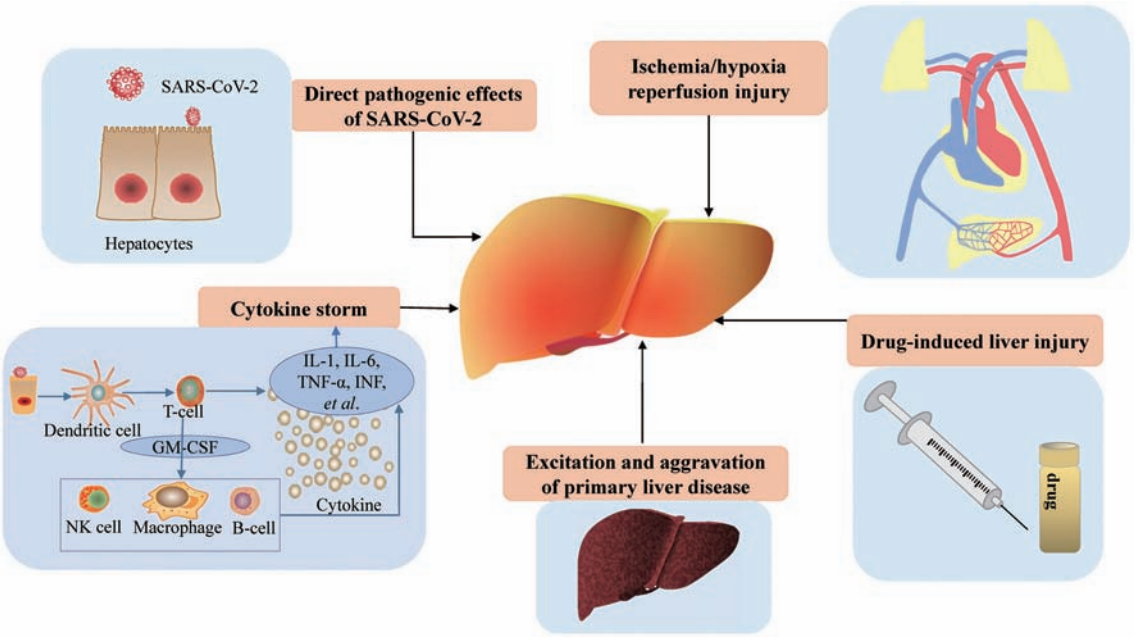

Fig. 1. Pathogenesis of COVID-19-related liver injury. GM-CSF, granulocyte-macrophage colony stimulating factor; NK, natural killer.

and mortality (all $p<0.05$).[52]

Notably, the interaction between COVID-19 and pre-existing CLD could cause liver injury and aggravate the course of these two diseases.[53–55] A study collected clinical data of 228 COVID-19 patients with CLD from 13 Asian countries, and found that among COVID-19 patients with CLD, 43% of CLD patients without cirrhosis developed acute liver damage, 20% of patients with compensated cirrhosis developed acute-on-chronic liver failure (ACLF) (11.6%) or acute decompensation (9.1%), 57% of patients with decompensated cirrhosis showed progressive aggravation of liver injury, and the mortality was as high as 43%.[53] The cases of ACLF secondary to SARS-CoV-2 infection have also been reported.[53,54,56,57] In addition, Iavarone *et al*.[54] found that mortality was significantly higher in COVID-19 patients with cirrhosis than in COVID-19 patients without cirrhosis (34% vs. 18%; $p=0.035$) and cirrhotic patients hospitalized for bacterial infections (34% vs. 17%; $p=0.03$). Similarly, a study including 2,780 COVID-19 patients found that patients with CLD were at an increased risk for mortality (relative risk: 2.8; 95% confidence interval: 1.9–4.0; $p<0.001$) compared to patients without CLD, and the relative risk was markedly higher in patients with cirrhosis (relative risk: 4.6; 95% confidence interval: 2.6–8.3; $p<0.001$).[55] The above studies suggest that COVID-19 can increase the liver burden of CLD patients and worsen the prognosis for patients with poor liver reserve capacity, and vice versa. Therefore, in order to avoid adverse clinical outcomes due to impaired liver reserve capacity, it is necessary to carry out risk stratification and personalization of the management for COVID-19 patients with CLD.

Pathogenesis of COVID-19-related liver injury

Although the mechanisms of liver injury in COVID-19 are not yet clear, multiple factors have been considered as the potential causes, including direct pathogenic effects of SARS-CoV-2, cytokine storm, hepatic ischemia and hypoxia, DILI, and aggravation of primary liver disease (Fig.1).

Direct pathogenic effects of SARS-CoV-2

Wander *et al*.[58] first reported a COVID-19 case with acute hepatitis as the initial manifestation, suggesting that liver could also be the target organ of SARS-CoV-2 infection. With the increasing studies of hepatic pathology in COVID-19, recently published data has indicated SARS-CoV-2 RNA and typical coronavirus particles in liver tissue,[36,37] which suggests that SARS-CoV-2 could directly infect hepatocytes. As the cellular receptor of SARS-CoV-2, angiotensin-converting enzyme 2 (ACE2) is mainly expressed in bile duct epithelial cells rather than liver tissue; thus, the route of SARS-CoV-2 infection to hepatocytes is not clear. In a mouse model of acute liver injury with partial hepatectomy established by Guan *et al*.,[59] the up-regulated expression of ACE2 mRNA after liver injury corresponds to the elevation of AST and ALT, suggesting that ACE2 expression in bile duct epithelial cells might be involved in the liver repair process, while those newborn hepatocytes might still maintain the characteristics of expressing ACE2, making them susceptible to SARS-CoV-2. Attention should also be paid to the non-ACE2-dependent manner of SARS-CoV-2 infection. The antibody-dependent enhancement (referred to as ADE) of virus infection is a phenomenon that virus-specific antibodies can enhance the replication and infection ability of the virus to enter monocytes/macrophages and granulocytes by interacting with Fc receptors and/or complement receptors.[60] A study showed that anti-SARS-CoV spike protein antibody can trigger ADE and mediate SARS-CoV entrance into immune cells that do not express ACE2.[61] Therefore, it is worth considering whether ADE can also mediate SARS-CoV-2's infection in hepatocytes in a non-ACE2-dependent manner and then cause liver damage. In addition, a study showed that hypoxia can induce an increase of ACE2 expression in human hepatocytes.[62] Since patients with COVID-19 often suffer from different degrees of hypoxia and systemic inflammatory reactions, hepatocytes may increase their susceptibility to SARS-CoV-2 through up-regulation of ACE2 expression under hypoxia conditions.

Cytokine storm

Laboratory examination of COVID-19 patients has mainly showed a decrease of lymphocytes, an increase of infection-related markers (including PCT, ESR, SF, and CRP) and inflammatory cytokines (including tumor necrosis factor-α [TNF-α], IL-2 receptor [IL-2R], and IL-6). These biochemical indicators were significantly increased in severe cases, which can lead to aggravation of inflammatory reactions and generation of cytokine storms.[63] Cytokine storm is the excessive activation of the immune system caused by infection, drugs, or certain diseases. It involves the continuous activation and proliferation of various lymphocytes and macrophages, and leads to a rapid and large secretion of various cytokines, including TNF-α, interferons (INFs, including INF-α, INF-β, INF-γ), IL-1, IL-6, IL-8, finally causing serious damage to vital organs through the inflammatory cascade reaction.[64] Similar to SARS, cytokine storm is a characteristic manifestation in critically ill COVID-19 patients, which is also the main cause of disseminated intravascular coagulation, acute respiratory distress syndrome, and multiple organ failure.[65] Phipps *et al*.[6] found that in COVID-19 patients, severe liver injury was associated with markers of end-organ dysfunction, including peak levels of high-sensitivity troponin, CK, and serum creatinine, as well as inflammatory markers, including peak PCT, CRP, D-dimer, SF and IL-6 levels (all $p<0.001$), suggesting that liver injury may be related to the inflammatory response and cytokine storm.[6] In addition, compared with ALT, elevated AST level seems to be more common in COVID-19 patients with liver injury and is closely related to poor prognosis. AST is widely distributed in various organs, such as liver, heart muscle, skeletal muscle, kidney, etc. Lei *et al*.[12] found that AST elevation was positively correlated with lymphocyte reduction and neutrophil elevation in COVID-19 patients, and the latter was a reliable indicator of disease severity, suggesting that multi-organ damage caused by cytokine storms may contribute to AST level abnormality.

Hepatic ischemia and hypoxia

Hypoxic hepatitis (HH), which is also known as ischemic hepatitis and shock liver, refers to reversible central necrosis of the hepatic lobule caused by severe hypotension and hypoxemia. It is characterized by sharp and transient elevation of serum transaminase, which usually reaches a peak level within 24 h (>20 ULN) and returns to normal within 1–2 weeks. HH is caused by many factors, with heart failure (39–70%) being the main cause, followed by septic shock (32%), septicemia (23%) and respiratory failure (15%).[66] Severe heart failure can cause liver congestion. The release of inflammatory mediators and endotoxin during septic shock may reduce the oxygen uptake ability of hepatocytes. When the above conditions are combined with oxidative stress induced by tissue ischemia-reperfusion, HH is more likely to occur.[66–68] A cohort study of 191 COVID-19 cases in Wuhan showed that the most common complication of COVID-19 patients was sepsis (59%), followed by respiratory failure (54%), acute respiratory distress syndrome (31%), heart failure (23%) and septic shock (20%).[69] Moreover, most COVID-19 patients have different degrees of hypoxemia and more than 40% of them need oxygen therapy.[38] The above complications are all risk factors for HH, indicating that COVID-19 patients may have a high risk of HH. In addition, in liver autopsy of a patient who died of COVID-19, a few hepatocytes with slight vesicular steatosis and watery degeneration as well as inflammatory cells were observed, which was considered to have been caused by hypoxia and ischemia-reperfusion.[5] Therefore, from the perspective of pathophysiology and histology, ischemia and hypoxia may be one of the pathogeneses of COVID-19-related liver injury. It should be noted that in the current reports of COVID-19-related liver injury, most cases present with mild to moderate elevation of liver enzymes, while cases of significant liver enzyme elevation (>10 ULN) are still uncommon. Therefore, further studies are needed to clarify HH as a possible mechanism to liver injury in COVID-19.

DILI

Currently, there are no specific antiviral drugs for COVID-19. Interferon-α, lopinavir/ritonavir, ribavirin, chloroquine phosphate and traditional Chinese medicine are the main drugs for antiviral treatment.[70] It has been reported that more than 50% of COVID-19 patients received antibiotic treatment during hospitalization.[38] In addition, it is relatively common for fever patients to take antipyretic-analgesics before admission. As the main component of antipyretic-analgesics, acetaminophen is the primary cause of acute liver failure in occident.[71] Of note, the above drugs have been reported to associate with abnormal liver enzymes in COVID-19 patients.[12,25,26,42,72] Piano *et al*.[26] found that de novo abnormalities of liver enzymes were more common in COVID-19 patients receiving lopinavir/ritonavir (64% vs. 48%, $p=0.045$), acetaminophen (63% vs. 47%, $p=0.048$), piperacillin/tazobactam (72% vs. 50%, $p=0.013$) and tocilizumab (82% vs. 52%, $p=0.009$). Another study showed that the use of lopinavir/ritonavir, ribavirin, interferon-beta and corticosteroids was independently associated with ALT/AST elevation in COVID-19 patients.[42] Falcão *et al*.[72] reported a significant liver enzyme elevation (~10 ULN) in a COVID-19 patient after using hydroxychloroquine, suggesting that hydroxychloroquine may have hepatotoxicity. Lopinavir/ritonavir is mainly metabolized by liver, and Fan *et al*.[25] found that the proportion of patients treated with lopinavir/ritonavir was significantly higher in the group with liver injury than the group without (57.8% vs. 31.3%, $p=0.01$), suggesting that liver injury may be related to the use of lopinavir/ritonavir.

Remdesivir has been reported to cause a significant increase in serum transaminase after usage in COVID-19 patients.[73,74] In a study, after remdesivir treatment, marked ALT/AST elevations and life-threatening elevations were observed in 6% and 2% of COVID-19 patients, respectively.[75] Furthermore, a pharmacovigilance analysis performed using VigiBase to summarize hepatic impairment showed that with remdesivir, increased liver enzymes were the most frequent adverse drug reactions (114, 88%), involving ALT/AST in 79 cases (61%) and bilirubin in 4 cases (3%).[76] According to Leegwater *et al*.,[77] who reported that one severe COVID-19 patient experienced an acute increase in ALT (1,305 IU/L) and AST (1,461 U/L) levels after receiving remdesivir treatment, and the interaction between P-glycoprotein inhibitors chloroquine and remdesivir had been considered to cause this acute toxic liver injury.

Muhović *et al*.[78] described the first case of a severe COVID-19 patient who developed DILI associated with the use of tocilizumab, marked by a 40-fold increase in transaminases levels. Notably, in this research, the diagnosis of DILI induced by tocilizumab was based on the Roussel Uclaf Causality Assessment Method (RUCAM). RUCAM is appreciated as a structured, standardized, validated, and liver-specific diagnostic approach that attributes scores to individual key items, providing final quantitative causality grading for each suspect drug/herb in a case report.[79] In brief, RUCAM quantifies the strength of association between a liver injury and the medication implicated as the cause of the injury, so it is recommended for assessing DILI cases.[80,81] In practice,

DILI commonly stands for the idiosyncratic DILI (iDILI) which lacks clear dose dependency and is caused by unpredictable events due to immunologic or metabolic drug reactions.[82] However, at present, not many studies have used RUCAM to evaluate iDILI in COVID-19. Considering the use of pharmacotherapy for COVID-19 may reflect disease severity, the diagnosis of iDILI in COVID-19 may be confounded. Thus, using the updated RUCAM to verify causality in suspected iDILI cases is necessary.

COVID-19 patients, especially critically ill patients, often need to take multiple drugs combined with therapy, which is more likely to increase the risk of DILI under the condition of systemic immune disorder. Therefore, the use of medicine should be rational to reduce the chance of DILI in clinical practice.

Excitation and aggravation of primary liver disease

Existing research has shown that COVID-19 patients with underlying liver disease have a higher risk of progressing to severe COVID-19 and could exacerbate original liver disease.[53,54] There are multiple reports of COVID-19 patients complicated with ACLF, emphasizing the influence of several factors on basic liver diseases during the course of COVID-19. Hepatitis B patients who are receiving antiviral therapy may have hepatitis attacks if they stop using anti-HBV drugs during the period of COVID-19. For those who have not received anti-HBV therapy, receiving high-dose hormone therapy may lead to hepatitis B virus reactivation (referred to herein as HBVr).[4] Therefore, as the American Gastroenterological Association recommends, antiviral prophylaxis should be provided for patients with high and moderate risk of HBVr treated with immunosuppressive drugs, while routine antiviral prophylaxis in patients with low risk of HBVr should be carefully considered.[83] In addition, attention should be paid to the potential impact of MAFLD on COVID-19-related liver injury. MAFLD is associated with extra-hepatic manifestations of metabolic syndrome. As an important immune organ of the human body, the liver contains a large number of macrophages. MAFLD patients usually have different levels of elevated inflammatory cytokines and SARS-CoV-2 infection can lead to immune stress; as such, it may accelerate the progression of MAFLD and lead to liver injury.[84]

The pathogenesis of COVID-19-related liver injury has not been clarified at present. In addition to the possible pathogenesis mentioned above, some scholars have also proposed the hypothesis that SARS-CoV-2 directly acts on hepatocytes or activates Kupffer cells through the "intestine-liver" axis after intestinal infection, and thus leads to liver injury.[85] In addition, some scholars believe that mechanical ventilation increases the positive end-expiratory pressure of the patient, causing an increase in right atrial pressure and obstructing venous return, thus leading to liver congestion, which may be one of the mechanisms of liver injury in COVID-19.[86] However, no research has confirmed the above views so far. Notably, the pathological changes of COVID-19-related liver injury are complex and diverse, which may be involved in a variety of pathogenesis factors, and therefore more related research is needed to explore its mechanism.

Treatment of COVID-19-related liver injury

At present, antiviral treatment for COVID-19 remains investigational, while etiological treatment is limited. Thus, for patients with COVID-19-related liver injury, the current therapy is mainly supportive treatment based on the severity of liver injury. Mild liver biochemical abnormalities in COVID-19 patients are usually transient and can recover without special treatment. However, for patients with acute severe liver injury, the probable causes of injury should be fully considered, including a history of preexisting liver disease, exposure to hepatotoxins, hypoxia, and circulation status. Meanwhile, liver biochemical indicators should also be closely monitored to prevent the occurrence of acute liver failure. Respiratory and circulatory support should be strengthened in patients with hypoxic hepatitis. For patients with suspected DILI, discontinuation or reduction of the use of suspected drugs should be considered.[4] As for patients with underlying liver disease, liver-protecting drugs should be given appropriately, and the combination of immunosuppressive drugs and antiviral therapy in those with viral hepatitis should be carefully considered. For liver transplant patients, although studies showed that reducing immunosuppressive agents did not increase the risk of mortality, the application of immunosuppressant should still be weighed.[87]

Conclusions

In conclusion, the overall incidence of abnormal liver enzymes is high in COVID-19 patients, especially in severe cases, and is associated with poor prognosis. Advanced age, male sex and high BMI are the predictors of abnormal liver enzymes in COVID-19. Pediatric patients with COVID-19-related liver injury usually experience minor symptoms. COVID-19 patients with CLD have a higher risk of developing liver injury and progressing to severe cases. COVID-19 may aggravate their primary liver diseases and the prognosis mainly depends on the liver reserve capacity. In clinical practice, extra attention should be paid to the potential impact of underlying diseases on COVID-19-related liver injury, although the clinical data is still insufficient.

So far, the understanding of COVID-19-related liver injury has been constantly increasing. Recent research has shown that SARS-CoV-2 can directly infect hepatocytes, providing a new vision for the pathogenesis of liver injury. In addition, close attention should be paid to the adverse effect of drugs on liver in the treatment for COVID-19, especially iDILI. In fact, there is compelling evidence that the liver injury observed in patients with COVID-19 is at least partially due to iDILI. To verify causality in suspected cases, the updated RUCAM published in 2016 should be used. Until there is verifiable evidence to support the theory, clinicians should carefully consider the risk of DILI and try to avoid the overuse of relevant drugs. At last, although COVID-19 vaccination has begun worldwide, the threat of COVID-19 and its hepatic damage should not be underestimated. Further research on effective treatment methods for COVID-19-related liver injury is essential. Clinical staff should continue to summarize the clinical characteristics and treatment experience of COVID-19-related liver injury to provide new theoretical basis for its standardized treatment and in-depth mechanism research.

