ISSN 2225-0719

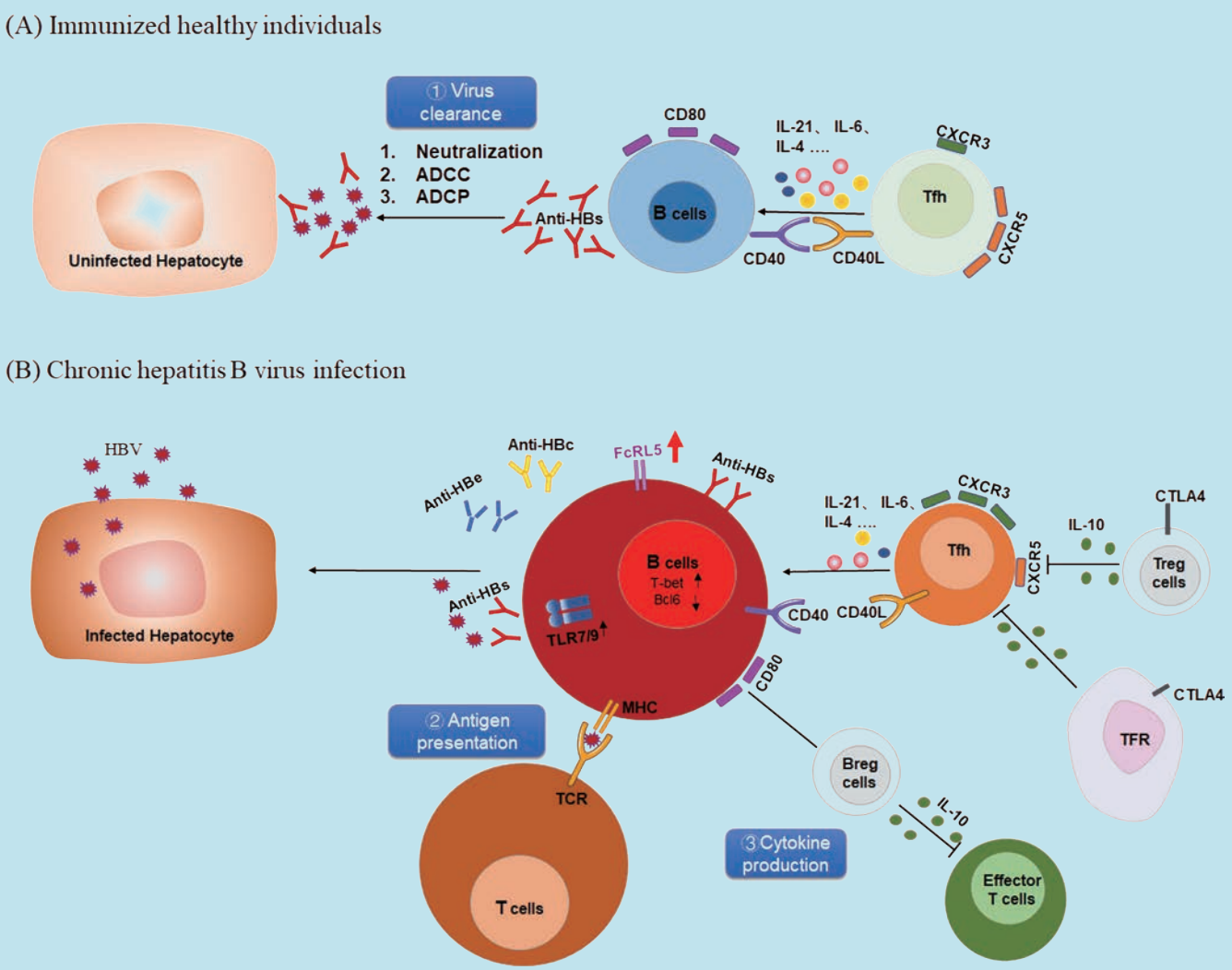
2021

Volume 9 Issue 4

July / August

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)

Contact Information

Editorial Office

Managing Editors: Dr. Huaidong Hu
Dr. Zhi Peng
Dr. Sandeep Kumar Karn
Telephone: +86-23-6370 1383
Fax: +86-23-6370 1383
E-mail: jcth@xiahepublishing.com
Postal Address: 74 Linjiang Road, Yuzhong District, 400010 Chongqing, CHINA

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 4
Publication date: August 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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DOI: 10.14218/JCTH.2021.ANNOUNCE

Announcement

Journal of Clinical and Translational Hepatology Receives Its First Impact Factor (4.108) and CiteScore (6.7)

Harry Hua-Xiang Xia[1], George Y. Wu[2] and Hong Ren[3*]

[1]*Department of Gastroenterology, First Affiliated Hospital, Guangdong Pharmaceutical University, Guangzhou, Guangdong, China;* [2]*Department of Medicine, Division of Gastroenterology-Hepatology, University of Connecticut Health Center, Farmington, CT, USA;* [3]*Institute for Viral Hepatitis, Department of Infectious Diseases, The Second Affiliated Hospital of Chongqing Medical University, Chongqing, China*

Citation of this article: Xia HHX, Wu GY, Ren H. *Journal of Clinical and Translational Hepatology* receives its first impact factor (4.108) and CiteScore (6.7). J Clin Transl Hepatol 2021;9(4):453. doi: 10.14218/JCTH.2021.ANNOUNCE.

We are pleased to announce that *Journal of Clinical and Translational Hepatology* (*JCTH*) has received its first impact factor – 4.108 – in the Journal Citation Report released by Clarivate Analytics on June 30, 2021. *JCTH* now ranks 42nd among 92 journals in the Gastroenterology and Hepatology category and is in the second quartile (Q2) in the category (Fig. 1). Moreover, *JCTH* has also received its first CiteScore – 6.7 – released by Elsevier on June 4, 2021 and ranks 16th among 62 hepatology journals according to that evaluation system (Fig. 2).

JCTH, which is owned by the Second Affiliated Hospital of Chongqing Medical University and published by Xia & He Publishing Inc., publishes articles on both basic and clinical liver disease research. The translational application of basic science is a distinctive feature of the Journal. *JCTH* was launched in 2013 and has been included in PubMed Central and PubMed since July 2015, in Scopus since May 2020, and in SCIE since November 2020.[1]

We would like to take this opportunity to express our heartfelt gratitude to our staff, associate editors, editorial board members, early-career editors, reviewers, authors, and readers, for their dedication, contributions, and attention to the Journal.

Having the first impact factor being over 4 and CiteScore being 6.7 is encouraging and inspiring. However, we recognize these achievements as representing a solid foundation from which we will continue to push forth our efforts toward greater academic goals. In these efforts, we will continue to be rigorous in both academic quality and ethical standards, ever-striving to bring timely research findings in hepatology to the scientific community as an internationally influential journal.

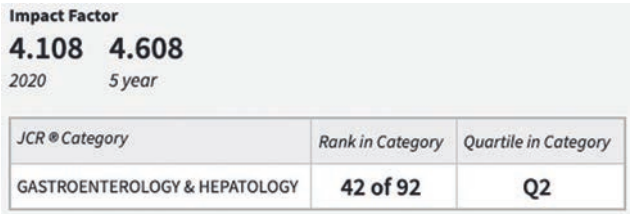

Fig. 1. Impact factor 2020 of *Journal of Clinical and Translational Hepatology* (From Web of Science, Clarivate Analytics).

CiteScore ↓	Highest percentile ↓	Citations 2017-20 ↓	Documents 2017-20 ↓	% Cited ↓
6.7	75% 16/62 Hepatology	1,445	215	68

Fig. 2. CiteScore 2020 of *Journal of Clinical and Translational Hepatology* (From Scopus, Elsevier).

Reference

[1] Xia HHX, Wu GY, Ren H. *Journal of Clinical and Translational Hepatology* has been indexed in SCIE: a milestone towards a greater academic goal. J Clin Transl Hepatol 2020;8(4):357–358. doi:10.14218/JCTH.2020.00138.

Abbreviation: *JCTH*, *Journal of Clinical and Translational Hepatology*.

***Correspondence to:** Hong Ren, The Second Affiliated Hospital of Chongqing Medical University, Chongqing 400010, China. ORCID: https://orcid.org/0000-0002-4557-0918. Tel: +86-23-6288-7083, Fax: +86-23-6370-3790, E-mail: renhong0531@vip.sina.com

Journal of Clinical and Translational Hepatology **2021** vol. 9(4) | 454–455
DOI: 10.14218/JCTH.2021.00236

Editorial

Serum Resistin as a Biomarker in Nonalcoholic Fatty Liver Disease: Is This a Road to be Taken?

Dimitrios Patoulias*

Second Propedeutic Department of Internal Medicine, Aristotle University of Thessaloniki, General Hospital "Hippokration", Thessaloniki, Greece

Received: 20 June 2021 | **Revised:** 5 July 2021 | **Accepted:** 12 July 2021 | **Published:** 3 August 2021

Citation of this article: Patoulias D. Serum resistin as a biomarker in nonalcoholic fatty liver disease: is this a road to be taken? J Clin Transl Hepatol 2021;9(4):454–455. doi: 10.14218/JCTH.2021.00236.

Nonalcoholic fatty liver disease (NAFLD), which encompasses a broad spectrum ranging from nonalcoholic fatty liver (NAFL) to nonalcoholic steatohepatitis (NASH), constitutes a global nightmare, since it affects up to one-fourth of people worldwide, representing a major cause of cirrhosis and hepatocellular carcinoma.[1] Besides the fact that NAFLD is usually thought to be closely related to obesity, almost 40% of affected individuals are classified as nonobese and 20% as lean, all of whom consequently suffer from excessive morbidity and mortality as well.[2] NAFLD is interconnected with significant comorbidities, namely atherosclerotic cardiovascular disease (although it is still a controversial area of knowledge), chronic kidney disease, type 2 diabetes mellitus (T2DM), atrial fibrillation, and obstructive sleep apnea.[3–8] Therefore, there is an urgent need for biomarkers that could contribute to the identification and risk stratification of subjects with NAFLD, especially those with or at high risk for major comorbidities, and their response to therapeutic management.

Recently, another disease profile has been proposed to replace the existing one for NAFLD, termed as "metabolic dysfunction-associated fatty liver disease" (MAFLD) with diagnosis being based upon histological criteria (liver biopsy), imaging or circulating biomarker evidence of fat accumulation in the liver (hepatic steatosis) in addition to one of the following three criteria, namely overweight/obesity, presence of T2DM, or evidence of metabolic dysregulation.[9] This definition, despite initial doubts, may be of better clinical utility, as demonstrated in recent cohort studies.[10]

In the present issue of *Journal of Clinical and Translational Hepatology*, Han and colleagues[11] performed a thorough and methodologically rigorous meta-analysis assessing the potential applicability of serum resistin, a pro-inflammatory adipokine, as a biomarker of NAFLD at its entire spectrum. The authors initially demonstrated that subjects with NAFLD have significantly higher serum resistin levels compared to controls [standardized mean difference (SMD) = 0.522, 95% confidence interval (CI): 0.004 to 1.040, I^2=95.9%] and subjects with NASH have lower serum resistin levels than the healthy controls (SMD = −0.44, 95% CI: −0.83 to −0.55, I^2=74.5%), while no significant difference was identified for patients with NAFL compared to controls and patients with NAFL compared to those with NASH. Based on the contradictory results, Han *et al*.[11] performed a meticulous sensitivity analysis, documenting that patients with NASH have lower resistin levels compared to healthy controls, whereas no significant difference between NAFL patients versus controls and NAFL versus NASH patients exists. Additionally, their thorough meta-regression analysis failed to identify any significant source of the high observed heterogeneity for the generated results. The high number of included studies and the extensive subgroup, sensitivity and meta-regression analyses attribute further power to the generated results, despite the significant heterogeneity.

As the authors state, in interpreting the retrieved results, "*resistin levels seem to rise with the progression of NAFLD, from healthy to NAFL, but decline when NAFL progresses to NASH*". The question that arises is then "*who and when should be monitored*"? According to these results, it seems that resistin may be inadequate to serve as a marker for the distinguishment of a patient with NAFLD from the general population but may be useful for fibrosis risk stratification of a patient with an established diagnosis of NAFLD, since lower levels might indicate more severe disease. Its association with liver tumorigenesis (as shown in hepatitis C virus-infected patients with liver cirrhosis) could enhance this diagnostic strategy, although further data is required.[12] In addition, circulating resistin might be useful for the early identification of patients with some form of NAFLD at high risk for developing other co-morbidities, such as cardiovascular disease and renal impairment.[13,14]

To sum up, the meta-analysis by Han *et al*.[11] provides new, significant insights into the role of circulating resistin as a biomarker in NAFLD. According to current knowledge, it seems that this adipokine could be useful for monitoring of liver fibrosis among NAFLD patients, while it might have a role in the early detection of NAFLD-related comorbidities. However, it seems that further research in the field is still required to determine its exact role in risk stratification of affected individuals. Cost-effectiveness analyses are required, in order to establish resistin as a biomarker of choice in NAFLD.

Abbreviations: CI, confidence interval; MAFLD, metabolic dysfunction-associated fatty liver disease; NAFL, nonalcoholic fatty liver; NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; SDM, standardized mean difference; T2DM, type 2 diabetes mellitus.

***Correspondence to:** Dimitrios Patoulias, Second Propedeutic Department of Internal Medicine, General Hospital "Hippokration", Konstantinoupoleos 49, Thessaloniki 54642, Greece. ORCID: https://orcid.org/0000-0002-6899-684X. Tel: +30-6946900777, Fax: +30-2310225083, E-mail: dipatoulias@gmail.com

Funding

None to declare.

Conflict of interest

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

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Journal of Clinical and Translational Hepatology **2021** vol. 9(4) | 456–457
DOI: 10.14218/JCTH.2021.00240

Editorial

A Novel Backward Stepwise Logistic Regression and Classification and Regression Tree Model to Predict 180-day Clinical Outcomes in Hepatitis B Virus-acute-on-chronic Liver Failure Patients

Shima Ghavimi

Division of Gastroenterology and Hepatology, Department of Medicine, Marshall University, Joan C. Edwards School of Medicine, Huntington, WV, USA

Received: 21 June 2021 | **Revised:** 1 July 2021 | **Accepted:** 7 July 2021 | **Published:** 22 July 2021

Citation of this article: Ghavimi S. A novel backward stepwise logistic regression and classification and regression tree model to predict 180-day clinical outcomes in hepatitis B virus-acute-on-chronic liver failure patients. J Clin Transl Hepatol 2021;9(4):456–457. doi: 10.14218/JCTH.2021.00240.

As the era of precision and personalized medicine is gaining exponential positive gain in the field of medicine, there is a positive shift towards a more evidence-based patient care approach for patients with hepatological diseases. One factor that is crucial in any physician's decision-making efforts involves the application of novel innovative approaches that can enhance predicting survival outcome. Acute-on-chronic liver failure (ACLF) is a perfect example of how liver can rapidly deteriorate, and the hepatitis B virus (HBV) is one crucial culprit. Patients can experience organ failure that leads to their mortality, and in this article the authors clearly described the use of backward stepwise logistic regression (LR) and classification and regression tree (CART) analysis to derive two predictive models and then compared them with the model of end-stage liver disease (MELD) score for novel prognostic models of the 180-day outcome for patients with HBV-ACLF.

This innovative study showed the novel predictive models to be superior to MELD score, providing Hepatologists and Gastroenterologists with a new guiding technique in their evidence-based patient-centered care approach for their clinical treatment decision-making. Of note, one of the leading causes of chronic liver failure in Asia is HBV[1] and the current leading therapeutical intervention for cure is liver transplantation (LT).[2–4]

However, due to decline of health of patients with ACLF, some of the patients become delisted from the organ transplantation list because they are not well enough to undergo LT. Currently, MELD score is the most used for patients on the LT list, although several other scoring modalities are available.[5] Current and recent advancements in Hepatology generated the finding for HBV-ACLF having a window of 30 to 90 days, with respect to the scoring system used. In the article of interest, the authors introduced a predictive module of 180-day outcome for patients based upon multiple variables that are not currently used in the MELD score. Also, there are other scoring systems that have been introduced for HBV-ACLF recently; although, the authors created a CART system scoring that is easier for the clinician to interpret and, in turn, for the patient to receive the paramount care they deserve. Ultimately, the research demonstrated that both LR and the CART model appeared to perform better than the MELD score.

There are, however, some factors and limitations within the study (as properly mentioned in the article), ranging from not having a mid-data report and not having a larger cohort for studying their novel scoring models. I believe if a consensus can be developed by having multiple LT centers around the globe adapt this novel scoring module, we can achieve a better understanding and more precise scoring system developed for patients with HBV-ACLF requiring LT. These novel models which the authors have described and investigated may be helpful for Transplant Hepatologists, Gastroenterologists and Health Care teams who need to make essential clinical decisions for patients with HBV-ACLF.

Funding

None to declare.

Conflict of interest

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

Abbreviations: ACLF, acute-on-chronic liver failure; CART, classification and regression tree; HBV, hepatitis B virus; LT, liver transplantation; MELD, model of end-stage liver disease.

*Correspondence to:** Shima Ghavimi, 1249 15th Street, 2nd & 3rd Floor, Huntington, WV 25701, USA. ORCID: https://orcid.org/0000-0002-1760-5925. Tel: +1-304-691-1000, E-mail: Ghavimi@marshall.edu

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Journal of Clinical and Translational Hepatology **2021** vol. 9(4) | 458–465
DOI: 10.14218/JCTH.2020.00122

Original Article

Role of Ras-related Nuclear Protein/Polypyrimidine Tract Binding Protein in Facilitating the Replication of Hepatitis C Virus

Jihua Xue[1], Jun Cheng[1], Xuejiao Ma[1], Yixian Shi[2], Huafa Yin[1], Yufeng Gao[1*] and Jiabin Li[1*]

[1]*Department of Infectious Diseases, The First Affiliated Hospital of Anhui Medical University, Hefei, Anhui, China;* [2]*Department of Hepatology, Mengchao Hepatobiliary Hospital of Fujian Medical University, Fuzhou, Fujian, China*

Received: 17 November 2020 | **Revised:** 16 February 2021 | **Accepted:** 11 March 2021 | **Published:** 18 June 2021

Abstract

Background and Aims: Ras-related nuclear (RAN) protein is a small GTP-binding protein that is indispensable for the translocation of RNA and proteins through the nuclear pore complex. Recent studies have indicated that RAN plays an important role in virus infection. However, the role of RAN in hepatitis C virus (HCV) infection is unclear. The objective of this study was to investigate the role and underlying mechanisms of RAN in HCV infection. ***Methods:*** Huh7.5.1 cells were infected with the JC1-Luc virus for 24 h and then were incubated with complete medium for an additional 48 h. HCV infection and RAN expression were determined using luciferase assay, quantitative reverse transcription-PCR and western blotting. Small interfering RNA was used to silence RAN. Western blotting and immunofluorescence were used to evaluate the cytoplasmic translocation of polypyrimidine tract-binding (PTB), and coimmunoprecipitation was used to examine the interaction between RAN and PTB. ***Results:*** HCV infection significantly induced RAN expression and cytoplasmic redistribution of PTB. Knockdown of RAN dramatically inhibited HCV infection and the cytoplasmic accumulation of PTB. Colocalization of RAN and PTB was determined by *immunofluorescence, and* a direct interaction of RAN with PTB was demonstrated by coimmunoprecipitation. ***Conclusions:*** PTB in the host cytoplasm is directly associated with HCV replication. These findings demonstrate that the involvement of RAN in HCV infection is mediated by influencing the cytoplasmic translocation of PTB.

Citation of this article: Xue J, Cheng J, Ma X, Shi Y, Yin H, Gao Y, *et al*. Role of ras-related nuclear protein/polypyrimidine tract binding protein in facilitating the replication of hepatitis C virus. J Clin Transl Hepatol 2021;9(4):458–465. doi: 10.14218/JCTH.2020.00122.

Keywords: Ras-related nuclear protein; HCV infection; Polypyrimidine tract-binding protein; Nucleo-cytoplasmic translocation; Novel anti-HCV therapeutics.

Abbreviations: ANOVA, analysis of variance; DAAs, direct-acting antiviral agents; DAPI, 4′,6′-diamidino-2-phenylindole dihydrochloride; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HCV, hepatitis C virus; UTR, untranslated region; PTB, polypyrimidine tract-binding protein; RAN, ras-related nuclear protein; RT, room temperature; si, small interfering.

[*]**Correspondence to:** Jiabin Li and Yufeng Gao, Department of Infectious Diseases, The First Affiliated Hospital of Anhui Medical University, No. 218, Jixi Road, Shushan District, Hefei, Anhui 230022, China. ORCID: https://orcid.org/0000-0002-6539-1802 (JL), https://orcid.org/0000-0003-1822-8161 (YG). Tel: +86-551-62923627, Fax: +86-551-62923617, E-mail: lijiabin@ahmu.edu.cn (JL) or Tel: +86-551-62922383, Fax: +86-551-62922096, E-mail: aygyf@126.com (YG)

Introduction

Hepatitis C virus (HCV) infection is a major cause of chronic hepatitis, liver cirrhosis and hepatocellular carcinoma, affecting approximately 71 million persons according to recent estimates.[1] HCV therapy has been revolutionized with the introduction of direct-acting antiviral agents (commonly known as DAAs), which can achieve viral eradication in >95% of cases with minimal toxicity and overall good tolerability.[2] However, the implementation of these approaches is complicated by the cost, potential for reinfection, generation of drug-resistant viruses, reactivation of other viruses, and end-stage liver disease progression that occurs even after virus clearance.[3,4] HCV also remains the sole hepatitis virus for which a vaccine is not yet available. Thus, novel prophylactic and therapeutic approaches for HCV are still necessary. Identification of host factors involved in HCV replication is critical to understand the molecular mechanism of the viral life cycle, which has significant implications for the development of host-directed strategies to interrupt this cycle.

The ras-related nuclear (RAN) protein is a small GTP-binding protein belonging to the RAS superfamily. It has a well-established role in regulating the transport of macromolecules across the nuclear envelope[5,6] and has also been implicated in mitotic spindle assembly, cell cycle progression, and nuclear envelope formation.[7,8] In eukaryotic organisms, the active transport of macromolecules between the nucleus and cytoplasm is an essential cellular process.[9] Dysregulated protein levels of RAN could cause aberrant nucleo-cytoplasmic transport of RNA and proteins, possibly leading to the initiation and progression of many diseases.

Cellular protein polypyrimidine tract-binding protein (PTB) has been shown to enhance HCV translation by binding to the 5′-untranslated region (UTR) and the 3′-end 98 nucleotides (X region) of HCV RNA.[10–12] The immunodepletion of PTB could completely inhibit HCV translation.[13] These results present evidence for the functional requirement of PTB during HCV translation initiation. In addition to the conserved X region of the 3′ UTR, PTB also binds to the poly(U) tract of the 3′UTR.[14,15] And UV cross-linking studies showed that the PTB-5′-UTR binding was much weaker than the PTB-3′-UTR binding.[16,17] The strong and preferential binding of PTB to the 3′ UTR suggests that it may be recruited to participate in the initiation of HCV RNA replication.[17] The earlier work found that silencing of PTB by small interfering RNA (siRNA) substantially blocked HCV replication.[18,19] And HCV RNA synthesis could be inhibited by anti-PTB antibody in a cell-free, de novo HCV RNA synthesis system.[18] The direct evidence that PTB is required for HCV RNA replication is that PTB co-

Table 1. Primer sequences used in the study

Gene	Upstream primer (5′→3′)	Downstream primer (5′→3′)
HCV	GCGTTAGTATGAGTGTCGTG	TCGCAAGCACCCTATCAG
RAN	GTGAAGGCGAAATCCATTGT	TCCTAGCAAGCCAGAGGAAG
GAPDH	GAAGGTGAAGGTCGGAGTC	GAAGATGGTGATGGGATTTC

localizes with the viral replicase complex.[20] These studies indicate that PTB is a part of the HCV RNA replication complex and participates in viral RNA synthesis. The above results together indicate that PTB has dual functions in HCV life cycle, including translation and RNA replication.

Our initial study showed that HCV infection significantly induced the expression of RAN. Additionally, infectious HCV cell culture systems have been developed, enabling further investigations of the molecular mechanism of HCV infection.[21,22] Thus, this study was conducted to investigate the role and underlying mechanisms of RAN in HCV infection in an HCV cell culture system using a JC1-Luc chimeric virus.

Methods

Cell culture and virus plasmids

Human hepatoma Huh7.5.1 cells were grown at 37°C in a 5% carbon dioxide atmosphere with Dulbecco's modified Eagle's medium supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, 1× nonessential amino acids, 100 U/mL of penicillin, 100 μg/mL of streptomycin, and 10% fetal bovine serum. The plasmid pFL-JC1 was a kind gift from Apath (St. Louis, MO, USA). The chimeric full-length construct pFL-JC1 has been described elsewhere.[21,23,24] To facilitate the detection of HCV infection, reporter viruses were constructed by inserting the firefly luciferase gene into the carboxyl-terminal region of NS5A in the JC1 genomes.[23,25]

RNA transfection, HCV infection, and titration

The production of infectious HCV in hepatocytes was performed as described previously. Briefly, Huh7.5.1 cells were mixed with *in vitro*-transcribed RNA and electroporated (Gene Pulser System; Bio-Rad Hercules, CA, USA) using a single square wave at 260 V and a 25-millisecond pulse length. The supernatant was harvested and concentrated using a centrifugal filter (Amicon 100K; Millipore, Billerica, MA, USA). Purified viruses were used for infection and titration.

HCV replication and treatment

Huh7.5.1 cells were seeded into 96-well plates at a density of 5×10^3 cells per well in 100 μL of medium. After incubating overnight for attachment, JC1-Luc virus was added to the wells. After 24 h, the medium was aspirated and replaced with 100 μL of complete medium, followed by an additional 48 h incubation. The HCV infection and RNA replication rates were quantified by measuring the luciferase activity using a microplate luminometer (Veritas microplate luminometer; Turner Biosystems, Sunnyvale, CA, USA).

Quantitative reverse transcription-PCR

Cells were collected by trypsinization, and the total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's protocol. cDNA was synthesized using the PrimeScript™ Reverse Transcription Reagent Kit with gDNA Eraser (Takara, Tokyo, Japan). The products were then used for analysis by the PRISM 7900 Sequence Detection System (Applied Biosystems Inc., Foster City, CA, USA) and the SYBR® Premix Ex *Taq*™ Kit (Takara). The samples were processed in triplicate and analyzed by the $2-\Delta\Delta Ct$ method. The primers were purchased from Sangon Biotech (Shanghai, China) and are listed in Table 1.

Western blotting

Whole-cell extracts were prepared using RIPA lysis buffer containing the protease inhibitor PMSF. Additionally, the cytoplasmic and nuclear extracts from cells were extracted using the Nuclear and Cytoplasmic Extraction Reagents (Product No. 78835; ThermoFisher Scientific, Waltham, MA, USA). Equivalent amounts of protein (20 μg) were separated by 12% SDS-PAGE and transferred to a polyvinylidene difluoride membrane (Millipore). The membrane was blocked in 5% nonfat milk in Tween 20 Tris-buffered saline and incubated with primary antibodies specific for HCV core protein (Abcam, Cambridge, UK), PTB (Abcam), RAN (Abcam), glyceraldehyde-3-phosphate dehydrogenase (GAPDH, a cytoplasmic protein marker; Cell Signaling Technology, Danvers, MA, USA) and proliferating cell nuclear Ag protein (PCNA, a nuclear protein marker; Cell Signaling Technology, Danvers, MA, USA) at 4°C overnight. After washing, the membrane was incubated with horseradish peroxidase-conjugated secondary antibodies (SouthernBiotech, Birmingham, AL, USA) for 1 h at room temperature (RT) and visualized with enhanced ECL (ThermoFisher Scientific) following exposure to X-ray films.

Immunofluorescence and confocal analysis

Huh7.5.1 cells plated on glass cover slips (BD Biosciences, San Jose, CA, USA) were infected with JC1-Luc virus for 24 h and then incubated with 100 μL of complete medium. The cells were harvested for immunofluorescence staining after 48 h of incubation. The cells were fixed in 4% paraformaldehyde for 30 m at RT, permeabilized in 0.5% Triton X-100/phosphate-buffered saline for 20 m, and then blocked in 1% bovine serum albumin in phosphate-buffered saline for 1 h at RT to minimize nonspecific adsorption of the antibodies. The cells were then incubated with primary antibodies (PTB or RAN: 1:100; Abcam) in 1% bovine serum albumin/Tween 20 Tris-buffered saline overnight at 4°C, followed by an additional incubation in Alexa fluor-conjugated secondary antibodies (1:500; Life Technologies, Gaithersburg, MD, USA) at RT for 1 h. The nucleus was stained with 4′,6′-diamidino-2-phenylindole dihydrochloride (commonly known as DAPI; Sigma-Aldrich, St. Louis, MO, USA), and then the cells were washed three times with phosphate-buffered saline. After the coverslips were mounted on glass slides with mounting medium, the glass slides were photographed using a confocal microscope (Olympus, Tokyo, Japan).

Table 2. siRNA sequences used in the study

siRNAs	Sense, 5′→3′	Antisense, 5′→3′
003	ACAGGAAAGUGAAGGCGAA dTdT	dTdT UGUCCUUUCACUUCCGCUU
004	GACCUUCGUGAAACGUCAU dTdT	dTdT CUGGAAGCACUUUGCAGUA
005	GUAUGUAGCCACCUUGGGU dTdT	dTdT CAUACAUCGGUGGAACCCA

Protein-protein interaction analysis

For coimmunoprecipitation, the cells were lysed with Cell Lysis Buffer (1×; Cell Signaling Technology) containing 1 mM PMSF, 10 mM nicotinamide and 10 μM trichostatin A The lysed cells were centrifuged (13,000 × *g*, 4°C, 10 m), and the supernatants were used for immunoprecipitation. Next, 50 μL of fresh protein G magnetic beads (Millipore) was incubated with anti-RAN or anti-PTB for 10 m with continuous mixing at RT. The lysates (400 μg) and the immobilized capture antibody were then incubated at 4 °C under constant rotation overnight. The beads were washed three times with 1 mL of cold lysis buffer containing protease inhibitors. Finally, the beads were resuspended in 80 μL of 2× Laemmli sample buffer and heated to 95°C for 10 m. The beads were then centrifuged for 1 m at 1,000 × *g*, and the supernatant was collected and used for western blotting.

RNA interference

Small interfering (si)RNAs against human RAN (Ribobio, Guangzhou, China) and control scrambled siRNA (Ribobio, Guangzhou, China) were predesigned and synthesized. Huh7.5.1 cells (at 30% to 40% confluence) were transfected with 100 nM RAN siRNAs using the Lipofectamine® RNAiMAX Reagent (Invitrogen), according to the manufacturer's protocol. Twenty-four hours after transfection, the cells were infected with JC1-Luc virus for 24 h and subsequently incubated with fresh medium for 48 h. The cells were then collected and lysed for luciferase assays, quantitative reverse transcription-PCR and western blotting. The sequences used here are listed in Table 2.