Funding

This work was supported by the Hubei Province Key Research and Development Project (No.2020BCB007).

Conflict of interest

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

Author contributions

Study conception and design (HW, SL, MC), acquisition of data (HW), analysis and interpretation of data (HW, SL, MC), drafting of the manuscript (HW, SL, HL), critical revision of the manuscript for important intellectual content (SL, HW, HL, MC), and administrative, technical, or material support, study supervision (MC).

Data sharing statement

All data are available upon request.

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Journal of Clinical and Translational Hepatology **2021** vol. 9(2) | 247–255
DOI: 10.14218/JCTH.2021.00006

Review Article

Coronavirus Disease-2019 (COVID-19) and the Liver

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Received: 2 January 2021 | **Revised:** 5 February 2021 | **Accepted:** 26 February 2021 | **Published:** 22 March 2021

Abstract

Within a year of its emergence, coronavirus disease-2019 (COVID-19) has evolved into a pandemic. What has emerged during the past 1 year is that, apart from its potentially fatal respiratory presentation from which the severe acute respiratory syndrome-coronavirus-2 (SARS-CoV-2) derives its name, it presents with a myriad of gastrointestinal (GI) and liver manifestations. Expression of the angiotensin-converting enzyme-2 (ACE-2) receptor throughout the GI tract and liver, which is the receptor for the SARS-CoV-2, may be responsible for the GI and liver manifestations. Besides acting directly via the ACE-2 receptor, the virus triggers a potent immune response, which might have a role in pathogenesis. The virus leads to derangement in liver function tests in close to 50% of the patients. The impact of these derangements in patients with a normal underlying liver seems to be innocuous. Severe clinical presentations include acute decompensation and acute-on-chronic liver failure in a patient with chronic liver disease, leading to high mortality. Evolving data suggests that, contrary to intuition, liver transplant recipients and patients with autoimmune liver disease on immunosuppression do not have increased mortality. The exact mechanism underlying why immunosuppressed patients fare well as compared to other patients remains to be deciphered. With newer variants of COVID-19, which can spread faster than the original strain, the data on hepatic manifestations needs to be updated to keep a step ahead of the virus.

Citation of this article: Elhence A, Vaishnav M, Biswas S, Chauhan A, Anand A, Shalimar. Coronavirus disease-2019 (COVID-19) and the liver. J Clin Transl Hepatol 2021;9(2): 247–255. doi: 10.14218/JCTH.2021.00006.

Keywords: Transaminitis; Cirrhosis; Vaccine; ACLF.

Abbreviations: ACE-2, angiotensin-converting enzyme-2; ACLF, acute-on-chronic liver failure; AIH, autoimmune hepatitis; ALD, alcoholic liver disease; ALF, acute liver failure; ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; CLD, chronic liver disease; COVID-19, coronavirus disease-19; CSS, cytokine storm syndrome; DILI, drug-induced liver injury; GGT, gamma glutamyl transferase; GI, gastrointestinal; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HCQ, hydroxychloroquine; HCV, hepatitis C virus; ICU, intensive care unit; IHC, immunohistochemistry; IL, interleukin; INR, international normalized ratio; LFT, liver function test; LMWH, low molecular weight heparin; MELD, model for end-stage liver disease; mTOR, mammalian target of rapamycin; NACSELD, North American Consortium For The Study Of End-Stage Liver Disease; NAFLD, non-alcoholic fatty liver disease; PBC, primary biliary cirrhosis; PSC, primary sclerosing cholangitis; RT-PCR, reverse transcription-polymerase chain reaction; S, spike protein; SARS-CoV-2, severe acute respiratory syndrome-coronavirus-2; SIC, sepsis-induced coagulopathy; SOFA, sequential organ failure assessment; TMPRSS2, transmembrane serine protease 2; UGI, upper gastrointestinal; ULN, upper limit of normal.

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Introduction

The first case of coronavirus disease-2019 (COVID-19) was reported from Wuhan, China, in December 2019. Since then, the severe acute respiratory syndrome-coronavirus-2 (SARS-CoV-2), responsible for COVID-19, has evolved into a pandemic, involving all continents to date (i.e. 31st January 2021).[1] SARS-CoV-2 is distinct from other coronavirus infections in that it manifests with a myriad of extra-pulmonary manifestations. Avid expression of the angiotensin-converting enzyme-2 (ACE-2) receptor throughout the gastrointestinal (GI) tract, including gastric, small intestinal and colonic mucosal cells, vascular endothelial cells, cholangiocytes and smooth muscle cells is the reason for the common occurrence of GI symptoms and hepatic manifestations.[2]

Pathogenesis of GI and liver manifestations

SARS-CoV-2 uses the spike protein (S) to bind to the ACE-2 receptor in target cells. The ACE-2 receptor is present on type 1 and 2 surface alveolar cells, leading to the predominant respiratory symptoms and the droplet mode of transmission. The ACE-2 receptor is also widely expressed throughout the GI tract (Fig. 1). On immunohistochemical (IHC) staining, Hamming *et al*.[2] demonstrated that the ACE-2 receptor is present in abundance in the vascular endothelium and smooth muscle cells of the vessels supplying the GI tract.

The pathophysiology of liver injury in COVID-19 is not as well established as its intestinal counterpart. In the liver, cholangiocytes and hepatic endothelial cells have been proposed to be the target cells for SARS-CoV-2.[3] Cholangiocytes express not only the ACE-2 receptor but also the transmembrane serine protease 2 (TMPRSS2), which cleaves the S protein of the virus prior to its entry into cells, thus providing the basis of cholangiocytes being highly vulnerable to SARS-CoV-2 damage.[4] It has also been shown in the liver ductal organoid model that SARS-CoV-2 leads to direct cytopathic changes in cholangiocytes, as hypothesized.[4] Histopathologic evaluation of autopsy and post-mortem biopsies reveal mild sinusoidal dilation with increased small lymphocyte infiltration. In addition, steatosis, multifocal hepatic necrosis without inflammatory cellular infiltration, and canalicular cholestasis have all been reported in the liver biopsies of patients with COVID-19 patients. Interestingly,

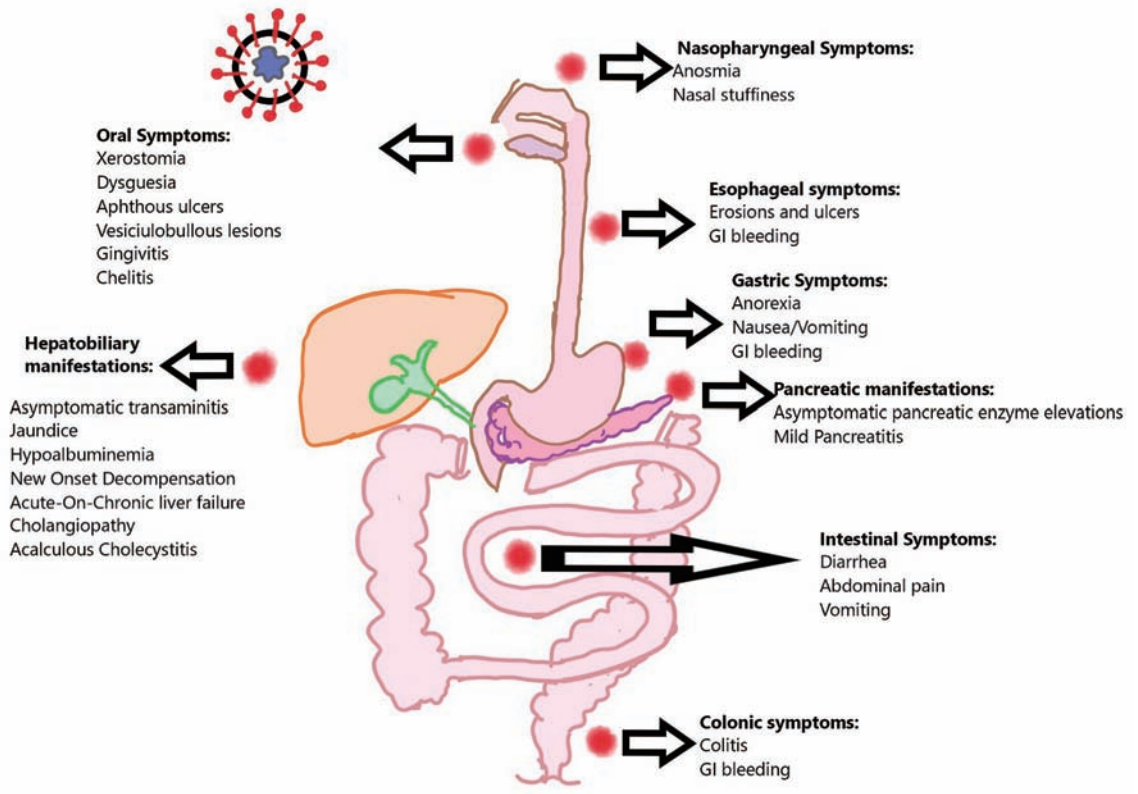

Fig. 1. GI and hepatic manifestations of COVID-19.

portal tract inflammation was not evident in these biopsies.[5] Sinusoidal dilation is attributed to cardiogenic venous outflow slowdown. It is well recognized that hypoxia and impaired cardiovascular function predispose the liver to injury. Both zones 1 and 3 show injury with no cellular infiltrate, ballooning, Mallory hyaline, or fibrosis. Several potential mechanisms have been postulated in the pathophysiology of liver manifestations, such as a direct viral insult, exacerbation of the underlying liver disease, hyperinflammatory states, and drug-induced injury, but evidence to support either mechanism is scanty.[6]

Liver manifestations of COVID-19

Hepatic injury is common in COVID-19 and is multifactorial. Possible reasons include direct hepatic involvement due to the virus, drug-induced liver injury (DILI) due to various therapeutic agents, hypotension, and the associated underlying liver disease (cirrhosis due to various etiologies, alcoholic steatohepatitis, non-alcoholic fatty liver disease, and viral hepatitis) (Fig. 2). The prevalence of GI and hepatic manifestations of COVID-19 is variable across studies from different regions, as highlighted in the data from meta-analyses (Table 1)[7–13] and individual studies (Table 2).[14–19] In addition, endemic areas are associated with co-infections, such as acute viral hepatitis, and tropical infections, such as malaria and dengue.[20–22]

Liver function test abnormalities

Alanine aminotransferase (ALT) elevations were seen in 4% to 33% of cases, according to China's initial reports,[23–25] and 39% of cases in a large study from New York, USA.[26] The prevalence of aspartate aminotransferase (AST) elevation ranged between 4% to 53% in a Chinese cohort and up to 58% in a USA cohort.[23,25,26] Both enzymes were mildly elevated in terms of absolute numbers and less than 5-times the upper limit of normal (ULN) in the majority. Kulkarni *et al*.[13] in their meta-analysis, placed the pooled incidence of AST and ALT elevation at 22.5% and 20.1%, respectively.

Elevation in gamma glutamyl transferase (GGT) has been reported in 13% to 54%, whereas elevation in alkaline phosphatase (ALP) is uncommonly elevated, in only 2% to 5% of cases.[27,28] In the meta-analysis by Kulkarni *et al*.,[13] the ALP and GGT elevation incidence was 6.1% and 21.1%, respectively. The rise in ALP may be disproportionate to other liver enzymes.

Hyperbilirubinemia may be seen in up to 18% of cases.[13,25,28] However, the derangement in the liver function tests can be multifactorial, as highlighted above, and it may be difficult to attribute to SARS-CoV-2-induced hepatic dysfunction alone.

Hypoalbuminemia has been described in severe COVID-19 patients and may not parallel changes in AST and ALT. In a retrospective cohort of 299 patients, 106 (35.5%) patients had low albumin, with significant differences in the albumin levels of survivors and non-survivors (37.6 g/L vs. 30.5 g/L).[29] Albumin levels have also been found to be an independent predictive factor for mortality.[29] In a meta-analysis of 1,990 patients across 14 studies, hypoalbuminemia was noted in 55.5%.[13] An important finding was that only 11% to 45.8% of patients with non-severe infection had hypoalbuminemia. In the severely ill, hypoalbuminemia was seen in up to 72.9%; whereas, among the deceased, hypoalbuminemia was reported in 78–100% cases, making

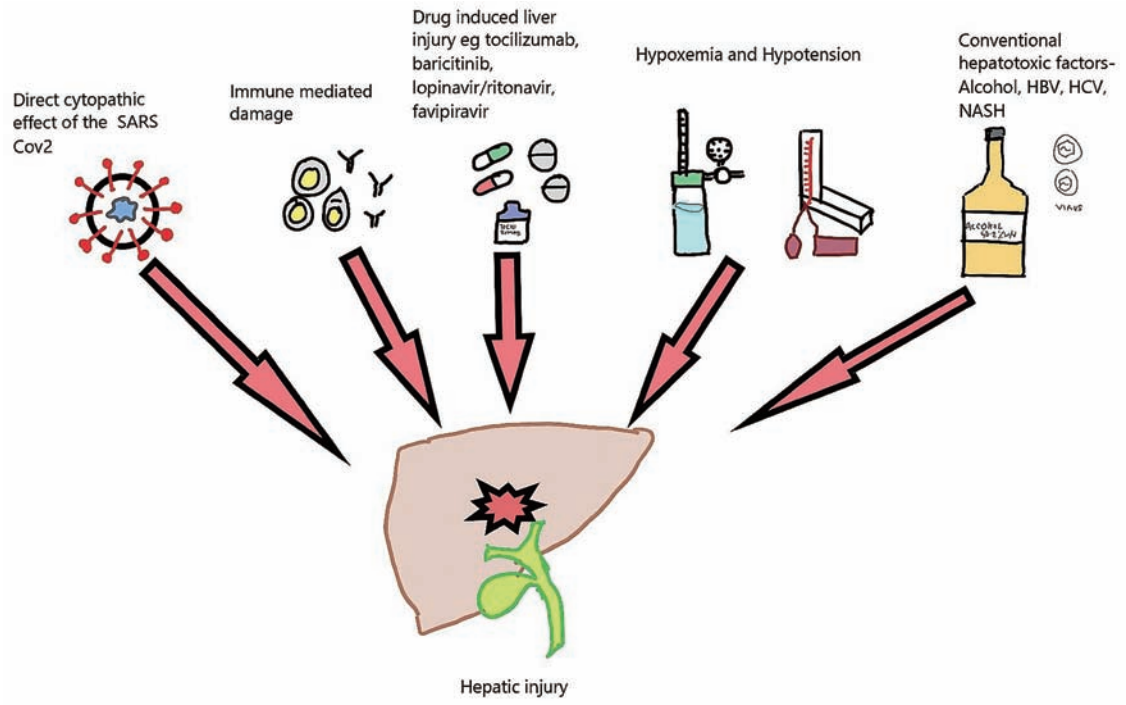

Fig. 2. Multifactorial nature of liver injury in COVID-19.

a case for the use of albumin levels as a prognostic marker in these patients.[13] Albumin is a negative acute phase reactant, and the clinical relevance of low albumin as a predictor of outcomes must be interpreted with caution.

Coagulation disturbances

Prothrombin time/international normalized ratio: Coagulation disturbances in COVID-19 may be due to either a dysregulated immune response or liver failure, with dysregulated immune response being more commonly encountered.[30,31] The cytokine storm syndrome (referred to herein as CSS) associated with COVID-19 leads to excessive pro-inflammatory cytokine release, which eventually results in endothelial injury, which may lead to disseminated intravascular coagulation, microvascular thrombotic angiopathy, and pulmonary embolism.[32,33] Several studies have described prolonged prothrombin times and D-dimer levels.[30,34,35]

Endotheliitis was observed in the liver of patients with COVID-19 and fibrin microthrombi were found in liver sinusoids.[36,37] The largest series of liver biopsies taken at autopsy (48 patients) showed massive dilation of portal vein branches, luminal thrombosis, portal tract fibrosis, and microthrombi in the sinusoids.[38]

The altered liver function tests (LFTs) could be related to CSS leading to shock and coagulopathy, affecting liver perfusion and resulting in cell death.[38,39] Klok *et al*.[40] reviewed 184 patients admitted in three intensive care units (ICUs) in the Netherlands and reported the composite incidence of thrombotic events (considering both arterial and/or venous) to be 49 % adjusted for competing risk of mortality. The most common thrombotic event was pulmonary thromboembolism, seen in 87% of patients. Tang *et al*.[41] determined that the administration of low molecular weight heparin (LMWH) for 7 days or longer was associated with lower 28-day mortality in patients with sepsis-induced coagulopathy

Table 1. Prevalence of liver manifestations in patients with COVID-19 as reported in meta-analyses

Author[Ref]	Mao *et al*.[7]	Sultan *et al*.[8]	Parasa *et al*.[9]	Kumar *et al*.[10]	Wan *et al*.[11]	Zarifian *et al*.[12]	Kulkarni *et al*.[13]
Patients included	6,686	10,676	4,805	4,676	15,141	13,251	20,479
Elevated AST	21%	15%	20%	25%	25.4%	22.8%	22.5%
Elevated ALT	18%	15%	14.6%	23%	25.3%	20.6%	20.1%
Elevated Bilirubin	6%	16.7%	NR	9%	8.8%	7.8%	13.4%
Prolonged INR	NR	NR	NR	7%	NR	18%	9.7%
Hypoalbuminemia	6%	NR	NR	60%	NR	39.8%	55.5%
ALP	NR	NR	NR	NR	NR	4.6%	6.1%
GGT	NR	NR	NR	NR	NR	NR	21.1%

INR, international normalized ratio; NR, not reported.