Data analysis

All the data were processed using SPSS 19.0 software and presented as mean ± standard error. Analysis of variance (commonly known as ANOVA) and the least significant difference test were used for comparisons among the groups. When the data were not normally distributed, the Mann-Whitney *U* test and Kruskal-Wallis test were used. A *p*-value less than 0.05 was considered significant.

Results

HCV infection induces RAN expression and cytoplasmic redistribution of PTB

After Huh7.5.1 cells were infected with the JC1-Luc virus, the HCV infection and replication efficiencies were analyzed using the luciferase assay and western blotting. The luciferase activity and core protein expression levels were markedly increased when the cells were infected with the JC1-Luc virus ($p<0.01$; Fig. 1A, B, D) compared with the control group. RAN expression was also examined by quantitative reverse transcription-PCR and western blotting. JC1 virus infection substantially induced the RAN expression compared with the control ($p<0.01$; Fig. 1B–D).

As mentioned above, studies have shown that PTB binds to HCV RNA at several different sites and participates in viral replication or translation. The cytoplasmic translocation of PTB was determined by western blotting in our study. The results revealed that PTB was predominantly localized in the nucleus in the absence of HCV infection; however, strong cytoplasmic accumulation of PTB was observed following HCV stimulation (Fig. 2A, C; $p<0.01$). The distribution of PTB in HCV-infected cells was also evaluated by immunofluorescence assay. The cytoplasmic distribution of PTB was identified when cells were infected with the JC1-Luc virus (Fig. 2B). These data together illustrated that HCV infection induced the cytoplasmic accumulation of PTB.

Knockdown of RAN inhibits HCV infection and cytoplasmic accumulation of PTB

The silencing effects of siRNAs on the mRNA and protein expression levels of RAN were evaluated using quantitative reverse transcription-PCR and western blotting. Compared with the MOCK-treated group, the RAN mRNA and protein levels in RAN siRNA-treated cells were significantly decreased ($p<0.01$; Fig. 3). Inhibition of the replication of HCV was measured using the luciferase assay, quantitative reverse transcription-PCR and western blotting. The luciferase activity and both HCV RNA and core protein expression levels were dramatically inhibited by RAN siRNA ($p<0.01$; Fig. 4A–C) compared with the MOCK-treated group. Additionally, the cytoplasmic accumulation of PTB in the RAN-silenced group was significantly decreased (Fig. 4D, E).

RAN colocalizes with PTB

To verify whether RAN colocalizes with PTB, the subcellular localization of RAN and PTB were evaluated by immunofluorescence staining with anti-RAN (red) and anti-PTB (green) antibodies in Huh7.5.1 cells infected with the JC1 virus. The merged image in yellow indicates the combination of PTB fluorescence intensity with the RAN fluorescence intensity. PTB and RAN staining was mainly distributed in the nucleus of Huh7.5.1 cells, but strong cytoplasmic RAN and PTB immunofluorescence was identified when cells were infected with the JC1 virus (Fig. 5). Additionally, double-labeling of RAN and PTB was observed in both the cytoplasm and nucleus. The above results revealed that HCV infection induces the cytoplasmic distribution of RAN and PTB and RAN colocalizes with PTB.

RAN directly interacts with PTB

We investigated the potential crosstalk between RAN and PTB in Huh7.5.1 cells. Therefore, endogenous protein-protein interaction in cells was examined by co-immunoprecipitation experiments using anti-RAN and anti-PTB antibodies. Cells were extracted and immunoprecipitated with anti-RAN anti-

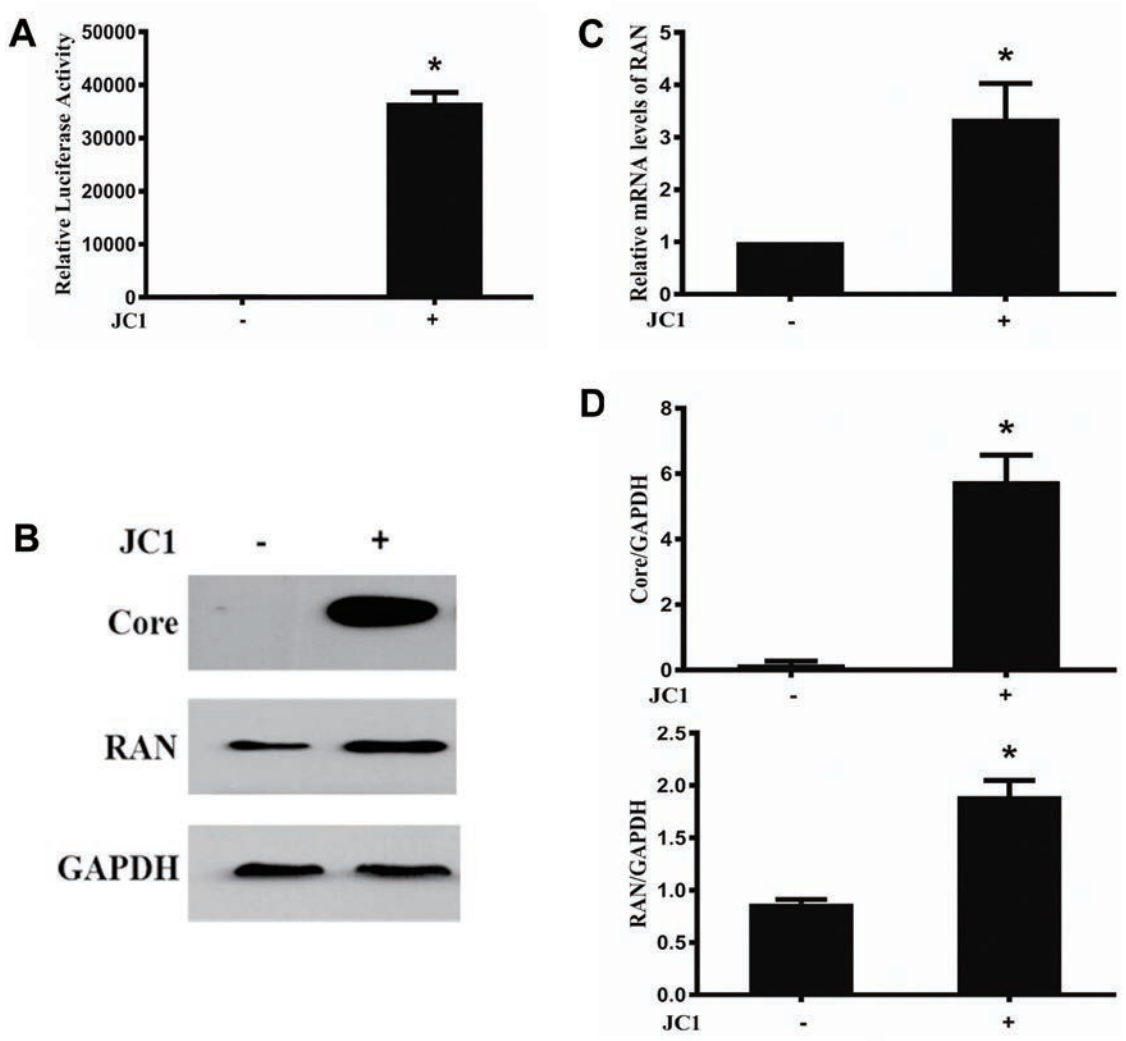

Fig. 1. HCV infection induces the expression of RAN. Huh7.5.1 cells were infected with the JC1 virus. Naïve Huh7.5.1 cells were used as a MOCK-infected control. (A) The replication of HCV was examined using the luciferase assay. (B) Intracellular core and RAN levels were analyzed using western blotting at 48 h post-infection. (C) Intracellular RAN levels were determined using quantitative reverse transcription-PCR at 48 h post-infection. (D) The ratios of core/GAPDH and RAN/GAPDH are shown. Representative images of three independent experiments are presented. $^{*}p<0.01$, compared with control cells.

body, and the immune complexes were analyzed by western blotting with the anti-PTB antibody. RAN interacted with PTB in Huh7.5.1 cells (Fig. 6), confirming their crosstalk.

Discussion

RAN is a critical player in nucleo-cytoplasmic transport that is mainly localized in the nucleus and cycles between the GDP-bound inactive and GTP-bound active state.[26] It is now well established that RAN plays an important role in cancer development and progression.[27,28] It is overexpressed in various cancers and correlated with increased aggressiveness of the cancer cells *in vitro* and *in vivo*.[29–31] Recent studies have indicated that RAN also plays an important role in virus infection. A study showed that the microRNA miR-134 regulated poliovirus replication via the modulation of RAN.[32] Additionally, the reduced production of RAN by RNA interference markedly reduced the synthesis of EV71-encoded viral proteins and virus titers.[33] However, the role of RAN in HCV infection remained unclear.

The above data prompted us to investigate the function of RAN in the regulation of HCV infection. Our results showed that HCV infection significantly induced RAN expression. Additionally, the knockdown of RAN expression with siRNAs significantly reduced HCV replication. RAN silencing has been shown to cause aberrant nucleo-cytoplasmic transport of tumor suppressors and oncogenes, possibly leading to the initiation of cancer.[34] Because RAN plays a key role in controlling nucleo-cytoplasmic trafficking, we hypothesized that HCV-induced upregulation of RAN expression might be involved in the development of HCV by influencing the essential viral proteins or host proteins for viral replication.

PTB is primarily localized in the nucleus. However, it can shuttle from the nucleus to the cytoplasm in response to specific signals, such as viral infection.[25] As a ubiquitous RNA-binding protein, PTB can function as both a repressor and activator of RNA metabolism by restructuring RNA to promote or inhibit the binding of other factors, processes known to be important for the life cycle of many viruses.[35] The necessity of PTB for HCV replication and translation has been proposed. Studies have shown that the recognition of

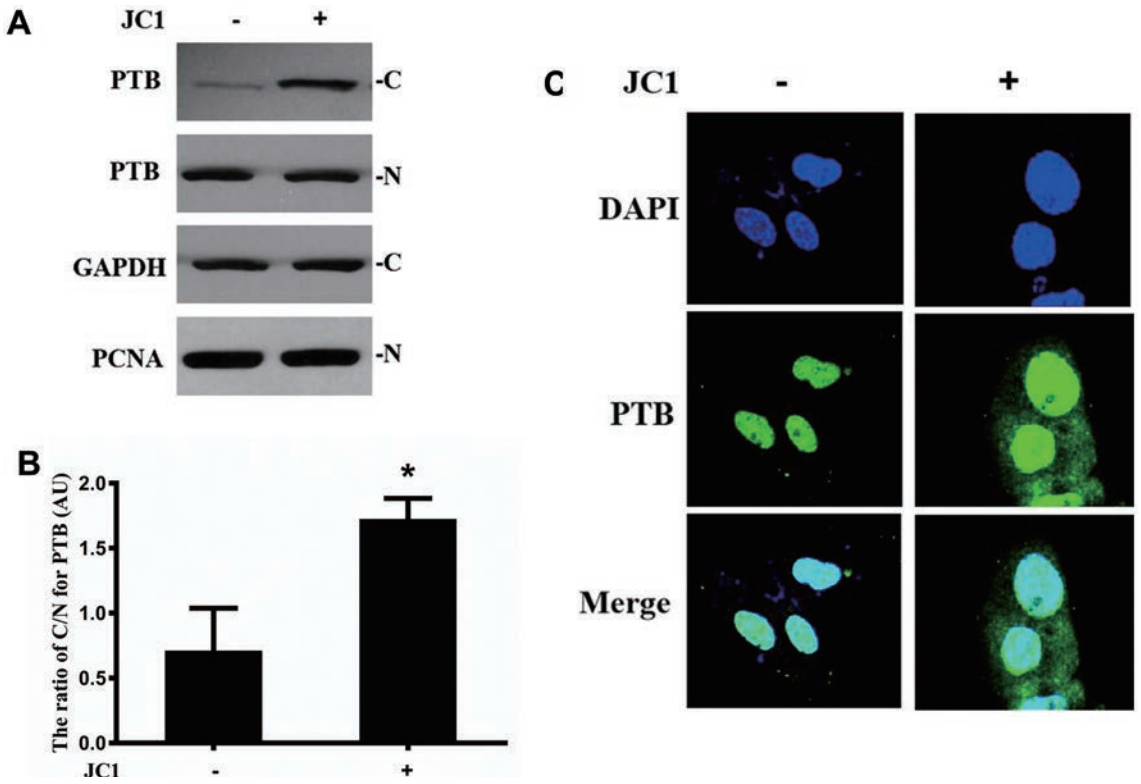

Fig. 2. HCV infection induces the nucleo-cytoplasmic shuttling of PTB. (A and B) The cytoplasm to nuclear ratio for PTB expression levels was detected using western blotting. C, cytoplasm; N, nucleus; PCNA, proliferating cell nuclear Ag. The representative blots of three independent experiments are shown. (C) Imaging of PTB fluorescence intensity. Cellular PTB was stained with anti-PTB mouse antibody and then stained with Alexa fluor 488 anti-rabbit secondary antibody (green color). The nucleus was stained with DAPI (blue color). Merge indicates the combination of PTB fluorescence intensity with nuclear fluorescence intensity. $^{*}p<0.01$, compared with control cells.

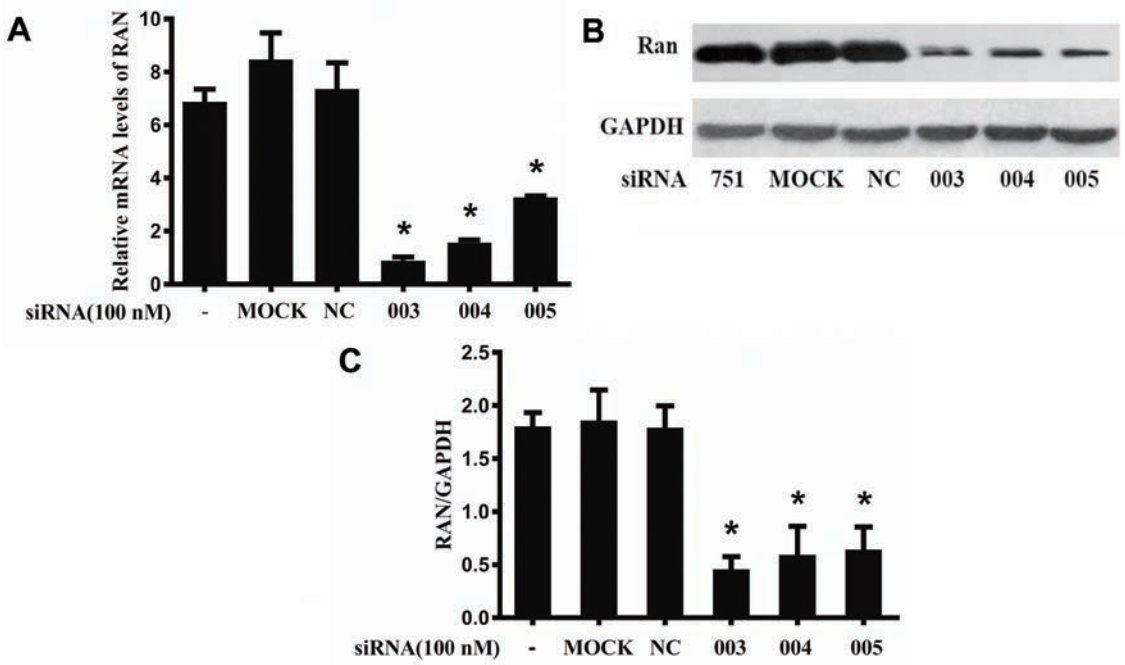

Fig. 3. Silencing effects of siRNAs on the mRNA and protein expression levels of RAN. Huh7.5.1 cells were treated with siRNAs specific to RAN (003, 004 and 005); an irrelevant siRNA (NC) was used as a control in each experiment. (A–B) The expression levels of RAN after siRNA treatment were determined by quantitative reverse transcription-PCR and western blotting. GAPDH mRNA and protein were used for normalization. (C) The ratio of RAN/GAPDH is shown. The results represent three independent experiments. $^{*}p<0.01$, compared with MOCK-treated cells.

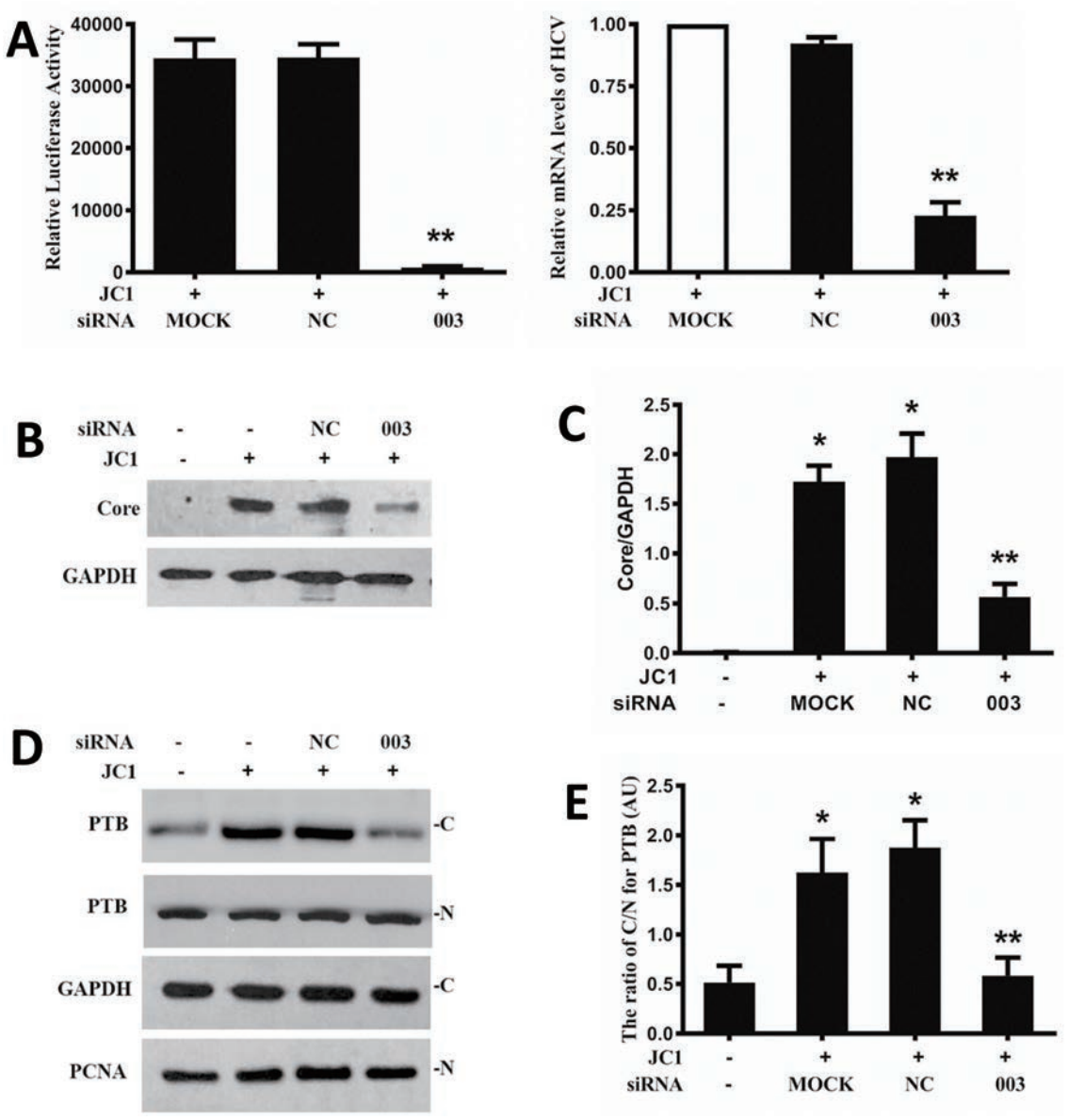

Fig. 4. Knockdown of RAN inhibits HCV replication and the cytoplasmic redistribution of PTB. (A–C) Inhibition of HCV replication was investigated using the luciferase assay, quantitative reverse transcription-PCR and western blotting. Naïve Huh7.5.1 cells were used as a control. (D–E) Inhibition of the cytoplasmic redistribution of PTB was studied using western blotting. $^{*}p<0.01$, compared with control cells. $^{**}p<0.01$, compared with JC1 virus-infected cells.

the 3′-UTR by PTB was necessary for the efficient replication of HCV RNA.[14,18,36] Other studies have also indicated that the interaction of PTB with the 5′-UTR and 3′-UTR of the HCV RNA was required for the initiation of translation.[12,13]

Our previous results indicated that the cytoplasmic accumulation of PTB was directly associated with HCV replication, and blocking the cytoplasmic redistribution of PTB could inhibit HCV replication.[37] However, the mechanism of nucleo-cytoplasmic translocation of PTB is not clear. As the major cellular function of RAN is to regulate nucleo-cytoplasmic transport of molecules through the nuclear pore complex, we consider that RAN might interact with PTB and facilitate its nucleo-cytoplasmic translocation.

Based on the above research assumptions, our research continued and found that increased nucleo-cytoplasmic translocation of PTB in response to HCV infection was dramatically inhibited by RAN silencing. RAN interacted with PTB, as demonstrated by coimmunoprecipitation studies, and facilitated its nucleo-cytoplasmic translocation. Because PTB in the host cytoplasm is directly associated with HCV replication, the involvement of RAN in HCV replication can be the result of the cytoplasmic accumulation of PTB.

Conclusions

In summary, our results demonstrate that the involvement of RAN in HCV infection is mediated by interacting with PTB and then influencing the cytoplasmic translocation of PTB. Our work uncovers a new mechanism responsible for host cellular factors involved in HCV infection and indicates that targeting of the nucleo-cytoplasmic translocation of the host PTB protein

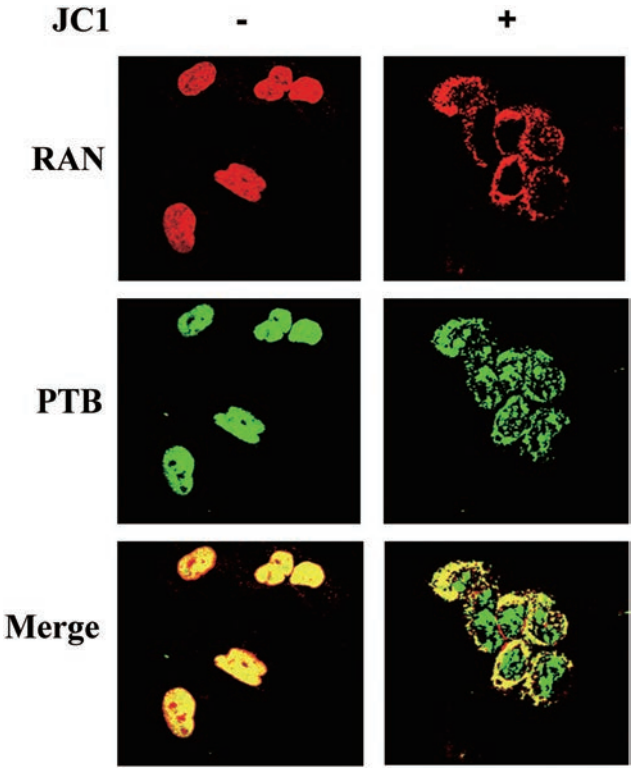

Fig. 5. Colocalization of RAN with PTB on Huh7.5.1 cells infected with JC1 virus. Cells infected with JC1 virus were fixed with ice-cold methanol at 48 h post-infection. The fixed cells were incubated with anti-RAN antibody or anti-PTB antibody followed by a secondary antibody labeled with Alexa fluor 647 (red, RAN) or Alexa fluor 488 (green, PTB). The colocalized signal (yellow) was visualized by merging the two images. All the experiments were performed in triplicate.

could be a novel strategy against HCV. Antiviral agents acting through this mechanism might inhibit viral infection with no or a decreased chance of drug-resistant mutations. Ideally, the combination of a RAN suppressor with known anti-HCV drugs might provide a variety of drug regimens that are appropriate for different patients and provide the potential advantage for preventing or decreasing drug-resistant mutations.

Acknowledgments

We thank Professor Zhi Chen (Zhejiang University, Hangzhou, China) for technical support.

Funding

The work was supported by the Anhui Provincial Natural Science Foundation (1608085QH172), States S&T Projects of 13th Five Year (2018ZX10302206), Chinese Foundation for Hepatitis Prevention and Control (TQGB20200151, TQGB20180367), and Anhui Key Program of Medical Scientific Research of China (#2010A010).

Conflict of interest

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

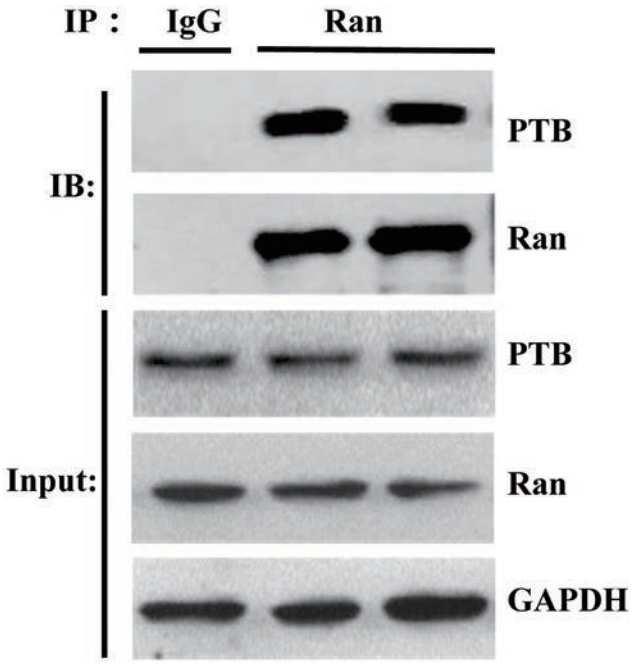

Fig. 6. RAN physically interacts with PTB in Huh7.5.1 cells. Cells were extracted and immunoprecipitated with the anti-RAN antibody, and the immunocomplexes were analyzed by western blotting using the anti-PTB antibody. The IgG antibody was used as a negative control.

Author contributions

Study concept and design (JL, YG), acquisition of data (JX, JC), analysis and interpretation of data (JX, XM), drafting of the manuscript (JX, YS), critical revision of the manuscript for important intellectual content (XM, JC), administrative, technical, or material support, and study supervision (HY).

Data sharing statement

All data are available upon request.

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Journal of Clinical and Translational Hepatology **2021** vol. 9(4) | 466–476
DOI: 10.14218/JCTH.2021.00036

Original Article

Interaction of Hepatitis B Virus X Protein with the Pregnane X Receptor Enhances the Synergistic Effects of Aflatoxin B1 and Hepatitis B Virus on Promoting Hepatocarcinogenesis

Yongdong Niu[1#*], Shaohua Fan[2#], Qin Luo[3], Liming Chen[4], Danmei Huang[1], Wenjun Chang[5], Wenxin Qin[3*] and Ganggang Shi[1*]

[1]Department of Pharmacology, Shantou University Medical College, Shantou, Guangdong, China; [2]Key Laboratory for Biotechnology on Medicinal Plants of Jiangsu Province, School of Life Science, Jiangsu Normal University, Xuzhou, Jiangsu, China; [3]State Key Laboratory of Oncogenes and Related Genes, Shanghai Cancer Institute, Renji Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai, China; [4]Department of Oncology, First Affiliated Hospital of Shantou University Medical College, Shantou, Guangdong, China; [5]Department of Environmental Hygiene, Second Military Medical University, Shanghai, China

Received: 20 January 2021 | **Revised:** 24 February 2021 | **Accepted:** 18 March 2021 | **Published:** 19 April 2021

Abstract

Background and Aims: Hepatitis B virus (HBV) infection has been found to increase hepatocellular sensitivity to carcinogenic xenobiotics, by unknown mechanisms, in the generation of hepatocellular carcinoma. The pregnane X receptor (PXR) is a key regulator of the body's defense against xenobiotics, including xenobiotic carcinogens and clinical drugs. In this study, we aimed to investigate the molecular mechanisms of HBV X protein (HBx)-PXR signaling in the synergistic effects of chemical carcinogens in HBV-associated hepatocarcinogenesis. ***Methods:*** The expression profile of PXR-cytochrome p450 3A4 (CYP3A4) signaling was determined by PCR, western blotting, and tissue microarray. Cell viability and aflatoxin B1 (AFB1) cytotoxicity were measured using the cell counting kit-8 assay. Target gene expression was evaluated using transient transfection and real time-PCR. The genotoxicity of AFB1 was assessed in newborn mice with a single dose of AFB1. ***Results:*** HBx enhanced the hepatotoxicity of AFB1 by activating CYP3A4 and reducing glutathione S-transferase Mu 1 (GSTM1) in cell lines. Activation of PXR by pregnenolone 16α-carbonitrile increased AFB1-induced liver tumor incidence by up-regulating oncogenic KRAS to enhance interleukin (IL)-11:IL-11 receptor subunit alpha-1 (IL11RA-1)-mediated inflammation in an HBx transgenic model. ***Conclusions:*** Our finding regarding AFB1 toxicity enhancement by an HBx-PXR-CYP3A4/ GSTM1-KRAS-IL11:IL11RA signaling axis provides a rational explanation for the synergistic effects of chemical carcinogens in HBV infection-associated hepatocarcinogenesis.

Citation of this article: Niu Y, Fan S, Luo Q, Chen L, Huang D, Chang W, *et al*. Interaction of hepatitis B virus X protein with the pregnane X receptor enhances the synergistic effects of aflatoxin B1 and hepatitis B virus on promoting hepatocarcinogenesis. J Clin Transl Hepatol 2021;9(4):466–476. doi: 10.14218/JCTH.2021.00036.

Keywords: Liver cancer; Hepatitis B virus X protein; Pregnane X receptor; Aflatoxin B1; Hepatotoxicity.

Abbreviations: AFB1, aflatoxin B1; BW, body weight; CCK-8, cell counting kit-8; CYP1A2, cytochrome p450 1A2; CYP3A4, cytochrome p450 3A4; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethyl sulfoxide; GSTM1, glutathione S transferase Mu 1; h, human; HBV, hepatitis B virus; HBx, hepatitis B virus X protein; HCC, hepatocellular carcinoma; IHC, immunohistochemistry; IL, interleukin; IL11RA-1, IL-11 receptor subunit alpha-1; PCN, pregnenolone 16α carbonitrile; PXR, pregnane X receptor; qRT-PCR, quantitative real-time polymerase chain reaction; RIF, rifampicin; Tg, transgenic.

[#]These authors contributed equally to this work.