Table 2. Prevalence of GI liver manifestations in patients with COVID-19 infection as reported in individual studies from across the countries to highlight the regional variation

Author[Ref]	Laszkowska *et al.*[14]	Guan *et al.*[15]	Aghemo *et al.*[16]	Moura *et al.*[17]	Docherty *et al.*[18]	Rivera *et al.*[19]
Patients included	2,804	1,099	292	400	20,133	76
Country	USA	China	Italy	Brazil	UK	Spain
Overall prevalence of GI symptoms	38.7%	NR	28.2%	33.4%	29%	59.2%
Diarrhea	23.4%	3.8%	27.1%	17.3%	20.4%	40.8%
Nausea/vomiting	23.2%	5%	4.0%	13.8%	19.8%	22.4%/9.2%
Abdominal pain	11.9%	NR	NR	11.5%	10.2%	27.6%
Anorexia	NR	NR	NR	6%	NR	15.8%
Elevated AST	NR	22.2%	26.7%	NR	NR	NR
Elevated ALT	NR	21.3%	18.5%	NR	NR	NR
Elevated bilirubin	NR	10.5%	10.6%	NR	NR	NR
Prolonged INR	NR	NR	NR	NR	NR	NR
Elevated ALP	NR	NR	9.6%	NR	NR	NR
Hypoalbuminemia	NR	NR	NR	NR	NR	NR

INR, international normalized ratio; NR, Not reported.

(SIC) score of ≥4 or a D-dimer value of > 6 times the ULN. They used a working definition of SIC as previously defined by the presence of infection-induced organ dysfunction as characterized using a composite score compiled using platelets, international normalized ratio (INR), and sequential organ failure (SOFA) score.[42]

SARS-CoV-2 and acute liver failure

As noted previously, a non-specific rise in AST and ALT levels up to 5 times the ULN can be seen in COVID-19 patients, along with hyperbilirubinemia. Acute liver failure (ALF) has been reported rarely. Of the five ALF cases reported, three were from the USA and one from Germany and Qatar each.[43–47] Two of these were young, aged 24 years and 35 years, while the other three were above 50 years of age. Most of these patients were critically ill and a single etiology could not be identified as a cause except in the patient with hepatitis B co-infection, who had acute fulminant hepatitis B infection but only mild COVID-19 pneumonia. Out of the five ALF patients, two survived, two expired, and one remained critically ill at the time of writing.

SARS-CoV-2 and hepatitis B

Hepatitis B virus (HBV) infection rates among patients with COVID-19 have been reported between 2.1% and 12.2% from China. Zou *et al*.[48] reported their clinical experience of 20 patients with COVID-19 and chronic HBV co-infection in a retrospective analysis, noting its severe illness and poor prognosis compared to 306 patients with only COVID-19 infection. They reported significantly lower prealbumin levels but no difference in levels of liver enzymes, length of hospital stay or discharge rates. Chen *et al*.,[49] in their retrospective analysis of 123 cases, including 15 cases with HBV, reported more severe disease in HBV-COVID-19 coinfection compared to HBV-negative cases (46.7% vs. 24.1%) as well as a higher mortality rate (13.3% vs. 2.8%). Zha *et al*.,[50] in their observational study of 31 cases, had 2 patients with HBV infection and found that they took a longer time to clear COVID-19 infection (mean difference of 10.6 days). Aldhaleei *et al*.[51] reported a case of hepatitis B flare due to COVID-19, although large-scale studies are required to validate these findings. There is a single case report of HBV induced ALF in a patient with mild SARS-CoV-2 infection.[47]

SARS-CoV-2 and hepatitis C

There is very limited data on hepatitis C virus (HCV) and COVID-19. Wang *et al*.,[52] in their case-control study of over 1 million patients with cirrhosis, included 16,530 with COVID-19 and 820 with COVID-19 and chronic liver disease (CLD) and reported higher odds for patients with HCV in acquiring COVID-19 than for those without [adjusted odds ratio of 12.9]. Thus, although these findings would support the notion that patients with HCV-related CLD are at a greater risk for acquiring COVID-19 infections, there is a dearth of data to validate this finding or identify the impact of COVID-19 on disease course, management and outcome.

SARS-CoV-2 and alcoholic liver disease

No studies have looked exclusively at outcomes of patients with alcoholic liver disease (ALD) with COVID-19. However, a retrospective study from our center reported that the fraction of patients with ALD had decreased in the early part of the pandemic compared to the pre-pandemic era, likely as a result of total lockdown imposed in India and decreased alcohol availability.[53,54] However, the outcome of these patients was not different from those with other etiologies.[53] Following lifting of the lockdown and increased availability and sale of alcohol, a center from the UK reported doubling of admissions due to ALD and an increase in the proportion of patients with severe alcoholic hepatitis and alcohol-related acute-on-chronic liver failure (ACLF).[55]

SARS-CoV-2 and autoimmune liver disease

Data on the impact of COVID-19 on primary biliary cirrho-

sis (PBC), primary sclerosing cholangitis (PSC) or autoimmune hepatitis (AIH) are evolving. Since COVID-19 is associated with transaminitis and hyperbilirubinemia, it may be confused with a flare of AIH. Thus, a liver biopsy may be mandated to confirm the diagnosis prior to initiation of therapy.[56] Gerussi *et al*.,[57] in their case series, described 10 patients across seven hospitals in Italy who were undergoing immunosuppression for AIH. Of the 10 patients, 2 had a recent flare for which they were on high dose steroids. Liver enzymes remained normal in all cases and improved in the two acute cases.

A recently published large retrospective study of 70 patients with AIH, 19 with PBC, 19 with PSC and 16 with variant syndromes were compared in a propensity-matched analysis to 862 non-AIH CLD and 769 patients without liver disease.[58] The cohort with AIH had no increase in ICU stay or mortality compared to patients with other liver disease etiologies or those without liver disease, although close to 80% of AIH patients were on immunosuppression.[58] The only significant factors for AIH mortality were age and baseline liver disease. The authors hypothesized that despite immunosuppression, patients with AIH have preserved immune responses to SARS-CoV-2 and hence are not at a disadvantage. This large study reassures patients and physicians alike and strengthens the already prevalent recommendation of not changing these patients' immunosuppressive therapy in the pandemic.[58]

SARS-CoV-2 and non-alcoholic fatty liver disease

Two large retrospective studies show that presence of non-alcoholic fatty liver disease (NAFLD) is a risk factor for development of severe COVID and mortality even after correcting for comorbidities, such as obesity and diabetes.[59,60] The authors hypothesized that the increased progression of COVID-19 in patients with NAFLD might be due to either exaggerated hepatic immune response contributing to systemic inflammation or the prothrombotic state in these patients contributing to disease progression.[61] However, a third large retrospective study failed to reach similar conclusions, possibly because of different criteria used to define COVID-19 progression and a higher fraction of patients with diabetes (50%) compared to the previous two studies, which might have negated the effect of NAFLD on multivariate analysis.[62]

SARS-CoV-2 and DILI

Although liver injury might occur in patients infected with SARS-CoV-2 due to many reasons, DILI should be considered among the important differentials of liver injury in these patients. The commonly implicated drugs include those which have been repurposed for use in COVID-19, such as hydroxychloroquine (HCQ), azithromycin, lopinavir/ritonavir, baricitinib, and those which been developed exclusively for COVID-19, such as remdesivir. Idiosyncratic DILI is a well-known but rare adverse effect of HCQ.[63] Azithromycin is also associated with rare idiosyncratic cholestatic hepatitis.[63] Lopinavir has been shown to cause both hepatocellular and cholestatic liver injury, leading to enzyme elevation up to > 5-times the ULN in 3% to 10% of patients.[63] The current information on remdesivir suggests that is an unlikely cause of clinically significant liver injury, as suggested by healthy volunteer studies and controlled studies.[64] The safety data on favipiravir, although sparse, appears to be reassuring.[65] Tocilizumab and other interleukin (IL)-6 antagonizing therapies, although frequently associated with elevated aminotransferases (10% to 50%), are rarely associated with elevations > 5-times the ULN (1–2%).[66] Tocilizumab and other immunosuppressants used in the treatment of COVID-19 are also theoretically associated with the risk of reactivation of viral hepatitis.[66] Baricitinib is an unlikely cause of DILI but has been associated with risk of reactivation of hepatitis B.[67]

Acute decompensation and ACLF

Patients with CLD and cirrhosis have systemic immunodeficiency, which places them at a higher risk for COVID-19. Data available from registries place the number of new decompensations at 45% and the mortality rate in such patients at 40%, which is higher in patients with advanced liver disease.[53,68,69] The clinical presentations include acute decompensation-jaundice, ascites, hepatic encephalopathy, and GI bleed.[53,68] Severe presentation includes ACLF with organ failure.

In their multicentric study, Bajaj *et al*.[68] reviewed 37 patients with cirrhosis and COVID-19 compared to a cohort of 127 patients with cirrhosis alone and 108 patients with COVID-19 alone. ACLF, as per North American Consortium for the Study of End-Stage Liver Disease (known as the NACSELD) criteria, was seen in 40 patients, with 11 in the cirrhosis-COVID-19 group and 29 in the COVID-19 group alone and with no difference in mortality across both groups (55% vs. 36%, p=0.25). The authors also reported higher mechanical ventilation and non-invasive ventilation use requirements, central line placement and ICU transfer in the cirrhosis and COVID-19 group compared to the cirrhosis only group. A study from our center compared 28 patients with cirrhosis and COVID-19 with 78 historical controls with cirrhosis matched for etiology and model for end-stage liver disease (commonly referred to as MELD) score. The overall mortality rate was higher in the cirrhosis and COVID-19 group, at 42.3% vs. 23.1%, p=0.07. The mortality was even higher in the sicker group with ACLF and COVID-19, 100% vs. 53.3%, p=0.01.[53]

Variceal bleeding

Although the data on upper gastrointestinal (referred to herein as UGI) bleeding in patients with COVID-19 continues to evolve, there are limited data on variceal bleed in patients with cirrhosis and COVID-19. In a study from our center evaluating the outcomes of cirrhosis in COVID-19 infection, variceal bleeding was the most common form of decompensation present in 11/16 (68%) of the patients.[53] In another study from our center, UGI bleeding was present in 24/1,342 (1.8%) of all patients hospitalized with COVID-19.[70] The majority (88%) of bleeding episodes represented variceal bleeds in patients with cirrhosis and had encouraging outcomes with no rebleed or death at 5 days with primary conservative management.[70]

Hepatocellular carcinoma

The presence of hepatocellular carcinoma (referred to herein as HCC) is associated with poor outcomes, with an increased risk of overall and COVID-19-related mortality in patients with CLD and COVID-19 infection.[71] In addition, the COVID-19 pandemic has also affected the standard of care of patients with HCC. In a large retrospective study, including more than 600 patients, a lower number of patients were evaluated during the pandemic period compared to the same period prior to the pandemic. More than 20% of patients experienced a treatment delay and 13.1% needed

a modification in the treatment strategy, both attributable to the COVID-19 pandemic.[72]

Liver transplant

A multicentric registry reported outcomes of patients with liver transplants (n=151) compared to controls (n=627).[73] GI symptoms (nausea, vomiting, abdominal pain, and diarrhea) were experienced by a greater proportion of patients in the transplant cohort than the comparison cohort- 30% vs. 12%, p<0.001. There was no difference in respiratory symptoms experienced (77% vs. 81%, p=0.248) or hospitalization rates (82% vs. 76%, p=0.106) between the two groups. However, the rates of ICU admission (28% vs. 8%, p<0.001) and the proportion receiving invasive ventilation (20% vs. 5%) were higher, and median hospital stay (11 days vs. 8 days, p=0.046) was longer in the liver transplant group. Surprisingly, the proportion of deaths in the transplant cohort was significantly less than the comparison cohort (19% vs. 27%, p=0.046) with the dominant cause of death being COVID-19 lung disease. The authors also reported no liver-related mortality, rejection, or re-transplant in the transplant group. Similar outcomes have been reported from a prospective study from Spain (18%) and UK national registries (20%).[73,74] The Spanish study reported on the prospective follow-up of 111 post-transplant recipients and showed that although chronically immunosuppressed patients are at increased risk of acquiring the infection yet, they are not at increased risk of mortality.[74] The analysis also reported no effect on immunosuppression on mortality, particularly calcineurin inhibitors and mammalian target of rapamycin (commonly known as mTOR) inhibitors, except mycophenolate, particularly in doses greater than 1 g per day (relative risk of 3.94).[74] The authors hypothesize that this effect might be due to the CD8+ depleting effect of mycophenolate.[74]

SARS-CoV-2 cholangiopathy

ACE-2 receptor and TMPRSS2 are highly expressed on cholangiocytes, and hepatic organoid models have been used to show the virus's direct cytopathic effect on cholangiocytes.[4] Recently, a small case series described an entity called post-COVID-19 cholangiopathy that is characterized by changes in both extrahepatic and intrahepatic biliary tree with microscopic features of severe vacuolization injury to cholangiocytes, along with microangiopathy and evidence of developing secondary biliary cirrhosis among three patients who initially had severe elevation of liver enzymes and acute hypoxemic respiratory failure, and prolonged hospitalization due to COVID.[75] However, the exact contribution of SARS-CoV-2 in the development of cholangiopathy is unclear, since a similar entity has been demonstrated in critically ill hospitalized patients. None of the patients in the above series had immunohistochemical evidence of SARS-CoV-2 infection on liver biopsy samples.[76]

Gallbladder

Few case reports exist which describe patients presenting with COVID-19 and acute cholecystitis with the disease attributed to the virus on the basis of positive quantitative reverse transcription-polymerase chain reaction (referred to herein as RT-PCR) of tissue samples in one patient, while the other two had positive RT-PCR results for nasopharyngeal swabs.[77–79] All three patients had acalculous cholecystitis with positive Murphy's sign, thickening of gallbladder wall and pericholecystic fluid on ultrasound. However, the significance of these findings in the pathogenesis of COVID-19 needs to be elucidated.

Evaluation of patients with liver manifestations of COVID-19

Patients presenting with acute febrile illness and respiratory symptoms, such as sore throat, nasal stuffiness, dry cough and breathlessness, should undoubtedly be evaluated for COVID-19. Patients presenting with transaminitis, either symptomatic or asymptomatic, should also be offered testing for COVID-19 apart from standard tests for viral hepatitis, autoimmune markers, copper studies and metabolic panel. Similarly, patients with underlying liver disease presenting with new decompensation or ACLF should also be tested for COVID-19.[53]

Despite the ongoing pandemic, the scourge of tropical diseases, such as dengue, malaria, chikungunya, typhoid, tuberculosis, and scrub typhus, should not be forgotten, as they may share certain symptoms with COVID-19 but treatment and prognosis differ. Moreover, co-infections with COVID-19 and these tropical illnesses have been frequently reported.[20–22] Hence, in patients presenting with acute febrile illness, these differentials should also be considered, apart from COVID-19.

Management of patients with liver manifestations

Management of GI symptoms does not require specific drugs, apart from those approved for management of COVID-19, which are in a state of continuous evolution. Transaminitis should trigger a search for reversible and alternate causes of liver injury along with a diligent search for a culprit drug among the drugs the patient is receiving. Management of patients with decompensated liver disease and ACLF should be done according to standard guidelines.[80] Patients with variceal bleed may require endoscopy, which should be done with all recommended precautions, including personal protective equipment, preferably in a negative pressure room.[81]

Lack of knowledge in the current literature

The GI and liver manifestations of COVID-19 have been described now in multiple studies. The clinical implications of the new strain of COVID-19, the VOC 202012/01 strain known to spread faster, need to be seen.[82] The effect of this new strain on hepatic manifestations remains to be explored. Emerging data also suggest that immunity to COVID-19 infection wanes rapidly, particularly in asymptomatic individuals.[83] In light of these findings, reinfection has also been reported.[84] Whether reinfection tends to be asymptomatic or presents with more severe hepatic manifestations remains to be seen.

Conclusion and points to focus on in future studies

A year or so into the COVID-19 pandemic, we have learned that liver involvement is common, but usually secondary, and seen more commonly in severe COVID-19.[13] The speculated mechanisms for hepatic injury, in addition to direct viral cytotoxicity, are immune injury, cytokine storm, ischemia and hypoxia reperfusion injury.[5] We need more studies to unravel the mystery of pathogenesis of liver involvement

in COVID-19. Multiple therapies have been recommended in COVID-19, with different efficacies and side effect profiles. The therapeutic armamentarium against COVID-19 is rapidly expanding but with modest evidence for the efficacy of remdesivir and dexamethasone in moderate to severe COVID-19.[85] Most drug trials have excluded patients with underlying CLD and GI disease. What needs to be looked at is the effect of these drugs on patients of cirrhosis and ACLF, where the immune system is already dysregulated. Vaccination has already started in North America, Europe and India, with many more vaccines still in preclinical development and some in clinical trials.[86] Vaccination forms the basis of exit strategy in this pandemic to return back to normal lives. There have been doubts about the duration of natural immunity in COVID-19 and speculation that vaccine-induced immunity will last longer. We need to study how long the immune response lasts in patients with liver disease, immune response generation to vaccines in these patients and what type of vaccine would be best suited for special populations, as different vaccines would have different storage requirements, cost, adverse effect profiles and efficacies.[87]

Funding

None to declare.

Conflict of interest

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

Author contributions

Study concept and design (S), acquisition of data (AE, MV, SB, AC, AA, S), analysis and interpretation of data (AE, MV, SB, AC, AA, S), drafting of the manuscript (AE, MV, SB, AC, AA, S), critical revision of the manuscript for important intellectual content (AA, AC, S), administrative, technical, or material support, study supervision (S)

Data sharing statement

All data are available upon request.

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Journal of Clinical and Translational Hepatology **2021** vol. 9(2) | 256–264
DOI: 10.14218/JCTH.2021.00055

Review Article

Cytokine Storm of COVID-19 and Its Impact on Patients with and without Chronic Liver Disease

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Received: 4 February 2021 | **Revised:** 11 March 2021 | **Accepted:** 1 April 2021 | **Published:** 19 April 2021

Abstract

The coronavirus pandemic has resulted in increased rates of hepatic decompensation, morbidity and mortality in patients suffering from existing liver disease, and deranged liver biochemistries in those without liver disease. In patients with cirrhosis with coronavirus disease 2019 (COVID-19), new onset organ failures manifesting as acute-on-chronic liver failure have also been reported. The severe acute respiratory syndrome-coronavirus-2 (SARS-CoV-2) also directly binds to enterocytes and cholangiocytes via the angiotensin converting enzyme receptor 2, although the lung remains the portal of entry. Superadded with the COVID-19 related bystander hepatitis, a systemic inflammatory response is noted due to unregulated macrophage activation syndrome and cytokine storm. However, the exact definition and diagnostic criteria of the 'cytokine storm' in COVID-19 are yet unclear. In addition, inflammatory markers like C-reactive protein, ferritin, D-dimer and procalcitonin are frequently elevated. This in turn leads to disease progression, activation of the coagulation cascade, vascular microthrombi and immune-mediated injury in different organ systems. Deranged liver chemistries are also noted due to the cytokine storm, and synergistic hypoxic or ischemic liver injury, drug-induced liver injury, and use of hepatotoxic antiviral agents all contribute to deranged liver chemistry. Control of an unregulated cytokine storm at an early stage may avert disease morbidity and mortality. Several immunomodulator drugs and repurposed immunosuppressive agents have been used in COVID-19 with varying degrees of success.