[*]**Correspondence to:** Yongdong Niu, Shantou University Medical College, Shantou, Guangdong 515041, China. ORCID: https://orcid.org/0000-0002-5392-3736. Tel: +86-754-88900432, Fax: +86-754-88557562, E-mail: ydniu@126.com; Wenxin Qin, Shanghai Cancer Institute, Shanghai 200032, China. ORCID: https://orcid.org/0000-0002-1989-3027. Tel: +86-21-68371105, Fax: +86-21-64432142, E-mail: wxqin@sjtu.edu.cn; Ganggang Shi, Shantou University Medical College, Shantou, Guangdong 515041, China. ORCID: https://orcid.org/0000-0002-6856-1192. Tel: +86-754-88900301, Fax: +86-754-88557562, E-mail: ggshi@stu.edu.cn

Introduction

Hepatocellular carcinoma (HCC) is a primary malignancy with a high incidence of mortality rate worldwide.[1,2] A specific association between hepatitis B virus (HBV) infection and aflatoxin B1 (AFB1) exposure in hepatocarcinogenesis has been suggested.[3,4] AFB1, a potent hepatotoxicant and carcinogen, requires the bioactivation of AFB1 to the 8,9-epoxide form of AFBO, which is the most important risk factor for HCC due to its irreversible genotoxic effects.[5,6] Epidemiological studies and a previous prospective case-control study[7] have suggested a strong interaction between HBV and AFB1 exposure in the etiology of HCC.[8] However, the mechanism underlying this synergistic interaction remains unclear.

The pregnane X receptor (PXR) is a ligand-dependent steroid and xenobiotic receptor that is responsible for the metabolic activation or detoxification of several carcinogens, and may play various roles in hepatocellular carcinogenesis.[9,10] The metabolism of AFB1 *in vivo* is closely related to PXR, cytochrome P450 3A4 (CYP3A4), and glutathione S-transferase

Mu 1 (GSTM1).[11] HBV X protein (HBx) is considered a key regulator in HCC, due to its capacity to function as a deregulated transcriptional activator. Although HBx alone rarely causes spontaneous liver cancer in our ATX-HBx/FXR$^{-/-}$ mouse model,[12] it increases the incidence of G/C-to-T/A transversion mutations by approximately 2-fold following AFB1 exposure in ATX-HBx transgenic mice.[13] In addition, p21-HBx knock-in transgenic (Tg) mice, in which HBx was knocked into the p21 allele, show spontaneous liver tumors at the age of 18 months, whereas p21 knockout does not increase their susceptibility to HCC.[14] Considering that the induction of CYP3A4 by HBx is PXR-dependent and HBx is a PXR cofactor,[15] we propose that HBx-PXR signaling increases the genotoxicity of AFB1 by disrupting the metabolism of AFB1 *in vivo*.

In this study, we demonstrate homeostatic disturbance of AFB1 metabolizing enzymes by HBx contributes to enhancing AFB1 genotoxicity, resulting in oncogenic KRAS signaling to induce hepatocarcinogenesis via activating a PXR signaling axis in HBx Tg mice. These results provide a possible mechanism for the synergism of HBV and AFB1 co-exposure in hepatocarcinogenesis.

Methods

Cell culture

HepG2 human HCC cells, Hepa1-6 murine hepatoma cells, and AML12 normal murine hepatocytes were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were maintained in Dulbecco's modified Eagle's medium (i.e. DMEM), RPMI-1640 medium, or DMEM:F12 medium containing 10% fetal bovine serum, at 37°C in a 5% CO_2 incubator.

Reagents and plasmids

Pregnenolone 16α-carbonitrile (PCN, a specific mPXR agonist), rifampicin (RIF, a human (h)PXR agonist), and AFB1 were obtained from the Sigma-Aldrich Corporation (St. Louis, MO, USA). The expression plasmids and vectors for mPXR, hPXR, and Flag-tagged HBx have been described previously.[15,16]

Transient transfection

HepG2 cells were grown to 70–80% confluence in 6-well plates. Cells were transiently transfected with HBx and/or PXR using Lipofectamine™ 2000, as previously described.[12] Transfected cells were treated with drugs for 24 h before being lysed and for RNA extraction as described. Transfection experiments were performed at least three times in triplicate. Data are presented as fold induction over empty vector alone.

Reverse-transcriptase polymerase chain reaction, quantitative real-time polymerase chain reaction (qRT-PCR), and western blotting

Total RNA was prepared using TRIzol reagent. SYBR Green-based qRT-PCR was performed with an ABI 7500 Real-Time PCR System (Applied Biosystems Inc., Hercules, CA, USA). Data were normalized against a cyclophilin control. The qRT-PCR primer sequences are presented in Supplementary Table 1.

Specific antibodies against HBx (MAB8419; Millipore, Danver, MA, USA), PXR (PP-H4417-00; R&D Systems, Minneapolis, MN, USA), MDR1 (ab3366; Abcam, Cambridge, UK), CYP3A4 (H00001576-B01P; Novus Biologicals, Littleton, CO, USA), SULT2A1 (ab38416; Abcam, Cambridge, UK), CYP3A (sc-30621; Santa Cruz Biotechnology, Dallas, TX, USA), cytochrome p450 1A2 (CYP1A2) (AP11325c; Abgent, Suzhou, China), and GSTM1 (AP6896b; Abgent, Suzhou, China) were used.

Cell proliferation assay

Cell viability and AFB1 cytotoxicity were measured using the cell counting kit-8 (CCK-8) assay (Dojindo Laboratories, Rockville, MD, USA), as described in the following methods. HepG2 or PXR knockdown (HepG2) cells[15] were trypsinized and seeded at 5×10^3 cells/well in 96-well plates. After 24 h, AFB1 was added and incubated for another 24–96 h at 37°C. The effects of different AFB1 concentrations were evaluated in HepG2 cells after various exposure times. For longer treatment with AFB1, mycotoxin was added every 48 h, with each medium renewal. Then, 10 μL of CCK-8 solution was added and plates were incubated for an additional 1–2 h. The optical density for each well was measured at a wavelength of 450 nm.

HBx Tg mice

Six- to eight-week-old HBx Tg mice were maintained on a 12 h light/12 h dark cycle. Animals were allowed food (standard chow) and water ad libitum. Mice were euthanized by CO_2 asphyxiation 24 h after treatment with 40 mg/kg of the mPXR agonist PCN by intraperitoneal injection. Livers were excised, snap-frozen on dry ice, and stored at -80°C until further analysis. All protocols and procedures were approved by the Institutional Animal Care and Research Advisory Committee of the Shanghai Cancer Institute. Expression analysis for PXR, MDR1, CYP3A11, SULT2A1, CYP3A, and GSTM1 was performed by qRT-PCR and immunohistochemistry (IHC).

Administration of toxins

Six pregnant HBx Tg female mice were used in the study. Pups (3–7 days of age) were inoculated intraperitoneally with a single injection of AFB1 at 7 mg/kg body weight (BW)[17] with or without 40 mg/kg PCN per week. All dosing was administered by intraperitoneal injection based on BW at the time of treatment. Animals treated with solvent vehicle (dimethyl sulfoxide, commonly known as DMSO) were used as the controls. In the transient genotoxicity study with AFB1, 3- to 7-day-old animals (n=20, 5 in each group) were treated with a single dose of AFB1, then euthanized either at 24 h or 7 days to check for AFB1:DNA adducts. Six- to eight-week-old animals (16 HBx Tg mice and 6 wild-type mice) were treated with a single dose of AFB1 at 7 days posttreatment with PCN to assess the activity of PXR signaling. For the chronic AFB1-induced liver cancer HBx Tg mice model, HBx Tg offspring (n=48) were randomized into four groups: (1) vehicle (n=7); (2) AFB1 (n=12); (3) PCN (n=15); or (4) AFB1+PCN (n=14). Mice were euthanatized at 14 months by CO_2 inhalation, and livers were collected for histopathology and qRT-PCR. All procedures were approved by the Ethics Committee of Shanghai Cancer Institute.

IHC and tissue microarray

Clinical samples were obtained from the First Affiliated Hospital and Second Affiliated Hospital of Shantou University Medical College after acquiring informed consent according to an established protocol approved by the Ethics Committee of Shantou University Medical College (SUMC-2015-07) and in conjunction with the ethical requirements of "The Operational Guidelines for Ethics Committees that Review Biomedical Research" and the Declaration of Helsinki.

Human HCC tissues and mouse liver tissues were analyzed using IHC, as previously described.[12] Tissues were first placed into paraffin blocks and dewaxed with xylene followed by rehydration, and antigen retrieval (1:25, DAKO) was performed according to standard procedures. The tissues were then sequentially blocked with 2% bovine serum albumin/0.1% Triton X-100 for 60 m at room temperature. Tissues were incubated with primary antibody against human PXR (1:40) (PP-H4417-00; R&D) or PXR (1:40) (H1-160, sc-25381; Santa Cruz Biotechnology, Dallas, TX, USA), HBx antibody (1:250) (ab39716; Abcam), CYP3A4 (1:500) (H00001576-B01P; Abnova), SULT2A1 (1:500) (sc-32941; Santa Cruz Biotechnology), CYP1A2 (1:50) (AP11325c; Abgent), GSTM1 (1:100) (AP6896b, Abgent), CYP3A (1:150) (L-14; (Santa Cruz Biotechnology), or AFB1 (1:150) (6A10, Novus Biologicals LLC) at 4°C overnight, followed with biotinylated horseradish-peroxidase secondary antibody for 1 h. For controls, tissues were incubated without primary antibody.

Immunostaining with antibody against hPXR was performed on a tissue assay of HBV-associated diseases, representing 16 normal liver tissues, 24 hepatitis liver tissues, 32 hepatic cirrhosis liver tissues, and 32 HCC liver tissues (duplicate cores per case), according to the method described above. The results were observed and photographed with an Axioskop 2 microscope (Carl Zeiss, Oberkochen, Germany) and DP70 Imaging System (Olympus, Tokyo, Japan). Slides were graded as follows: −, 0–5% cells stained; +, 5–10% cells stained; ++, 10–50% cells stained; +++, >50% cells stained.

Data mining

Expression patterns of AFB1 metabolic and PXR signaling genes were analyzed in our previous data (GSE84402), which included 13 matched HCC samples and corresponding adjacent liver tissues with HBV infection. Similar information can also be found in the GSE14520 dataset, which includes 35 paired clinical early-stage HBV-associated HCC samples and corresponding adjacent liver tissue.[2] The relative genes were downloaded and parsed into Excel for analysis. To uncover the prognostic value of AFB1 metabolism relative to PXR signaling genes in the cohort, a Kaplan-Meier plotter was used (http://kmplot.com/analysis/). Additionally, the HCCDB3 (GSE25097) dataset (http://lifeome.net/database/hccdb/home.html), which includes 268 HCC tissues, 243 adjacent liver tissues, 40 cirrhotic liver tissues, and 6 healthy tissue samples, was used.[18]

Statistical analysis

Data were expressed as the mean±standard deviation of three independent experiments. Significance of the differences was determined by Student's *t*-test. Clinical data were analyzed using the Wilcoxon signed-rank and Mann-Whitney *U* tests. The Kaplan-Meier method with log-rank analysis was used to obtain estimates of overall survival. A *p*-value <0.05 indicated significant mean differences.

Results

Deregulation of PXR-CYP3A4 signaling is involved in HBV-associated hepatocellular carcinogenesis

We have previously reported that the *PXR* mRNA level is substantially lower in intermediate and end-stage HBV-associated HCC samples compared to normal and adjacent noncancerous tissue samples.[15] To determine whether PXR signaling is dysregulated in HCC, the dynamic expression of PXR was examined in 104 HBV-associated clinical samples by tissue array. Lower expression was observed in HCC samples than compared to normal controls, adjacent noncancerous tissues, and early small tumors. At the advanced or end stage, there was a general decrease or even absence of PXR in tumor tissues (Fig. 1A and Supplementary Table 2). These results suggested that *PXR* or *PXR* signaling is dysregulated in HCC.

We also evaluated the *PXR* mRNA levels in fresh samples from patients with HBV-associated liver disease (Fig. 1B). The association of PXR with pathological stage was analyzed in six pairs of HCC stage I and HCC stage II tissue samples, by western blotting (Supplementary Fig. 1A). HBx shared similar strong costaining in cirrhosis but showed low co-expression with PXR in HCC sections, as observed by IHC in serial sections (Fig. 1C). Down-regulation of both PXR and CYP3A4 was observed in 24 paired HBV-associated intermediate and advanced HCC tissues compared to corresponding adjacent noncancerous tissues (Fig. 1D and Supplementary Fig. 1B). Moreover, a clinical association between PXR and CYP3A4 with liver disease statuses was found in the HCCDB dataset HCCDB3 (Fig. 1E and Supplementary Fig. 1C; $p<0.0001$).

Activation of PXR by its agonist and/or HBx enhances the toxicity of AFB1

The contribution of HBV infection to hepatocarcinogenesis following AFB1 exposure is related to the sensitization of hepatocyte susceptibility to AFBO cytotoxicity.[19] We have previously reported that CYP3A4 is up-regulated in the pre-HCC stage and adjacent nontumor tissues, and that induction of CYP3A4 by HBx is PXR-dependent.[15] This suggests that the activation of PXR by HBx will affect the metabolism of AFB1 in the liver. As shown in Figure 2A, prolonged exposure to AFB1 led to a significant increase in the sensitivity toward of AFB1 toxicity. Treatment of HepG2 cells with RIF, to activate the PXR, increases AFB1 metabolism by inducing CYP3A4.[20] Activation of PXR by HBx produced a similar effect, i.e. the toxicity of AFB1 was enhanced by RIF (Fig. 2B). PXR and HBx co-overexpression also increased AFB1 hepatoxicity, especially in the presence of RIF (Fig. 2C). Moreover, this effect was abolished by PXR knockdown (Fig. 2D). These results suggest that atypical activation of PXR by HBx is a potent promoting factor that affects AFB1 hepatoxicity.

Molecular mechanism of AFB1 toxicity enhancement by HBx-PXR interaction

To understand the induction of AFB1 hepatotoxicity by HBx-PXR interaction, we measured the expression of AFB1 metabolizing enzymes in HepG2 cells transfected with PXR and/or HBx. Figure 3A shows the key AFB1-metabolizing

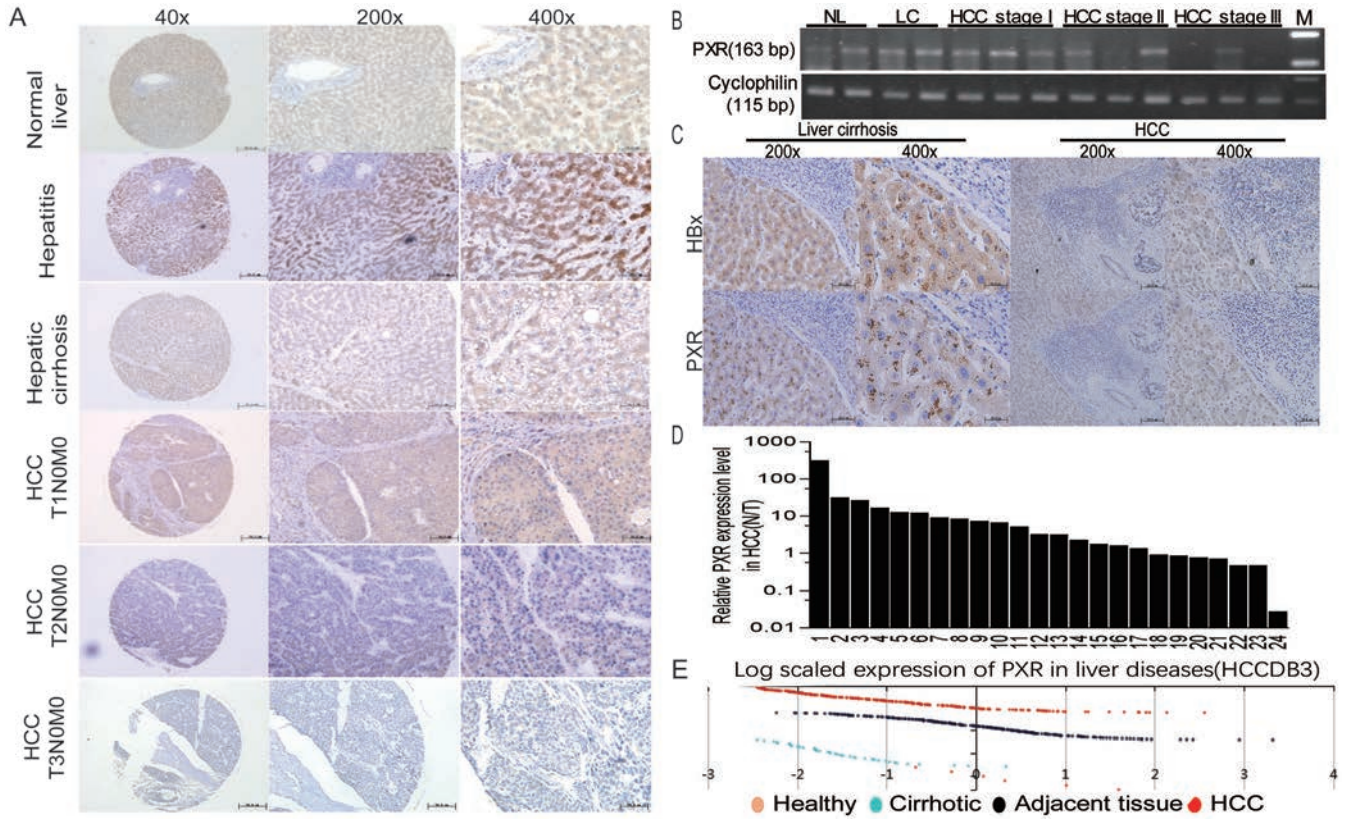

Fig. 1. Ectopic expression model of PXR in liver specimens of HBV-associated liver diseases. (A) IHC of PXR in HBV-associated liver diseases (n=104). Representative samples for each stage are shown, and include normal liver tissues, hepatic cirrhosis liver tissues, and HCC clinical pathology stage I–III liver tissues. Scale bar, 100 μm. Original magnification, × 40, 200 and 400. (B) Relative PXR mRNA level in HBV-associated liver diseases. RT-PCR was performed from two normal livers (NL), two cirrhotic livers (LC), three HCC stage I, three HCC stage II, and three HCC stage III cases. (C) IHC showing the same expression pattern of HBx and PXR in hepatic cirrhosis and HCC tissues. Scale bar, 100 μm. (D) PXR expression in 24 paired HCC and non-tumor tissues by qRT-PCR. (E) PXR mRNA levels in a cohort from the HCCDB database (Accession No. HCCDB3).

enzymes involved in hepatocarcinogenesis. CYP3A4, the most important enzyme involved in converting AFB1 to AFBO, was significantly up-regulated in HepG2 cells in the presence of RIF/PXR and AFB1 (Fig. 3B–D). High expression of *CYP3A4* and cytoplasmic immunoreactivity was also shown in our previous studies on HBV[15] and HCV cirrhosis, respectively. *CYP1A2*, which is also responsible for the formation of AFBO, remained unchanged, in urine of patients with HCC and chronic HBV, based on comparisons of the amounts of the AFB1 metabolic product AFM1 and AFB1-N7 guanine adducts. *CYP1A2* remained unchanged or slightly decreased in HepG2 cells overexpressing PXR and/or HBx after AFB1 exposure (Supplementary Fig. 2A), and *GSTM1*, a major factor responsible for the enzymatic detoxification of AFBO,[21] dramatically decreased (Fig. 3C). *EPHX1*, which is responsible for AFB1 8,9-epoxide hydrolysis, has been proposed to be important. However, the rapid nonenzymatic hydrolysis is difficult to perform *in vitro*.[22]

Additionally, the key enzymes metabolizing AFB1 in the presence of RIF were analyzed in HepG2 cells. Dramatically increased CYP3A4 was found in both the RIF and HBx treatment groups, whereas CYP1A2 was induced by HBx but reduced by HBx and RIF. The change in GSTM1 was not significant (Fig. 3D).

To determine whether PXR signaling indeed regulates key AFB1-metabolizing enzymes, we used short hairpin RNA targeting human PXR. We failed to observe a significant induction of *CYP3A4* by HBx in PXR knockdown cells, even upon RIF treatment. In contrast, we observed the induction of *CYP3A4* but not *GSTM1* in rescue experiments in PXR knockdown cells by HBx-PXR interaction (Fig. 3E).

Activated HBx-PXR signaling promotes AFB1: DNA adducts by disrupting key AFB1-metabolizing enzymes

To explore whether the activation of PXR by HBx can induce AFB1 genotoxicity *in vivo*, we analyzed the expression of AFB1-metabolizing genes. *CYP3A11*, *GSTM1,2* AND *CYP2E1* levels were dramatically increased, while those of *Gsta2*, which is the murine glutathione S-transferase isozymes alpha class 2, and *Gsta3* decreased in mouse hepatoma Hepa1-6 cells transfected with hPXR and HBx, and treated with RIF (Fig. 4A, B). Additionally, the expression of CYP3A11 and GSTM1 increased, but that of GSTA3 decreased in AML12 cells transfected with HBx and treated with AFB1 compared to cells transfected with the empty vector (Supplementary Fig. 2B). To demonstrate the activation of PXR by HBx *in vivo*, we showed that there is no difference in *mPXR* and *mRXR* between wild-type and HBx Tg mice (Fig. 4C), and the expression of *CYP3A11* was increased in both HBx Tg and wild-type mice (Fig. 4D, E). To further confirm the activation by HBx on mPXR, the expression of Mdr1 and Sult2a1 was observed in both HBx Tg and control mice (Fig. 4D, E).

To determine the genotoxicity of AFB1-exposed mice, we

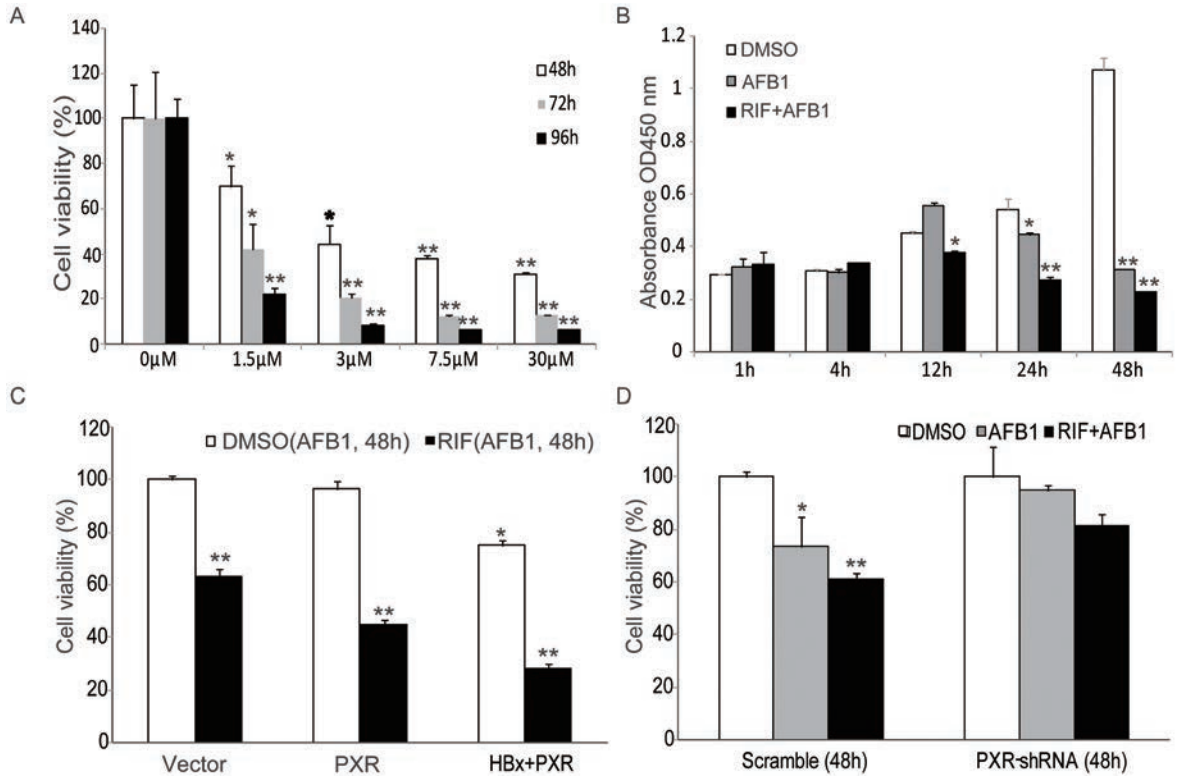

Fig. 2. Activated PXR signaling enhances the liver toxicity of AFB1. (A) Dose-dependent and time-dependent response curves of AFB1-induced toxicity in HepG2 cells. (B) Activated endogenous PXR signaling by RIF enhanced liver cell toxicity of AFB1. (C) Co-overexpressed hPXR and HBx enhanced the liver toxicity of AFB1. HepG2 cells were treated with RIF (10 μM) and AFB1 (3 μM) after transient transfection of PXR and/or HBx. (D) Toxicity of AFB1 can be blocked by knockdown of PXR. Comparative toxicity of AFB1 in PXR knockdown cells. AFB1 cytotoxicity was assayed using a CCK-8 assay. Three independent experiments were performed. *$p<0.05$, **$p<0.01$, treated vs. control.

measured AFB1:DNA adducts by histological evaluation of newborn mice with and without PCN treatment (Fig. 4F). We failed to find significant differences between the AFB1 and AFB1+PCN groups in terms of forming AFB1:DNA adducts in the liver biopsies of HBx Tg mice; although, there could have been a marginal increase in AFB1:DNA adducts in the HBx Tg (AFB1+PCN) group compared to that in the wild-type (AFB1+PCN) group (Fig. 4F).

CYP1A2 and CYP3A11 increased, while GSTM1 decreased in HBx Tg mice compared to wild-type mice after treatment with a single dose of AFB1 (Fig. 4G). Notably, adult mice could better tolerate aflatoxin-induced genotoxicity, possibly due to the increase in the number of GST genes. Here, Gsta1, Gsta2 and Gsta3, which are responsible for AFB1 detoxification in mice,[23] dramatically increased in HBx Tg mice (Fig. 4G). Also, a significant change occurred in AFB1-metabolizing enzymes in both AFB1-exposed HBx Tg mice and control mice, especially after PCN treatment (Fig. 4H, J), confirming that PXR signaling is involved in the potentiation of AFB1 genotoxicity *in vivo*, and suggesting that HBx-PXR-AFB1:DNA adduct signaling might serve as a driver in hepatocarcinogenesis.

Activated PXR signaling promotes AFB1-induced liver tumorigenesis in HBx transgenic mice

To determine the possible effect of HBx in potentiating the ability of AFB1 to induce tumorigenesis via PXR signaling, we established a chronic AFB1-induced liver tumor HBX Tg mouse model (Fig. 5A). We treated newborn mice with a single dose of AFB1, PCN, or vehicle neonatally. A total of 48 HBx Tg mice were euthanized and examined for liver tumors at 14 months of age. As shown in Figure 5B, approximately 42.9% (6/14) of mice treated with AFB1+PCN and 20.0% (3/15) of those treated with only AFB1 developed identifiable liver tumors, whereas no visible neoplasms or preneoplastic lesions were observed in the vehicle or PCN group for up to 14 months. Tumorigenesis in both the AFB1 and AFB1+PCN groups were confirmed by hematoxylin and eosin staining (Fig. 5C). Tumor numbers (6/6) and liver index (ratio of liver weight to BW) in male mice in the AFB1+PCN group were higher than the tumor numbers (3/8) of their male counterparts in the AFB1 group (Fig. 5D, E). Similarly, tumor incidence in the female AFB1+PCN group was higher than that in their female counterparts in the AFB1 group (Fig. 5F).

To gain insight into the heightened carcinogenesis in the AFB1+PCN group, we observed the up-regulation of the oncogene KRAS, which is consistent with tumor incidence. Oncogenic Ras can induce interleukin (IL)11 production in both mouse NIH3T3 and human pancreatic carcinoma cells.[24] However, we failed to show a significant change in IL11 (Fig. 5G). Considering the function of IL11 is dependent on the binding of IL-11 receptor subunit alpha-1 (IL11RA-1), which is reportedly involved in liver fibrosis, hepatocyte death, and inflammatory response,[25] we assessed the expression of IL11RA-1 and showed a similar pattern between *KRAS* and *IL11RA-1* in AFB1-induced liver tumorigenesis animal models (Fig. 5G). Since *IL11* is a canonical member of the *IL6* family, which has been identified as a key driver of hepatocarcinogenesis,[26,27] we also examined the expression of *IL6*, which is substantially increased in mouse-liver tumori-

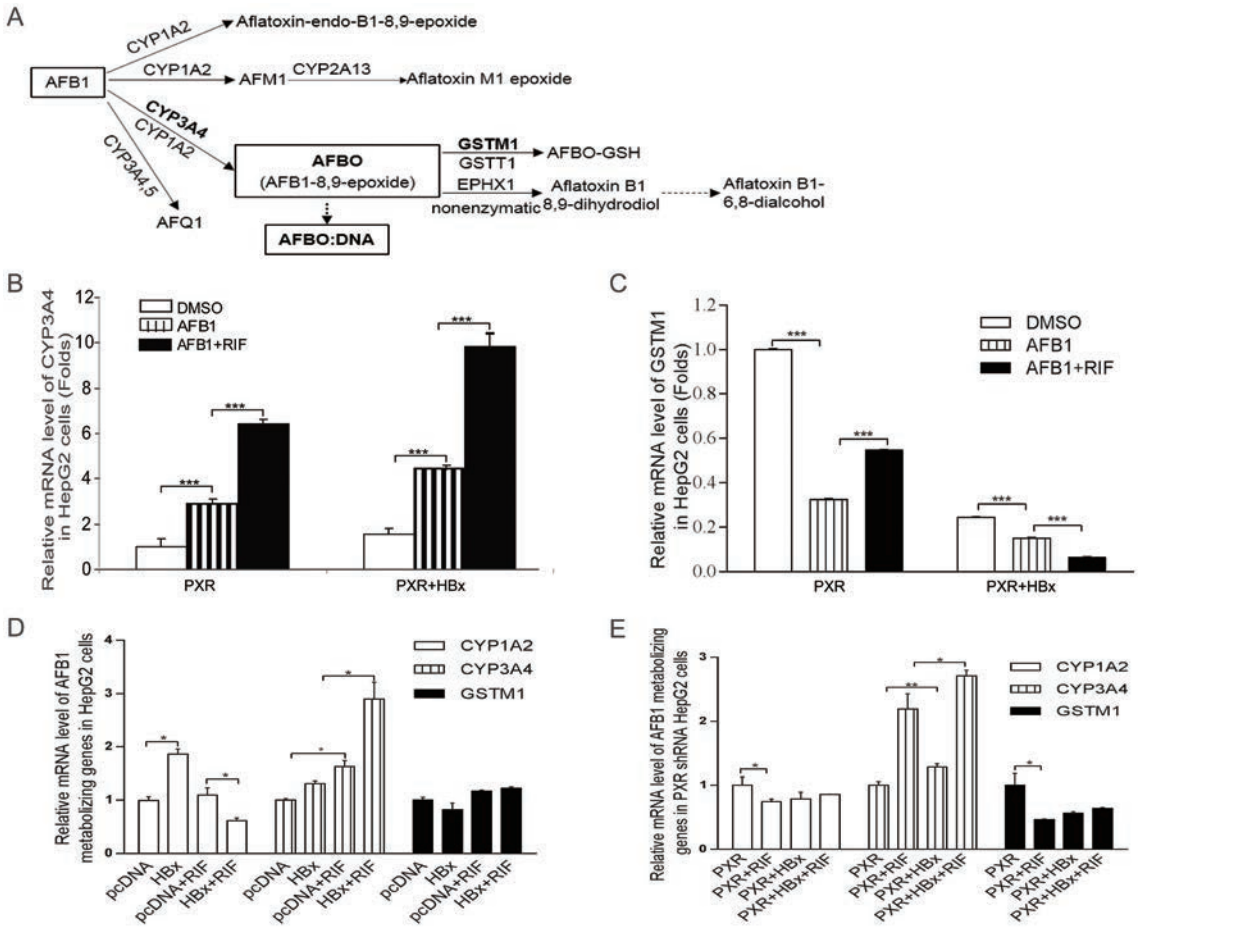

Fig. 3. Relationship of AFB1-metabolizing genes and the activated hPXR signaling pathway in HCC. (A) Schematic showing the key enzymes or steps in the metabolism of AFB1 in liver. (B,C) AFB1 and/or RIF enhanced CYP3A4 and GSTM1 expression. HepG2 cells were transiently transfected by PXR alone or PXR and HBx, and treated with RIF and/or AFB1 (3 μM), and then mRNA was isolated for qRT-PCR analysis of CYP3A4 and GSTM1. (D) Relationship of AFB1 metabolizing genes and the activated endogenous PXR signaling pathway in HepG2 cells. (E) Expression of genes related to AFB1 metabolism in cells overexpressing PXR with or without HBx.

genesis. The expression of *IL6* receptors showed either an opposite or no change (Fig. 5H).