Citation of this article: Premkumar M, Kedarisetty CK. Cytokine storm of COVID-19 and its impact on patients with and without chronic liver disease. J Clin Transl Hepatol 2021;9(2):256–264. doi: 10.14218/JCTH.2021.00055.

Keywords: Chronic liver disease; COVID-19; Cytokines; Hepatitis; Gut-liver axis.

Abbreviations: ACE2, angiotensin converting enzyme 2; ACLF, acute-on chronic liver failure; ALT, alanine transaminase; ARDS, acute respiratory distress syndrome; AST, aspartate transaminase; COVID-19, coronavirus disease-19; CRP, C-reactive protein; IL, interleukin; MAPK, mitogen-activated protein kinase; NAFLD, nonalcoholic fatty liver disease; NET, neutrophil extracellular trap; NK, natural killer; SARS-CoV-2, severe acute respiratory syndrome coronavirus-2; SIRS, systemic inflammatory response syndrome; TNF-α, tumor necrosis factor-alpha.

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Introduction

The novel coronavirus 2019 (COVID-19) disease, caused by the severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2), has resulted in a devastating global pandemic, with 113,989,973 confirmed COVID-19 cases, which include 2,531,542 deaths reported by the World Health Organization[1] as of March 2, 2021. COVID-19 has been reported as an air- and surface-borne contagious disease with features of viral pneumonia (fever, cough, lymphopenia, prothrombotic tendency, ground glass opacities on chest radiology) and hypoxemia.[2,3] In addition, alterations in liver chemistries have been reported in patients with and without liver disease, with some reports of increasing severity, complication, and new decompensation, while others refute this possibility. Liver chemistry changes are more likely in those with severe disease and those who have received multiple therapies, requiring high flow oxygen or invasive ventilation.[4] This presents an interesting clinical conundrum, as we need to assess the immunological injury, alteration in liver chemistries and varied clinical course in such patients.[5] We require predictive models of severity of disease, which enable us to prognosticate patients with cirrhosis during the COVID-19 pandemic.[6] In addition, the association between liver chemistries, need for invasive ventilation and COVID-19-associated hospital deaths remains controversial and despite availability of breakthrough vaccines, the pandemic is likely to continue claiming more lives.[7] Given the heterogenous clinical presentation, the spectrum of liver involvement varies from altered liver chemistry in patients without underlying liver disease to progressive decompensation in patients with cirrhosis.[8]

In this review, we have summarized relevant information related to the cytokine storm and pathophysiological basis of liver injury in COVID-19 in those with or without chronic liver disease. The mechanisms of liver injury in COVID-19 are crucial to planning strategies to ameliorate the direct viral, immunological or drug-related liver injury.

Cytokine storm and immune activation in COVID-19

The body's immune system can identify epitopes of the viral antigens of the SARS-CoV-2 via the antigen presenting cells (APCs), like dendritic cells and macrophages, that process the viral antigens and present them to the natural killer (referred to as NK) cells, CD4+ T helper cells and other lymphocytes, which in turn activate CD8+ cytotoxic T cells and B cells. The presentation of viral antigens using the major

histocompatibility complex ensures activation of both the innate and acquired immunity, resulting in proinflammatory cytokines, chemokines, and coagulation enzymes.[9,10] These inflammatory pathways, if dysregulated result in massive activation and 'cytokine storm', a prothrombotic tendency culminating into multiple organ failures and likely death. Pyroptosis is a form of programmed cell death which is an inflammatory caspase-1 dependent type, that occurs in response to infection with intracellular pathogens, such as SARS-CoV-2. Rapid viral replication can result in increased pyroptosis, which can be a precursor for massive release of inflammatory mediators.[10]

In COVID-19, uncontrolled immune response can lead to secondary hemophagocytic lympho-histiocytosis or macrophage activation syndrome, which presents as a life-threatening condition, in the form of persistent fever and pancytopenia quickly progressing into multi-organ failure and increased mortality.[11,12] Macrophage activation syndrome is diagnosed on the basis of clinical and laboratory diagnostic criteria which include fever, increased ferritin, triglyceride levels, pancytopenia, consumptive coagulopathy with hypofibrinogenemia, and splenomegaly.[13,14] Hemophagocytosis is defined as the engulfment of red blood cells, leucocytes, and platelets by macrophages (detected on histology).[13] Besides these features, low or absent NK cell activity and serum CD25 ≥2,400 units/mL is noted.[14] The cytokine storm refers to elevated interferon-alpha, interleukin (IL)-6, IL-1, CCL-5, CXCL8, and CXCL-10. In addition, inflammatory markers like C-reactive protein (CRP) and procalcitonin are frequently elevated.[15,16] Viral features, low interferon levels, increased neutrophil extracellular traps (NETs), and increased pyroptosis lead to impaired SARS-CoV-2 clearance and create the setting for macrophage activation syndrome and cytokine storm. Certain genetic mutations predispose to this condition.[17–19] Once the inflammation sets in, anti-viral treatment will be insufficient to control the disease severity and anti-inflammatory or immunomodulatory drugs are required. Normal antiviral response requires activation of controlled inflammatory syndrome but it is usually overtaken by systemic inflammatory response syndrome (commonly known as SIRS) due to uncontrolled inflammation. Cytokines are the signaling molecules of this response, which are produced by a multitude of immune cells, like dendritic cells, macrophages, neutrophils, NK cells, and adaptive T and B cells.[20] Binding of the COVID-19-associated damage-associated molecular patterns or pathogen-associated molecular patterns to pattern recognition receptors on the immune cells, like lymphocytes and antigen presenting cells, trigger signaling pathways that lead to the cytokine storm.[21,22] Various signaling pathways, including the mitogen-activated protein kinase (MAPK) pathway with Jun NH_2-terminal kinase, extracellular signal-regulated kinase, p65 and p38 MAPK, lead to elution of transcription factors and induce gene expression of several immune regulatory genes encoding proinflammatory cytokines.[23] The MAPK pathways modulate apoptosis and cross-talk between the p38 MAPK pathway and other pathways that can induce cell death.

Other downstream signaling pathways involve JAK1 and 2, Tyk2, and STAT3. Activation of the PI3 kinase/Akt pathway is essential to establish persistent infection with SARS-CoV2.[24]

The major triggered transcription factors are interferon response factors 3 and 7, nuclear factor KB, activation protein 1, and so on.[25] These in turn lead to expression of chemokines, cytokines, and adhesion molecules. The cascade of signaling events leads to recruitment of leucocytes, plasma cells and T cells to the site of infection, where they assist the innate response by macrophages to perform effector function and clear SARS-CoV-2. A counterbalancing mechanism for immune modulation is the negative feedback by the cytokines IL-10 and IL-4, which is often down-regulated in severe COVID-19. This leads to an unregulated and excessive cytokine storm resulting in secondary organ failures (Fig. 1).[26]

COVID-19, cell entry and angiotensin converting enzyme receptor

Although the spread of the SARS-CoV-2 infection is by droplet infection and the primary entrance is the respiratory tract, it also infects the gastrointestinal tract directly. The angiotensin converting enzyme 2 (ACE2) receptor is present on type II alveolar cells in the lung, esophageal epithelium, enterocytes of the ileum and colon, pancreas, hepatocytes and cholangiocytes, myocardium, proximal tubular cells of the nephron, and the pancreas.[27,28] The resultant inflammatory response may lead to viral clearance but when uncontrolled (in the form of cytokine storm), it can lead to vascular barrier damage, alveolar membrane integrity damage, multiorgan failure, and ultimately death.[29,30] Although the primary site affected is the lung, with acute lung injury and acute respiratory distress syndrome (ARDS), the liver is also affected by a similar mechanism (Fig. 1).

The ACE2 receptor is highly expressed on well-differentiated enterocytes, and this explains why fecal shedding of the virus is detected and diarrhea is a symptom of COVID-19. Gastrointestinal manifestations are noted in up to 61% of COVID patients. ACE2 receptors are present at various gastrointestinal sites, like gastric and duodenal glands and distal enterocytes. COVID-19 can present as malabsorption, altered intestinal permeability, and activation of the enteric nervous system. SARS-CoV-2 is a systemic infection and the intracellular vesicles containing the virus remain *in situ* for long after apparent recovery from the disease. Pathological examination of patients with liver disease shows the liver histology has microvesicular steatosis, as well as areas of focal necrosis with lymphocyte infiltration like reports of bystander hepatitis caused by immunological injury attributable to influenza virus. SARS-CoV-2 could also cause direct cytopathic injury to the liver, other than hypoxic, or free radical-mediated injury. The virus has also been detected in up to 41% of autopsied livers with a viral load of 1.6×10^6 copies per gram of liver tissue.[31,32] Down-regulation of the negative feedback counterregulatory IL-10 and IL-4 mechanism results in a hyperinflammatory cytokine storm.[9]

ACE2 expression in cell clusters is higher in cholangiocytes than in hepatocytes (59.7% vs. 2.6%), but immune-mediated hepatitis is more likely to be the explanation for deranged liver chemistries, as with other respiratory tract viruses.[33,34] With such a broad infection footprint, many drugs affecting the immune cascade have been tried. Use of the anti-IL-6 agent tocilizumab, hydroxychloroquine and steroids are examples, which have shown a varied efficacy.

Viral kinetics of SARS-CoV2 in cirrhosis

Cirrhosis is an immunocompromised state, and it appears there is impaired viral clearance of the SARS-CoV-2. The virus resides in double membrane vesicles, which prevent creation of pattern recognition receptors, and even after the PCR test being negative, the lung alveolar cells and macrophages can show tell-tale signs of these viral vesicles, even after 2 weeks of apparent resolution of disease.[2,21]

In cirrhosis, there is also a lower level of type I interferon, which results in impaired viral response. Neutrophils also contribute to viral clearance by release of free radicals, degranulation of vesicles, and secretion of antimicrobials through the formation of unique NETs.[35] Neutrophils are activated by IL-8, CXCL8, leukotriene B4 or lipopolysaccha-

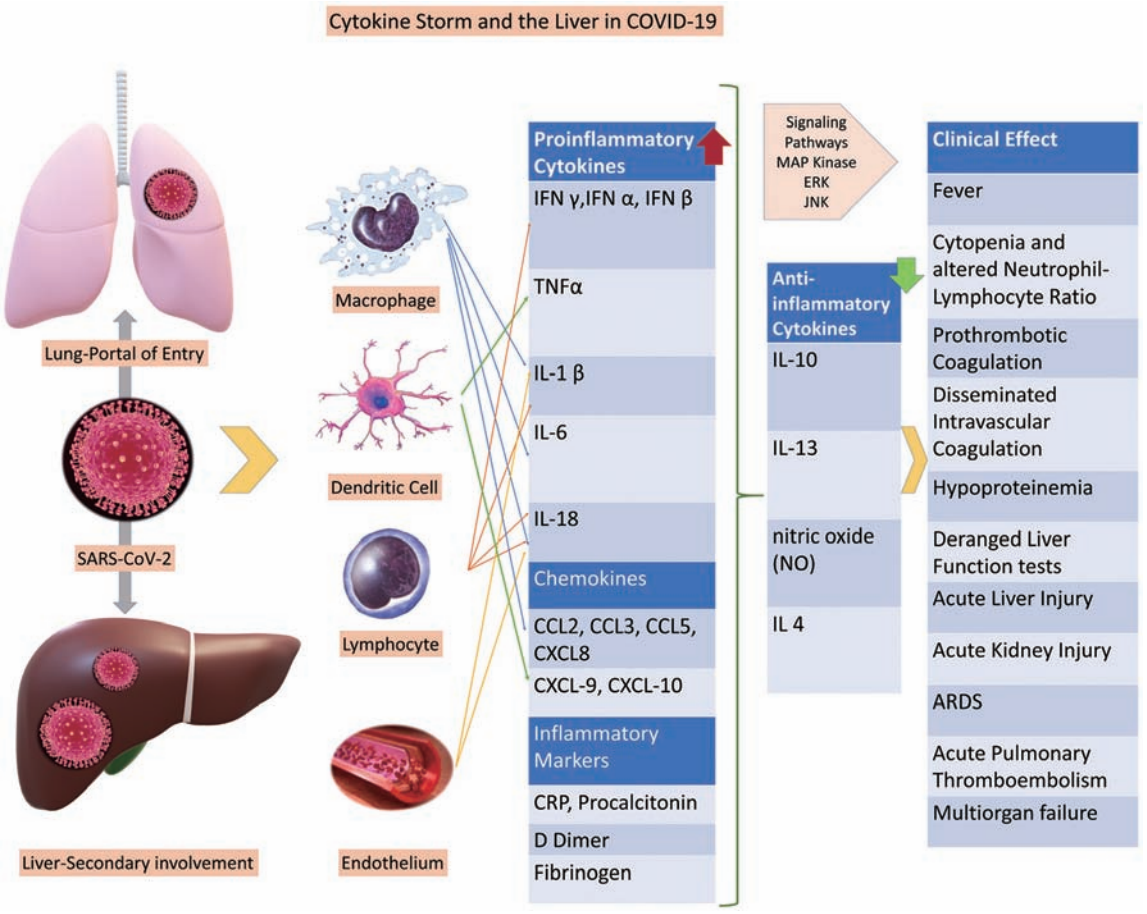

Fig. 1. COVID-19, cytokine storm and immune-mediated organ failure.

ride, and generate a programmed cell death with a chromatin reticular framework covered with neutrophil granule-derived peptides and proteolytic enzymes. This generates a net-like structure, in which pathogens get trapped, aptly called NETs. The positively charged histones of the chromatin network of NETs can bind to and immobilize negatively charged viral envelope of the SARS-CoV-2 particles.[36,37]

COVID-19 and hepatic involvement in people without liver disease

COVID-19 results in liver injury, transaminitis and even impending liver failure in patients without liver disease, especially those with moderate to severe illness. Hypoxemia, impaired cardiac function, and reduced tissue perfusion in severely ill COVID-19 patients can lead to increased vulnerability of an apparently healthy liver. The mechanisms of liver injury in a native 'healthy' liver are multifactorial. Direct viral cytopathic effects, hypoxic injury, hepatotoxicity from therapeutic drugs, and secondary damage due to multiple organ dysfunction are the most likely underlying mechanisms for liver injury. On histopathology, a mild increase in sinusoidal lymphocytic infiltration, sinusoidal dilatation, mild steatosis, and multifocal hepatic necrosis are noted. Direct cytopathic effects of the SARS-CoV-2 are multiple foci of necrosis in the periportal area (zone 1) and adjacent to terminal hepatic veins (zone 3), with minimum inflammation. The described histology is like non-viral related acute liver injury. Conspicuous absence of dense inflammation, widespread necrosis, ballooning, Mallory hyaline, or pericellular fibrosis, cholestasis or lack immune mediated damage differentiates it from viral hepatitis.[38,39]

Biochemically, abnormal liver chemistries in COVID-19 include elevation of aspartate transaminase (AST), alanine aminotransferase (ALT), and gamma-glutamyl transpeptidase, but bilirubin and alkaline phosphatase changes are seen rarely. In absence of liver disease, liver failure is rare. A recent meta-analysis[5] showed that the liver chemistries per se did not affect outcomes in patients with mild and moderate COVID-19 infection.

Table 1 shows the list of studies which provided data on liver injury in patients without underlying liver disease.[40–42]

COVID-19, inflammation, coagulation, and liver disease

The cytokine storm triggered by COVID-19 has several implications in those with liver disease. Firstly, patients with cirrhosis are already in a procoagulant rebalanced state and are predisposed to pulmonary microthrombosis.[43] The systemic inflammatory state is difficult to diagnose, as patients frequently have an elevated CRP and a preexisting cytopenia and splenomegaly attributable to portal hypertension. Due to the hyperdynamic circulation, patients with decompensated cirrhosis already have endothelial inflammation, elevated baseline norepinephrine, and are at increased risk

Table 1. List of studies which provided data on liver injury in patients without underlying liver disease

No.	Reference	Study type	No. of patients with COVID-19	Pre-existing liver diseases	Hepatobiliary function markers	Inflammatory markers and other relevant blood tests	Proposed possible theories of hepatic injury
1	Xie H[40]	Retrospective case series	79	Patients with previous liver diseases were excluded	31.6%, 35.4% and 5.1% of patients had elevated ALT, AST and TBIL, respectively. Median (range) values were 36.5 (17.5–71.5) U/L, 34.5 (25.3–55.3) U/L and 12.7 (8.1–15.4) mmol/L, respectively	CRP (max.,79.6 µmol/L) and ESR (max., 58 mm/h) increased; while LYM reduced (min., 0.9×10^9/L)	Overall disease exacerbation; disease severity. Males were more likely to have liver injury when infected with COVID-19 ($p<0.05$); compared with patients without liver injury, patients with liver injury had increased levels of white blood cell counts, neutrophils, CRP and CT score ($p<0.05$) and had a longer length of stay ($p<0.05$)
2	Zhang Y[41]	Retrospective case series	115	Two patients had chronic hepatitis B (excluded)	ALT and AST increased in 9.57% and 14.78% patients, respectively on admission. TBIL elevation was rarely observed. Mean levels higher in severe cases	54.78% had reduced ALB, significantly lower in severe cases. 57.39% had increased CRP, higher in severe cases (80.75+69.18). LDH level (mean+SD:346.10+257.26) significantly elevated in severe cases	Dysfunction of immune system. Levels of ALT, AST, TBIL, LDH and INR showed statistically significant elevation in severe COVID-19 cases compared with that in mild cases
3	Huang C[3]	Prospective case series	41	Chronic liver disease in one patient	AST (max., 48.0 U/L) increased in 37%, more in the ICU group	73% had LDH >245 U/L (max., 408 U/L). 37% had LYM $>1.0\times10^9$/L (max., 1.1×10^9/L)	Overall disease exacerbation: cytokine storm. Compared with non-ICU patients, ICU patients had higher plasma levels of IL2, IL7, IL10, GSCF, MCP1, MIP1A, and TNF-α
4	Fan Z[42]	Retrospective case series	148	None described	55 patients (37.2%) had abnormal liver function at hospital admission. Elevated ALT ($n=27$; 41–115 U/L), AST ($n=32$; 37–107 U/L), γ-glutamyl transferase ($n=26$; 48–159 U/L), ALP ($n=6$; 102–144 U/L), and total bilirubin ($n=9$; 21–46.6 µmol/L)	PCT and CRP elevated in those with abnormal liver function	More patients with abnormal liver function (57.8%) received treatment with lopinavir/ritonavir compared with those with normal liver function (31.3%; $p=0.01$)

ALP, alkaline phosphatase; ALT, alanine transaminase; AST, aspartate transaminase; CRP, C Reactive protein; G-CSF, granulocyte colony stimulating factor; ICU, intensive care unit; LDH, lactate dehydrogenase; LYM, lymphocyte; MCP, monocyte chemoattractant protein; MIP, macrophage inflammatory protein; PCT, procalcitonin; SD, standard deviation; TBIL, total bilirubin.

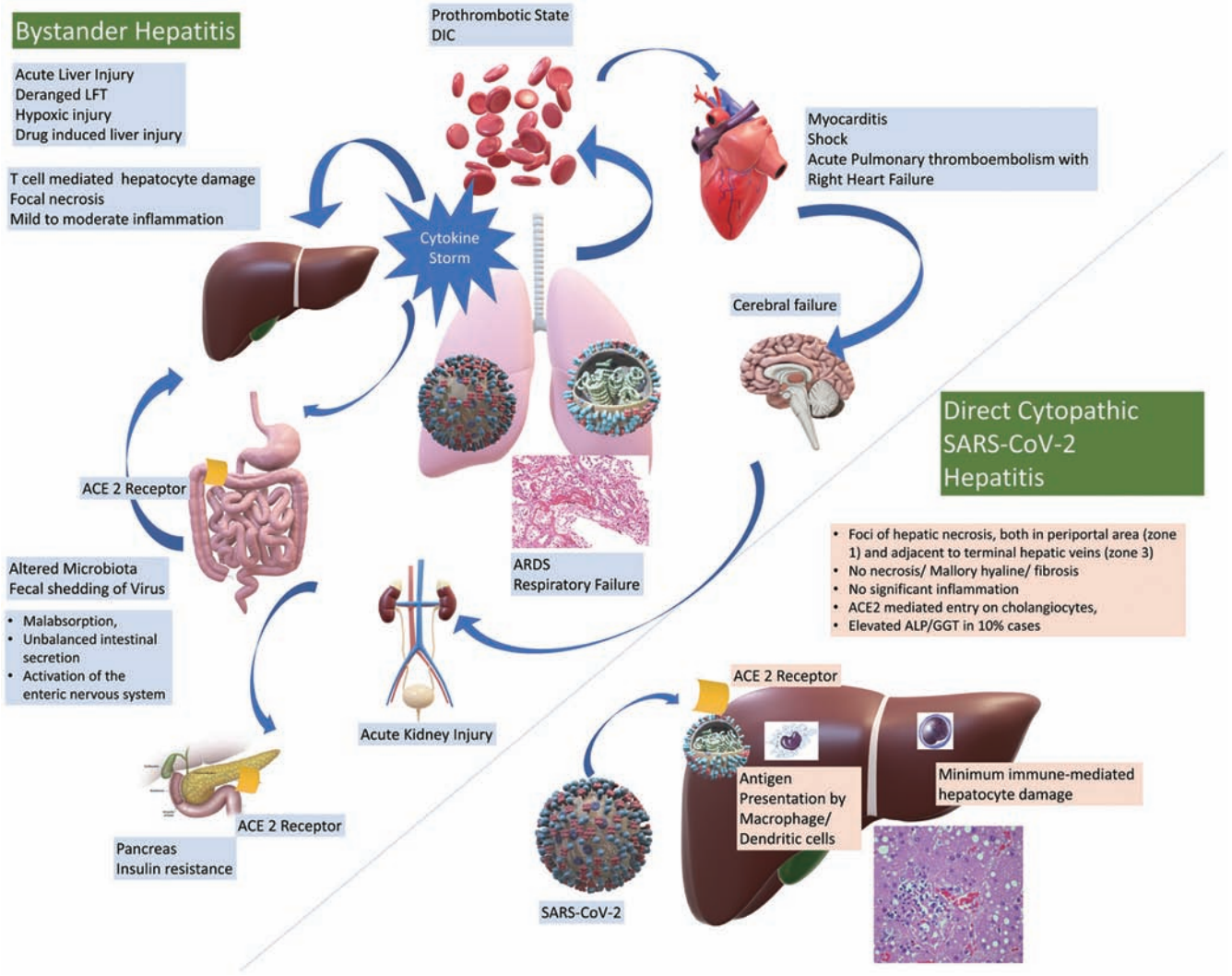

Fig. 2. Etiopathogenesis of liver injury in COVID-19.

of thromboembolism and ARDS.[44,45] The procoagulant tendency of COVID-19 is due to systemic endothelial activation, or damage mediated by viral binding to the ACE2 receptor on the endothelium and other organs. In addition, presence of comorbidities, mechanical ventilation, and bedridden state favor venous thromboembolism.[42]

The resultant influx of inflammatory mediators raises blood viscosity, and in presence of venous catheters or dialysis access, there is possibility of deep vein thromboses in cirrhosis and liver failure. Patients with predisposing risk factors like coronary artery disease and stroke are also affected by the cytokine storm and have increased risk for cardiac or cerebrovascular events when they have metabolic liver disease. Patients with liver disease often fare poorly in an intensive care setting during for viral pneumonia with respiratory failure, like COVID-19 and H1N1 influenza infections.[42,46–48] Although patients with cirrhosis are likely to benefit from prophylactic anticoagulation with low molecular weight heparin, they are also at increased risk of variceal bleeding due to increased portal pressure triggered by new onset bystander hepatitis. (Fig. 2). Also, endogenous heparinoids are produced in patients with liver failure with cytokine storm or cirrhosis with systemic inflammation, which affect coagulation and predispose to bleeding. Therefore, using balancing anticoagulants in patients with cirrhosis with COVID-19 with a cytokine storm harbors inherent bleeding risk on one hand and pulmonary microthrombosis on the other.[49] The terminal events in these patients with cirrhosis with COVID-19 have been progressive respiratory failure, with secondary organ failures like cardiac or renal failure requiring inotropic support, secondary sepsis, variceal bleeding, or sometimes sudden cardiac events.[50,51] In the multicentric APCOLIS study[52] of 288 patients, 43% of patients with liver disease presented as acute liver injury, 20% presented as acute-on-chronic liver failure (commonly referred to as ACLF) or acute decompensation (9%). A Child Turcotte Pugh score >9 predicted mortality with hazard ratio of 19.2 (95% confidence interval: 2.3–163.3), with sensitivity of 85.7% and specificity of 94.4%. Patients with liver disease have poor outcomes in the setting of invasive ventilation. Improved intensive care, timely interventions and monitoring altered liver chemistries can improve outcomes.[53]

Other associations, such as presence of chronic hepatitis B, did not increase the mortality risk. Therefore, it appears that the cytokine storm is one of the important defining fac-

tors contributing to morbidity and mortality in those with liver disease.[54] A raised AST and direct bilirubin at baseline were independent predictors of COVID-19 mortality.

When acute liver injury and ACLF were reported in patients with liver disease, it was typically seen in the setting of multiple organ failures, severe pneumonia or ARDS. After propensity matching, the baseline and peak values of liver function tests, the trajectory of COVID-19 and severity of liver scores and outcomes are often equivalent in those with compensated cirrhosis.[54,55] In contrast, in decompensated liver disease, there is a marked risk of COVID-19-associated liver and coagulation failure. Particularly, studies have reported such events in patients with Child-Turcotte-Pugh B and C cirrhosis with increased decompensation events like ascites, coagulopathy, and hepatic encephalopathy and in-hospital mortality. In view of the increased morbidity and mortality, it is essential to protect patients with decompensated cirrhosis and provide guidance to better manage and evaluate patients with COVID-19 and its complications.[56]

Table 2 shows the list of studies which included patients with underlying liver disease, and significant findings.[57–63]

COVID-19, obesity and fatty liver disease

A recent paper by Bramante *et al*.[64] showed that presence of nonalcoholic fatty liver disease (NAFLD) is associated with increased risk of hospital admission [odds ratio: 2.04 (1.55, 2.96, $p<0.01$)]. In another study[65] on 202 NAFLD patients with COVID-19, altered liver chemistries were noted in 75% during hospital stay. About a third of patients with NAFLD continued to have raised transaminases even on follow up, suggesting a long-lasting superadded insult to the fatty liver. Male sex, age >60 years, high body mass index, presence of comorbidities and NAFLD were associated with progression to severe COVID-19 disease. On logistic regression, NAFLD was an independent risk factor for COVID-19 progression, high likelihood of ongoing liver injury and raised liver chemistries during hospital stay, and prolonged duration of viral shedding. It appears that presence of obesity, NAFLD and metabolic syndrome are associated with an increased risk of COVID-19 progression.[66]

Drugs targeting the cytokine storm

Several drugs have been tested in COVID-19 based on the assumption that dysregulated immune responses need to be curbed. One of the main therapies includes the use of steroids, either prednisolone or methylprednisolone or intravenous hydrocortisone, which act through the glucocorticoid receptor and effector genes. As per the World Health Organization guidelines, systemic corticosteroid therapy is not for routine use. It should only be given to those with cytokine storm, ARDS, acute heart failure, acute kidney injury, and high serum levels of D-dimer.[67] Anti-rheumatic drugs, hydroxychloroquine, chloroquine, JAK inhibitors, IL-1 and IL-6 inhibitors, anti-tumor necrosis factor-alpha (commonly referred to as TNF-α) drugs, corticosteroids, colchicine, and intravenous immunoglobulin. The use of chloroquine and hydroxychloroquine was reported to reduce COVID-19-mediated injury, by arresting the cytokine storm or the activation of CD8+ cells, or by preventing endocytosis-mediated uptake of the virus. Chloroquine and hydroxychloroquine act by accumulating in lysosomes, increasing the pH of the endosome, thereby interfering with viral entry or exit from the cells. Also, these drugs interfere with the ACE2 receptor, preventing entry of the SARS-CoV-2. Chloroquine and hydroxychloroquine may reduce glycosylation of the ACE2 receptor which prevents the virus binding to and entering the new cells. However, major trials have found no putative benefit for prophylaxis of COVID-19, and gradually these drugs have been disregarded.[68] Similarly, other direct antivirals like remdesivir and favipiravir also failed to show significant efficacy or survival benefit.[69,70] Tocilizumab, a humanized IgG1 monoclonal antibody to the IL-6 receptor, has been used with limited success in COVID-19. The recommended dose of tocilizumab is 8 mg/kg intravenous as single or two divided doses at 12 to 24 h intervals, with a maximum dose of 800 mg. However, the adverse events include increased propensity of infection, hypertriglyceridemia, diverticulitis, and hepatotoxicity.[71]

Several repurposed drugs have been adopted from rheumatology practice to assess amelioration of the cytokine storm in COVID-19. Colchicine has been recommended as potential therapy for complications of COVID-19, as an IL-1 inhibitor.[72] Other drugs include a recombinant humanized anti-IL6 receptor antibody called sarilumab, a recombinant human mouse chimeric monoclonal antibody called siltuximab, and an IL-1 blocker called anakinra.[73,74] Anakinra, an anti-rheumatic drug, was studied in the trial setting to inhibit pathological effects of IL-1 alpha and IL-1 beta.[75] Other than drugs that directly inhibit the immune response, cytokine dialysis has also been tried, using blood ultrafiltration, diffusion, and adsorption circuits in dialysis machines. Restoration of the immune IL-6/IL-1 levels and other proinflammatory molecules theoretically protects against organ failures but clinical efficacy is still unclear, and the immune dysregulation is only one problem of many. A novel treatment approach for preventing and managing the cytokine storm using mesenchymal stem cell-based immunomodulators has been proposed. Intravenous transplantation of mesenchymal stem cells was shown to be effective in COVID-19 in a trial.[76]

Relevance of the cytokine storm in COVID-19

After describing the various aspects of the cytokine storm, it is important to emphasize that the condition has no definition. In most studies on COVID-19, it is described as a hyperimmune response characterized by the release of ILs, interferon, TNF, chemokines, and other mediators. These represent a normal response to a variety of pathogens, and the term 'cytokine storm' implies that these released cytokines are injurious to the host; furthermore, there is no consensus yet as to the levels of permissible cytokines that distinguish a well-conserved innate immune response from a dysregulated hyperinflammatory immune response. In addition, all the signaling pathways described have regulatory and counterregulatory responses and pleiotropic downstream mediators that may be acting in complex dependent activities that cannot be easily predicted. To complicate matters, it is unclear if the cytokine storm is pathogenic or protective in an individual patient.[77] The abject failure of some drugs like tocilizumab, anakinra, etc. is a case in point and should dampen the enthusiasm displayed globally for applying drugs for one condition without much success in another. It is time to reinterpret and define the cytokine storm. The role of T cells that exert protective functions by reigning in on overactive innate immunity is important, as lymphopenia is associated with ARDS.[78] The important role of microthrombosis in the pathogenesis of severe pneumonia and outcomes related to hypoxemia with secondary organ failures is often under-played. The failure of the use of immunomodulators used in rheumatological conditions should make us reassess the degree of cytokine storm and possibly use therapy in patients with demonstrated high levels of cytokines. The cytokine release syndrome was described by Maude *et al*.[79] in recipients of chimeric an-

Table 2. List of studies which included patients with underlying liver disease, and significant findings[61–67]

No.	Reference	Study type	No. of patients with COVID-19	Pre-existing liver diseases	Hepatobiliary function markers	Inflammatory markers and other relevant blood tests	Proposed possible theories of hepatic injury
1	Wang D[57]	Retrospective case series	138	Chronic liver disease in 2.9% of patients	No significant liver abnormalities	LYM (median: 0.8×10^9/L) reduced in 70.3% of cases, and LDH (median: 261 U/L) increased in 39.9% of patients	Overall disease exacerbation
2	Cai Q[58]	Retrospective case series	298	2.7% had liver disease. CHB (1.7%). NAFLD (4.7%). ALD (3%)	14.8% experienced liver injury, with ALT max., 59.5 U/L and AST max., 65 U/L: 8.7%, respectively	CRP (max., 47.13 mg/dL) increased in 70% cases	Overall disease exacerbation. Liver injury mainly occurred in severe patients (36.2% vs. 9.6%, $p<0.001$)
3	Xu XW[59]	Retrospective case series	62	12% had underlying liver disease	AST (max., 32 U/L) increased in 16% of patients	42% showed LYM reduction	None described
4	Shi H[60]	Retrospective case series	81	Hepatitis or liver cirrhosis in 9% of cases	AST (>40 U/L) increased in 53% of patients, lower in asymptomatic patients	CT imaging described	None described
5	Zhang B[61]	Retrospective case series with the data of non-survivors	82	Liver diseases in 2.4% cases. Patients who died had comorbidities (76.8%), including hypertension (56.1%), heart disease (20.7%), diabetes (18.3%), cerebrovascular disease (12.2%), and cancer (7.3%)	ALT (>40 U/L), AST (>40 U/L), and TBIL (> 20.5 mmol/L)	LYM ($<1.0\times10^9$/L), ALB (<40 g/L) and CD8+ cells ($<220\times10^9$/L). CRP (100%), lactate dehydrogenase (93.2%), and D-dimer (97.1%). IL-6 >10 pg/mL used as cut-off	–
6	Guan WJ[62]	Retrospective case series	1,099	Hepatitis B in 2.1% of patients	AST >40 IU/L (22.2%). ALT >40 IU/L (21.3%)	PCT >0.5 ng/mL (5.5%)	–
7	Li L[63]	Retrospective case series	85	Hepatitis B, alcoholic liver disease, and fatty liver disease ($n=2$ in each category)	24.7% had ALT elevation at admission	CRP ≥20 mg/L and LYM count $<1.1\times10^9$/L were independent risk factors for hepatic injury. ALB (mean: 33.4 g/L) in the ALT-elevated group was significantly lower	Inflammatory cytokine storm. Deterioration of the disease with a dynamic process. Limitation: 6 in the elevated-ALT group ($n=33$) had a history of liver disease (i.e. HBV infection, alcoholic liver disease, fatty liver)

ALP, alkaline phosphatase; ALT, alanine transaminase; AST, aspartate transaminase; CRP, C Reactive protein; HBV, hepatitis B virus; LDH, lactate dehydrogenase; LYM, lymphocyte; NAFLD, nonalcoholic fatty liver disease; PCT, procalcitonin; TBIL, total bilirubin.

tigen receptor T cell therapy, where the peak plasma IL-6 level was approximately 10,000 pg/mL, which was almost 1,000-times higher than the level reported in severe COVID-19. Hence, a consensus definition and diagnostic criteria for the cytokine storm is the need of the day.[80]

Summary

The COVID-19 crisis has presented an enormous challenge to the medical community, as it is a multisystem disease with high mortality and secondary attack rate in predisposed individuals, requiring a multidisciplinary approach for diagnosis, prognostication, and management decision plans. Several therapeutic agents have been tried to manage the hyperinflammatory cytokine storm which leads to immune-mediated organ damage. The trial and failure of several agents like hydroxychloroquine, remdesivir, chloroquine, etc. underlines the fact the evidence-based practice is still unable to provide an answer for controlling the cytokine storm. Strategic vaccination is now a reality, but the story of COVID-19 suggests that we need to be prepared to provide treatments which can manage and control the deleterious effects of our immune reaction, while retaining the viral clearance and disease-controlling immune mechanisms.

Funding

None to declare.

Conflict of interest

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

Author contributions

Conceptualization and original draft preparation (MP), conceptualization, reviewing and editing of the manuscript (CKK). Both authors approved the final version of the manuscript.