Clinical implications of an HBx-PXR-AFB1 metabolizing signaling axis in patients with virus-associated HCC

To assess the clinical significance of AFB1-metabolizing genes in HBV-associated HCC, we compared the ratio of AFB1-metabolizing genes in 24 paired HBV-associated intermediate and advanced HCC clinical samples (Fig. 6A). The expression patterns of *PXR, CYP3A4, GSTM1, KRAS,* and *IL11RA* in HBV-associated HCC were further confirmed by data mining in the GSE84402 dataset (Fig. 6B). Consistently, we observed possible clinically associated expression patterns of *PXR*, *CYP3A4*, *GSTM1*, *KRAS,* and *IL11RA* in GSE14520, which included 35 paired clinical early-stage HBV-associated HCC samples and corresponding adjacent liver tissue samples (Fig. 6C and Supplementary Fig. 3A–J). Additionally, to further uncover the possible prognostic value of PXR, CYP3A4, GSTM1, KRAS, IL11, and IL11RA in the cohort, RNA-Seq data from 93 Asian patients with HCC and hepatitis virus infection were analyzed by Kaplan-Meier plots with a log-rank test. We found that poor overall survival of HCC patients was associated with high CYP3A4 and GSTM1 expression levels (Fig. 6D, E; log-rank $p<0.05$) but not with PXR, KRAS, IL11, and IL11RA (Supplementary Fig. 4A–D).

Discussion

Viral infection alone is rarely oncogenic in hepatocarcinogenesis,[28] although integration sites in the human genome of HBV have been observed more frequently in tumors.[29] The TERT promoter, Wnt, TP53, and MLL4 confer risk for HBV-related HCC, as determined through a genome-wide association study[30] and exome sequencing.[31] However, HBV and its DNA replication fail to increase DNA damage and TP53 mutation by AFB1 in HepaRG cells, which have hepatocyte-like morphology, effectively metabolize AFB1 and support HBV infection.[32] Aflatoxin exposure to individuals with HBV infection increases the risk of HCC by at least 3-fold.[33] Hepatic oval cells, considered to be liver stem cells, cause liver tumors in an overexpressing HBx xenograft tumor model combined with AFB1 exposure.[34] The collaborative effect of HBx and AFB1 in causing hepatic steatosis in zebrafish and HBV transgenic mice has been reported.[35] Our results suggest an HBx-PXR-CYP3A4/GSTM1-AFB1 genotoxicity-KRAS-IL11:IL11RA signaling axis to explain the synergistic effect of chemical and infectious liver carcinogens

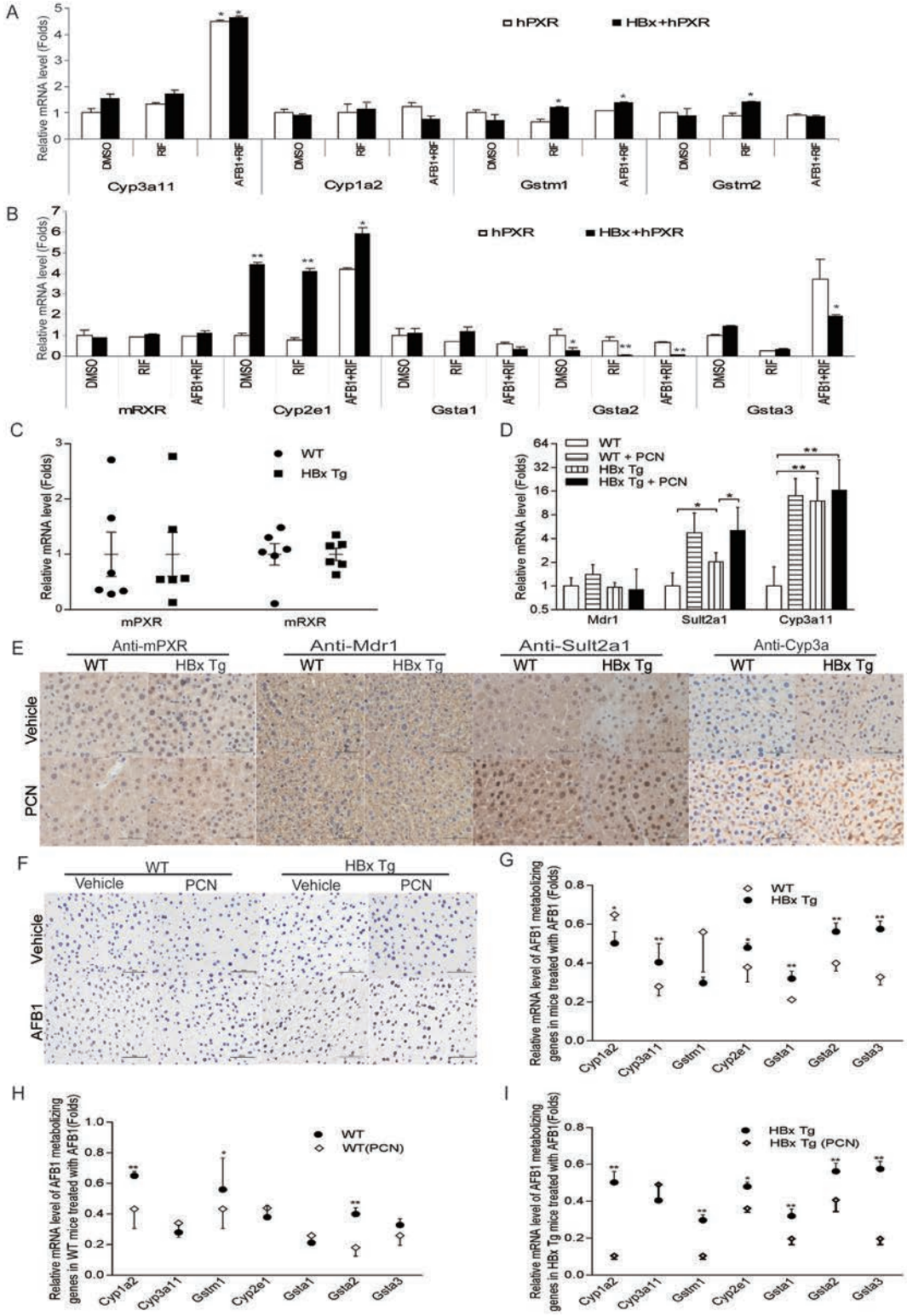

Fig. 4. PXR-AFB1-metabolizing genes (Cyp3a11/Gstm1)-AFB1:DNA adduct signaling in HBx Tg mice. (A, B) Relationship of AFB1 metabolizing genes and HBx. Mice Hepa1-6 cells were treated with RIF and AFB1 after transient transfection of HBx and hPXR. Expression of CYP3A11, CYP1A2, GSTM1, mRXR, CYP2E1, Gsta1, Gsta2, and Gsta3 was analyzed by qRT-PCR. (C) Expression of mPXR and mRXR in HBx Tg mice treated with PCN. (D, E) Expression of PXR signaling genes in HBx Tg mice after PCN treatment, determined by qRT-PCR and IHC. Representative immunostaining of mPXR, Mdr1, Sult2a1 and CYP3A (instead of CYP3A11) in control mice and HBx Tg mice. (F) IHC showing induction of AFB1:DNA adducts by activated PXR in newborn HBx Tg mice. (G–I) Representative expression of genes responsible for AFB1 metabolism in both control mice and HBx Tg mice with or without PCN treatment. *$p<0.05$, **$p<0.01$, treated vs. control.

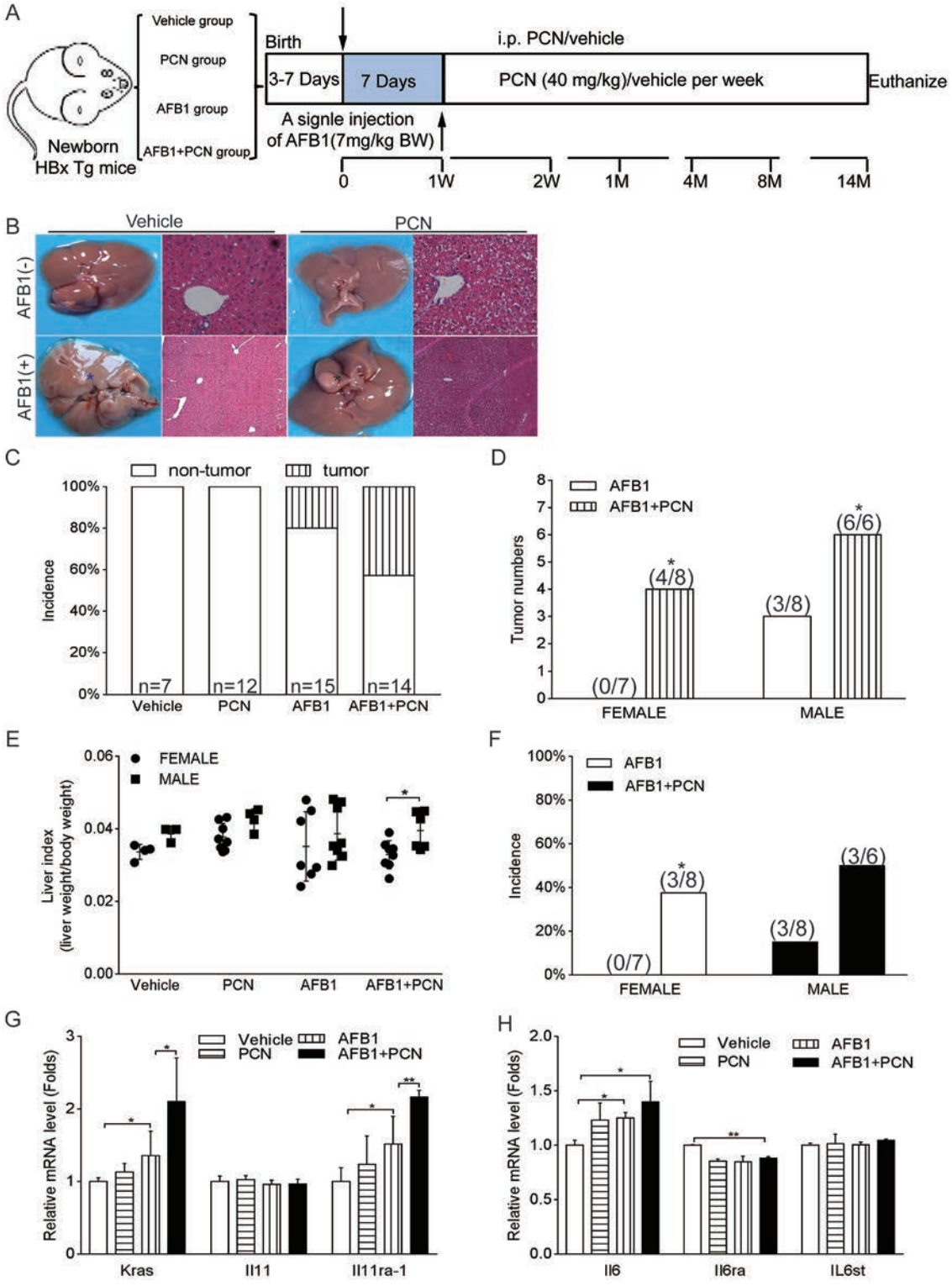

Fig. 5. Molecular mechanism of tumorigenesis due to HBx and AFB1 co-exposure in mice. (A) Scheme of the experimental protocol for a single injection of AFB1 (7 mg/g) in newborn HBx Tg mice. All mice were first treated with PCN (40 mg/kg of BW) or vehicle on day 7 post-injection of AFB1, and then were administered intraperitoneal injections every 7 days. Mice were euthanized at 14 months. (B) Representative gross appearance and hematoxylin and eosin-stained sections (×100 magnification) of livers from 14-month-old HBx Tg mice. Asterisks denote visible tumors between the groups as indicated. Representative hematoxylin and eosin staining from HBx Tg mice/(AFB1, AFB1+PCN) groups showing liver tumorigenesis *in situ*. (C) Prevalence of tumorigenesis in vehicle (n=7), PCN (n=12), AFB1 (n=15), and AFB1+PCN (n=14) HBx Tg mice. (D–G) Tumor number, liver index and prevalence of tumorigenesis in male and female HBx Tg mice. (H) Expression of inflammatory genes was measured by qPCR. *p<0.05, **p<0.01.

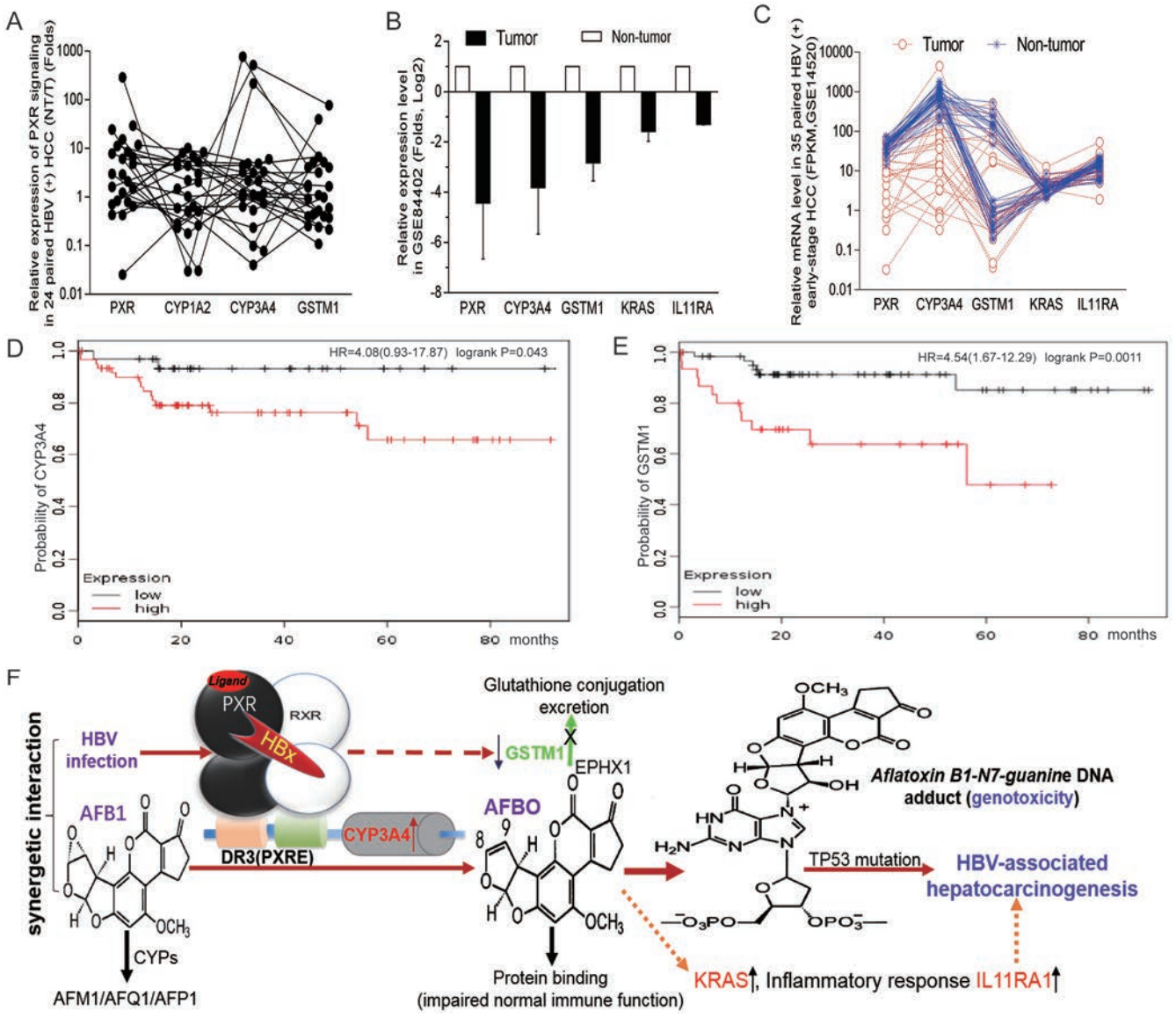

Fig. 6. Clinical correlation of the HBx-PXR-AFB1-metabolizing signaling axis in HBV-associated HCC. (A) Expression of PXR, CYP1A2, CYP3A4 and GSTM1 in 24 paired HBV-associated HCC samples. The value represents the ratio of gene expression in adjacent nontumor tissues to that in the corresponding HCC. (B) Expression of PXR, CYP3A4, GSTM1, KRAS and IL11RA in HBV-associated HCC from the GSE84402 dataset. (C) Expression of PXR, CYP3A4, GSTM1, KRAS and IL11RA in the early-stage HBV-associated HCC dataset (GSE14520). (D, E) Prognostic value for survival time of CYP3A4 and GSTM1 in the Asian HCC cohort with hepatitis. (F) Schematic diagram illustrating AFB1 exposure combined with ectopic activation of PXR signaling by HBx in hepatocarcinogenesis. Under the conditions of AFB1 and HBV co-exposure, the binding of agonist-activated PXR with RXRα to the PXRE region of the CYP3A4 promoter is enhanced by HBx coactivation. Bioactivation of AFB1 to AFBO, which is catalyzed by CYP3A4, is augmented as a result of enhanced transactivation of the CYP3A4 gene. Alternatively, the detoxification pathway through EPHX and GSTM1 is compromised by the molecular interaction of PXR and HBx due to transcriptional suppression of GSTM1. As a result, genotoxic AFBO accumulates to form DNA adducts or binds to proteins and causes genomic instability, which may lead to mutations in p53 and/or an increased activity of the KRAS-IL11:IL11RA-1 axis.

in hepatocarcinogenesis (Fig. 6G), although the findings are not likely the only reasonable explanation for hepatitis virus-associated HCC (Fig. 6F).

PXR can increase toxic xenobiotic-induced hepatotoxicity in the liver.[10,36] CYP3A4 increases liver toxicity by metabolizing xenobiotics to carcinogens, which has been shown to have clinical significance.[37] PXR-CYP3A11 might be involved in the inflammatory response to tumorigenesis in AFB1-induced liver tumor models.[38] HepG2 cells with activities of various phase I, II, and III enzymes represent a good cellular model to investigate the activation and detoxification of genotoxic procarcinogens.[39] Our findings that RIF increases susceptibility to AFB1 in HepG2 cells but not PXR knockdown cells also support our hypothesis that PXR is involved in the activation and detoxification of AFB1. Iterative HBV infection leads to interrelated changes in CYP3A4 involved in the carcinogenic activation of AFB1 in patients with hepatitis or cirrhosis.[40] The induction of P450s by HBx has been associated with the bioactivation of AFB1 to AFBO.[41] Subsequently, we showed that HBx increases AFB1 cytotoxicity via PXR-CYP3A4 signaling. Moreover, chromatin immunoprecipitation- sequencing data show GSTM1, a phase II metabolism gene closely related to HCC,[42] is regulated by PXR.[43] Of note, in China

in early 1995, individuals from a high AFB1-exposed population with mutant genotypes at EPHX1 and GSTM1 were reported to be at greater risk for HCC due to p53 mutations at codon 249. EPHX1 is speculated be a novel PXR target gene.[44] However, the EPHX1 locus did not appear to be related to HCC in a human study including 231 HCC cases and 256 controls.[45] Despite the fact that HBx might be involved in the induction of phase I and II metabolic enzymes responsible for the bioactivation and elimination of AFB1,[46] HBx alone is insufficient to induce most CYPs and GSTs to alter the antioxidant system in most cell studies and animal models.[47] Mariana *et al*.[48] reported that, via a meta-analysis, the evidence for HBV-aflatoxin interaction with TP53 mutation in HCC is weak. Sulforaphane inhibits the formation of AFB1:DNA adducts, which is dependent on transcriptional repression of AFB1 metabolic enzymes rather than direct inhibition of catalytic activity, and GSTM1 has strong protective effects against DNA damage by AFB1 in the human liver.[49] Similarly, the synergistic interaction between virus and AFB1 exposure does not provide direct evidence to elucidate the synergistic effect of co-exposure of HBx and chemical carcinogens.

Based on our observations, HBx-PXR signaling can increase AFB1 hepatotoxicity in HepG2 cells, and activated PXR can promote the incidence and number of liver malignancies resulting from AFB1-induced carcinogenesis in HBx Tg mice by the age of 14 months, as observed in comparison to our cohort of paired only AFB1 exposure, vehicle, and PCN groups. Therefore, we propose that abnormal transactivation of PXR by HBx and/or AFB1 may promote subsequent carcinogenesis, despite the fact that we did not show whether there is a difference in AFB1:DNA adducts between the AFB1+PCN group and AFB1 treatment only group in the initiation stage of carcinogenesis. Moreover, sex disparity is a remarkable feature of inflammation-driven HCC.[1] IL-6 has been identified as a key regulator of male-predominant liver carcinogenesis.[26] We also showed that males develop liver neoplasms with much higher frequency than females, and KRAS-mediated canonical IL-6 family member IL11:IL11RA-1 signaling may be a major driver of hepatocellular carcinogenesis in our animal models; although, we still cannot discount the interference from IL6:IL6RA signaling.

In conclusion, by exploring the relationship between HBx-PXR interaction and AFB1 metabolism, we propose that sustained activation of PXR, especially by HBx, might aggravate the hepatotoxicity or genotoxicity of AFB1 by inducing CYP3A4 and reducing GSTM1. Furthermore, increased tumor development may be linked to an oncogene KRAS-mediated IL11:IL11RA inflammatory response to induce HCC. Overall, we uncovered an HBx-PXR-CYP3A4/GSTM1-AFB1 genotoxicity-KRAS-IL11:IL11RA signaling axis to explain the synergistic effect of chemical and infectious liver carcinogens in hepatocarcinogenesis, though the exact relationship of IL11RA signaling, synergistic effects of aflatoxin B1 and HBV in promoting hepatocarcinogenesis remains complicated.

Funding

This study was funded by the National Natural Science Foundation of China (Grant Nos. 81772972, 81672731, 81572703, 81572451), Natural Science Foundation of Guangdong Province (Grant Nos. 2021A1515010776, 2015A030313449), Science and Technology Planning Project of Guangdong Province “Public Research and Capacity Building” Special Project Fund (Grant No. 2014A020212285), Department of Education, Guangdong Government under the Top-tier University Development Scheme for Research and Control of Infectious Diseases (Grant Nos. 2016026, 2015060, 2015089).

Conflict of interest

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

Author contributions

Conceived the project (YN, WX, GS), designed the experiments (YN, SF), performed the experiments (QL, LC, DH, SF, WC), performed analyses (YN, SF, WC), oversaw the writing of the manuscript (YN, WX, GS). All authors read and approved the final manuscript.

Data sharing statement

All data are available upon request.

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Journal of Clinical and Translational Hepatology **2021** vol. 9(4) | 477–483
DOI: 10.14218/JCTH.2020.00150

Original Article

Effects of Bivalirudin and Unfractionated Heparin on Liver and Renal Function in Chinese Patients with Coronary Artery Disease Undergoing Coronary Angiography with/without Percutaneous Coronary Intervention

Qiaowei Jia[1,2#], Jia Hu[1#], Wenfeng Ji[1], Liansheng Wang[1] and Enzhi Jia[1*]

[1]*Department of Cardiovascular Medicine, First Affiliated Hospital of Nanjing Medical University, Nanjing, Jiangsu, China;*
[2]*Department of Cardiovascular Medicine, Liyang People's Hospital, Liyang, Jiangsu, China*

Received: 4 December 2020 | **Revised:** 13 February 2021 | **Accepted:** 19 March 2021 | **Published:** 9 April 2021

Abstract

Background and Aims: Unfractionated heparin (UFH) and bivalirudin are widely used as anticoagulants in cardiovascular medicine, including for thrombosis prevention during coronary angiography (CAG) and percutaneous coronary intervention (PCI). Little is known of the effects of UFH and bivalirudin on liver and kidney function in patients subjected to these procedures. This study compared the effects of bivalirudin and UFH on liver/renal function in patients with coronary artery disease who underwent CAG, with or without PCI. ***Methods:*** The study comprised 134 consecutive patients (40–89 years-old), who underwent CAG (or CAG and PCI); among them, 66 and 68 patients were subject to, respectively, bivalirudin or UFH. The following indicators of liver/renal function were measured before and after the procedures: plasma alanine aminotransferase (ALT), aspartate aminotransferase (AST), blood urea nitrogen, estimated glomerular filtration rate (eGFR), creatinine clearance, and serum creatinine. Patients were further stratified by severity of chronic kidney disease (CKD), based on original eGFR. ***Results:*** Relative to baseline, in the bivalirudin group, ALT and AST were higher after CAG (p=0.005, 0.025), while blood urea nitrogen and serum creatinine were lower (p=0.049, <0.001). In the UFH group, ALT, AST, eGFR, and creatinine clearance were lower after CAG ($p \leq 0.001$, all). Patients given bivalirudin with moderate or severe CKD, but not those with mild CKD, gained significant improvement in kidney function. ***Conclusions:*** Relative to UFH, bivalirudin may better safeguard the renal function of patients with coronary artery disease who undergo CAG, especially patients with moderate-to-severe renal insufficiency. UFH may cause less liver damage than bivalirudin.

Citation of this article: Jia Q, Hu J, Ji W, Wang L, Jia E. Effects of bivalirudin and unfractionated heparin on liver and renal function in Chinese patients with coronary artery disease undergoing coronary angiography with/without percutaneous coronary intervention. J Clin Transl Hepatol 2021;9(4):477–483. doi: 10.14218/JCTH.2020.00150.

Introduction

Coronary angiography (CAG) and percutaneous coronary intervention (PCI) are recommended for patients with a high risk of acute coronary syndrome. These procedures require adjunctive antithrombotic therapy with anticoagulants and antiplatelet agents.[1] However, there is no gold standard antithrombotic agent, with both optimal clinical benefits and acceptable risk of complications.

Unfractionated heparin (UFH) is one of the oldest agents applied for prevention and treatment of arterial and venous thromboembolism, and is used widely as an anticoagulant during CAG and PCI for its convenience, safety, and low cost. In addition, many new anticoagulation agents have appeared in clinical practice in recent decades. Bivalirudin is a direct thrombin inhibitor, extracted from the derivative hirudin fragment, which is widely used in patients undergoing PCI. Compared with UFH or glycoprotein IIb/IIIa inhibitors, the clearance of bivalirudin is less dependent on renal function,[2] and bivalirudin is characterized by rapid onset and fewer complications, with a short half-life of 25 minutes under normal renal function.[3]

Bivalirudin is currently considered an alternative for patients with progressed and advanced chronic kidney disease (CKD).[4] CKD is prevalent among patients with coronary artery disease (CAD) and has been associated with shorter survival, bleeding, and thrombosis as a complication of PCI.[5–7] This may be due to the multiple hemostatic perturbations in patients with CKD.[8–9]

Hemostasis is largely modulated by protein synthesis

Keywords: Bivalirudin; Unfractionated heparin; Coronary artery disease; Coronary angiography; Percutaneous coronary intervention; Liver function.

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; BUN, blood urea nitrogen; CAD, coronary artery disease; CAG, coronary angiography; CCr, creatinine clearance; CKD, chronic kidney disease; CK-MB, myoglobin isoenzyme of creatine phosphokinase; FBG, fasting blood glucose; eGFR, estimated glomerular filtration rate; HDL, high-density lipoprotein cholesterol; LDL, low-density lipoprotein cholesterol; LMWH, low-molecular-weight heparin; MHB, myohemoglobin; PCI, percutaneous coronary intervention; SCr, serum creatinine; TC, total cholesterol; TG, triglyceride; UA, uric acid; UFH, unfractionated heparin.

[#]These authors contributed equally to this work.

[*]**Correspondence to:** Enzhi Jia, Department of Cardiovascular Medicine, First Affiliated Hospital of Nanjing Medical University, Guangzhou Road 300, Nanjing, Jiangsu 210029, China. Tel: +86-13951623205, E-mail: enzhijia@njmu.edu.cn, enzhijiacn@aliyun.com

and degradation in the liver. In patients with severe liver disease, the hemostatic system is always dysfunctional because of hepatic protein synthesis disorders.[10] Yet, studies concerning the effects of anticoagulants on liver function are limited.

To aid clinicians' selection of anticoagulant, the present study evaluated the relative effects of bivalirudin and UFH on the liver and kidney functions of patients with CAD who underwent CAG, with or without PCI.

Methods

Participants

Participants were recruited from consecutive patients who underwent CAG with or without PCI at the First Affiliated Hospital of Nanjing Medical University from 8 July 2017 to 30 June 2020. Patients with any of the following were excluded: massive hemorrhage within 1 month; severe thrombocytopenia (blood platelet count $<20\times10^9$/L); dialysis-dependent end-stage renal failure; or allergy to bivalirudin or hirudin. Massive hemorrhage sufficient for exclusion was defined as clinically overt bleeding, accompanied by a decrease in hemoglobin ≥2 g/dL, requiring a transfusion of ≥2 U of packed red blood cells, and occurring at a site of concern (intracranial, intraocular, intraspinal, intra-articular, intramuscular with compartment syndrome, pericardial, or retroperitoneal), or resulting in death.[11]

Finally, the study population consisted of 134 patients, aged 40 to 89 years. Among them, 66 and 68 were administered, respectively, bivalirudin and UFH as antithrombotic therapy during CAG.