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Journal of Clinical and Translational Hepatology **2021** vol. 9(2) | 265–268
DOI: 10.14218/JCTH.2020.00094

Case Report

Checkpoint Inhibition in the Treatment of Unresectable, Advanced Lymphoepithelioma-like Hepatocellular Carcinoma

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Received: 19 October 2020 | **Revised:** 13 December 2020 | **Accepted:** 5 January 2021 | **Published:** 1 February 2021

Abstract

Lymphoepithelioma-like hepatocellular carcinoma (LEL-HCC) is a very rare neoplasm, with distinct epidemiologic, morphologic and clinical characteristics. Molecular mechanistic insight into the pathogenesis of this carcinoma suggests a pivotal role for the host immune system in the proliferation and progression of this tumor. However, while detailed genomic profiling of these hepatic tumors have revealed an intra-tumoral inflammatory mutational signature that may predispose to immune checkpoint inhibitor efficacy, no published report has described their use in this tumor type. Unfortunately, with near 100 cases of LEL-HCC reported in the literature to date and the majority of cases confined to localized and resectable disease, current evidence-based practices in the unresectable setting are lacking, with unknown benefit of chemotherapy or immunotherapy. We report on the case of a 68 year-old man with unresectable, advanced LEL-HCC who had evidence of disease stability after starting on the immune checkpoint inhibitor nivolumab. His disease response persisted off therapy for over a year and was potentially augmented by radiotherapy at the site of local progression. For this extremely rare tumor subtype, this case highlights the potential efficacy and safety of immune checkpoint blockade in LEL-HCC and reinforces the need for more robust, large-scale analysis of patients with these rare tumors to better evaluate treatment strategies and outcomes.

Citation of this article: Hermel DJ, Du EZ, Lin R, Frenette CT, Sigal DS. Checkpoint inhibition in the treatment of unresectable, advanced lymphoepithelioma-like hepatocellular carcinoma. J Clin Transl Hepatol 2021;9(2):265–268. doi: 10.14218/JCTH.2020.00094.

Keywords: Immune checkpoint inhibitor; Lymphoepithelioma-like hepatocellular carcinoma; Stereotactic body radiation therapy; Immunotherapy; LEL-HCC.

Abbreviations: HCC, hepatocellular carcinoma; HCV, hepatitis C virus; ICI, immune checkpoint inhibitor; LEL-HCC, lymphoepithelioma-like hepatocellular carcinoma; LELC, lymphoepithelial-like carcinomas; MELD, model for end-stage liver disease; PD-1, programmed death-1; PDL-1, programmed death ligand 1; SBRT, stereotactic body radiation therapy; TACE, trans-arterial chemoembolization.

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Introduction

Lymphoepithelioma-like hepatocellular carcinoma (LEL-HCC) is a rare hepatic neoplasm that is histologically characterized by a prominent reactive lymphoid infiltrate interspersed among undifferentiated carcinoma cells.[1] Tumors with similar histopathologic features have been described in a variety of anatomical regions, yet primary liver involvement is an extremely rare entity that is traditionally classified into cholangiocarcinoma and HCC variants with unique etiological and clinical features.[2] While the majority of reported cases of LEL-HCC tend to be early-stage, associated with preceding viral hepatitis and amenable to surgical resection, metastatic unresectable disease is seldom described and lacks clear evidence-based treatment guidelines.[3] Incorporation of immune checkpoint inhibitors (ICIs), specifically agents that block inhibitory immune signaling through disruption of the interaction between programmed death-1 (PD-1) and its ligand (PD-L1), into the treatment paradigm of this disease has not been previously described. However, its use in this lymphocyte-enriched tumor type has a strong rationale for efficacy given the well-described correlation between ICI response and intra-tumoral lymphocyte concentration.[4] Additionally, these novel agents have shown durable efficacy in a range of tumor types, with a safe toxicity profile.[5]

We report on a case of a patient with unresectable primary LEL-HCC who had a potential protracted and durable response to the PD-1 inhibitor nivolumab. Experiencing minimal toxicity, the patient had stable disease on nivolumab for approximately 3 years, with an isolated area of progression for which he received localized radiotherapy with appropriate tumor shrinkage. He completed a total of 63 doses of nivolumab and has continued to have stable disease off all therapy for more than 1 year. Our patient's clinical response demonstrates the potential efficacy and tolerability of single agent ICIs in this lymphocyte-enriched tumor subtype and suggests the potential benefit of localized radiotherapy to supplement ICI response in localized progression.

Case report

A 68 year-old man with a prior history of cirrhosis secondary to hepatitis C virus (HCV) presented to our clinic with a new enhancing 2.4 cm mass in segment 8 of the liver that was

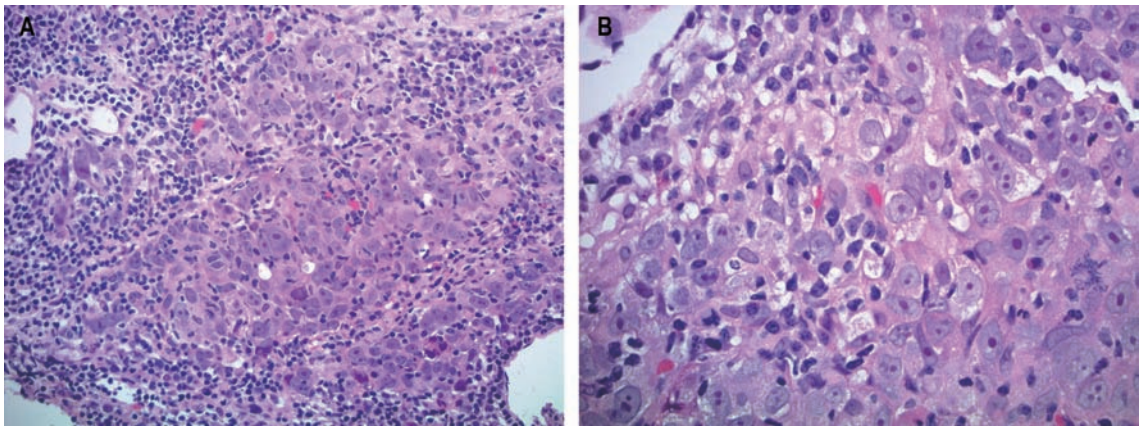

Fig. 1. Hematoxylin-eosin stain of the patient's tumor specimen. The morphology depicts clusters of large malignant cells with prominent nucleoli and eosinophilic cytoplasm with prominent infiltrating lymphocytes, viewed through (A) low and (B) high power.

detected on abdominal magnetic resonance imaging. The decision was made to pursue trans-arterial chemoembolization (referred to as TACE) of the lesion given tumor accessibility and his overall well-compensated, Child-Pugh A, model for end-stage liver disease (commonly known as MELD) 6 cirrhosis without associated portal hypertension. Despite his good initial radiographic response to TACE, follow-up imaging 2 months later revealed evidence of new periesophageal, gastrohepatic, and periportal lymphadenopathy.

He underwent a core needle biopsy of an enlarged subhepatic lymph node, which showed histologic evidence of LEL-HCC. Morphology was notable for large cells with abundant eosinophilic cytoplasm and prominent nucleoli associated with frequent lymphocytes (Fig. 1). Tumor cells expressed glutamine synthetase and CD10 by immunohistochemistry. Further immunostaining examinations showed negativity for CK7, CK19, CK20, and CK5 and positivity for the epithelial marker OSCAR (Fig. 2). The tumor tissue showed no evidence of microsatellite instability by polymerase chain reaction, and staining with the anti-PD-L1 clone SP142 showed PD-L1 expression in neoplastic cells at a tumor proportional score of 10% (Fig. 3). Serum alpha-fetoprotein level was 4 ng/mL. Due to the relatively rapid tumor progression, large tumoral lymphoid component and known efficacy data in HCC, the patient was initially started on a chemotherapy regimen of fluorouracil, folinic acid and oxaliplatin and underwent 7 cycles of therapy with stable disease but ongoing neuropathy limiting chemotherapy tolerance. Concomitantly, the patient underwent treatment for relapsed HCV with a 12-week course of sofosbuvir and velpatasvir and achieved a sustained virologic response.

He was then switched to single-agent nivolumab at 240 mg every 2 weeks and had stable porta hepatis lymphadenopathy for approximately 2 years on this ongoing therapy. His periesophageal, intra-abdominal and mediastinal lymphadenopathy regressed while on the therapy. The drug was well-tolerated, with development of a lichenoid-rash that improved with topical treatment. After 2 years of relatively stable disease, he had evidence of subsequent progression in the porta hepatic lymph nodes and received palliative stereotactic body radiotherapy (referred to as SBRT) with significant localized regression on follow-up imaging. He continued nivolumab for 1 more year, which was ultimately discontinued due to patient preference and lack of clear evidence from early clinical trials that ICI treatment over 2 years improves overall survival.[6] See Fig. 4 for complete treatment sequencing and relevant corresponding radiographic changes throughout treatment. To date, sur-

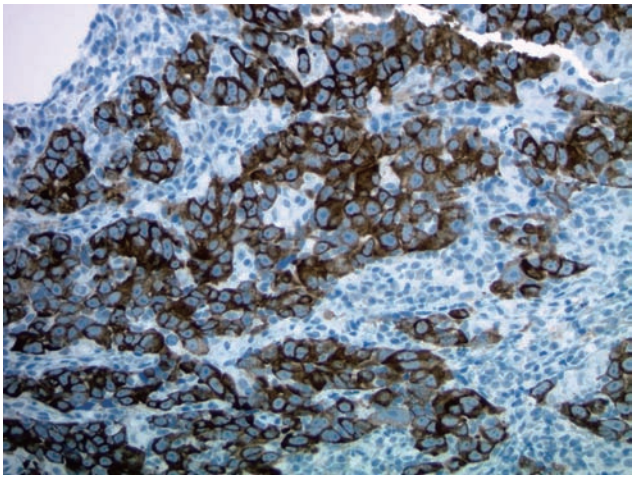

Fig. 2. Tumor stained with the anti-cytokeratin clone OSCAR. This epithelial marker is strongly positive in the tumor cells and supports the diagnosis of LEL-HCC.

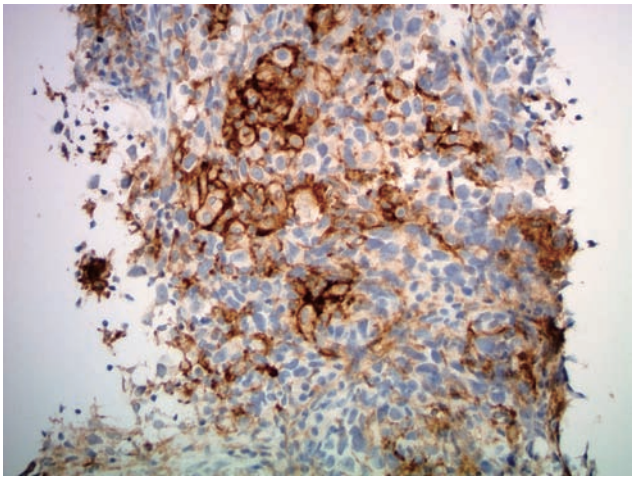

Fig. 3. Tumor tissue stained with the anti-PD-L1 clone SP142. The staining shows low level of PD-L1 expression, with tumor proportional score (10%, 2+).

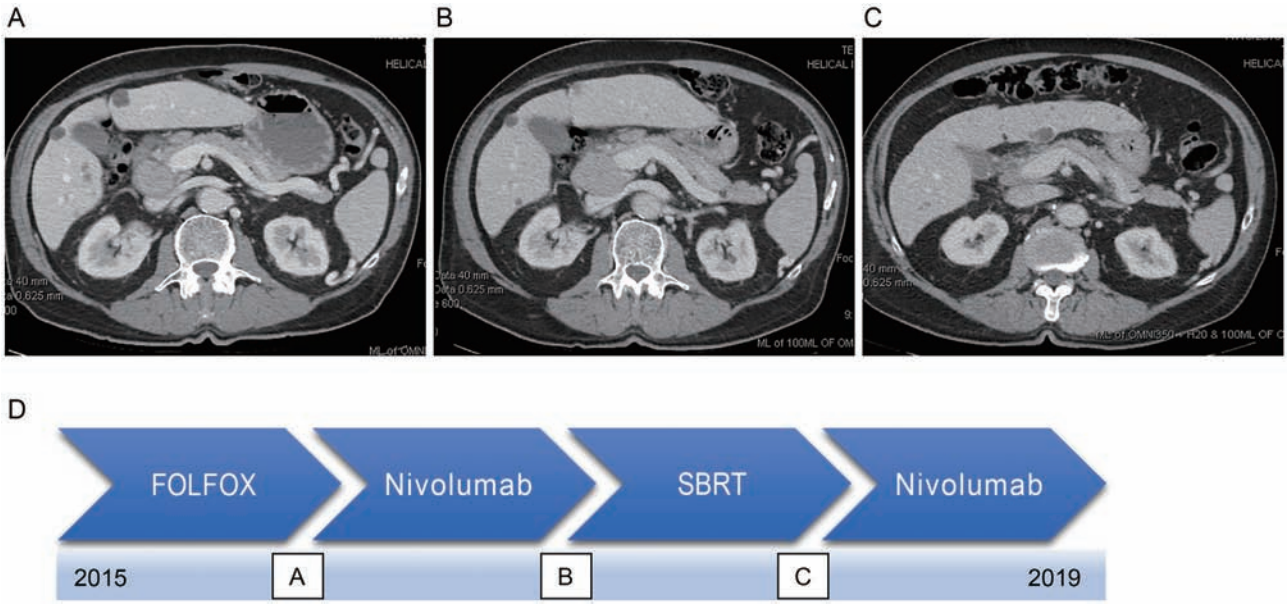

Fig. 4. Timeline of treatment course and corresponding radiographic findings. The following is a timeline of the different treatment modalities from the initiation of chemotherapy in 2015 to completion of nivolumab in the mid-2019s. At corresponding treatment points, there are computed tomography images showing changes in the patient's porta hepatic lymphadenopathy. These time points occur prior to initiation of nivolumab therapy (A), prior to initiation of SBRT (B), and after completion of SBRT (C). In each intervening computed tomography scan, the conglomerate porta hepatis lymph nodes change from 6.8 cm × 4.6 cm to 7.7 cm × 5.9 cm to 3.7 cm × 2.9 cm, respectively.

veillance imaging continues to show stable disease without new or progressive disease over 1 year from his last dose of nivolumab and over 2 years since completion of SBRT.

Discussion

LEL-HCC represents a unique and rare subtype of HCC, with limited clinical evidence to guide systemic treatment options in advanced unresectable disease. The use of ICIs in this tumor type has not previously been described, though its potential efficacy has been previously postulated based on the distinct pathologic features of this tumor that are known to promote an immune-sensitizing tumoral milieu as well as potentiate ICI response in other tumor types. We describe a case of a patient with LEL-HCC who had durable disease control on ICI therapy, with stable disease persisting for over 4 years since ICI initiation and continuing off this therapy for over a year. The clinical course of his disease also demonstrates the potential utility of radiotherapy at local progression as a useful modality to supplement ICI treatment in LEL-HCC.

As an entity first described in 1995, LEL-HCC is defined by its histologic findings of undifferentiated large epithelial cells with prominent nucleoli that commonly stain positive for pankeratin (i.e. AE1/AE3) and hepatocyte specific antigen (i.e. HepPar1) and are intermixed with abundant lymphocytes primarily comprised of CD4 and CD8 T cells.[7,8] Clinically, patients with LEL-HCC have been thought to have a more favorable prognosis than those with HCC, though our understanding of this disease is limited by its rarity and lack of published data. In the largest analysis of known cases, the epidemiologic, demographic, clinical characteristics and outcomes of 66 patients with LEL-HCC were evaluated.[8] In this retrospective review of published cases, the median age at diagnosis was 58 years and the majority of patients were male (64%) and white (65%). Likewise, half of the patients with LEL-HCC had liver cirrhosis, 40% had hepatitis B virus infection, and 34% had HCV infection. Most patients had very early stage disease, with 88% having a single focal lesion (the median size of which was 38 mm), and consequently 91% of patients underwent surgical resection, with the remaining undergoing orthotopic liver transplantation. Though outcome data in this study are not available for all patients, review of individual retrospective case-series included in this study suggests a relatively low recurrence rate after surgery (of less than 9.1%) and 5-year survival rate of 94.1% to 100%.[9,10]

While LEL-HCC is associated with a relatively favorable prognosis when diagnosed at early stages, there is a lack of clinical evidence guiding the disease course or treatment strategies in the advanced, non-operable setting. In the surgically operable setting, attempts have been made to incorporate post-operative chemotherapy; however, the benefit of this therapy is unclear in the adjuvant setting and cannot be reliably extrapolated to the metastatic setting.[11–13] Nonetheless, chemotherapy has been proven to be efficacious in lymphoepithelial-like carcinomas (LELCs) of other anatomic locations. For example, a patient with LELC of the breast had a profound response to docetaxel, doxorubicin, and cyclophosphamide and incorporation of chemotherapy with surgery and radiation has yielded complete, durable responses in head and neck LELC patients.[14,15]

The use of ICIs in the treatment paradigm of LEL-HCC has not been previously described. One case report describes a 37 year-old woman with pulmonary LELC who was treated with nivolumab after progressive disease on systemic chemotherapy.[16] Unfortunately, she died from complications shortly after starting nivolumab, so the effectiveness of this therapy is difficult to discern. Despite the paucity of clinical evidence, there are biologically plausible reasons to suggest potential efficacy of ICIs in LEL-HCC. Unlike conventional HCC, LEL-HCC has an increased intratumoral proportion of predominantly cytotoxic lymphocytes, which have been predictive of ICI response in various tumor subtypes. Likewise, although PD-L1 tumor expression has not been predictive of benefit of ICIs in HCC, there is

evidence of elevated expression in LELCs, as in our patient, which may underlie potential ICI effectiveness as in other specific tumor subtypes.[17]

In addition to the above histologic findings that could suggest plausible benefit from ICIs in LEL-HCC, genomic comparisons between LEL-HCC and HCC have revealed mutational differences that suggest LEL-HCC may be more immunogenic and therefore potentially more susceptible to ICIs. Whole exome sequencing comparisons between conventional HCC and LEL-HCC have identified decreased overall nucleotide variants in specific genes of the Wnt/β-catenin and Notch signaling pathways as well as focal amplification of chromosome 11q13.3 in LEL-HCC.[18] The Wnt/β-catenin and Notch signaling pathways are mechanistically important in driving an immunosuppressive tumoral milieu, and chromosome 11q13.3 is strongly associated with an immune checkpoint efficacy signature.[19,20] These somatic alterations in LEL-HCC suggest the potential benefit of immunotherapy. Together, the histopathologic, molecular and genomic features of LEL-HCC suggest a strong rationale for applicability and feasibility of ICIs in this tumor type.

Conclusions

We describe a patient with unresectable, advanced LEL-HCC who had evidence of sustained disease stability after starting nivolumab monotherapy. This case highlights a novel, previously undescribed treatment approach for patients with LEL-HCC in whom no supporting treatment data exist in the inoperable and non-transplant setting. The patient's durable disease control on ICI therapy in the context of the distinctive immunogenic pathologic features of this tumor suggest that there may be a broader role for ICI therapy in patients with LEL-HCC. However, as with any single case report in a rare, poorly-understood disease, it is difficult to definitely distinguish between causative treatment effect or the clinical spectrum of disease phenotype. Going forward, a collaborative, multi-institutional registry database is needed for patients with LEL-HCC to better understand the clinical course of this extremely rare hepatic neoplasm as well as the uptake and effectiveness of potential treatments.

Funding

None to declare.

Conflict of interest

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

Author contributions

Concept (DDS), data collection (DJH, DDS), drafting and writing of the manuscript (DJH), critical revision of the manuscript for important intellectual content (EZD, RL, CTF, DSS).

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Journal of Clinical and Translational Hepatology **2021** vol. 9(2) | 269–273
DOI: 10.14218/JCTH.2020.00129

Case Report

Rapid Recovery in COVID-19 Patients with Chronic Hepatitis B Virus Infection Treated with Tenofovir Disoproxil Fumarate

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Received: 25 November 2020 | **Revised:** 26 December 2020 | **Accepted:** 20 February 2021 | **Published:** 9 April 2021

Abstract

The coronavirus disease 2019 (COVID-19) pandemic continues worldwide. We report here two cases of chronic hepatitis B patients with acute respiratory syndrome coronavirus 2 infection treated with tenofovir disoproxil fumarate who demonstrated a favorable outcome. This report adds some evidence that concurrent HBV infection may not worsen COVID-19 infection and tenofovir disoproxil fumarate treatment may have partial positive effect on COVID-19 rapid recovery.

Citation of this article: Chen X, Liu D, Yang D, Zheng X. Rapid recovery in COVID-19 patients with chronic hepatitis B virus infection treated with tenofovir disoproxil fumarate. J Clin Transl Hepatol 2021;9(2):269–273. doi: 10.14218/JCTH.2020.00129.

Introduction

The coronavirus disease 2019 (COVID-19) pandemic, caused by acute respiratory syndrome coronavirus 2 (SARS-CoV-2), continues worldwide. As of on February 2, 2021, there are 102,942,987 confirmed cases and 2,232,233 deaths according to a report from the World Health Organization (WHO).

COVID-19 patients present with fever, cough, and dyspnea, with computed tomography scan displaying ground glass opacities (GGO) and bilateral lung infiltrates.[1] The critical factors associated with the severity and mortality in COVID-19 patients were considered to be advanced age and underlying diseases, such as diabetes and cardiovascular and cerebrovascular diseases.

The WHO has estimated that 257 million people had chronic hepatitis B infection by 2015, and many of them have progressed to end-stage liver disease. Data from two large cohorts showed that 0.1–2.1% of COVID-19 patients have hepatitis B virus (HBV) coinfection;[1,2] however, the clinical evidence of SARS-CoV-2 and HBV coinfection on the severity and outcome of COVID-19 is very limited. Most studies have illustrated that HBV coinfection does not aggravate the disease, while a few studies reported adverse results.[3,4] Here, we report two cases of chronic hepatitis B (CHB) patients with SARS-CoV-2 infection treated with tenofovir disoproxil fumarate (TDF) who demonstrated a favorable outcome.

Case report

Case 1

A 76 year-old male who lived in Wuhan developed a fever on February 6, 2020, with a maximum body temperature of 39.5°C, accompanied by chills, cough, chest distress and fatigue. He first presented to the fever clinic on February 11 because the symptoms had not self-resolved. Admission test results showed that his white blood cell (WBC) count was normal, while his lymphocyte count was low (decreased to 1.0×10^9/L); the chest computed tomography scan demonstrated typical viral pneumonia features. He was prescribed ceftezole, ribavirin, and lianhua qingwen capsules for 2 days. On February 13, his oropharyngeal swab test for SARS-CoV-2 RNA was positive, and he was admitted to the isolation ward. Based on his Wuhan resident history, fever symptoms, SARS-CoV-2 nucleic acid test result, and the computed tomography report, the patient was diagnosed with COVID-19.

The patient had a history of bronchiectasis and chronic obstructive pulmonary disease (COPD) for more than 30 years, type 2 diabetes for 15 years, and CHB for more than 20 years. He had undergone successive treatment with lamivudine (LAM), then LAM plus adefovir dipivoxil (ADV) for more than 10 years, and then switched to TDF within the last 2 years. Serologic tests showed that he was HBV DNA-negative, and hepatitis B surface antigen-, hepatitis B e antibody-, and hepatitis B core antibody-positive.

Keywords: COVID-19; SARS-CoV-2; Chronic hepatitis B; Tenofovir disoproxil fumarate.

Abbreviations: ADV, adefovir dipivoxil; ALT, alanine aminotransferase; ANPs, acyclic nucleoside phosphonates; AST, aspartate aminotransferase; CHB, chronic hepatitis B; CK, creatine kinase; COPD, chronic obstructive pulmonary disease; COVID-19, coronavirus disease 2019; CRP, c-reactive protein; ESR, erythrocyte sedimentation rate; GGO, ground glass opacities; HBV, hepatitis B virus; hsCRP, hypersensitivity CRP; IFN, interferon; IL, interleukin; ISGs, interferon-stimulated genes; LAM, lamivudine; LDH, lactate dehydrogenase; LPS, lipopolysaccharide; Myo, myoglobin; RdRp, RNA complex RNA polymerase; SARS-CoV-2, acute respiratory syndrome coronavirus 2; TDF, tenofovir disoproxil fumarate; TNF, tumor necrosis factor; WBC, white blood cell; WHO, World Health Organization.

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On admission, the patient complained of dyspnea and chest distress after exercise. He showed a pulse of 106 beats per minute and oxygen saturation of 92% while breathing ambient air. After administration of oxygen therapy, delivered by nasal cannula at 3 L per minute, his oxygen saturation values increased up to 97%.

After admission, the patient's lymphocyte count was decreased to 0.89×10^9/L and the D-dimer level was elevated to 4.52 mg/L. Alanine aminotransferase (ALT) level was 60 U/L and aspartate aminotransferase (AST) level was 135 U/L. The levels of myocardial enzymes, including creatine kinase (CK), lactate dehydrogenase (commonly referred to as LDH), and myoglobin (referred to as Myo), were elevated (CK of >1,300 U/L, LDH of 497 U/L, and Myo of 287.7 μg/L). C-reactive protein (commonly referred to as CRP) levels were increased significantly (to 168 mg/L), as was erythrocyte sedimentation rate (commonly referred to as ESR) (to 61 mm/h); procalcitonin was normal. Cytokine test indicated that the level of interleukin (IL)-6 was elevated (to 45.84 ng/mL) (Table 1). The levels of lymphocyte subsets, immunoglobulin, and complement were normal. Computed tomography scan of the lungs and the abdomen demonstrated that scattered GGO were present in both lungs, especially in the subpleural area; dense strips were seen in the middle lobe of the right lung and the lingula of the left lung. Emphysema of bilateral lungs and atherosclerosis of coronary and aortic vessels were observed. Liver cirrhosis was suspected in this patient, due to widening of liver fissures and atrophy of the left lobe of the liver (Fig. 1A, D).

The patient had moderate fever in the first 4 days after admission, with a maximum body temperature of 38.5°C, which was controlled by physical cooling. The patient's symptom of chest distress and dyspnea lasted for 6 days, with the oxygen saturation remaining above 95% on nasal oxygen delivery at 3 L/min. The patient demonstrated intermittent cough and gradual improvement. He was re-tested for SARS-CoV-2 nucleic acid on February 20 and February 22 respectively, and the results were both negative. His chest computed tomography scans on February 22 and February 28 indicated that the lesion was gradually decreasing in size (Fig, 1B, C). Laboratory results demonstrated an improvement in lymphocyte count, ALT, AST, CK, hypersensitivity CRP (referred to as hsCRP), IL-6 (Table 1), and SARS-CoV-2-IgM; IgG test on February 29 was positive. On March 2, the patient was discharged after 18 days of hospitalization and was recommended self-isolation for at least 14 days.

In hospital, he was administered arbidol (200 mg three times daily, oral) as antiviral therapy, ceftizoxime (2 g every 8 h, intravenous) to control lung infection, vitamin C (2 g once daily, intravenous) as antioxidant, and magnesium isoglycyrrhizinate (150 mg once daily, intravenous) to improve liver function. He was also administered acarbose (50 mg three times daily, oral) and gliclazide (60 mg once daily, oral) to control blood glucose level, TDF (300 mg once daily, oral) as an anti-HBV medicine, and traditional Chinese medicine No. 2 according to the Guidelines of the Diagnosis and Treatment of COVID-19 (version 5) published by the National Health Commission of China.

Case 2

A 32 year-old male community worker in Wuhan, who had close contact with COVID-19 patients for 3 weeks due to work requirements, was required by the government to visit a fever clinic to rule out SARS-CoV-2 infection on February 13. He did not exhibit any symptoms of fever, cough, or fatigue. His computed tomography scan showed single small GGO under the pleura in the middle lobe of the right lung (Fig. 1E). Counts for WBC and lymphocytes, and tests for liver function, kidney function, and hsCRP on February 14 were normal. SARS-CoV-2 nucleic acid and IgM tests for mycoplasma pneumoniae, chlamydia pneumoniae, syncytial virus, adenovirus, and coxsackie virus were negative. The SARS-CoV-2 nucleic acid was rechecked on February 15, and the results suggested that the 2019-nCoV open reading coding frame lab (nCoVORFlab) was positive and 2019-nCoV-N gene was negative. Thus, the patient was classified with asymptomatic COVID-19 infection and was admitted to the mobile cabin hospital for isolation.

The patient had received hepatitis B e antigen-positive CHB diagnosis decades prior. He had showed elevated ALT and AST, jaundice, and HBV DNA up to 10^7 IU/mL the past June, when computed tomography of the abdomen had also suggested fatty liver. He underwent antiviral treatment with TDF (300 mg once daily, oral) last June and his HBV DNA load had dropped to less than 100 IU/mL last November. After that, he continued to take TDF daily.

After admission, the patient was administered arbidol (200 mg three times daily, oral) as antiviral therapy, moxifloxacin (400 mg once daily, oral) to prevent secondary infection, lianhua qingwen capsules (4 capsules three times daily, oral) and traditional Chinese medicine No. 2 (twice daily, oral). His SARS-CoV-2 nucleic acid tests on February 29, March 1, and March 2 were negative and lung computed tomography performed on March 2 demonstrated no obvious abnormalities (Fig. 1F). He was discharged on March 3 and recommended self-isolation for at least 14 days.

Discussion

The two cases we report herein involve CHB patients taking TDF with concurrent COVID-19 infections. Case 1 was an elderly male with multiple underlying illnesses, and the patient had an immunocompromised status. On admission, the low oxygen saturation values and significantly abnormal laboratory results classified this patient as a severe case, while the lung lesions on computed tomography were relatively less severe. After 7 days of hospitalization, his SARS-CoV-2 nucleic acid had rapidly changed to negative, with an improvement in laboratory results and lung lesions. Prior to discharge, his SARS-CoV-2-IgM and IgG statuses were confirmed to be positive as well. As an immunocompromised patient, his progression of COVID-19 was not very severe and he achieved a relatively quick recovery. Case 2 was an asymptomatic infection diagnosed by positive SARS-CoV-2 nucleic acid and single small GGO on lung computed tomography. His SARS-CoV-2 nucleic acid results changed to negative and the GGO in lung computed tomography disappeared after treatment, while laboratory results, such as those for lymphocyte count, liver and renal function, and hsCRP, were normal throughout the course of his illness. Although the patient had underlying liver disease, his condition was very mild.

TDF, a nucleotide reverse transcriptase inhibitor recommended as one of the most potent drugs to suppress HBV and a first-line anti-human immunodeficiency virus drug by the WHO, has been well-documented to have a role in regulating immunity. Studies have shown that after 12 months of TDF treatment, the frequency and function of natural killer (CD56+CD3-) cells in CHB patients were significantly increased compared with baseline,[5] suggesting that TDF might contribute to the activation of natural killer cells. In addition, studies have found that the nucleotide analogues (TDF and ADV) might have an effect on inducing interferon (IFN)-λ3 compared to nucleoside analogs (LAM and entecavir) both *in vitro* and *in vivo*.[6] IFN-λ3 can induce phosphorylation and up-regulate the expression of interferon-stimulated genes (commonly referred to as ISGs)

Table 1. Symptoms, treatments and laboratory results of case 1

Date	2.13	2.14	2.15	2.16	2.17	2.18	2.19	2.20	2.21	2.22	2.23	2.24	2.25	2.26	2.27	2.28	2.29	3.1	3.2
Symptom																			
Fever, °C	38.1	38.3	38.5	37.7															
Dyspnea	√	√	√	√	√	√													
Cough	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√		
Treatment																			
Ceftizoxime		√	√	√	√	√	√	√	√	√	√	√	√	√	√				
Arbidol		√	√	√	√	√	√	√	√	√	√								
Vitamin C		√	√	√	√	√	√	√	√	√	√	√	√	√	√				
Magnesium isoglycyrrhizinate		√	√	√	√	√	√	√	√	√	√	√	√	√	√				
Chinese herbal											√	√	√	√	√	√	√	√	√
Acarbose and gliclazide										√	√	√	√	√	√	√	√	√	√
TDF	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√
Oxygen therapy at 3 L/m	√	√	√	√	√	√	√	√	√	√	√	√	√	√					
Laboratory results																			
WBC count as $\times 10^9$/L	–	7.68	–	–	–	–	–	5.74	–	–	–	7.04	–	5.88	–	–	–	–	–
Neutrophil count as $\times 10^9$/L	–	6.31	–	–	–	–	–	4.31	–	–	–	5.04	–	4.14	–	–	–	–	–
Lymphocyte count as $\times 10^9$/L	–	0.89*	–	–	–	–	–	0.76*	–	–	–	1.28	–	1.11	–	–	–	–	–
Albumin	–	31.9*	–	–	–	–	–	32.7*	–	–	–	–	–	–	–	–	–	–	–
Globulin	–	30.3#	–	–	–	–	–	32.7#	–	–	–	–	–	–	–	–	–	–	–
ALT in U/L	–	60#	–	–	–	–	–	49#	–	–	–	–	–	–	–	–	–	–	–
AST in U/L	–	135#	–	–	–	–	–	37	–	–	–	–	–	–	–	–	–	–	–
CK in U/L	–	>1300#	–	–	–	–	–	107	–	–	–	–	–	–	–	–	–	–	–
LDH in U/L	–	497#	–	–	–	–	–	255#	–	–	–	–	–	–	–	–	–	–	–
Myo in μg/L	–	287.7#	–	–	–	–	–	–	–	–	–	28.3	–	–	–	–	–	–	–
CRP in mg/L	–	168#	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
hsCRP in mg/L	–	–	–	–	–	–	–	28.86#	–	–	–	0.95	–	5.95#	–	–	–	–	–
Procalcitonin in μg/L	–	<0.13	–	–	–	–	–	0.07	–	–	–	–	–	–	–	–	–	–	–
ESR in mm/h	–	–	–	–	–	–	–	61#	–	–	–	–	–	60#	–	–	–	–	–
IL-2 in mg/L	–	2.88	–	–	–	–	–	–	–	–	–	–	–	5.22#	–	–	–	–	–
IL-4 in mg/L	–	1.95	–	–	–	–	–	–	–	–	–	–	–	9.75#	–	–	–	–	–
IL-6 in mg/L	–	45.48#	–	–	–	–	–	–	–	–	–	–	–	10.4#	–	–	–	–	–
IL-10 in mg/L	–	3.98	–	–	–	–	–	–	–	–	–	–	–	6.64#	–	–	–	–	–
IFN-α in mg/L	–	2.96	–	–	–	–	–	–	–	–	–	–	–	4.79	–	–	–	–	–
IFN-γ in mg/L	–	4.9	–	–	–	–	–	–	–	–	–	–	–	5.53	–	–	–	–	–

√ represents the duration of symptoms and treatments. * represents values below limits. # represents values above limits.