Application of bivalirudin and UFH during CAG

Bivalirudin was given intravenously at a loading dose of 0.75 mg/kg before CAG, and then at 1.75 mg/kg/h as intravenous drip until the end of the surgery, with an additional 4 h intravenous drip for those who underwent PCI. During CAG, patients with creatinine clearance (CCr <30 mL/m and not on dialysis were given bivalirudin at a rate of 1.0 mg/kg/h. UFH was given intravenously at a dose of 2,000 U before angiography, with an additional 0-14,000 U of UFH during the operation on an as-needed basis for those undergoing PCI. Iodixanol injection was used as contrast agent for CAG and PCI.[12]

Clinical design

Demographic data, medical history, and the results of laboratory measurements of the patients, including alanine aminotransferase (ALT, in U/L), aspartate aminotransferase (AST, in U/L), blood urea nitrogen (BUN, in mmol/L), serum creatinine (SCr, in μmol/L), total cholesterol (TC, in mmol/L), triglyceride (TG, in mmol/L), fasting high-density lipoprotein (HDL) cholesterol (in mmol/L), fasting low-density lipoprotein (LDL) cholesterol (in mmol/L), fasting blood glucose (FBG, in mmol/L), uric acid (UA, in μmol/L), myoglobin isoenzyme of creatine phosphokinase (CK-MB, in ng/mL), myohemoglobin (MHB, ng/mL), red blood cell count ($\times10^{12}$/L), white blood cell count ($\times10^9$/L), platelet count ($\times10^9$/L), hemoglobin (in g/L), and the Gensini score, were collected and sorted in a dedicated database. The differences in the following laboratory parameters before (baseline) and after CAG were compared between the bivalirudin and UFH groups: ALT, AST, creatinine clearance (CCr), and estimated glomerular filtration rate (eGFR). The results of CAG were reported by at least two experienced cardiologists immediately at the end of the procedure. The Gensini score was used to evaluate the severity of CAD,[13] after all procedures and other data collection.

The CCr was estimated using the Cockcroft-Gault equation, as follows: CCr in mL/m=(140-age, y)×(weight in kg)×(0.85, if female)/(72×SCr in mg/dL).[14] The eGFR in this Chinese population was calculated using the "CKD-EPI" equation as follows, with the GFR expressed as mL/m/1.73 m^2, SCr as mg/dL. and age in years. For females with SCr ≤(>)0.7, then $eGFR=(144)\times(SCr/0.7)^a\times(0.993)^{age}$, where a=−0.329 (−1.209). For males with SCr ≤(>)0.9, then $eGFR=(141)\times(SCr/0.9)^a\times(0.993)^{age}$, where a=−0.411 (−1.209).[15]

Patients were stratified according to eGFR as having mild (≥60 mL/m), moderate (30–69 mL/m), or severe (<30 mL/m) CKD.[16]

Ethical approval and consent to participate

All patients provided written informed consent. The ethics committee of Nanjing Medical University approved all the experimental protocols.

Data analysis

The data analysis was performed using the Statistical Package for Social Sciences software (ver. 16.0; SPSS, Chicago, IL, USA). Skewed data are presented as median (interquartile range), normal data as mean±standard deviation, and categorical data as absolute values. Data analyses utilized chi-squared tests to determine differences in sex, smoking status, drinking status, and medical history. Independent samples *t*-tests, one-way analysis of variance, and paired samples *t*-tests were applied to normal data, as appropriate. Other baseline characteristics (non-normal data) were examined by Mann-Whitney and Wilcoxon rank tests. Multi-factor logistic regression analysis was applied to identify the risk factors to liver function and kidney function. A *p*-value of <0.05 was considered significant in the 2-tailed tests.

Results

Baseline characteristics of the subjects

Compared with the patients given UFH in this study, the patients in the bivalirudin group were significantly older ($p<0.001$), and with higher levels of ALT, SCr, BUN ($p<0.001$, each), and AST ($p=0.002$). In addition, patients in the bivalirudin group had significantly higher rates of hypertension, cerebral infarction ($p=0.002$, both) and CAD ($p=0.011$). The HDL cholesterol ($p=0.280$), LDL cholesterol ($p=0.274$), and FBG ($p=0.836$) (Table 1).

Baseline characteristics of the bivalirudin group stratified by CKD severity

Renal function was judged prior to CAG as mild, moderate, or severe based on eGFR, according to the international standard (Table 2).[16] Among all the baseline characteristics considered, levels of only the following increased significantly with classification of severity: SCr, BUN, UA, and MHB ($p<0.001$, all). Only FBG decreased with severity of CKD ($p=0.032$).

Table 1. Baseline characteristics of the subjects by the anticoagulants used in CAG and PCI

	Bivalirudin	UFH	*p*
Subjects, *n*	66	68	—
Age, years	71.09±11.53	62.68±9.18	<0.001
Sex, M/F	51/15	50/18	0.615
Weight, kg	69.39±11.05	66.72±7.82	0.109
Hypertension, Y/N	55/11	40/28	0.002
Diabetes mellitus, Y/N	26/40	19/49	0.160
Cerebral infarction, Y/N	23/43	8/60	0.002
Smoke, Y/N	25/41	31/37	0.327
Drink, Y/N	15/51	11/57	0.338
ALT, U/L	22.65 (14.28–34.23)	35.00 (27.93–44.00)	<0.001
AST, U/L	22.65 (17.95–31.10)	27.85 (20.93–40.78)	0.002
SCr, μmol/L	117.00 (80.85–186.95)	61.20 (51.83–73.13)	<0.001
BUN, mmol/L	8.36 (6.10–13.95)	5.69 (4.75–6.81)	<0.001
TC, mmol/L	4.04±1.23	3.95±1.05	0.664
TG, mmol/L	1.20 (0.93–1.62)	1.39 (0.94–2.04)	0.177
HDL, mmol/L	0.98±0.29	1.02±0.23	0.280
LDL, mmol/L	2.48±0.90	2.32±0.78	0.274
FBG, mmol/L	5.03 (4.34–6.13)	4.99 (4.48–6.26)	0.836
UA, μmol/L	427.29±134.88	318.81±99.09	<0.001
CK-MB, ng/mL	3.79 (2.39–12.18)	2.05 (1.66–3.62)	<0.001
MHB, ng/mL	24.00 (11.30–43.64)	13.18 (10.36–19.95)	0.003
Gensini score	86.00 (37.75–126.00)	48.00 (12.88–93.00)	0.011

Skewed data are presented as median (interquartile range), normal data as mean±standard deviation, and categorical data as absolute values. N, no; Y, yes.

Liver and renal function tests before and after CAG

To evaluate the potential benefits of bivalirudin for patients with CKD, the differences in ALT, AST, BUN, and SCr from baseline after CAG were examined (Table 3). For patients given bivalirudin, the serum levels of ALT and AST were significantly higher after CAG (p=0.005, 0.025, respectively), which indicated possible liver injury, while BUN and SCr were lower (p=0.049, <0.001), suggesting a renoprotective effect. Significant increases in the calculated CCr (p=0.001) and eGFR (p=0.022) also indicated improvement in renal function.

In the UFH group, the serum levels of ALT and AST significantly declined after CAG compared with the baseline (p<0.001, =0.001); while BUN (p=0.009), SCr (p<0.001), CCr (p<0.001) and eGFR (p<0.001) decreased. Thus, UFH may exert some positive effects on the liver but not on the kidney.

Differences in eGFR after CAG according to eGFR and Gensini score

To explore the renal benefits of bivalirudin among patients with different original renal functions, patients were apportioned to three groups according to eGFR; as mild, moderate or severe CKD (Table 4). Patients with moderate or severe CKD gained significant renal benefits (p=0.018, 0.039), while patients with mild CKD failed to show obvious improvements in kidney function (p=0.890). This suggested that bivalirudin may be more likely to exert renoprotective effects in patients with moderate-to-severe renal insufficiency.

Gensini scoring is widely used for determining the severity of CAD (Table 4). To investigate further the renal benefits of bivalirudin in patients with different severities of CAD, patients were apportioned to three groups according to the range interquartile of Gensini score. The eGFR data after CAG in patients with different severities of CAD showed no significant difference, suggesting that the renal benefits of bivalirudin may be not related to the severity of CAD.

Risk factors of liver and renal effects based on multi-factor logistic regression analysis

To identify risk factors of liver and renal effects among the overall population, a multi-factor logistic regression analysis was conducted (with the forward selection-conditional method; Table 5). The following were determined to affect renal function independently: the anticoagulant used in PCI (p<0.001); weight (p=0.001); and, Gensini score (p=0.030). Bivalirudin increased the probability of improvement in renal function by 82.7% compared with UFH.

Similarly, UFH exerted a hepatoprotective effect that was independent of other potentially confounding factors. In the UFH group, the plasma levels of ALT and AST were, respectively, 82.2% and 65.8% in the bivalirudin group.

Discussion

In this study, we compared the effects of bivalirudin and UFH on liver and renal function in patients with CAD who

Table 2. Baseline characteristics of patients' prior bivalirudin by CKD severity

	Mild	Moderate	Severe	*p*
Subjects, *n*	26	25	15	—
Age, years	67.92±11.92	72.84±12.21	73.67±8.76	0.195
Sex, M/F	19/7	19/6	13/2	0.595
Weight, kg	71.87±11.41	68.00±10.58	67.43±11.11	0.343
HTN, Y/N	19/7	22/3	14/1	0.179
Diabetes mellitus, Y/N	8/18	9/16	9/6	0.165
CI, Y/N	11/15	9/16	3/12	0.348
Smoke, Y/N	10/16	9/16	6/9	0.966
Drink, Y/N	5/21	7/18	3/12	0.726
ALT, U/L	26.75 (15.18–39.45)	19.60 (13.95–31.25)	24.00 (12.50–31.60)	0.216
AST, U/L	25.35 (18.35–35.23)	23.50 (18.80–29.25)	18.20 (14.00–21.90)	0.091
SCr, μmol/L	74.07±19.33	139.50±30.40	286.75±93.49	<0.001
BUN, mmol/L	6.32±2.83	10.26±4.07	16.76±4.48	<0.001
TC, mmol/L	3.92±1.24	4.10±1.24	4.15±1.25	0.818
TG, mmol/L	1.55±0.78	1.26±0.43	1.18±0.52	0.112
HDL, mmol/L	0.89±0.28	1.08±0.28	0.95±0.27	0.068
LDL, mmol/L	2.36±0.83	2.48±1.00	2.70±0.89	0.517
FBG, mmol/L	5.46 (4.84–6.69)	4.91 (4.37–5.96)	4.47 (4.04–5.35)	0.032
UA, μmol/L	346.01±103.48	468.71±118.81	499.13±142.44	<0.001
CK-MB, ng/mL	3.75 (2.39–11.35)	3.15 (2.06–7.70)	6.07 (2.52–14.99)	0.426
MHB, ng/mL	14.50 (8.54–22.28)	25.00 (12.26–39.44)	78.88 (34.78–116.36)	<0.001
Gensini score	101.13±85.63	91.26±60.57	81.90±80.82	0.730

Data points are as reflected by eGFR. Skewed data are presented as median (interquartile range), normal data as mean±standard deviation, and categorical data as absolute values. CI, cerebral infraction; HTN, hypertension; N, no; Y, yes.

underwent CAG, with or without PCI. For data analysis, the subjects were apportioned to either the bivalirudin or UFH group, as appropriate. After rigorous laboratory measurements, data collection, and statistical comparisons, we made some surprising and interesting discoveries.

In the group given UFH, the ALT and AST levels after CAG

Table 3. Laboratory parameters reflecting liver and renal functions before and after CAG in the bivalirudin and UFH groups

	Before PCI	After PCI	*p*
Bivalirudin			
ALT, U/L	22.65 (14.28–34.23)	27.00 (21.55–36.00)	0.005
AST, U/L	22.65 (17.95–31.10)	25.50 (19.20–32.95)	0.025
BUN, mmol/L	10.18±5.43	9.53±5.10	0.049
SCr, μmol/L	147.19±94.99	136.68±84.91	<0.001
CCr, mL/m	53.54±33.61	58.02±38.56	0.001
eGFR, mL/m	55.21±31.49	57.79±32.19	0.022
UFH			
ALT, U/L	35.00 (27.93–44.00)	28.70 (19.43–40.58)	<0.001
AST, U/L	27.85 (20.93–40.78)	27.00 (19.48–38.48)	0.001
BUN, mmol/L	5.81±1.52	5.37±1.62	0.009
SCr, μmol/L	64.43±17.20	68.83±17.44	<0.001
CCr, mL/m	99.37±26.69	92.07±21.93	<0.001
eGFR, mL/m	100.53±15.41	96.80±15.53	<0.001

Table 4. Baseline and postoperative[a] eGFR values according to severity of CKD and Gensini score in the bivalirudin group

	Subjects, *n*	Baseline	Postoperative	*p*
CKD[b]				
Mild	26	88.73±19.43	90.82±22.32	0.406
Moderate	25	41.89±7.80	45.03±9.73	0.018
Severe	15	19.33±6.57	21.80±7.31	0.039
Gensini score				
<37.5	16	55.31±37.62	56.50±36.01	0.542
37.5–126	35	52.33±28.23	55.35±30.47	0.099
≥126.5	15	61.86±32.91	64.84±33.13	0.064

Data are presented as mL/m. [a]Before and after CAG; [b]eGFR ranges for mild, moderate, and severe CKD were ≥60, 30–60, and <30 mL/m, respectively.

were significantly lower compared with the baseline levels. This appears to conflict with previous studies. According to the National Library of Medicine's LiverTox database, hepatotoxicity is the most frequently reported adverse event associated with heparins,[17–24] and 8% of the events were due to UFH.[18] The association between UFH and elevations in serum AST was first reported in 1975.[19] However, although AST levels were higher after heparin administration, such elevations were asymptomatic and did not lead to severe liver injury. Conjectured mechanisms included non-hepatic sources for the enzymes,[25] induction of these enzymes in hepatocytes,[26] reduction in the clearance of these enzymes from circulation, and hepatocellular membrane modification.[27,28] In a recent randomized study, circulating mir-122 was selected as a biomarker to identify liver cell necrosis. The researchers opined that heparins, including UFH, may cause a transient, low-level death of hepatocytes, and the subsequent activation of innate immune response may promote the injury.[29]

For clarification, we explored the data further. Among the 68 patients in the UFH group, 8 had higher pre-CAG ALT levels than normal and the remaining 60 had normal pre-CAG ALT levels. While among the eight patients who had higher pre-CAG ALT levels, 4 showed ALT descent to a normal level after CAG. Besides, 14 patients had higher pre-CAG AST levels among the 68 subjects, and only 3 patients' AST level descended to a normal level after CAG. After taking an intersection, we found that only two patients with both higher pre-CAG ALT and AST levels among the 68 subjects achieved improved ALT and AST levels, which descended to normal (where elevation of ALT and AST was defined as >69 and >45 U/L).

On the other hand, among the 66 patients given bivalirudin, 64 had normal pre-CAG ALT levels and only 2 had higher pre-CAG ALT levels than normal. While among the 64 patients who had normal pre-CAG ALT levels, 4 patients' ALT rose to an abnormal level after CAG. Besides, 62 patients had normal pre-CAG AST levels among the 66 subjects, and 7 patients' AST level rose to an abnormal extent after CAG. After taking an intersection, two patients with both normal pre-CAG ALT and AST levels in the bivalirudin group showed worse ALT and AST levels, which became abnormal (where normal ALT and AST was considered 13–69 U/L and ≤45 U/L).

It was reported that cardiac hepatopathy, which is used to describe any liver damage caused by cardiac disorders in the absence of other possible causes of liver damage, can be examined as congestive hepatopathy and acute cardiogenic liver injury. Furthermore, acute cardiogenic liver injury is

Table 5. Multi-factor logistic regression analysis of associations between anticoagulant (bivalirudin or UFH) and basic characteristics of patients and renoprotective effects[a], ΔALT[b], and ΔAST[c]

	OR (95% CI)	*p*
Renoprotective effects[a]		
Anticoagulant	0.173 (0.073–0.409)	<0.001
Weight	0.922 (0.878–0.968)	0.001
Gensini score	1.007 (1.001–1.013)	0.030
ΔALT[b]		
Anticoagulant	0.178 (0.078–0.404)	<0.001
TG	0.478 (0.244–0.936)	0.031
ΔAST[c]		
Anticoagulant	0.342 (0.155–0.755)	0.008
Sex	0.395 (0.159–0.980)	0.045
Gensini score	1.011 (1.005–1.018)	0.001

[a]The renoprotective effect was calculated as $\Delta eGFR=eGFR_2-eGFR_1$; where $eGFR_1$ and $eGFR_2$ are the eGFR values before and after CAG, respectively. ΔeGFR >0 (<0) indicates positive (negative) renoprotective effects. The covariates were age, sex, weight, medical history, smoking and drinking status, plasma levels of ALT, AST, BUN, TC, TG, HDL, LDL, FBG, UA, CK-MB and MHB, and Gensini score. [b]$\Delta ALT=ALT_2-ALT_1$; where ALT_1 and ALT_2 are the ALT values before and after CAG, respectively. The covariates were age, sex, weight, medical history, smoking and drinking status, plasma level of AST, SCr, BUN, TC, TG, HDL, LDL, FBG, UA, CK-MB and MHB, and Gensini score. [c]$\Delta AST=AST_2-AST_1$; where AST_1 and AST_2 are the AST values before and after CAG, respectively. The covariates were age, sex, weight, medical history, smoking and drinking status, plasma level of ALT, SCr, BUN, TC, TG, HDL, LDL, FBG, UA, CK-MB and MHB, and Gensini score.

most commonly associated with acute cardiocirculatory failure caused by acute myocardial infarction, acute decompensated hepatic failure, or myocarditis.[30] In acute cardiogenic liver injury patients, the laboratory measurements showed elevation in transaminase and lactate dehydrogenase levels.[30–32] Thus, we hypothesize that the decline in transaminase in the UFH group was mainly due to the improvement in coronary circulation and myocardial oxygen delivery after the CAG; the liver benefited as well, and the mild liver injury from the UFH was more than compensated for.

Thus, regarding liver function, patients undergoing CAG and PCI may benefit more from UFH, relative to bivalirudin. Notably, heparins were shown to alleviate liver injury in several animal studies.[33,34]

In addition, significant renal improvement was observed in the bivalirudin group compared with the UFH (Supplementary Fig. 1 and 2). This was especially true for patients suffering from moderate or severe CKD; patients with eGFR ≥60 mL/m showed no significant renal benefits from bivalirudin. The paired-samples tests suggested that the renoprotective effects of bivalirudin may not be associated with the severity of CAD. In other words, the renal benefits of bivalirudin may be enjoyed by patients with either mild or severe CAD.

This study has several limitations. First, the sample size is small, which may lead to inaccuracy of the results and conclusions. Further studies with large samples are warranted. Second, the results would be more convincing if patients with similar renal function were matched with the bivalirudin group as a control group. The mechanisms of the effects on liver and kidney of bivalirudin and UFH have not been clarified, and we intend further explorations of these questions in the future.

Despite its limitations, this study is the first to discuss the renal benefits of bivalirudin, and to suggest a possible liver benefit associated with UFH, in patients undergoing CAG and PCI. This report may help physicians choose anticoagulants for patients with abnormal liver and kidney function. We have planned a future multicenter, large-sample, and multi-ethnic study to verify these conclusions and explore the mechanisms.

Conclusions

As anticoagulants used for CAG and PCI procedures, bivalirudin may provide better benefit to renal function compared with UFH, especially in patients with moderate-to-severe renal insufficiency. On the other hand, UFH is less likely to cause liver injury than bivalirudin.

Acknowledgments

The authors are grateful for the support provided by the First Affiliated Hospital of Nanjing Medical University and Liyang People's Hospital.

Funding

This study received support from the National Natural Science Foundations of China (No. 81970302).

Conflict of interest

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

Author contributions

Guarantor (EJ), conception of the study (EJ), initial drafting of the paper (QJ), enrollment of participants and collection of data (JH), supervision of the enrollment of patients and collection of data (EJ), and data analysis and review of the manuscript for important intellectual content (EJ, QJ, JH).

Data sharing statement

All data are available upon request.

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Journal of Clinical and Translational Hepatology **2021** vol. 9(4) | 484–493
DOI: 10.14218/JCTH.2021.00018

Original Article

Serum Resistin Levels in Adult Patients with Nonalcoholic Fatty Liver Disease: A Systematic Review and Meta-analysis

Dongli Han[1#], Jie Chen[2#], Shousheng Liu[3], Zengzhi Zhang[2], Zhenzhen Zhao[3], Wenwen Jin[1*] and Yongning Xin[1,4*]

[1]Department of Infectious Disease, Qingdao Municipal Hospital, Dalian Medical University, Dalian, Liaoning, China; [2]Qingdao City Shinan District Centers for Disease Control and Prevention, Qingdao, Shandong, China; [3]Clinical Research Center, Qingdao Municipal Hospital, Qingdao, Shandong, China; [4]Digestive Disease Key Laboratory of Qingdao, Qingdao, Shandong, China

Received: 7 January 2021 | **Revised:** 19 March 2021 | **Accepted:** 9 April 2021 | **Published:** 17 May 2021

Abstract

Background and Aims: Previous studies reported that serum resistin levels were remarkably changed in patients with nonalcoholic fatty liver disease (NAFLD) but the conclusions were inconsistent. The aim of this study was to investigate accurate serum resistin levels in adult patients with NAFLD. ***Methods:*** A complete literature research was conducted in the PubMed, Embase, and Cochrane Library databases, and all the available studies up to 7 May 2020 were reviewed. The pooled standardized mean difference (SMD) values were calculated to investigate the serum resistin levels in patients with NAFLD and healthy controls. ***Results:*** A total of 28 studies were included to investigate the serum resistin levels in patients with NAFLD. Patients with NAFLD had higher serum resistin levels than controls (SMD=0.522, 95% confidence interval [CI]: 0.004–1.040, I^2=95.9%). Patients with nonalcoholic steatohepatitis (NASH) had lower serum resistin levels than the healthy controls (SMD=−0.44, 95% CI: −0.83–0.55, I^2=74.5%). In addition, no significant difference of serum resistin levels was observed between patients with NAFL and healthy controls (SMD=−0.34, 95% CI: −0.91–0.23, I^2=79.6%) and between patients with NAFL and NASH (SMD=0.15, 95% CI: −0.06–0.36, I^2=0.00%). Furthermore, subgroup and sensitivity analyses suggested that heterogeneity did not affect the results of meta-analysis. ***Conclusions:*** This meta-analysis investigated the serum resistin levels in adult patients with NAFLD comprehensively. Patients with NAFLD had higher serum resistin levels and patients with NASH had lower serum resistin levels than healthy controls. Serum resistin could serve as a potential biomarker to predict the development risk of NAFLD.

Citation of this article: Han D, Chen J, Liu S, Zhang Z, Zhao Z, Jin W, *et al*. Serum resistin levels in adult patients with nonalcoholic fatty liver disease: A systematic review and meta-analysis. J Clin Transl Hepatol 2021;9(4):484–493. doi: 10.14218/JCTH.2021.00018.

Introduction

Nonalcoholic fatty liver disease (NAFLD) is defined as hepatic steatosis by imaging or histology without secondary factors of hepatic fat aggregation, such as significant alcohol consumption and long-term use of a steatogenic medication.[1] NAFLD ranges from nonalcoholic fatty liver (NAFL), which is characterized as simple benign hepatic steatosis, to nonalcoholic steatohepatitis (NASH), the histologic features of which are macrovesicular steatosis, hepatocellular ballooning, lobular inflammation, and pericellular fibrosis. NASH can progress to the more severe fibrosis, that is defined as the accumulation of extracellular matrix proteins in the liver interstitial space, cirrhosis, and even the hepatocellular carcinoma.[2] Nowadays, NASH-associated cirrhosis has become the second leading cause for liver transplantation in the USA. Meanwhile, NAFLD increases the risk of developing type 2 diabetes, cardiovascular disease, and chronic kidney disease.[3]

NAFLD has been certainly become the most predominant chronic liver disease in the world, with the highly shocking prevalence of 25.24% among the global population. In fact, the prevalence is predicted to become even higher in the next decade.[4] Up to now, the diagnostic golden standard for NAFLD is liver biopsy. As an invasive technology, some adverse events can occur during liver biopsy diagnosis of patients, such as pain, infection, bleeding and even death.[5] Therefore, there is an urgent need to develop a novel biomarker to predict and diagnose NAFLD conveniently and accurately.

Resistin belongs to the family of resistin-like molecules, also known as “found in inflammatory zone” (FIIZ), and functions as a pro-inflammatory adipokine.[6] Resistin is mainly produced by adipose tissue, inflammatory cells, such as macrophages and monocytes, and hepatic stellate cells.[7] Previous reports have suggested that resistin could be up-regulated by proinflammatory cytokines, including TNF-α, IL-6, IL-1β. In turn, resistin can activate the nuclear factor-kappa B (NF-κB) signaling pathway and promote the synthesis of TNF-α, IL-6 and other pro-inflammatory agents.[8]

Regarding the association of serum resistin levels in pa-

Keywords: Resistin; Nonalcoholic fatty liver disease; Nonalcoholic steatohepatitis; Biomarker.

Abbreviations: CI, confidence interval; FIIZ, found in inflammatory zone; NAFL, nonalcoholic fatty liver; NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; NOS, Newcastle-Ottawa scale; SD, standard deviation; SMD, standardized mean difference.

[#]These authors contributed equally to this work.

[*]**Correspondence to:** Yongning Xin and Wenwen Jin, Department of Infectious Disease, Qingdao Municipal Hospital, 1 Jiaozhou Road, Qingdao, Shandong 266011, China. Tel: +86-532-82789463, Fax: +86-532-85968434, E-mail: xinyongning@163.com (YX) or 18669726268@163.com (WJ)

tients with NAFLD, the studies showed conflicting results so far. Some researchers have reported that serum resistin levels are high in patients with NAFLD, NAFL, and NASH compared to healthy subjects.[9] However, other researchers have suggested that no significant difference exists for serum resistin levels in patients with NAFLD, NAFL, NASH, and healthy controls.[10,11] In the comparison between patients with NASH and NAFL, some studies have found higher serum resistin levels in patients with NASH, whereas others studies found similar levels of serum resistin in patients with NASH and NAFL.[10–12] Meanwhile, some researchers have reported lower serum resistin levels in patients with NASH compared to patients with NAFL or healthy controls.[10,13]

In consideration of the inconsistence of serum resistin levels in patients with NAFLD, it is worthwhile to investigate the exact performance of serum resistin levels in patients with NAFLD according to the available studies. The aim of this study was to conduct a systematic review of the available studies and comprehensively analyze the relationship between serum resistin levels and the degree of NAFLD.

Methods

Search strategy

To obtain the relevant studies for this meta-analysis, a complete literature search was conducted in the databases of PubMed, Embase, and Cochrane Library by the following strategy: ((((((((((((Nonalcoholic Fatty Liver Disease) OR Non alcoholic Fatty Liver Disease) OR NAFLD) OR Nonalcoholic Fatty Liver Disease) OR Fatty Liver, Nonalcoholic) OR Fatty Livers, Nonalcoholic) OR Liver, Nonalcoholic Fatty) OR Livers, Nonalcoholic Fatty) OR Nonalcoholic Fatty Liver) OR Nonalcoholic Fatty Livers) OR Nonalcoholic Steatohepatitis) OR Nonalcoholic Steatohepatitides) OR Steatohepatitides, Nonalcoholic) OR Steatohepatitis, Nonalcoholic) AND (Resistin) OR Adipocyte Cysteine-Rich Secreted Protein FIIZ3) OR Adipocyte Cysteine Rich Secreted Protein FIIZ3. All the potentially relevant studies in English language and published before 7 May 2020 were reviewed. In case of data missed, we tried to contact the corresponding authors to obtain the original data.

Inclusion and exclusion criteria

Clinical studies which performed comparison of serum resistin levels between NAFLD (NAFL or NASH) patients and healthy controls were suitable for this meta-analysis. Studies were included if they conformed to the following criteria: (1) original full-text publications; (2) NAFLD diagnosed with biopsy, ultrasound, liver enzymes or computerized tomography; and (3) serum resistin levels compared. Studies were excluded according to the following principles: (1) patients with other causes of chronic liver disease (alcoholic fatty liver disease, viral or autoimmune hepatitis); (2) subjects included in more than one study; (3) some necessary data missing and not obtainable from the authors; (4) quality of publication too low; (5) reviews, editorials, case reports, letters, hypotheses, book chapters, studies on animals or cell lines, and unpublished data or abstracts; or (6) participants with other medical conditions, such as diabetes and coronary heart disease.

Data extraction and quality assessment

Two authors (HDL and CJ) evaluated each article and extracted the data independently. The controversy was solved by discussion with a third author (LSS). The study quality was evaluated using the Newcastle-Ottawa scale (NOS), as approved by the Cochrane Collaboration. The NOS uses a star system to decide the quality of a study in three realms: collection, comparability, and outcome/exposure. The NOS assigns four stars for selection, two stars for comparability, and three stars for outcome/exposure. Any study that received a score of 6 or more stars was regarded as being at low risk of bias (the highest quality), and lesser stars indicated a risk of bias.[14]

Statistical analysis

The meta-analysis was conducted using Stata/SE 15.0. Serum resistin levels in the NAFLD group and controls were extracted as mean difference±standard deviation (SD) and the pooled values were expressed as standardized mean difference (SMD) with 95% confidence interval (CI). Forest plots were constructed to evaluate the heterogeneity of included studies by I^2 statistic. According to Higgins and Thompson, I^2 values of approximately 25% represented low heterogeneity, approximately 50% represented medium heterogeneity, and approximately 75% represented high heterogeneity. In this meta-analysis, continuous-weighted fixed-effects model analysis was used when the $I^2 \leq 50\%$. Otherwise, the random-effects model was used. The possibility of publication bias was evaluated using funnel plot and the Egger's regression asymmetry test. The sensitivity analysis, subgroup analysis, and meta-regression analysis were conducted to explore the possible sources of (expected) heterogeneity among the eligible studies. The GRADE approach was used to evaluate the quality of the pooled results of serum resistin levels in the NAFLD group vs. controls, NASH group vs. controls, NAFL group vs. controls, and NAFL group vs. NASH group.

Results

Characteristics of the included studies

According to the search strategy, a total of 448 studies were obtained ((PubMed (n=103), Cochrane (n=328), and Embase (n=13)). After removing 109 duplicates, 339 articles were retrieved. After removing reviews, conference abstracts, letters, editorials, conference papers, notes and short surveys, 159 potential studies were retrieved. After full-text evaluation, 28 studies were included eventually for this meta-analysis (Fig. 1).

The main characteristics of the included studies are summarized in Table 1. All the included studies were cross-sectional or case-control studies. Patients with NAFLD in 22 studies[9,10,12,15–33] were assessed by liver histology, and 5 studies[34–38] evaluated NAFLD by ultrasonography. One study did not specifically describe the diagnosis of NAFLD.[39] Among these studies, 10 were carried out in Asia, 6 in North America, and 10 in Europe. Two studies were carried out in South America. Among the 28 included studies, 25 had no the risk of bias and 3 had risk of bias.

Comparison of the serum resistin levels in NAFLD patients and controls

A total of 1,934 patients with NAFLD and 1,240 controls were included in this study. Only 18 of the included 28 studies investigated the serum resistin levels in NAFLD patients

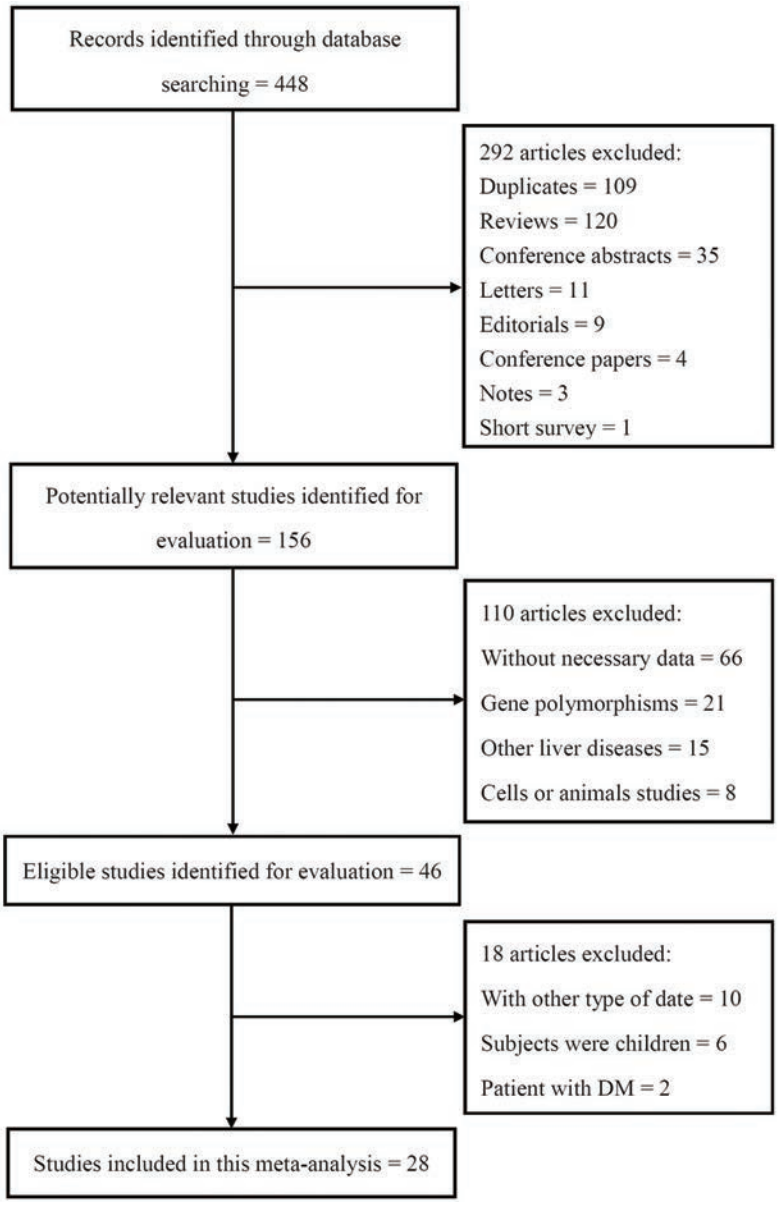

Fig. 1. Flow chart of the literature search process.

(NAFLD patients were not divided into the NAFL or NASH) and healthy controls. Random-effects model was used to conduct the meta-analysis and the results showed that patients with NAFLD had higher serum resistin levels than controls (SMD=0.522, 95% CI: 0.004–1.040, I^2=95.9%) (Fig. 2A). Ten studies investigated the serum resistin levels in patients with NASH and healthy controls. Random-effects model was used to conduct the meta-analysis and the results showed that patients with NASH had lower serum resistin levels than the healthy controls (SMD=−0.44, 95% CI: −0.83–0.55, I^2=74.5%) (Fig. 2B). Seven studies investigated the serum resistin levels in patients with NAFL and healthy controls. Random-effects model was used to conduct the meta-analysis and no significant difference of serum resistin levels was observed between patients with NAFL and healthy controls (SMD=−0.34, 95% CI: −0.91–0.23, I^2=79.6%) (Fig. 2C). Nine studies investigated the serum resistin levels in patients with NAFL and NASH. Fixed-effects model was used to conduct the meta-analysis and the results showed that there was no significant difference of serum resistin levels between patients with NAFL and NASH (SMD=0.15, 95% CI: −0.06–0.36, I^2=0.00%) (Fig. 2D).

Sensitivity and subgroup analyses

In consideration of significant heterogeneity existing between the NASH group vs. controls, NAFL group vs. controls, and NAFL group vs. NASH group, sensitivity analysis was carried out to explore the possible sources of heterogeneity in the included studies. Each study was evaluated by exclusion in turn, and then the summarized SMD of the remaining studies were calculated. Only when the

Table 1. Main characteristics of studies included in the meta-analysis

First author, Year	Group	*n* (M/F)	Age in years	BMI in kg/m^2	Country	Study design	Diagnose of NAFLD	Biopsy on controls	Measurement method of resistin	NOS
Argentou *et al*. 2009[10]	Control	9 (2/7)	37.11±9.78	55.22±8.6	Greece	Cross sectional	Liver biopsy	Yes	ELISA	8
	NAFLD	41 (15/26)	38.88±9.19	56.70±8.06						
	SS	31 (9/22)	38.06±9.23	56.27±8.45						
	NASH	10 (6/4)	41.04±9.07	58.02±6.99						
Auguet *et al*. 2013[20]	Control	19	44.1±10.7	49.5±7.0	Spain	Case-control	Liver biopsy	Yes	ELISA	6
	NAFLD	69	46.79±10.3	48.2±6.6						
Auguet *et al*. 2014[26]	Control	16	44±3.2	48.6±2.6	Spain	Cross sectional	Liver biopsy	Yes	ELISA	7
	SS	28	47.4±3.5	48.1±7.8						
	NASH	28	45.9±1.4	47.5±5.4						
Bostrom *et al*. 2011[19]	Control	40 (10/30)	44 (24–67)	NA	Sweden	Case-control	Liver biopsy	No	ELISA	4
	NAFLD	50 (37/13)	48 (24–65)	NA						
Pagano *et al*. 2006[15]	Control	33 (30/3)	42±3	26.9±1.0	Italy	Case-control	Liver biopsy	No	ELISA	8
	NAFLD	28 (26/2)	45±2	27.3±0.6						
Cengiz *et al*. 2010[18]	Control	24	38±10	25.6±1.1	Turkey	Case-control	Liver biopsy	No	ELISA	7
	NAFLD	76	39±9	30.1±4.5						
Eminler *et al*. 2014[21]	Control	40 (18/22)	NA	NA	Turkey	Cross sectional	Liver biopsy	No	ELISA	6
	NAFLD	40 (21/19)	NA	NA						
Floreani *et al*. 2008[32]	Control	137 (12/125)	60.2±10.4	NA	USA	Case-control	Liver biopsy	No	ELISA	4
	NASH	30 (0/30)	49.9±3.7	24.5±2.8						
Musso *et al*. 2005[28]	Control	25 (23/2)	38±2	25.2±0.6	Italy	Case-control	Liver biopsy	No	ELISA	7
	NASH	25 (23/2)	37±2	25.3±0.2						
Jarrar *et al*. 2008[12]	Control	38 (5/33)	40±9.5	47.5±9.4	USA	Case-control	Liver biopsy	Yes	ELISA	6
	NAFLD	45 (13/32)	NA	NA						
	SS	19 (2/17)	37±9.2	47.2±7.5						
	NASH	26 (11/15)	43.9±11.4	47.5±8.3						
Jiang *et al*. 2009[34]	Control	43	51.1±12.5	24.81±1.91	China	Case-control	Ultrasound	No	ELISA	7
	NAFLD	43	52.6±10.8	25.75±1.91						
Jamali *et al*. 2016[31]	Control	18 (13/5)	30.44±10.11	29.28±3.89	Iran	Case-control	Liver biopsy	No	ELISA	7
	NAFLD	18 (13/5)	34.5±8.85	31.58±3.94						
Krawczyk *et al*. 2009[33]	Control	16	NA	22.6±2.5	Poland	Case-control	Liver biopsy	No	ELISA	6
	NASH	18 (16/2)	42.55±21	26.6±4						
Musso *et al*. 2013[35]	Control	51 (33/18)	56±1	26±0.3	Italy	Cross sectional	Ultrasound	No	ELISA	7
	NAFLD	161 (101/60)	56±1	27.3±0.5						

(continued)

Table 1. *(continued)*

First author, Year	Group	*n* (M/F)	Age in years	BMI in kg/m²	Country	Study design	Diagnose of NAFLD	Biopsy on controls	Measurement method of resistin	NOS
Magalhaes *et al*. 2014[36]	Control	36	37.9±1.3	36.7 (30.3–55.4)	Brazil	Cross sectional	Ultrasound	No	ELISA	5
	NAFLD	24	39.5±1.6	39.4 (30.3–63.2)						
Musso *et al*. 2017[38]	Control	75 (61/14)	50±1	25.9±0.2	Italy	Cross sectional	Ultrasound	No	ELISA	7
	NAFLD	230 (59/171)	49±1	25.7±0.3						
Musso *et al*. 2012[25]	Control	40	50±3	25.1±1.6	USA	Case-control	Liver biopsy	No	ELISA	7
	SS	20	47±4	25.1±1.5						
	NASH	20	47±4	25.2±1.6						
Perseghin *et al*. 2006[39]	Control	47 (38/9)	36±8	26.8±3	USA	Case-control	NA	No	ELISA	6
	NAFLD	28 (24/4)	35±8	27.1±3.9						
Polyzos *et al*. 2016[27]	Control	25 (5/20)	53.6±1.8	30.5±0.8	Greece	Cross sectional	Liver biopsy	No	ELISA	7
	SS	15 (5/10)	53.9±2.6	31.9±1.3						
	NASH	14 (2/12)	54.8±1.6	33.9±1.6						
D'Incao *et al*. 2017[29]	Control	4	38.5±10.85	49±6.73	Brazil	Cross sectional	Liver biopsy	Yes	ELISA	7
	SS	9	39.08±9.63	53.19±9.44						
	NASH	12	49.45±6.71	47.53±6.33						
Jamali *et al*. 2016[23]	SS	2 (2/0)	27±2.82	28.09±7.77	Iran	Cross sectional	Liver biopsy	Yes	ELISA	6
	NASH	28 (17/11)	35±8.47	29.92±3.79						
Sanal *et al*. 2009[17]	Control	18	44±8	NA	India	Case-control	Liver biopsy	No	ELISA	6
	NAFLD	56	43±14	NA						
Senates *et al*. 2012[9]	Control	66 (33/33)	39±9	23±4	Turkey	Case-control	Liver biopsy	No	ELISA	7
	NAFLD	97 (55/42)	41±10	31±6						
Shen *et al*. 2014[22]	Control	43 (29/14)	45±14	22±1.8	China	Cross sectional	Liver biopsy	No	ELISA	7
	NAFLD	58 (38/20)	NA	NA						
Wong *et al*. 2006[16]	Control	41 (17/24)	42±10	24.1±6.8	China	Case-control	Liver biopsy	No	ELISA	7
	NAFLD	80 (52/28)	45±9	29±4.8						
Younossi *et al*. 2011[30]	SS	39 (3/36)	40.51±10.28	NA	USA	Cross sectional	Liver biopsy	Yes	ELISA	7
	NASH	40 (15/25)	44.08±10.05	NA						
Zhu *et al*. 2016[37]	Control	86 (57/29)	52.98±13.07	22.86±2.94	China	Case-control	Ultrasound	No	ELISA	7
	NAFLD	86 (57/29)	53±13.24	26.16±3.33						
Younossi *et al*. 2008[24]	Control	32 (13/19)	39.3±9.8	47±9.1	USA	Cross sectional	Liver biopsy	Yes	ELISA	7
	SS	15 (1/14)	37.4±8.3	45.7±4.8						
	NASH	22 (9/13)	42.5±10.4	48.2±8.7						

Data are presented in numbers or mean±SD or medians and interquartile ranges. BMI, body mass index; ELISA, enzyme-linked immunosorbent assay; NA, not available.

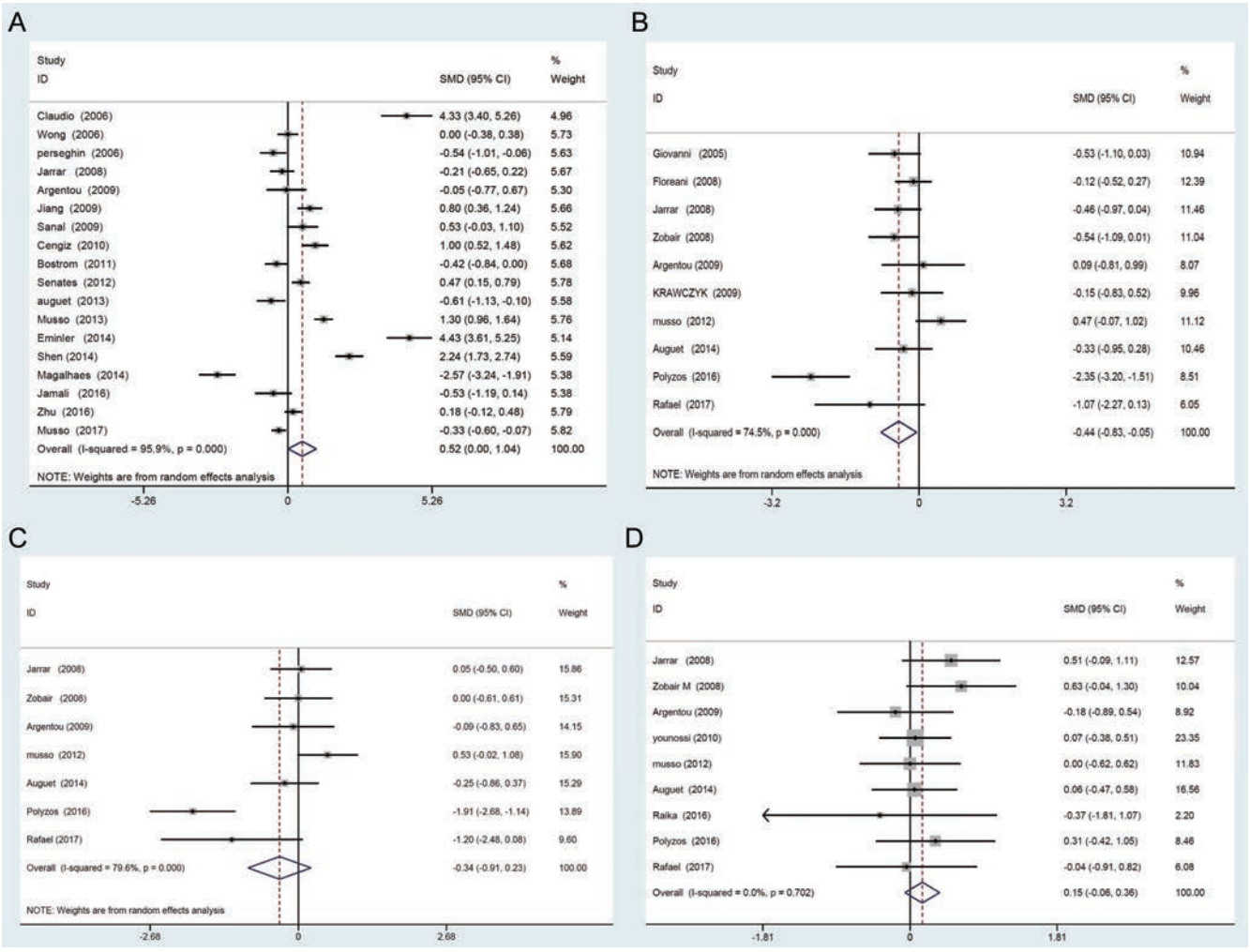

Fig. 2. Forest plots of serum resistin levels between (A) NAFLD patients vs. controls, (B) NASH patients vs. controls, (C) NAFL patient vs. controls, (D) NAFL patients vs. NASH patients.

study conducted by Polyzos *et al*.[27] was removed, the heterogeneity was significantly reduced, which indicated that this study was the main source of heterogeneity. In order to investigate whether this study affected the results of meta-analysis, the meta-analysis were reperformed after removal of the study (Polyzos *et al*. 2016) with the fixed-effects model. The results showed that patients with NASH had lower serum resistin levels than controls (SMD=−0.23, 95% CI: −0.43–0.04) (Fig. 3A); there was no significant difference of serum resistin levels between patients with NAFL vs. controls (SMD=0.03, 95% CI: −0.24–0.29) (Fig. 3B), and between patients with NAFL vs. NASH patients (SMD=0.14, 95% CI: −0.09–0.36) (Fig. 3C). These results indicated that the heterogeneity did not affect the results of meta-analysis.

The same method was used to explore the source of heterogeneity in the meta-analysis of studies for NAFLD patients vs. controls, but no study was found to contribute to the heterogeneity. In addition, the subgroup analysis was conducted according to the diagnosis methods, ethnicity, mean age, types of study design, and mean body mass index, but all of them failed to be the obvious source of heterogeneity. Funnel plots were constructed using the Egger's regression asymmetry test to investigate the possible publication bias in the NAFLD patients vs. controls, NASH patients vs. controls, NAFL patient vs. controls, and NAFL patients vs. NASH patients. As Figure 4 shows, no obvious publication bias was observed.

Meta-regression and quality evaluation

To further explore the source of heterogeneity between NAFLD and control groups, the effect of potential confounders were evaluated by meta-regression analysis (based upon random-effects) when ≥10 comparisons were available. Diagnosis methods, ethnicity, mean age, types of study design, mean body mass index, biopsy on controls, and NOS scores were entered separately as covariates. As Table 2 shows, all of these factors failed to account for the heterogeneity between NAFLD and controls (Table 2). The GRADE approach was used to evaluate the quality of the evidence, and the results showed that the quality of results of serum resistin levels in NAFLD patients vs. controls was low, and moderate in NASH patients vs. controls, NAFL patient vs. controls, and NAFL patients vs. NASH patients, which suggested that further research is likely to have an important impact on the present results and may change the present results (please see the Supplementary Tables 1–5).

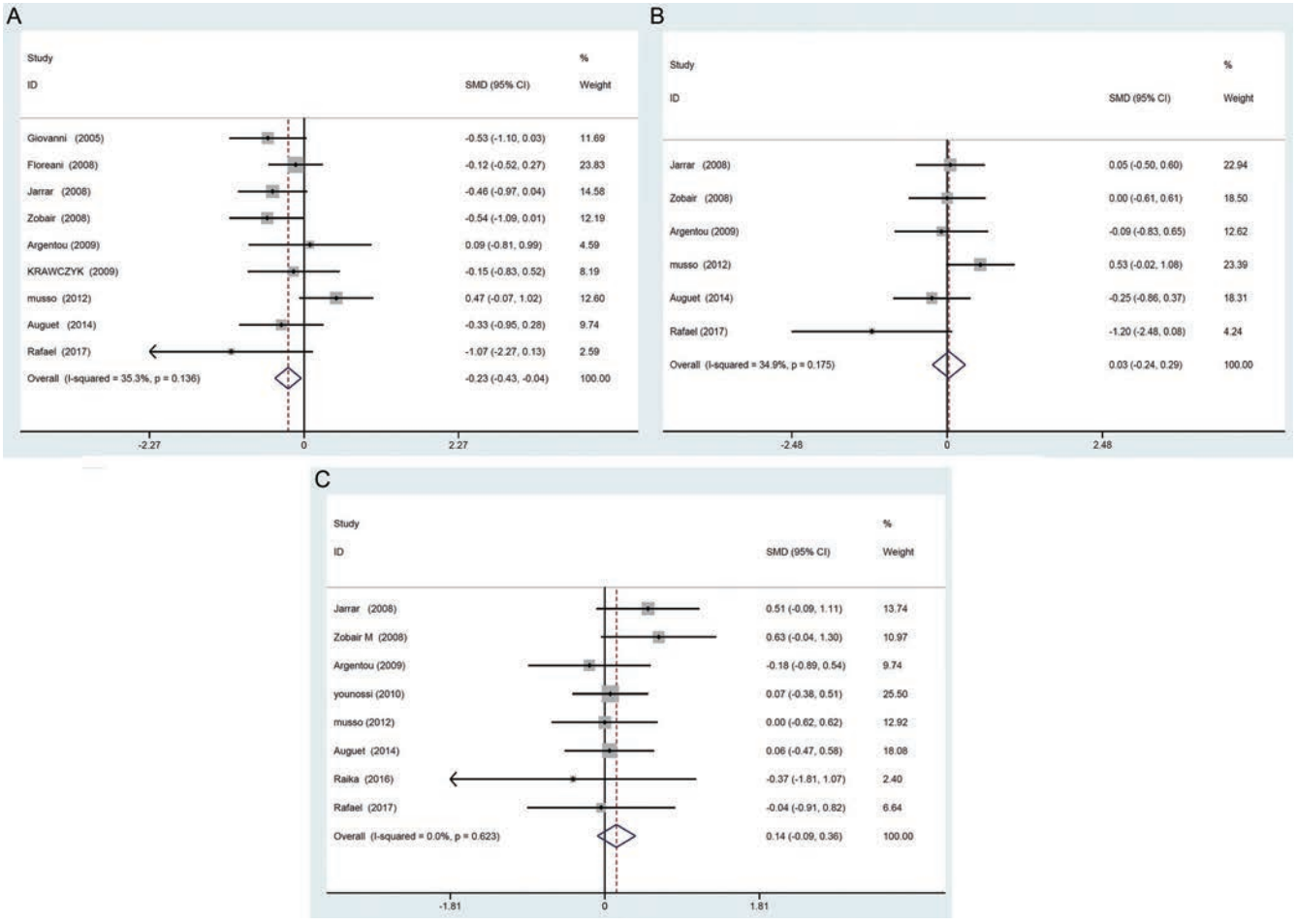

Fig. 3. Forest plots of serum resistin levels between (A) NASH patients vs. controls, (B) NAFL patient vs. controls, (C) NAFL patients vs. NASH patients after removed the study by Polyzos *et al*. (2016).

Discussion

Resistin is a significant pro-inflammation adipokine and the role of its serum levels in patients with NAFLD remain controversial. This study systematically analyzed the serum levels of resistin in patients with NAFLD, especially in those with NAFL and NASH. The results suggested that patients with NAFLD had higher serum resistin levels than healthy controls, but low serum resistin levels were observed in patients with NASH when compared to healthy controls. In addition, no significant difference of serum resistin levels was observed between patients with NAFL and healthy controls, and between patients with NAFL and NASH. A reasonable explanation may be that all the patients with NASH and NAFL were diagnosed by liver biopsy, and patients with NAFLD were diagnosed by liver biopsy or ultrasound. The difference of diagnostic methods may contribute to these outcomes.

Some previous studies reported that serum levels of resistin in patients with NAFLD were higher,[15] lower,[39] or of no significant difference[40] compared to healthy controls, accompanied by the different diagnosis methods used for NAFLD. Zhu *et al*.[37] investigated the levels of serum protein as the diagnostic biomarkers for NAFLD, and they found that serum resistin was significantly higher in patients with NAFLD than in healthy controls. However, Magalhaes *et al*.[36] investigated the serum levels of resistin in obese NAFLD patients and controls, but they found that the serum levels of resistin were negatively associated with the risk of NAFLD; that is, the serum resistin levels were low in NAFLD patients compared to controls. Except for the above reports, other research investigations also provided findings that precluded making a definitive conclusion. In this meta-analysis, we analyzed all the available studies which investigated the serum resistin levels in patients with NAFLD and controls, and we found that serum resistin levels were significant higher than in the healthy controls. Notably, all the patients with NAFLD were diagnosed by liver biopsy or ultrasound, and the NAFLD patients were not divided by NAFL and NASH stage. In consideration of the high heterogeneity in the meta-analysis, sensitivity analysis was conducted. Interestingly, when the study by Polyzos *et al*.[27] (2016) was removed, the heterogeneity was markedly decreased, but the results of meta-analysis were unchanged. These results indicated that an individual study may contribute to the heterogeneity, but whether the results of meta-analysis were affected should be further investigated.

Resistin up-regulates the expression of proinflammatory cytokines such as TNF-α, IL-6, IL-12, and monocyte chemoattractant protein-1 in monocytes, macrophages, and hepatic stellate cells via the NF-κB pathway.[41] Serum resistin levels in patients with NASH and the association of serum resistin levels with the risk fibrosis remains inconsistent. Argentou *et al*.[10] investigated the relationship of serum resist-

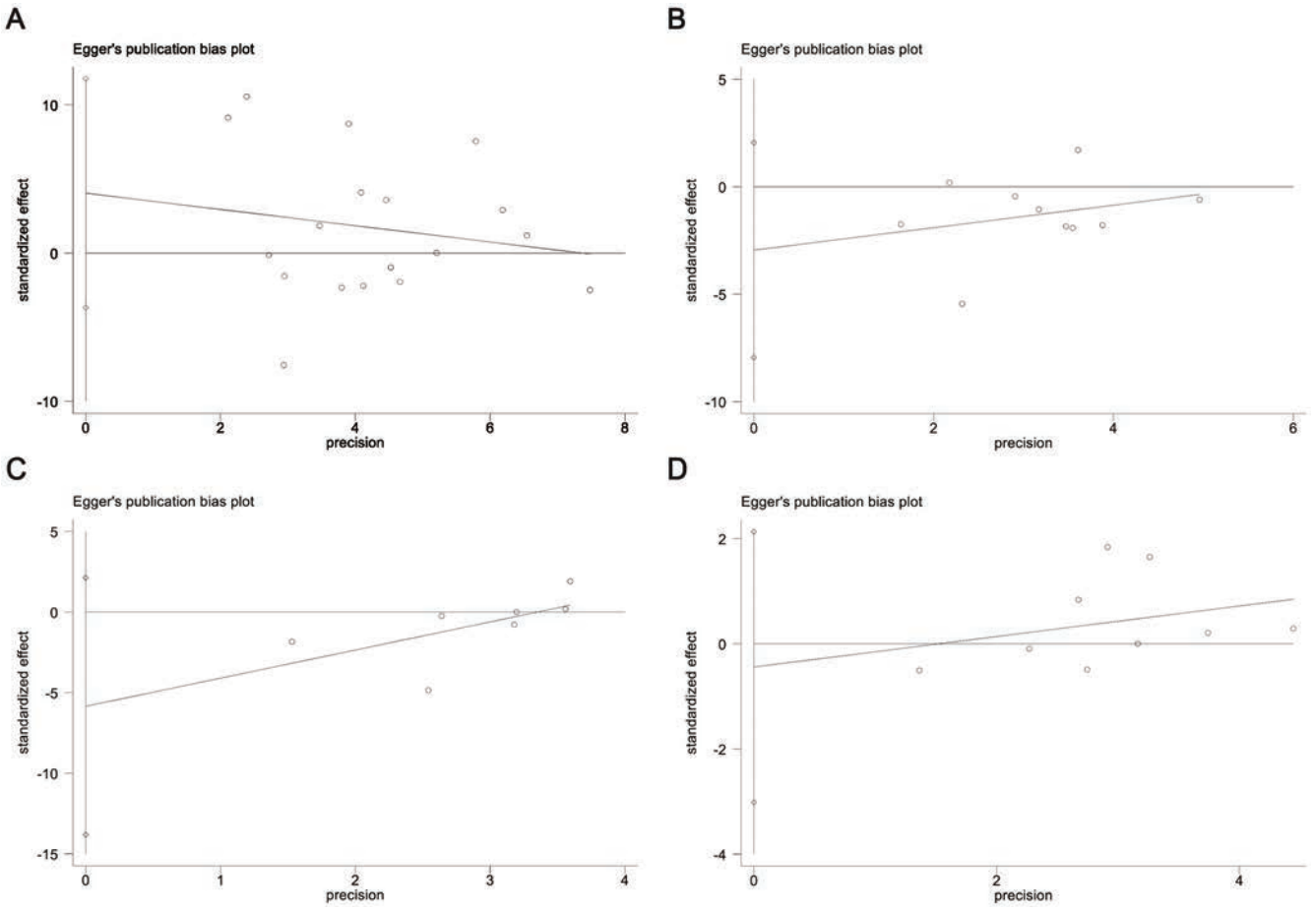

Fig. 4. Egger's funnel plots for publication bias for (A) NAFLD patients vs. controls, (B) NASH patients vs. controls, (C) NAFL patient vs. controls, (D) NAFL patients vs. NASH patients.

in levels with some individual histopathological parameters, global activity grade, and fibrosis stage in NASH patients, but no significant association was observed. However, Tsochatzis *et al*.[42] reported the serum levels in chronic hepatitis B and chronic hepatitis C patients; they also found that low resistin levels were associated with moderate/severe fibrosis in chronic hepatitis B/C patients, which suggested that serum resistin levels were negatively related to the degree of fibrosis. In this meta-analysis, the serum resistin levels in patients with NASH were significantly lower than in healthy controls, which was consistent with the previous study by Tsochatzis *et al*.[42] to some degree. The probable reason may be that patients with NASH possess different degrees of fibrosis, usually, and the serum resistin levels could be negatively associated with the fibrosis. In this study, however, all the patients with NASH had NAFLD-related NASH, and the cause of fibrosis in NASH patients was different from that of the chronic hepatitis B/C patients. Whether the relationship of serum resistin levels with fibrosis was affected by the cause of fibrosis remains unknown and further studies are needed to clarify it.

Our results suggested that patients with NAFLD had higher serum resistin levels than healthy controls, but low serum resistin levels were observed in the patients with NASH compared to healthy controls. This is an interesting finding because resistin levels seem to rise with the progression of NAFLD, from healthy to NAFL, but decline when NAFL progresses to NASH. The same phenomenon occurred in patients with type 2 diabetes. In 2020, Galla *et al*.[43] reported that patients with prediabetes had higher levels of resistin than patients with type 2 diabetes and healthy controls, as found in their 20-year follow-up study. In addition, a large number of cohort studies and meta-analysis suggested that resistin is a risk factor for cardiovascular disease.[44] Acute coronary syndromes often occur in patients with high resistin levels, while chronic stable angina pectoris is more common in patients with low resistin levels.[45] Given that pre-diabetes and coronary heart disease are a large part of the hidden population,[43,46] patients with NAFLD are more likely to suffer from the type 2 diabetes and coronary heart disease, which may have affected the results of this study. In addition, whether reduced resistin levels will reduce the risk of NAFL, type 2 diabetes and coronary heart disease is unknown, and more research is needed in the future.

This meta-analysis has strengths and limitations that may have affected its conclusions. This is the first meta-analysis to systematically investigate the serum resistin levels in patients with NAFLD. The serum resistin levels were evaluated in patients with NAFLD, including patients with NAFL and NASH. In addition, this work is based on 28 high-quality studies. The limitations, however, include that some NAFLD patients were diagnosed by ultrasound other than liver biopsy in the included studies. Second, higher heterogeneity may disturb the accuracy of the results. Third, the association of serum resistin levels with fibrosis was not investigated in detail in this study. Fourth, although every

Table 2. Meta regression analysis of possible sources of heterogeneity in NAFLD vs. control group (18 studies)

Effect size	Coefficient	Standard error	*t*	*p*>| t |	95% CI
Diagnosis methods	0.403	0.325	−1.13	0.276	0.073–2.225
Ethnicity	0.175	0.223	−1.58	0.133	0.175–1.287
Types of study design	1.527	1.318	0.49	0.631	0.245–9.513
Mean age (30–40, 40–50, ≥50)	0.814	0.346	−0.48	0.635	0.331–2.003
Mean BMI (>30)	0.467	0.246	−1.44	0.168	0.153–1.428
Biopsy on controls	0.367	0.393	−0.94	0.363	0.038–3.552
NOS score	1.996	0.783	1.76	0.097	0.869–4.586

step of this meta-analysis was carried out in strict accordance with the requirements, this meta-analysis was not registered on relevant websites in advance.