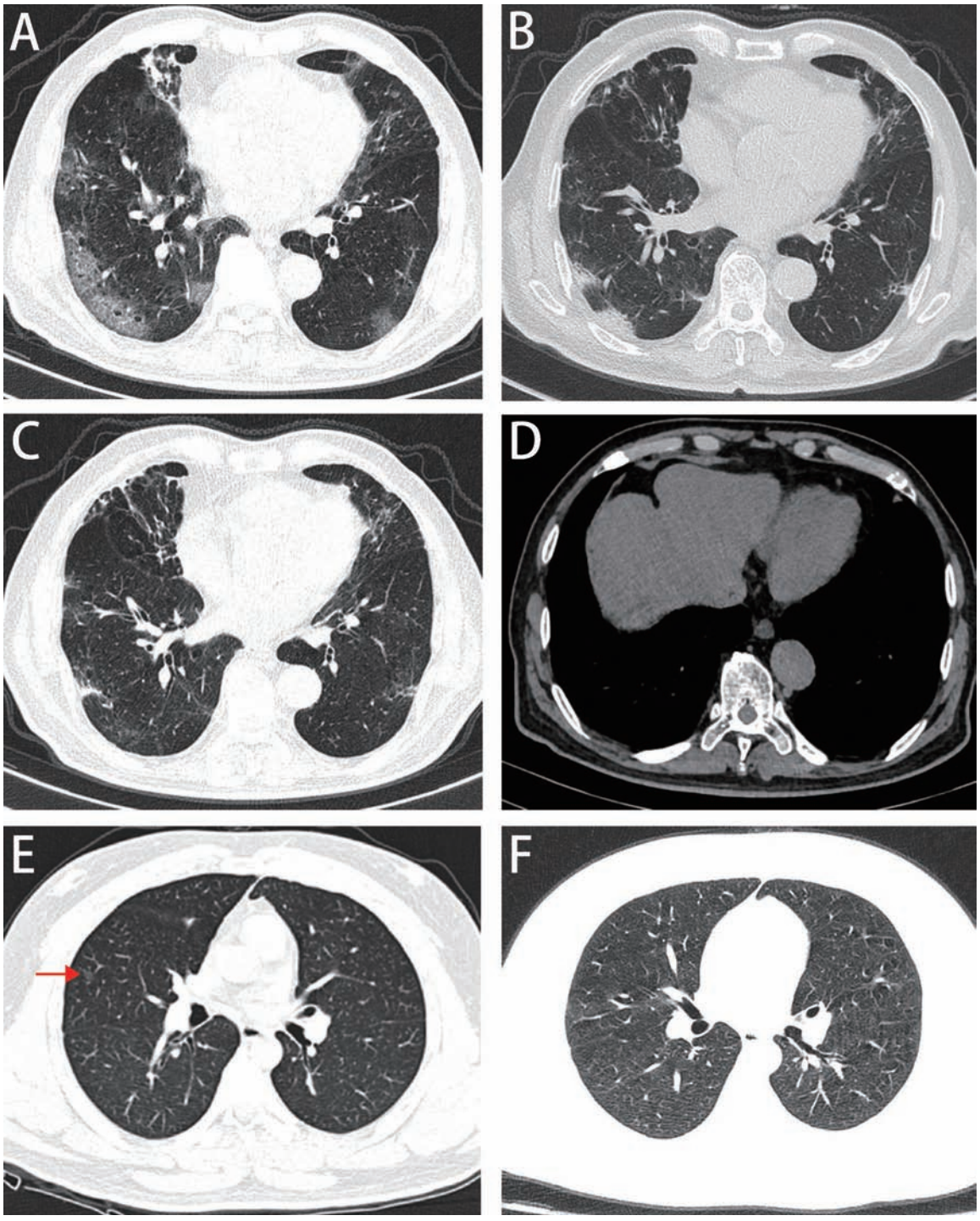

Fig. 1. Lung and abdomen CT scans of case 1 and case 2. (A–D) Case 1: Lung CT scans on February 14 (A), February 22 (B), and February 28 (C), and abdomen CT scan on February 14 (D). (E–F) Case 2: Lung CT scans on February 13 (E) and March 2 (F). CT, computed tomography.

and produce some antiviral proteins to exert antiviral effects.[7] Studies have revealed that cellular metabolites of acyclic nucleoside phosphonates (commonly referred to as ANPs, including TDF and ADV) can inhibit lipopolysaccharide (commonly referred to as LPS)-mediated production of IL-10 and, thus, induce the production of IL-12p70 and tumor necrosis factor-α (commonly known as TNF-α) in a dose-dependent manner, and plays an immunoregulatory role in HBV patients, with antiviral and antihepatocellular carcinoma properties.[8]

A recent study showed that tenofovir binds tightly to the SARS-CoV RNA complex RNA polymerase (commonly known as RdRp) and terminates the RNA synthesis catalyzed by SARS-CoV-2 RdRp,[9] providing the molecular basis

for tenofovir to be considered as a potential therapeutic for COVID-19. In addition, a large-scale cohort study conducted in Spain found that the incidence of SARS-CoV-2 infection in patients with CHB treated with tenofovir decreased (0.4%, 8/1,764), which indirectly reflects TDF's positive effect on the resistance to SARS-CoV-2.[3]

Lastly, patients with COVID-19 often exhibit immune system dysfunction, such as lymphopenia, decreased number of CD4+ T cells, and abnormal levels of cytokines (including cytokine storms), and this might be an indicator related to severity and mortality of the disease. Immunocompromised patients, such as the elderly, and patients with other comorbidities, might be more susceptible to SARS-CoV-2. We speculated that according to our SARS-CoV-2-infected patients with CHB treated with TDF; in addition to inhibiting the SARS-CoV-2 RdRp, the TDF medication might also improve the immune functions of these patients by restoring the activity levels of T cells and natural killer cells, inducing IFN-λ3 production, inhibiting IL-10 secretion, and inducing IL-12 production, thereby suppressing a cytokine storm caused by SARS-CoV-2. It might also have certain antiviral properties. Ultimately, TDF might alleviate the symptoms and shorten the course of COVID-19 in patients with CHB.

In conclusion, our observation from these two cases provided more evidence that concurrent HBV infection may not worsen COVID-19 infection and TDF treatment may partially contribute to a more rapid recovery from COVID-19. Large-cohort clinical studies are needed to subsequently explore the effects of HBV and TDF treatment on the clinical outcomes of COVID-19.

Funding

This work was supported by the Applied Basic and Frontier Technology Research Project of Wuhan (grant no. 2020020601012233) and the Fundamental Research Funds for the Central Universities (grant no. 2020kfyXGYJ016).

Conflict of interest

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

Author contributions

Study concept and design (XZ), acquisition of data (XC), analysis and interpretation of data (DY, XZ), drafting of the manuscript (XC), critical revision of the manuscript for important intellectual content (DL, XZ), administrative, technical, or material support, study supervision (XZ).

Data sharing statement

All data are available upon request.

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Journal of Clinical and Translational Hepatology **2021** vol. 9(2) | 274–275
DOI: 10.14218/JCTH.2021.00074

Letter to the Editor

Celiac Disease and Elevated Liver Enzymes: A Still Not Fully Defined Pathogenesis

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Received: 20 February 2021 | **Revised:** 13 March 2021 | **Accepted:** 21 March 2021 | **Published:** 7 April 2021

Citation of this article: Beenet L, Tonesi D. Celiac disease and elevated liver enzymes: A still not fully defined pathogenesis. J Clin Transl Hepatol 2021;9(2):274–275. doi: 10.14218/JCTH.2021.00074.

Dear Editor,

We read, with great interest, the comprehensive review by Villavicencio Kim J and Wu GY that systematically addressed the issue of liver enzyme elevation in celiac disease (CD) patients.[1]

The Authors reviewed, in detail, the most relevant studies reporting the frequency of liver enzyme elevation in CD patients and the possible causes, discussing the hypothesis that this elevation may be a clue to associated liver disease or an epiphenomenon, possibly secondary to the increased intestinal permeability that is known to characterize CD patients, especially at diagnosis, before starting a gluten-free diet.[2]

We would like to add some considerations that, in our opinion, could have implications in the pathogenesis of hepatic injury in CD.

As known, it has been reported that CD is frequently associated with other extraintestinal autoimmune diseases or even with the mere presence of autoantibodies without concomitant autoimmune pathology.[3–6]

Among the autoimmune diseases potentially associated with CD, autoimmune hepatitis (AIH) is worthy of mention, as previously reported.[1,3,7]

Of considerable interest, it has been reported that celiac patients frequently have anti-filamentous actin IgA antibodies that have shown reliable and significant correlation with villous atrophy.[8] These autoantibodies, although of IgG class, are also known to have very high specificity for AIH.[7,9]

This similarity between the two autoimmune diseases could be a clue that also supports possible immune-mediated pathogenesis of hypertransaminasemia in CD patients. Therefore, it would be relevant and worthy of study to analyze the presence of anti-actin antibodies in CD patients to verify whether these antibodies are markers of liver injury.

Finally, it should not be overlooked, the very remarkable issue of the potential development of hepatic steatosis, which, as appropriately mentioned by the Authors, is not uncommon in CD patients with celiac disease after starting a gluten-free diet.[10]

Funding

None to declare.

Conflict of interest

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

Author contributions

Performance of the research, collection and analysis of the data, writing of the paper, and approving the final version of the article, including the authorship list (LB, DT), guarantor of the article (LB).

Data sharing statement

All data are available upon request.

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Abbreviations: CD, celiac disease; AIH, autoimmune hepatitis.

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Journal of Clinical and Translational Hepatology **2021** vol. 9(2) | 276–278
DOI: 10.14218/JCTH.2021.00011

Letter to the Editor

Favipiravir-induced Liver Injury in Patients with Coronavirus Disease 2019

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Received: 4 January 2021 | **Revised:** 10 March 2021 | **Accepted:** 23 March 2021 | **Published:** 15 April 2021

Citation of this article: Kumar P, Kulkarni A, Sharma M, Rao PN, Reddy DN. Favipiravir-induced liver injury in patients with coronavirus disease 2019. J Clin Transl Hepatol 2021;9(2):276–278. doi: 10.14218/JCTH.2021.00011.

Favipiravir, an antiviral, was given restricted emergency use approval to treat coronavirus disease 2019 (COVID-19) in many countries. While the clinical efficacy of favipiravir in COVID-19 remains uncertain, the approval was based on findings from *in vitro* studies and a clinical trial.[1] Limited data from studies of the Ebola virus and influenza disease showed a favorable safety profile.[2] Herein, we provide the first report of drug-induced liver injury (DILI) due to favipiravir in patients treated for COVID-19.

The first patient is a 70-year-old female who presented with 4 days of abdominal pain and jaundice. Historically, she received tab Favipiravir for mild COVID-19 illness for 2 weeks. She denied having taken any other medications or herbal supplements, or alcohol intake. She was icteric, and laboratory evaluation revealed a cholestatic liver chemistry pattern (Table 1). Hepatitis A/B/C/E serologies, autoimmune markers, ceruloplasmin, and serologies for Epstein-Barr/Herpes simplex/cytomegalovirus, hepatic Doppler ultrasound were all negative/normal. A percutaneous liver biopsy showed moderate hepatocellular cholestasis with bilirubinostasis and mild inflammation comprised of lymphocytes with few eosinophils in the portal tracts (Fig. 1). The patient was treated with ursodeoxycholic acid (15 mg/kg), and liver biochemistry normalized after 10 weeks. In the absence of other etiologies, bland cholestasis on liver biopsy, and Roussel Uclaf causality assessment method (RUCAM) score of 7, consistent with probable DILI, the diagnosis of favipiravir-induced acute cholestatic jaundice was made.

The second patient is a 52-year-old female with essential hypertension, who presented with 5 days of jaundice and fatigue. She was treated with 12 days of tab favipiravir for mild COVID-19 illness. Aside from tablet paracetamol, the patient cited not taking any other medications or alcohol. Laboratory evaluation revealed markedly elevated liver enzymes, and workup for other causes of liver injury, as described previously, were negative (Table 1). The patient denied a liver biopsy and was treated with ursodeoxycholic acid (15 mg/kg). The patient made an uneventful recovery, and liver chemistries normalized after 4 weeks. Diagnosis of favipiravir-induced acute hepatitis was made with a RUCAM score of 7, consistent with probable DILI.

The third patient is a 50-year-old male with hepatitis B-related cirrhosis on tab entecavir, who presented with a 2-week history of abdominal distension and jaundice. The patient received tab favipiravir for 2 weeks for mild COVID-19 illness. The patient denied having taken any other medications or alcohol. The evaluation showed cholestatic liver chemistry, with a negative hepatitis B DNA titer. A computerized tomography scan showed evidence of cirrhosis with portal hypertension (Table 1). Workup for other causes of liver injury, as described for the first case, was negative. The patient was managed with diuretics, ursodeoxycholic acid, and other supportive medication. His symptoms and liver chemistries improved over the next 6 weeks. The diagnosis of acute decompensation of hepatitis B-related cirrhosis with acute cholestatic jaundice due to favipiravir was made with a RUCAM score of 7, consistent with probable DILI.

The unprecedented COVID-19 global pandemic has led to the rapid repurposing of investigational antiviral drugs, like favipiravir. The oral prodrug favipiravir is a purine nucleoside analogue; tje active metabolite favipiravir ibofuranosyl-5′-triphosphate inhibits RNA-dependent RNA polymerases of systemic acute respiratory syndrome coronavirus-2 (SARS-CoV-2).[3] It is metabolized in the liver by aldehyde oxidase and partially to a hydroxylated form by xanthine oxidase. Mild self-limiting transaminase elevation was reported in 2.1% of patients.[2,4] However, icteric presentation has never been reported in the English literature, to our knowledge. We suspected favipiravir-induced DILI in our cases because of latency timing, liver biopsy findings, exclusion of alternative causes, and a complete resolution with dechallenge. Liver enzyme abnormalities are also common in patients with COVID-19 and rarely progress to acute hepatitis. However, our patient's delayed presentation after COVID-19-related symptom resolution and normal liver biochemistry at baseline rule out this possibility. Although the exact mechanism of liver injury is unknown, the liver injury could be due to an idiosyncratic reaction to favipiravir or its metabolites. Also, we speculate that a higher dose might be responsible for liver injury. The wide gap between half-cytotoxic concentration (>400 μM) and half-maximal effective concentration (61.88 μM) against SARS-CoV-2 gives a comfortable safety margin, even with a high dose of favipiravir.[3] However, an increased intracellular con-

Abbreviations: COVID-19, coronavirus disease 2019; DILI, drug-induced liver injury; RUCAM, Roussel Uclaf causality assessment method; SARS-CoV-2, systemic acute respiratory syndrome coronavirus-2.

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Table 1. Laboratory findings at presentation for the patients with favipiravir-induced liver injury

Parameter	Patient 1	Patient 2	Patient 3
Hemoglobin in g/dL	9.6	12.6	12.2
Total leucocyte count/μL	10,500	11,900	5,000
Differential count, %	N68/L26/E2/M4	N84/L8/E1/M7	N64/L24/E4/M8
Platelets/μL $\times 10^3$	3.3	2	1.4
Urea in mg/dL	42	40	45
Creatinine in mg/dL	1.4	0.9	1.1
Total /direct bilirubin in mg/dL	29.8/21	12.5/9.3	4.7/2.7
Aspartate transaminase <40 U/L	200	1,265	456
Alanine transaminase <40 U/L	352	2,031	337
Alkaline phosphatase 30-120 U/L	606	362	804
Protein in g/dL	5.2	6.2	5.3
Albumin in g/dL	3.5	3.8	2.8
International normalized ratio	1.2	1	1.1
Hospitalization	Yes	Yes	Yes
Liver chemistry before starting tab favipiravir	Normal	Normal	Normal
Liver injury pattern	Cholestatic	Hepatocellular	Cholestatic
Latency period in days	18 days	12 days	14 days
Favipiravir dose & duration	3,600 mg on day 1 followed by 1,600 mg/day for 14 days	3,600 mg on day 1 followed by 1,600 mg/day for 12 days	3,600 mg on day 1 followed by 1,600 mg/day for 10 days
RUCAM score	7: Probable DILI	7: Probable DILI	7: Probable DILI
DILI severity index	Moderate-severe	Moderate-severe	Moderate-severe
Outcome	Resolution 10 weeks	Resolution 4 weeks	Resolution 6 weeks

DILI, Drug induced liver injury; NA, Not available; RUCAM, Roussel Uclaf causality assessment method.

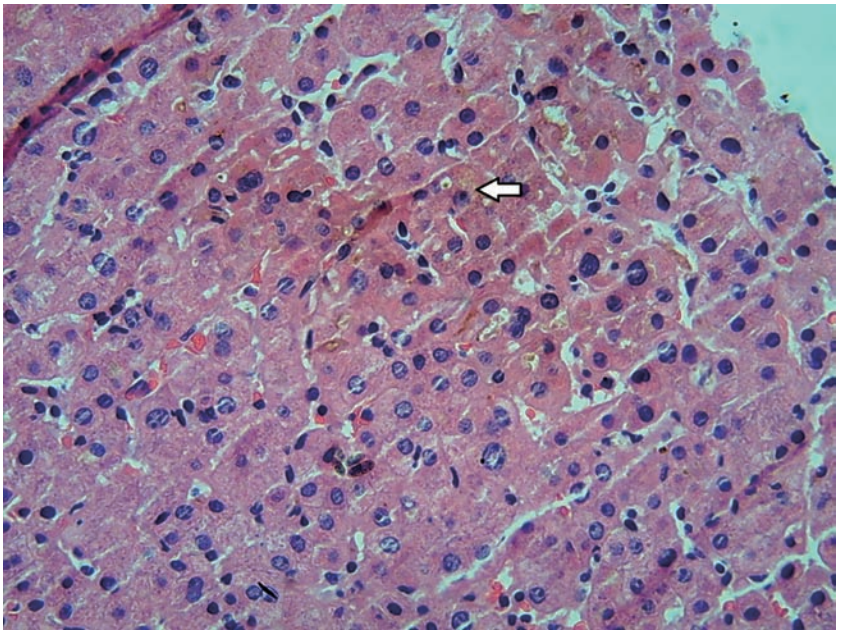

Fig. 1. Liver biopsy with a high-power view of moderate hepatocellular cholestasis (white arrow) with bilirubinostasis.

centration above the toxicity threshold cannot be ruled out owing to more considerable favipiravir plasma exposure in the Asian population, suggesting possible regional or ethnic differences in its pharmacokinetics.[3,5] Besides, continuous use causes self-inhibition of its liver metabolism, which may increase the favipiravir/inactive metabolite ratio. More than a two-fold increase in favipiravir plasma concentrations over half-maximal effective concentration are also predicted.[6] So, close monitoring of cardiac and hepatic function as well as of favipiravir blood concentration is recommended during the treatment period because of a lack of pharmacokinetics and safety data for higher doses.

In conclusion, we present the first report of hepatotoxicity cases in COVID-19 that were most likely due to favipiravir. Further research is needed to identify the related risk factors and mechanisms of liver injury.

Acknowledgments

We thank Dr. Gautam Bachu, Department of Pulmonary Medicine, Dr. Mohan Ramchandani, Department of Gastroenterology, and Dr. Anuradha, Department of Pathology, Asian Institute of Gastroenterology Hospitals, Hyderabad, India.

Funding

None to declare.

Conflict of interest

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

Author contributions

Study conception and design (PK, AK), compilation of information and initial drafting of the manuscript (PK), final editing and critical revision of the manuscript (PK, MS, AK, PNR, DNR). All authors approved the final draft.

Informed patient consent

Provided by all patients presented in this report.

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JCTH

Journal of Clinical and Translational Hepatology [Print ISSN: 2225-0719; Online ISSN: 2310-8819] is owned by the Second Affiliated Hospital of Chongqing Medical University and published by XIA & HE Publishing Inc.

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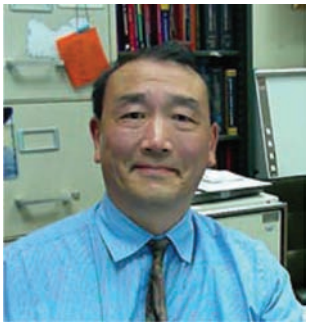

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Updated on April, 2021

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