Conclusions

In summary, this study systematically investigated the serum resistin levels in adult patients with NAFLD for the first time. The results suggest that patients with NAFLD have higher serum resistin levels than healthy controls, but patients with NASH have lower serum resistin levels than healthy controls. In addition, no significant differences of serum resistin levels were observed between the patients with NAFL and controls, nor the patients with NAFL and NASH. Although a little inconsistence between the results of this study and several previous studies existed, it remains reasonable to illustrate the variation of serum resistin levels in patients with NAFLD. In consideration of the present results, serum resistin possesses the potential to serve as a biomarker to predict the development risk of NAFLD, and the diagnostic sensitivity and specificity should be improved by excluding the interference of other factors. Further studies should be conducted to clarify the serum resistin levels in healthy controls and patients with NAFLD that is diagnosed by liver biopsy.

Funding

This study was supported by a grant from the National Natural Science Foundation of China (No. 31770837), which plays a significant role in the design of the study and data collection, analysis nor interpretation, nor in the writing of the manuscript.

Conflict of interest

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

Author contributions

Study concept and design (YX, WJ), acquisition and analysis of data (DH, JC, SL, ZeZ, ZhZ), drafting and writing of the manuscript (DH, JC), and revision of the manuscript (YX, WJ). All authors approved the final manuscript.

Data sharing statement

All data are available upon request.

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Journal of Clinical and Translational Hepatology **2021** vol. 9(4) | 494–502
DOI: 10.14218/JCTH.2021.00066

Original Article

Metabolic-associated Fatty Liver Disease as Assessed by the Fatty Liver Index Among Migrant and Non-migrant Ghanaian Populations

Anne-Marieke van Dijk[1#*], Sjoerd Dingerink[1#], Felix Patience Chilunga[2], Karlijn Anna Catharina Meeks[2,3], Silver Bahendeka[4], Matthias Bernd Schulze[5,6], Ina Danquah[6,7], Tracy Bonsu Osei[7], Erik Serné[8], Charles Agyemang[2] and Adriaan Georgius Holleboom[1]

[1]Department of Vascular Medicine, Amsterdam UMC, location AMC, Amsterdam, the Netherlands; [2]Department of Public Health, Amsterdam UMC, location AMC, Amsterdam Public Health Research Institute, Amsterdam, the Netherlands; [3]Center for Research on Genomics and Global Health, National Human Genome Research Institute, National Institutes of Health, Bethesda, MD, USA; [4]MKPGMS-Uganda Martyrs University, Kampala, Uganda; [5]Department of Molecular Epidemiology, German Institute of Human Nutrition Potsdam-Rehbruecke, Nuthetal, Germany; [6]Institute of Nutritional Science, University of Potsdam, Nuthetal, Germany; [7]Heidelberg Institute of Global Health (HIGH), Universitaetsklinikum Heidelberg, Heidelberg, Germany; [8]Department of Vascular Medicine, Amsterdam UMC, location VUmc, the Netherlands

Received: 9 February 2021 | **Revised:** 30 March 2021 | **Accepted:** 12 April 2021 | **Published:** 8 May 2021

Abstract

Background and Aims: Metabolic-associated fatty liver disease (MAFLD) is driven by high caloric intake and sedentary lifestyle. Migration towards high income countries may induce these driving factors; yet, the influence of such on the prevalence of MAFLD is clearly understudied. Here, we investigated the Fatty Liver Index (FLI), a proxy of steatosis in MAFLD, after migration of Ghanaian subjects. ***Methods:*** Cross-sectional data of 5282 rural, urban and migrant participants from the Research on Obesity and Diabetes among African Migrants (also known as RODAM) study were analyzed with logistic regression for geographical differences in FLI and associations with type 2 diabetes mellitus (T2DM), waist-to-hip ratio, and 10-year predicted risk of atherosclerotic cardiovascular disease (ASCVD). ***Results:*** Both FLI and the proportion with an FLI indicative of MAFLD steatosis (FLI ≥60) were higher in migrants compared with non-migrants. Prevalence of elevated FLI (FLI ≥60) in non-migrant males was 4.2% compared to 28.9% in migrants. For females, a similar gradient was observed, from 13.6% to 36.6% respectively. Compared to rural residents, the odds for a FLI ≥60 were higher in migrants living in urban Europe (odds ratio [OR] 9.02, 95% confidence interval [CI]: 5.02–16.20 for men, and 4.00, 95% CI: 3.00–5.34 for women). Compared to controls, the ORs for FLI ≥60 were 2.43 (95% CI: 1.73–3.41) for male T2DM cases and 2.02 (95% CI: 1.52–2.69) for female T2DM cases. One-unit higher FLI was associated with an elevated (≥7.5%) 10-year ASCVD risk (OR: 1.051, 95% CI: 1.041–1.062 for men, and 1.020, 95% CI: 1.015–1.026 for women). ***Conclusions:*** FLI as a proxy for MAFLD increased stepwise in Ghanaians from rural areas, through urban areas, to Europe. Our results clearly warrant awareness for MAFLD in migrant population as well as confirmation with imaging modalities.

Citation of this article: van Dijk AM, Dingerink S, Chilunga FP, Meeks KAC, Bahendeka S, Schulze MB, *et al*. Metabolic-associated fatty liver disease as assessed by the fatty liver index among migrant and non-migrant Ghanaian populations. J Clin Transl Hepatol 2021;9(4):494–502. doi: 10.14218/JCTH.2021.00066.

Keywords: Fatty liver; Non-invasive test; Migration; African population.

Abbreviations: γGT, gamma-glutamyltransferase; ACC, American College of Cardiology; AHA, American Heart Association; ALT, alanine aminotransferase; APRI, AST to platelet ratio; ASCVD, atherosclerotic cardiovascular disease; AST, aspartate aminotransferase; BMI, body mass index; BP, blood pressure; carotid IMT, carotid intima-media thickness; CCTA, coronary computed tomography angiography; CI, confidence interval; CVD, cardiovascular disease; FLI, Fatty Liver Index; GPAQ, Global Physical Activity Questionnaire; HDL, high-density lipoprotein; IQR, interquartile ranges; LDL, low-density lipoprotein; MAFLD, metabolic-associated fatty liver disease; MRI-PDFF, Magnetic Resonance Imaging Proton Density Fat Fraction; NASH, non-alcoholic steatohepatitis; OR, odds ratio; PNPLA3, patatin-like phospholipase domain containing 3; RODAM, Research on Obesity and Diabetes among African Migrants; SD, standard deviation; T2DM, type 2 diabetes mellitus; TG, triglycerides; VLDL, very low-density lipoprotein; WHR, waist-to-hip ratio.

[#]Contributed equally to this work.

[*]**Correspondence to:** Anne-Marieke van Dijk, Department of Internal and Vascular Medicine, Amsterdam University Medical Centres, location AMC, Meibergdreef 9 room M0-118, 1105 AZ Amsterdam, the Netherlands. ORCID: http://orcid.org/0000-0003-0831-527X. Tel: +31-20-5665-973, E-mail: a.vandijk2@amsterdamumc.nl

Introduction

In recent years, the prevalence of metabolic-associated fatty liver disease (MAFLD) has clearly increased, with an estimated prevalence of 25% worldwide.[1] The MAFLD prevalence increases coincide with the global increasing prevalence of type 2 diabetes mellitus (T2DM) and obesity, especially central obesity.[2] MAFLD is a spectrum of liver disease ranging from hepatic steatosis through non-alcoholic steatohepatitis (NASH), to fibrosis and cirrhosis.[3] MAFLD has a complex pathophysiology, but its root cause is insulin

resistance and MAFLD is therefore seen as the hepatic component of the metabolic syndrome.[4,5] In turn, MAFLD itself may contribute to the clinical manifestations of the metabolic syndrome and therefore to atherosclerotic cardiovascular disease (ASCVD), potentially by inducing dyslipidemia through increased secretion of triglyceride (TG)-rich lipoproteins, in combination with low-grade inflammation and hypercoagulability.[6]

Of note, the prevalence of MAFLD varies among ethnic groups. Despite a higher prevalence of obesity among ethnic minority groups, especially in women,[7] individuals of African descent are relatively less prone to MAFLD compared to individuals of European, Asian, or Hispanic descent, as assessed in the Multiethnic Cohort in the USA.[8] Variance in PNPLA3 has been established to influence susceptibility for MAFLD and may contribute to the observed ethnic difference in MAFLD. The PNPLA3-453I allele, which occurs at a higher frequency in African-ancestry populations (12.5%) compared with other populations (< 1%), is associated with lower hepatic fat content.[4,9,10]

Migration-related environmental changes and urbanization may facilitate sedentary lifestyle and obesity, potentially driving MAFLD.[11] Yet, the influence of migration on the prevalence and severity of MAFLD is understudied. In order to address this relevant question, homogeneity of the studied population is imperative to reduce the effects of differences in genetic background. To achieve this, we used data from the Research on Obesity and Diabetes among African Migrants (RODAM) study, representing a relatively homogenous group of Ghanaians which comprises adults originating from the Ashanti Region in Ghana, living in different environmental contexts.[12] Direct measures of hepatic fat content, such as liver biopsy, ultrasound, or Magnetic Resonance Imaging Proton Density Fat Fraction (MRI-PDFF) were unavailable in our study population. Therefore, we used a well-established non-invasive composite proxy to assess the steatotic component of MAFLD: the Fatty Liver Index (FLI).[13] We aimed to study the prevalence of MAFLD as assessed by the FLI among Ghanaian residents in rural and urban Ghana and among Ghanaians in three European cities.

Additionally, we assessed the associations of the FLI with T2DM, as well as predicted 10-year risk of ASCVD. Patients with MAFLD are at 1.5- to 2-fold increased risk for atherosclerotic cardiovascular disease (ASCVD), potentially via the mixed hyperlipidemia often observed in MAFLD patients.[6] Therefore, we included predicted 10-year risk of ASCVD in our study.

Methods

Study design and population

The RODAM study aims to gain knowledge on the development of obesity and diabetes mellitus among African migrants. Full details of the multicenter RODAM study, initiated in 2012, are published elsewhere.[12] In brief, 5,898 Ghanaian men and women aged 25–70 years, were recruited and physically examined from a population residing in rural Ghana, urban Ghana, and Ghanaians residing in three different European cities (Amsterdam, Berlin, and London). As we aim to study the effects of migration on the FLI, Ghanaians living in Europe were categorized as migrants, of which 97% were first-generation migrants. Ethical approval of the study protocols has been received at all sites. All authors had access to the study data and reviewed and approved the final manuscript.

Details on data acquisition, blood sampling and processing procedures can be found in the Supplementary File 1.

FLI

The FLI is validated for people aged 18–75 and is calculated by taking into account body mass index (BMI), waist circumference, TGs, and gamma-glutamyltransferase (γGT) according to the algorithm by Bedogni *et al*.[14] In the RODAM database, TG are measured in mmol/L, whereas the formula for the FLI requires TG expressed as mg/dL. For conversion, the factor 88.57 was used.[15] The FLI varies between 0 and 100, a FLI <30 is validated for predicting absence of MAFLD with a sensitivity of 91.5%.[13,14] Elevated FLI was defined as a score of ≥60, since a cut-off of 60 predicts presence of hepatic steatosis, with a specificity of 82.3%.[13] In our aim to capture MAFLD in the RODAM study, we excluded participants in case of retroviral therapy or treatment for hepatitis C and/or excessive alcohol use, defined as >21 units (168 gram alcohol) per week for men and >14 units (112 gram alcohol) per week for women from the current analysis.

10-year predicted risk of ASCVD

The standardized and clinically oriented American College of Cardiology (ACC)/American Heart Association (AHA) ASCVD risk score was applied to calculate the 10-year risk of clinically manifest ASCVD. This score can be used to calculate total 10-year cardiovascular disease risk percentages.[16,17] It accurately predicts ASCVD risk also in non-European populations.[18] It is scored as a percentage; a score of ≥7.5% is considered elevated risk of developing ASCVD in the next 10 years based on the prior work by Goff *et al*.[16] More information can be found in the Supplementary File 1. This risk score has been validated for subjects between 40–79 years of age, without prior history of ASCVD. Thus, for the ASCVD analysis, participants below 40 years of age and those with a history of ASCVD were excluded. The RODAM database contained self-reported information on stroke, heart attack, other heart conditions, and peripheral arterial disease. The risk score was calculated with an algorithm that combines age, sex, use of antihypertensive medication, presence of diabetes mellitus, systolic blood pressure, total cholesterol, high-density lipoprotein (i.e. HDL) cholesterol, and smoking status.

Data analysis

Data were analyzed using SPSS Statistical software, version 26 (IBM Corp., Armonk, NY, USA). All analyses were conducted separately for men and women due to a statistically significant interaction between FLI and sex. Normally distributed continuous variables were presented as means and standard deviations. Skewed continuous variables were presented as medians and interquartile ranges (IQRs). Categorical variables were presented as proportions. Differences between rural, urban, and migrant participants were assessed by ANOVA, Kruskal-Wallis tests, and χ^2 tests as appropriate. Three models were fitted to adjust for possible confounders. Model 1 was adjusted for age; model 2: model 1 + education; model 3: model 2 + physical activity, alcohol, and T2DM. The results are presented as odds ratios (ORs) and the corresponding 95% confidence intervals (CIs). A p-value of <0.05 was considered statistically significant. In addition, the associations between FLI and elevated 10-year risk of ASCVD were calculated, in which the 10-year risk of ASCVD was the dependent variable. For this ASCVD association, nearly identical models to adjust for potential confounding were used as in the assessment for

differences between rural, urban and migrated populations. The only difference between these models in correcting for confounding was the replacement of T2DM by smoking in model 3.

Results

General characteristics

From a total of 5,898 RODAM participants who underwent physical examination, 5,282 were included in the current analysis, based on age and the criteria of assessing FLI. Of these participants, 951 (18.0%) were rural Ghanaians, 1,432 (27.1%) were urban Ghanaians, and 2,899 (54.9%) were migrants. The majority of participants were female (62.7%). Mean age was higher in men than in women in urban participants and migrants. However, in rural participants, mean age was higher in women than in men. The mean duration of living in urban Europe was 18.6 years (standard deviation [SD]: 9.6) for males and 19.0 years (SD: 9.5) for females, calculated from 2,529 out of 2,899 migrants. Migrants had the highest levels of education and rural participants had the lowest levels, in both men and women. The prevalence of any alcohol consumption was higher in rural participants in men (53.1%) compared to urban participants (40.6%) and migrants (41.4%). In women, alcohol consumption was lower in urban participants (26.1%) compared to rural participants (30.9%) and migrants (31.2%). The proportion of current smokers was higher in migrants than in non-migrants (6.3% in migrant men, and 2.1% in migrant women). Compared to the two other groups, rural participants had the highest levels of physical activity, irrespective of sex. Differences in general characteristics are shown in Table 1.

FLI and its determinants by location of current residency

In both men and women, TG levels were highest among urban participants and the lowest among migrants, and γGT was the highest in urban participants and the lowest in rural participants. FLI, BMI, and waist circumference increased stepwise after migration form rural Ghana, through urban Ghana to Europe, whereby the Ghanaian homogenous population living in three distinct environments can be seen as a proxy for migration (as given in Table 1). Median FLI with corresponding IQR per location of residency at inclusion are shown in Figure 1a for men and Figure 1b for women. There was a positive gradient in the prevalence of elevated FLI (FLI ≥60) in males from rural (4.2%) through urban (16.3%) to Europe (28.9%), ($p<0.001$). A similar positive gradient was observed in females, with 13.6% in rural, 32.3% in urban, and 36.6% in Europe, respectively ($p<0.001$; Table 1). These differences retained statistical significance even after adjustment for age, education level, physical activity level, alcohol use, and T2DM for urban Ghana (adjusted OR: 4.09, 95% CI: 2.19–7.67 for men and 3.29, 95% CI: 2.47–4.39 for women) and migrants (adjusted OR: 9.02, 95% CI: 5.02–16.20 for men and 4.00, 95% CI: 3.00–5.34 for women), compared to rural Ghana (Fig. 2 for men and Fig. 3 for women).

Associations of T2DM and 10-year predicted ASCVD risk with FLI

Median FLI in participants with and without T2DM are shown in Figure 4. We found a positive association between T2DM and an elevated FLI (FLI ≥60) (adjusted OR: 2.43, 95% CI: 1.73–3.41 for men and 2.02, 95% CI: 1.52–2.69 for women; Supplemental Table 1).

In total, 2,611 RODAM study participants were included in the analysis for FLI and 10-year ASCVD risk after further exclusion of participants with an age below 40 and those with a history of clinically present CVD (Supplemental Fig. 1). As we hypothesized that MAFLD drives ASCVD, we used the continuous FLI as an independent variable and ASCVD as the dependent variable in this analysis. FLI was positively associated with elevated 10-year ASCVD risk (≥7.5%) among rural participants (adjusted OR: 1.05, 95% CI: 1.01–1.10 for men and 1.03, 95% CI: 1.02–1.05 for women), urban participants (1.08, 95% CI: 1.04–1.11 for men and 1.02, 95% CI: 1.01–1.02 for women), and migrants (men 1.04, 95% CI: 1.03–1.06 and women 1.02, 95% CI: 1.01–1.03). In the total study population, the adjusted OR was 1.05 per 1 unit increase in FLI (95% CI: 1.04–1.06) for men and 1.02 (95% CI: 1.02–1.03) for women (Supplemental Table 2).

Discussion

Key findings

Here, we shed light on the influence of migration on the prevalence of MAFLD by studying the FLI in a homogenous Ghanaian population living in rural Ghana, urban Ghana and Europe. The homogenous Ghanaian population living in three distinct environments is used as a proxy for migration. This study has three important findings. First, the prevalence of an elevated FLI (FLI ≥60) as an indicator of hepatic steatosis increased from rural participants, through urban participants, to European migrants, irrespective of sex. Second, T2DM was positively associated with higher odds for FLI in both Ghanaian men and women. Third, an elevated FLI (FLI ≥60) was associated with an higher odds for 10-year risk of ASCVD (≥7.5%) in both men and women, with a more pronounced effect in men.

Discussion of the key findings

Studies of human migration and features of cardiometabolic disease are scarce. Several studies have reported differences in the prevalence of MAFLD among different ethnicities; however, these were performed in participants residing in a single country.[16,19] For instance, multiple studies report a lower prevalence of MAFLD in African Americans compared to Hispanic Americans.[19–21] The prevalence of MAFLD in migrants of African descent is often lower than the prevalence of MAFLD in the general population of the host country.[20,21] Interestingly, this contrasts with the prevalence of morbidities with a close relation to MAFLD, i.e. obesity, T2DM, and hypertension, which are found to be more prevalent among ethnic minorities in Europe, including African groups.[20]

Factors driving variation in cardiometabolic health across geographical locations are thought to include changes in nutritional patterns, physical inactivity, and stress, in combination with genetic susceptibility and gene-environment interactions.[20,22] As MAFLD is driven by insulin resistance and obesity, one would expect the aforementioned factors to play a similar role in the effects of migration on the prevalence of MAFLD.[23] Interestingly, in our models, the effect sizes only slightly changed upon adjustment for lifestyle factors, such as physical activity, alcohol consumption, and T2DM. This may fit with the 'multiple hit' hypothesis which

Table 1. General characteristics of rural and urban residing, and migrant participants

Variables	Rural (*n*=951)	Urban (*n*=1,432)	Migrated (*n*=2,899)	Total (*n*=5,282)	*p*
Men, *n*	356	406	1,209	1,971	
Age, years	45.8±12.9	46.7±11.8	47.0±10.4	46.7±11.2	0.193
Education, *n* (%)					<0.001
Elementary	136 (38.2)	89 (21.9)	144 (11.9)	369 (18.7)	
Lower secondary	128 (36.0)	173 (42.6)	453 (37.5)	754 (38.3)	
Higher secondary	47 (13.2)	85 (20.9)	279 (23.1)	411 (20.9)	
Tertiary	21 (5.9)	37 (9.1)	250 (20.7)	308 (15.6)	
Alcohol consumption, *n* (%)	189 (53.1)	165 (40.6)	500 (41.4)	854 (43.3)	<0.001
Smoking, *n* (%)					0.023
Current	17 (4.8)	12 (3.0)	76 (6.3)	105 (5.3)	
Past	48 (13.5)	61 (15.0)	131 (10.8)	240 (12.2)	
Physical activity, *n* (%)					<0.001
Low levels	37 (10.4)	85 (20.9)	287 (23.7)	409 (20.8)	
Medium levels	54 (15.2)	72 (17.7)	176 (14.6)	302 (15.3)	
High levels	238 (66.9)	223 (54.9)	457 (37.8)	918 (46.6)	
WHR	0.89±0.06	0.90±0.06	0.93±0.07	0.91±0.07	<0.001
WHR ≥0.90, *n* (%)	126 (35.4)	222 (54.7)	785 (64.9)	1,133 (57.5)	<0.001
T2DM, *n* (%)	15 (4.2)	46 (11.3)	158 (13.1)	219 (11.1)	<0.001
BMI, kg/m^2	21.0±3.0	24.1±3.8	27.0±3.9	25.4±4.4	<0.001
AST/ALT ratio	1.99±0.82	1.70±0.72	1.44±0.56	1.60±0.68	<0.001
Waist circumference, cm	76.9±8.4	84.7±10.3	92.6±10.9	88.1±12.0	<0.001
TG, mmol/L, median (IQR)	0.96 (0.7–1.3)	1.02 (0.8–1.3)	0.89 (0.67–1.19)	0.92 (0.70–1.23)	<0.001
γGT, mmol/L, median (IQR)	33.1 (24.3–51.6)	39.5 (27.8–56.6)	36.7 (27.7–50.1)	36.5 (27.0–51.5)	0.003
FLI, median (IQR)	11.7 (6.6–21.8)	27.0 (12.3–47.5)	42.6 (21.8–64.6)	31.8 (14.3–56.7)	<0.001
FLI, categorized					<0.001
<30, *n* (%)	298 (83.7)	214(52.7)	426 (35.2)	938 (47.6)	
≥60, *n* (%)	15 (4.2)	66 (16.3)	349 (28.9)	430 (21.8)	
Women, *n*	595	1,026	1,690	3,311	
Age, years	46.9±12.6	44.7±11.2	46.1±9.5	45.8±10.7	<0.001
Education, *n* (%)					<0.001
Elementary	367 (61.7)	520 (50.7)	427 (25.3)	1,314 (39.7)	
Lower secondary	154 (25.9)	367 (35.8)	571 (33.8)	1,092 (33.0)	
Higher secondary	18 (3.0)	87 (8.5)	362 (21.4)	467 (14.1)	
Tertiary	11 (1.8)	28 (2.7)	186 (11.0)	225 (6.8)	
Alcohol consumption, *n* (%)	184 (30.9)	268 (26.1)	527 (31.2)	951 (28.7)	0.014
Smoking, *n* (%)					<0.001
Current	0 (0)	0 (0)	21 (2.1)	21 (0.6)	
Past	5 (0.8)	21 (2.0)	67 (4.0)	93 (2.8)	
Physical activity, *n* (%)					<0.001
Low levels	130 (21.8)	406 (39.6)	406 (24.0)	942 (28.5)	
Medium levels	126 (21.2)	158 (15.4)	289 (17.8)	582 (17.6)	
High levels	294 (49.4)	434 (42.3)	577 (34.1)	1,305 (39.4)	

(continued)

Table 1. *(continued)*

Variables	Rural (*n*=951)	Urban (*n*=1,432)	Migrated (*n*=2,899)	Total (*n*=5,282)	*p*
WHR	0.89±0.07	0.90±0.06	0.88±0.08	0.89±0.07	<0.001
WHR ≥0.85, *n* (%)	444 (74.6)	829 (80.8)	1,153 (68.2)	2,426 (73.3)	<0.001
T2DM, *n* (%)	35 (5.9)	87 (8.5)	154 (9.1)	276 (8.3)	0.049
BMI, kg/m^2	23.7±4.5	28.0±5.5	30.3±5.1	28.4±5.6	<0.001
AST/ALT ratio	1.96±0.75	1.87±0.62	1.62±0.49	1.76±0.60	<0.001
Waist circumference, cm	83.8±11.2	90.4±11.9	95.7±12.0	92.1±12.6	<0.001
TG, mmol/L, median (IQR)	0.97 (0.74–1.34)	1.01 (0.74–1.36)	0.73 (0.57–0.98)	0.85 (0.64–1.16)	<0.001
γGT, mmol/L, median (IQR)	26.6 (20.5–36.7)	29.5 (22.8–38.5)	27.3 (21.3–36.9)	27.9 (21.6–37.3)	<0.001
FLI, median (IQR)	16.7 (8.3–36.2)	40.9 (19.3–69.6)	46.6 (25.4–71.7)	39.1 (18.5–67.4)	<0.001
FLI, categorized					<0.001
<30, *n* (%)	402 (67.6)	388 (37.8)	514 (30.4)	1,304 (39.4)	
≥60, *n* (%)	81 (13.6)	331 (32.3)	619 (36.6)	1,031 (31.1)	

Data presented as mean±SD unless stated otherwise. SD, standard deviation; WHR, waist-to-hip ratio; T2DM, type 2 diabetes; BMI, body mass index; AST, aspartate aminotransferase; ALT, alanine aminotransferase; TG, triglycerides; γGT, gamma-glutamyltransferase; FLI, fatty liver index; IQR, interquartile ranges.

postulates that multiple factors act together in inducing MAFLD, and adjustment for a few of these factors would not have a major effect.[24] In addition to physical inactivity and genetic susceptibility, gut microbiota have been suggested to play a role. Several studies report the interaction between liver and gut as a critical player in the onset of MAFLD.[25–27] Dietary factors may alter the composition of the gut microbiota, which in turn may contribute to the development of MAFLD.[28] Evidence for these changed dietary factors in an urban population compared to a rural population was found in a prospective cohort study that was conducted in South Africa. The nutrition intakes of urban-residing men and women were consistently higher than those of their rural counterparts.[29] This is often accompanied by a nutrition transition to a Westernized diet, frequently high in fat and sugar.[30] This reported change in dietary factors could also play a role in our study population. Taken together, the sizeable disparity in FLI in our study between similar populations living in different environments suggests a more significant role for environmental factors such as dietary changes and alterations in the gut microbiome, in driving the prevalence of MAFLD than for genetic susceptibility.

Of note, we found a strong relation of the FLI with the presence of T2DM. The interplay between T2DM and MAFLD is complex. T2DM is an important risk factor for developing MAFLD, and vice versa, MAFLD may contribute to insulin resistance. Insulin resistance is a central mechanism that leads to lipolysis in peripheral adipose tissue and an increased hepatopetal flux of free fatty acids, driving lipotoxicity in the liver, with subsequent inflammation and hepatocyte injury.[4,31] A higher prevalence of MAFLD in patients with T2DM has been found.[23,32] In turn, ectopic fat accumulation in MAFLD is thought to affect T2DM. This ectopic fat accumulation is associated with increased gluconeogenesis, decreased glycogen synthesis and inhibition of insulin signalling.[32]

We observed a significant association of FLI with 10-year risk of ASCVD, bolstering the notion that patients with MAFLD may have increased ASCVD. The relation between MAFLD and ASCVD is supported by studies of subclinical atherosclerosis, such as carotid intima-media thickness

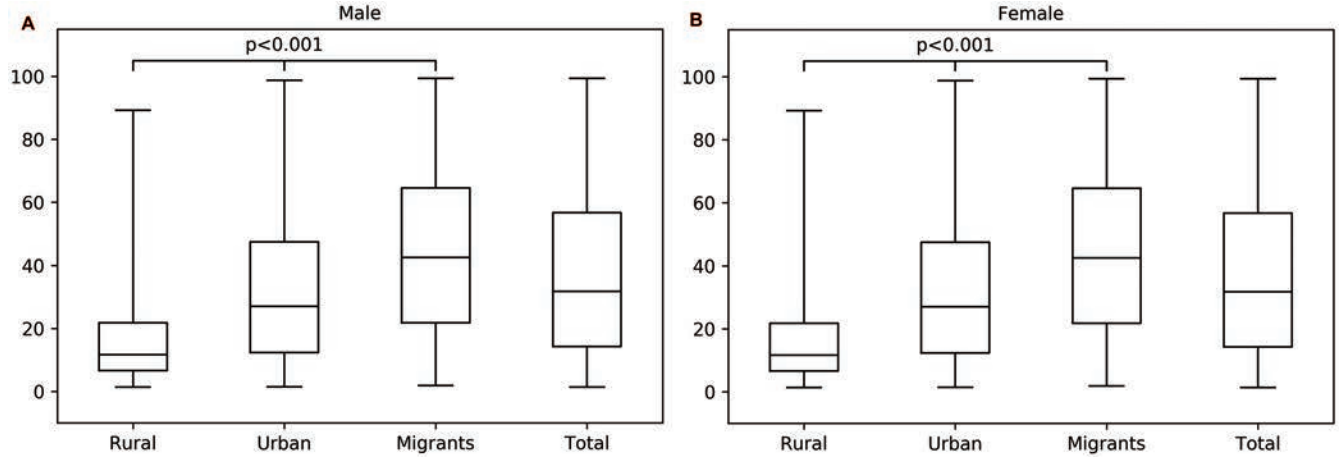

Fig. 1. Continuous FLI. (A) FLI in rural Ghana, urban Ghana and Ghanaian migrants in males. (B) FLI in rural Ghana, urban Ghana and Ghanaian migrants in females. FLI, fatty liver index.

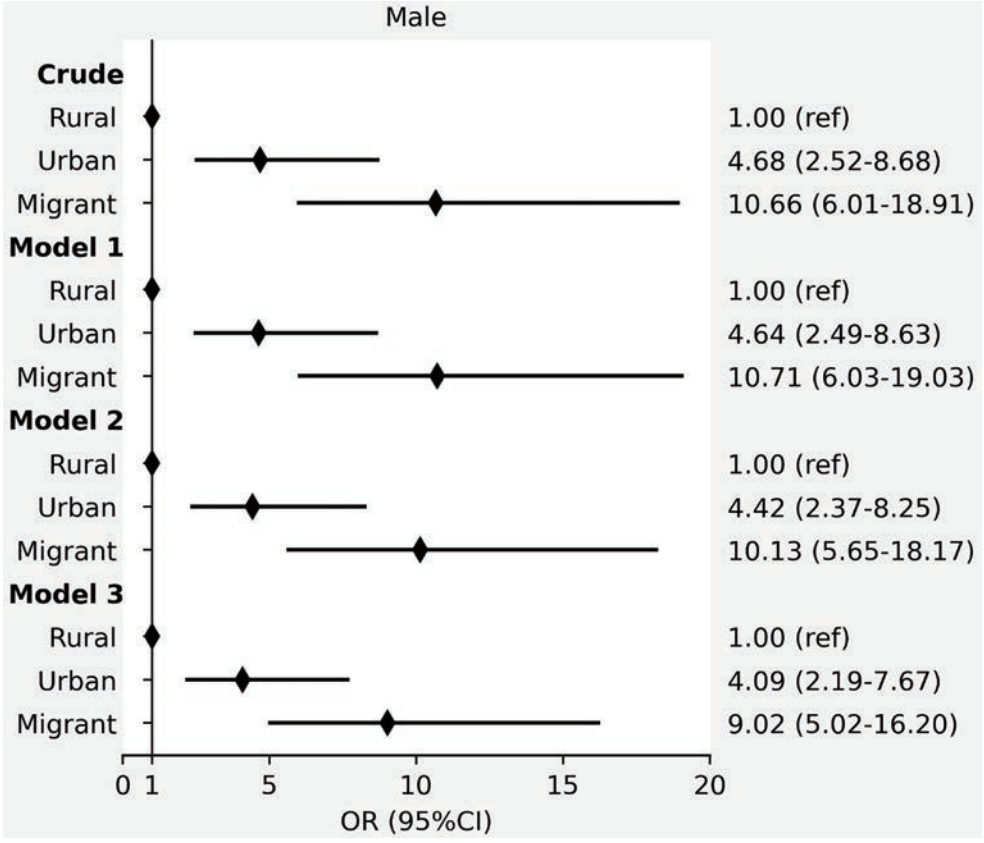

Fig. 2. ORs with 95% CIs for elevated FLI (FLI ≥60) in urban Ghana and Ghanaian migrants compared with rural Ghana in men. Model 1 adjusted for age; model 2: model 1 + education; model 3: model 2 + physical activity, alcohol, and T2DM. OR, odds ratio; CI, confidence interval; FLI, fatty liver index; T2DM, type 2 diabetes.

(commonly known as carotid IMT) and coronary calcification.[33,34] Lee *et al*.[35] conducted a cross-sectional study to investigate the influence of MAFLD on subclinical coronary atherosclerosis as detected by coronary computed tomography angiography (commonly referred to as CCTA). Fatty liver was assessed by ultrasound. In patients with MAFLD, ORs after adjustment for cardiovascular risk factors were higher for atherosclerotic plaques (OR: 1.18). In addition, there was a significant association of FLI ≥30 with non-calcified plaque (OR: 1.37). In addition, meta-analyses of studies with cardiovascular events also support the relation of MAFLD and ASCVD.[36] The underlying pathways are likely complex and difficult to decipher since many co-morbid factors may co-exist in these patients, such as hypertension, T2DM and obesity. Low grade inflammation[38] and hypercoagulable state[39] have also been implicated to mediate the relation between NAFLD and asCVD. Yet, evidence from Mendelian randomization studies most strongly supports that the MAFLD may drive ASCVD by mixed hyperlipidemia, through very low-density lipoprotein (i.e. VLDL) hypersecretion.[6,37]

Strengths and limitations

The FLI is a surrogate marker validated against ultrasonography by Bedogni *et al*.[14] in a Caucasian population and replicated by others.[13,40] Potential anthropometric and laboratory data were used in a logistic regression model to obtain a simple and accurate algorithm for the prediction of increased liver fat content, after exclusion of participants with hepatitis B and C. Due to this anthropometric and laboratory data, FLI is not directly based on liver fat content; yet, Bedogni *et al*.[14] reported an area under the receiver operating characteristic curve of 0.85. In the RODAM study, no imaging modalities of hepatic steatosis, such as abdominal ultrasound or MRI-PDFF,[41] or liver biopsies, were available to validate our findings with the FLI. When using the applied cut-off value of 60, in order to validate the FLI in a population-based study, the likelihood ratio was 5.10 for the presence of MAFLD. Additionally, a cut-off of ≥60 showed a specificity of 91%.[42] Unfortunately, blood platelets were not included in the RODAM study; hence, liver fibrosis proxies, such as Fibrosis-4 and aspartate to aminotransferase (i.e. AST) to platelet ratio (APRI) could not be included in our current analysis. We excluded other liver conditions in our calculations, as much as possible. We were able to exclude participants that used an excessive amount of alcohol and participants that used medication for hepatitis B and retroviral therapy. However, no data on untreated participants was available in the RODAM study. A great strength of the study is the homogeneity of the studied population, which provides a unique opportunity to investigate the metabolic effects of migration.

Conclusion

In conclusion, our study shows that, compared to rural areas, the prevalence of MAFLD as assessed by the FLI in the

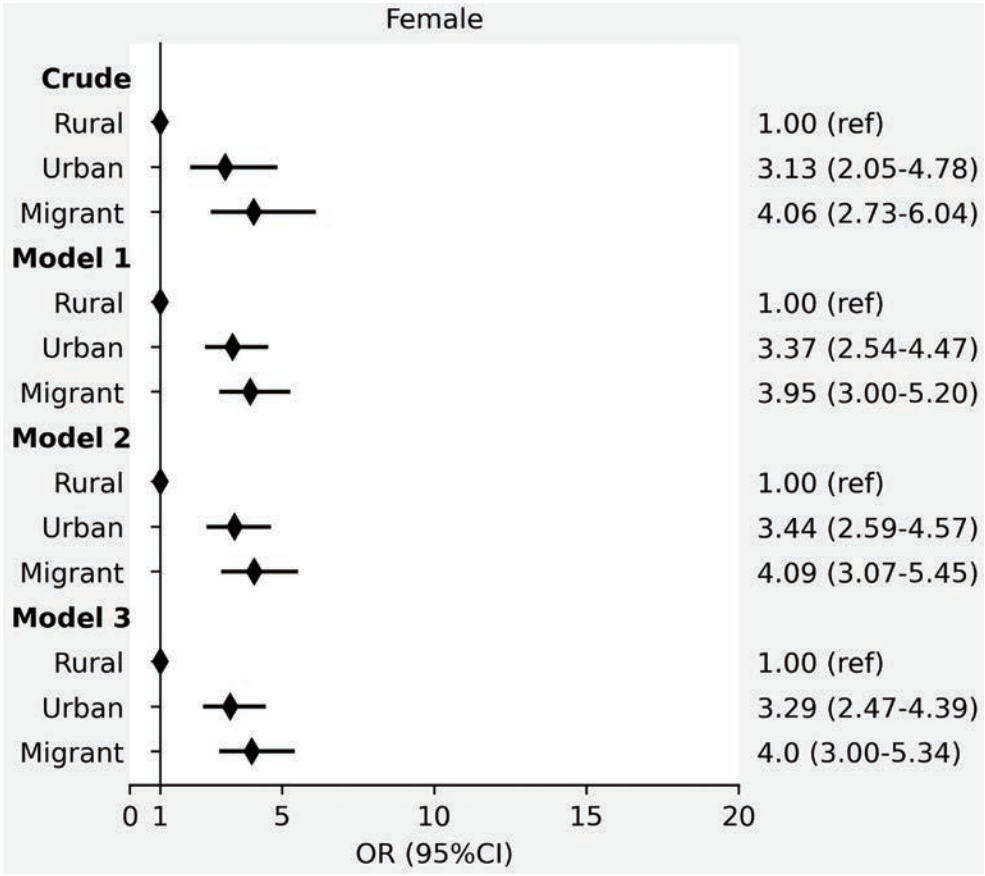

Fig. 3. ORs with 95% CIs for elevated FLI (FLI ≥60) in urban Ghana and Ghanaian migrants compared with rural Ghana in women. Model 1 adjusted for age; model 2: model 1 + education; model 3: model 2 + physical activity, alcohol, and T2DM. OR, odds ratio; CI, confidence interval; FLI, fatty liver index; T2DM, type 2 diabetes.

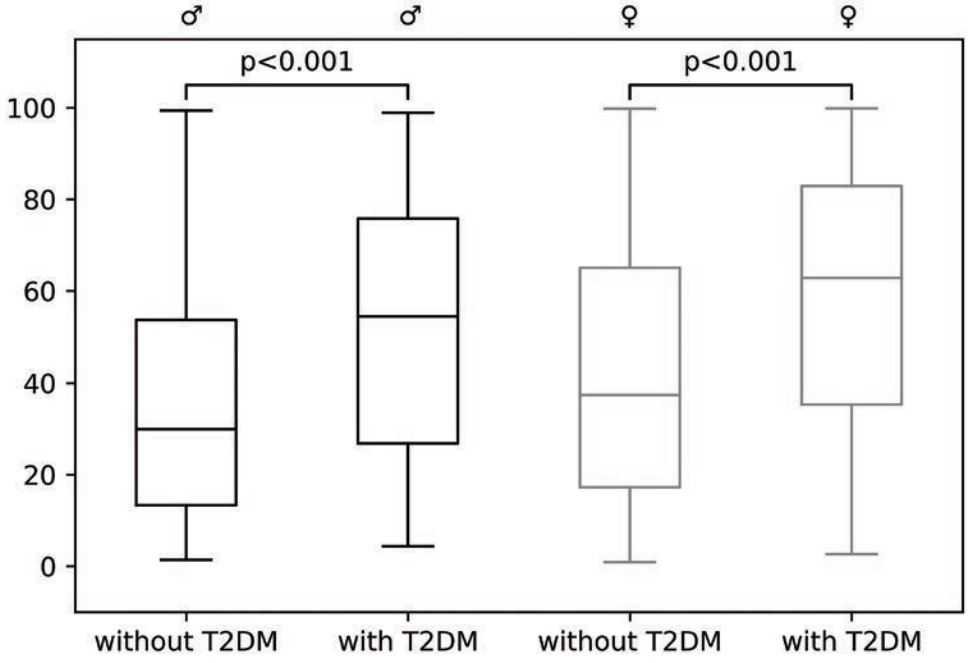

Fig. 4. FLI in male (black) and female (grey) participants with and without T2DM. FLI, fatty liver index; T2DM, type 2 diabetes.

Ghanaian RODAM population was higher in urban areas and even higher in Europe. In addition, FLI was strongly correlated with T2DM and ASCVD risk. This sheds light on MAFLD in this African population, and highlights the possible influence of migration on the prevalence of MAFLD, providing a clear rationale for future prospective studies with imaging modalities.

Acknowledgments

The authors are very grateful to the advisory board members and Ghanaian volunteers for participating in this project. We thank Jan van Straalen from the Academic Medical Centre for standardization of the lab procedures, and the Academic Medical Centre Biobank for their support in biobank management and high-quality storage of collected samples. We thank Erik Beune, from the department of Public Health at the Amsterdam UMC, the Netherlands, for his input in this project.

Funding

This work was supported by the European Commission under the Framework Programme (Grant No. 278901). The study sponsor was not involved in the design of the study; the collection, analysis and interpretation of data; writing the report; nor the decision to submit the report for publication. AGH was supported by the Amsterdam UMC Fellowship grant, a Holland Health TKI-PPP grant and by research grants from Gilead and Novo Nordisk. KACM was supported by the Intramural Research Program of the National Institutes of Health in the Center for Research on Genomics and Global Health (CRGGH). The CRGGH was supported by the National Human Genome Research Institute, the National Institute of Diabetes and Digestive and Kidney Diseases, the Center for Information Technology, and the Office of the Director at the National Institutes of Health (No. 1ZIAHG200362).

Conflict of interest

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

Author contributions

Design of the study (AMD, SD, FPC, CA, AGH), preparation of the draft of the protocol and drafting of the manuscript (AMD, SD), statistical analyses and interpretation (AMD, SD, FPC, CA), and revision of the manuscript (AMD, SD, AGH, CA). All authors reviewed and critically revised the manuscript.

Data sharing statement

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

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Journal of Clinical and Translational Hepatology **2021** vol. 9(4) | 503–513
DOI: 10.14218/JCTH.2021.00014

Original Article

Serum from Acute-on-chronic Liver Failure Patients May Affect Mesenchymal Stem Cells Transplantation by Impairing the Immunosuppressive Function of Cells

Yongyuan Zheng[1†], Shu Zhu[1†], Xingrong Zheng[1], Wenxiong Xu[1], Xuejun Li[1], Jianguo Li[1], Zhiliang Gao[1,2], Chan Xie[1,2*] and Liang Peng[1,2*]

[1]*Department of Infectious Diseases, The Third Affiliated Hospital of Sun Yat-sen University, Guangzhou, Guangdong, China;* [2]*Guangdong Province Key Laboratory of Liver Disease Research, The Third Affiliated Hospital of Sun Yat-sen University, Guangzhou, Guangdong, China*

Received: 7 January 2021 | **Revised:** 22 February 2021 | **Accepted:** 11 March 2021 | **Published:** 30 March 2021

Abstract

Background and Aims: The safety and efficacy of mesenchymal stem cells (MSCs) in the treatment of acute-on-chronic liver failure (ACLF) have been validated. However, the impact of the pathological ACLF microenvironment on MSCs is less well understood. This study was designed to explore the changes in the functional properties of MSCs exposed to ACLF serum. ***Methods:*** MSCs were cultured in the presence of 10%, 30% and 50% serum concentrations from ACLF patients and healthy volunteers. Then, the cell morphology, phenotype, apoptosis and proliferation of MSCs were evaluated, including the immunosuppressive effects. Subsequently, mRNA sequencing analysis was used to identify the molecules and pathways involved in MSC functional changes in the context of ACLF. ***Results:*** In the presence of ACLF serum, MSC morphology significantly changed but phenotype did not. Besides, MSC proliferation activity was weakened, while the apoptosis rate was lightly increased. Most importantly, the immunosuppressive function of MSCs was enhanced in a low-concentration serum environment but transformed into a proinflammatory response in a high-concentration serum environment. RNA sequencing indicated that 10% serum concentration from ACLF patients mediated the PI3K-Akt pathway to enhance the anti-inflammatory effect of MSCs, while the 50% serum concentration from ACLF patients promoted the conversion of MSCs into a proinflammatory function by affecting the cell cycle. ***Conclusions:*** The 50% ACLF serum concentration is more similar to the environment in the human body, which means that direct peripheral blood intravenous infusion of MSCs may reduce the effect of transplantation. Combining treatments of plasma exchange to reduce harmful substances in serum may promote MSCs to exert a stronger anti-inflammatory effect.

Citation of this article: Zheng Y, Zhu S, Zheng X, Xu W, Li X, Li J, *et al*. Serum from acute-on-chronic liver failure patients may affect mesenchymal stem cells transplantation by impairing the immunosuppressive function of cells. J Clin Transl Hepatol 2021;9(4):503–513. doi: 10.14218/JCTH.2021.00014.

Introduction

Acute-on-chronic liver failure (ACLF) is a distinct clinical syndrome, characterized by liver failure due to acute hepatic injury and underlying chronic liver disease; it has a high 28-day mortality. Liver transplantation is the only treatment that has proven beneficial, but the rapid disease progression and lack of donors limit the application of this treatment.[1,2] The infusion of mesenchymal stem cells to treat liver failure has been verified as safe and effective in clinical trials[3,4] as well as by animal experiments based upon acute liver failure models.[5–7] In clinical practice, the treatment of patients with ACLF with infusions of mesenchymal stem cells (MSCs) significantly increased the 24-week survival rate by improving liver function and decreasing the incidence of severe infections.[4] In the treatment of fulminant hepatic failure in large animal (pig) models, immediate intraportal transplantation of MSCs quickly participated in liver regeneration via proliferation and transdifferentiation into hepatocytes.[5] MSCs harbor anti-inflammatory, immunomodulatory, antiapoptotic and proliferative properties and hold great promise in the treatment of both acute and chronic liver diseases.[2] However, studies generally have focused on the effectiveness and mechanism of MSCs in the treatment of liver failure, while the impact of the pathological ACLF microenvironment on MSCs has received little attention thus far.

The impact of the pathological microenvironment on MSC

Keywords: Mesenchymal stem cells; Acute-on-chronic liver failure; Immunomodulatory; Cell therapy; Microenvironment.

Abbreviations: ACLF, acute-on-chronic liver failure; AP, ACLF patient; DEGs, differentially expressed genes; GO, Gene Ontology; GSEA, Gene Set Enrichment Analysis; HC, healthy control; KDA, Key driver analysis; KEGG, Kyoto Encyclopedia of Genes and Genomes; MSCs, mesenchymal stem cells; PPI, protein-protein interaction.

[†]Both authors contributed equally to this work.

[*]**Corresponding Authors:** Liang Peng, 600[#] Tianhe Road, Tianhe district, Guangzhou, Guangdong 510530, China. ORCID: https://orcid.org/0000-0001-6184-5750. Tel/Fax: +86-20-8525-2372, E-mail: pliang@mail.sysu.edu.cn; Chan Xie, 600[#] Tianhe Road, Tianhe district, Guangzhou, Guangdong 510530, China. ORCID: https://orcid.org/0000-0002-0225-5483. Tel/Fax: +86-20-8525-2372, E-mail: xchan@mail.sysu.edu.cn

Table 1. Clinical characteristics of the participants enrolled in the study

Group	HBV-ACLF, *n*=20	HC, *n*=20
Male sex	18	14
Age in years	35.10±6.03	36.6±7.42
WBC as 10^9/L	5.88±2.81	6.17±1.46
PLT as 10^9/L	120.35±64.65	240.55±37.03
ALT in U/L	171.10±214.34	19.05±13.55
AST in U/L	130.65±192.93	19±5.53
PTA, %	32.2±5.03	N.D.
INR	2.42±0.43	N.D.
Albumin in g/L	36.96±5.39	N.D.
TBil in μmol/L	311.48±132.24	N.D.
CR in μmol/L	63.94±12.16	70.23±14.24
Na in μmol/L	137.10±4.06	N.D.
HBeAg-positive	4	0
Complication	11	0
Ascites	6	0
SBP	5	0
Hepatic encephalopathy	0	0
Hepatorenal syndrome	0	0
UGB	0	0
MELD score	23.49±3.74	N.D.
MELD-Na score	20.15±8.36	N.D.
COSSH-ACLF Grade	1	N.D.

Data are shown as means±standard deviations. ACLF, acute-on-chronic liver failure; ALT, alanine aminotransferase; AST, aspartate aminotransferase; COSSH-ACLF, Chinese Group on the Study of Severe Hepatitis B-ACLF; CR, creatinine; HBeAg, hepatitis B e antigen; HC, healthy control; INR, international normalized ratio; MELD, model for end-stage liver disease; N.D., not determined; PLT, platelet; PTA, prothrombin time activity; SBP, spontaneous bacterial peritonitis; TBil, total bilirubin; UGB, upper gastrointestinal bleeding; WBC, white blood cell.

function is extremely important. In the treatment of inflammatory diseases, the therapeutic effect of MSCs is mainly the result of immunomodulation and this function is mediated by the inflammatory microenvironment, which means that these cells have immunoregulatory plasticity. In response to different amounts and kinds of inflammatory mediators, MSCs produce ample amounts of immunoregulatory factors, cell-mobilization factors and growth factors, thereby facilitating tissue repair by tissue-resident stem cells.[8,9] In organismal aging, hormonal, immunologic, and metabolic factors are the critical microenvironmental signals that trigger MSC dysfunction, particularly the shift in differentiation from osteoblasts to adipocytes that occurs following the activation of key signaling pathways, such as intracellular oxidative stress and posttranscriptional regulation.[10] In addition, in systemic sclerosis, patient serum mediates oxidative stress effects on MSC function, such as increasing the apoptosis rate and osteoblastic/adipogenic potential, whereas the immunosuppressive function of MSCs becomes reduced.[11] Although the influence of the pathological ACLF microenvironment on MSCs has never been reported, our previous studies have examined the changes in the functional properties of heterologous umbilical cord (UC)-MSCs exposed to ACLF serum and aimed to simulate the pathological microenvironment *in vitro*, as well as to determine the molecular mechanisms of MSC plasticity.

Methods

Human serum sample collection

Serum was collected from 20 patients and 20 healthy volunteers, who were included as healthy controls (HCs). The clinical and biological characteristics of the participants are shown in Table 1. The inclusion criterion and the exclusion criteria of ACLF were based on the Asian Pacific Association for the Study of the Liver.[12] The ACLF grade was based on Chinese Group on the Study of Severe Hepatitis B-ACLF.[13] Blood from ACLF patients and healthy volunteers was centrifuged at 2,000 *g* for 15 m and the serum samples were stored at −80°C. The isolation and culture of UC-MSCs were performed according to Good Manufacturing Practice (referred to as GMP) protocols in our GMP laboratory, as previously described.[14] In addition, human peripheral blood mononuclear cells from healthy donors were isolated by Ficoll, according to standard procedures. The samples were obtained with written informed consent from all subjects, in accordance with the Declaration of Helsinki. This study was carried out in accordance with the

recommendations of the ethics committee of our hospital (Third Affiliated Hospital of Sun Yat-sen University, Guangzhou, China).

Serum pretreatment scheme for MSCs

Before analyzing the functional characteristics of MSCs (except for proliferation assay), different concentrations of ACLF patient (AP) sera and HC sera were added to normal MSC culture medium for 48 h. According to the treatment concentrations (10%, 30% and 50%), the groups were named AP10, AP30, AP50, HC10, HC30, and HC50, and the normal cultured MSC group was a blank control.

Surface marker expression

When the MSCs had grown to 80% confluence, the cells from different groups were harvested, and the positive and negative expression of the surface markers (CD14, CD34, CD45, HLA-DR, CD29, CD44, CD105 and CD166; all relevant monoclonal antibodies were purchased from Biolegend, San Diego, CA, USA) was examined. The staining was carried out according to the manufacturer's instructions, and the data were analyzed with Kaluza software (Beckman Coulter, Brea, CA, USA) and FlowJo 7.5 (Treestar, Ashland, OR, USA).

Proliferation assay

MSCs were plated at 2,000 cells/well in 96-well plates and then treated with different serum concentrations from ACLF patients and HC volunteers. Ten microliters of cell counting kit 8 (CCK-8) solution (Dojindo, Kumamoto, Japan) was added at the indicated time points on days 1, 3, 5, and 7. Proliferation was measured using the IncuCyte HD imaging system (Essen BioScience, Tokyo, Japan) by measuring the optical density value at 450 nm. The results are expressed as the percentage of proliferation±standard error of the mean and normalized to 100% as the initial number of cells plated.

Apoptosis assay

MSCs were plated at 1×10^6 cells/well in six-well plates. After adding different concentrations of serum to stimulate the cells for 48 h, the number of apoptotic cells was evaluated by annexin V and propidium iodide (BD Biosciences, Franklin Lakes, NJ, USA) labeling, according to the manufacturer's instructions. The labeled cells were analyzed using Kaluza software (Beckman Coulter) and FlowJo 7.5 (Treestar). The results are expressed as the percentage of annexin V+ cells.

T lymphocyte proliferation assay

For cell sorting, peripheral blood mononuclear cells were stained with human CD3 microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany), according to the manufacturer's instructions, and then sorted by a Miltenyi magnetic bead sorter to harvest CD3+ T cells. The purified CD3+ T cells were stained with 5 mM 5-(and-6)-carboxyfluorescein diacetate succinimidyl ester (Cell Trace; Invitrogen, Carlsbad, CA, USA). MSCs (1×10^5 cells/well) were seeded in 24-well flat-bottom plates and incubated for 24 h. After serum pretreatment, T cells were added at a MSC/T cell ratio of 1:10 and were cocultured with MSCs for 5 days in RPMI-1640 containing 10% (v/v) fetal bovine serum, 50 U/mL penicillin and 50 U/mL streptomycin; a 5 μg/mL aliquot of phytohemagglutinin (Sigma-Aldrich, St. Louis, MO, USA) was added to activate T cell proliferation. The results are expressed as the percentage of proliferation±standard error of the mean.

RNA sequencing

The total RNA from MSCs, which had been pretreated with serum from each group, were isolated by Trizol (Invitrogen), following the manufacturer's protocol. The transcripts were sequenced using the BGISEQ-500 sequencing platform (BGI Tech Company, Guangdong, China). Essentially, differential expression analysis was performed using the DESeq2 (v1.4.5) with Q value <0.05. To obtain insight into the change of phenotype, Gene Ontology (GO; http://www.geneontology.org/) and Kyoto Encyclopedia of Genes and Genomes (KEGG; https://www.kegg.jp/) and Gene Set Enrichment Analysis (GSEA) of annotated different expression gene was performed by Phyper (https://en.wikipedia.org/wiki/Hypergeometric_distribution) based on hypergeometric test. The significant levels of terms and pathways were corrected by Q value with a rigorous threshold (Q value <0.05) by Bonferroni. The protein-protein interaction (PPI) analysis was conducted by DIAMOND and STRING. Key driver analysis (KDA) was performed according to Tran's methods. All the analyses were conducted with the online bioinformatic platform Dr. Tom (biosys.bgi.com/) provided by BGI.[15]

Statistical analysis

The results are presented as the means±standard deviations of the independent experiments. Comparisons were made using a two-tailed *t*-tests (between two groups), one-way ANOVA (for multigroup comparisons) or Kruskal-Wallis and Mann-Whitney *U* tests (for nonnormally distributed data). A *p* value of <0.05 was considered to represent a significant difference. The statistical analyses were performed using SPSS v. 22.0 (IBM Corp., Armonk, NY, USA). Graphing was performed using Prism 6.01 software (GraphPad, San Diego, CA, USA).

Results

ACLF serum pretreatment significantly changed MSC morphology but not phenotype

First, we observed the morphology of MSCs under the microscope after serum pretreatment and then detected their phenotypes. Microscopic analysis (Fig. 1A) showed that the cells were no longer arranged in a spindle-like manner in the AP groups, as compared with that of the MSC group in normal culture conditions and the HC group. With increasing serum concentrations in the AP groups, the gaps between cells became larger and many coarse particles appeared around the nuclei. In the HC group, when the serum concentration increased to 50%, the cell morphology also changed slightly. Second, we analyzed the phenotypes of MSCs treated with different types of serum and found no large changes (Fig. 1B). It suggested that when MSCs entered patients with ACLF, the cells might be adversely affected, although the phenotypes

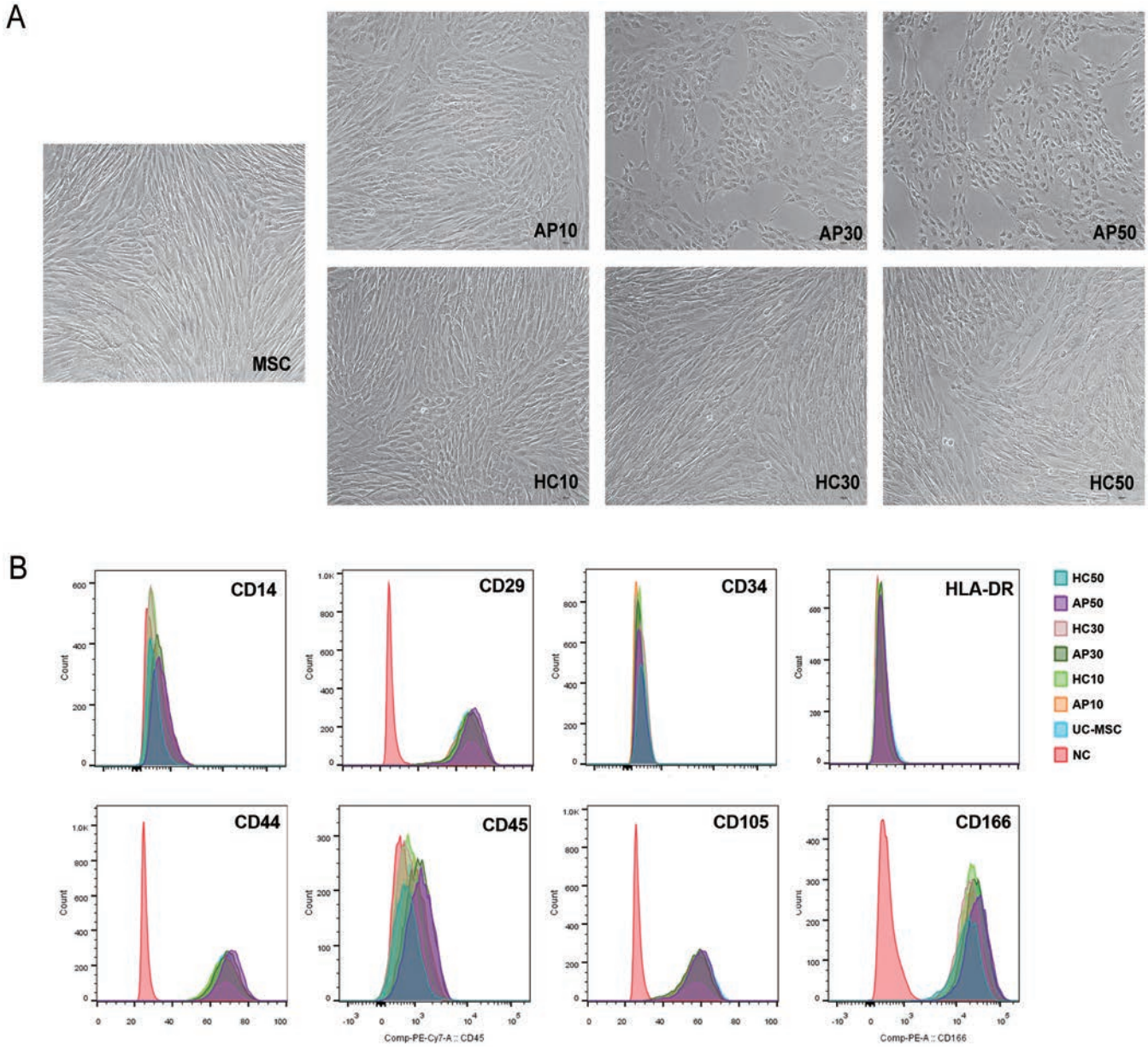

Fig. 1. ACLF serum pretreatment significantly changed MSC morphology but not phenotype. (A) Representative photographs of MSCs cultured after serum treatment for 48 h in different groups. Scale bars, 200 μm. (B) UC-MSC phenotype showed no differences after serum pretreatment among groups. Flow cytometry analysis showed that the cultured UC-MSCs were positive for CD29, CD105, CD166 and CD44 but negative for CD34, CD45, CD14, CD164 and HLA-DR. ACLF, acute-on-chronic liver failure; AP, ACLF patient; HC, healthy control; MSCs, mesenchymal stem cells.

were still maintained.

ACLF serum dose-dependently decreased the proliferation rate of MSCs but significantly induced apoptosis only at 50% concentration

Since ACLF serum pretreatment significantly changed the morphology of MSCs, we wondered whether the proliferation and apoptosis of MSCs would be affected. Therefore, we evaluated the proliferation rate of MSCs cultured for 7 days and the apoptosis level after serum pretreatment. In AP groups, only 10% serum significantly reduced MSC proliferation, as compared to that of the MSC group and HC group (Fig. 2A). In addition, as the serum concentration increased, the MSC proliferation rate remained sluggish (Fig. 2B). However, we found that it did not cause obvious apoptosis, regardless of whether the serum was from HC donors or ACLF patients, when the pretreatment concentration was 10% (Fig. 2C–D). In the AP group, only when the serum pretreatment concentration was 50%, the percentage of apoptotic MSCs increased. To our surprise, with increase in serum concentration in the HC group, the proportion of early and late apoptotic cells increased, and there was a significant difference (Fig. 2D). Altogether, the data indicated that there might be certain harmful substances existing in the serum of ACLF patients, which could inhibit the proliferation of MSCs

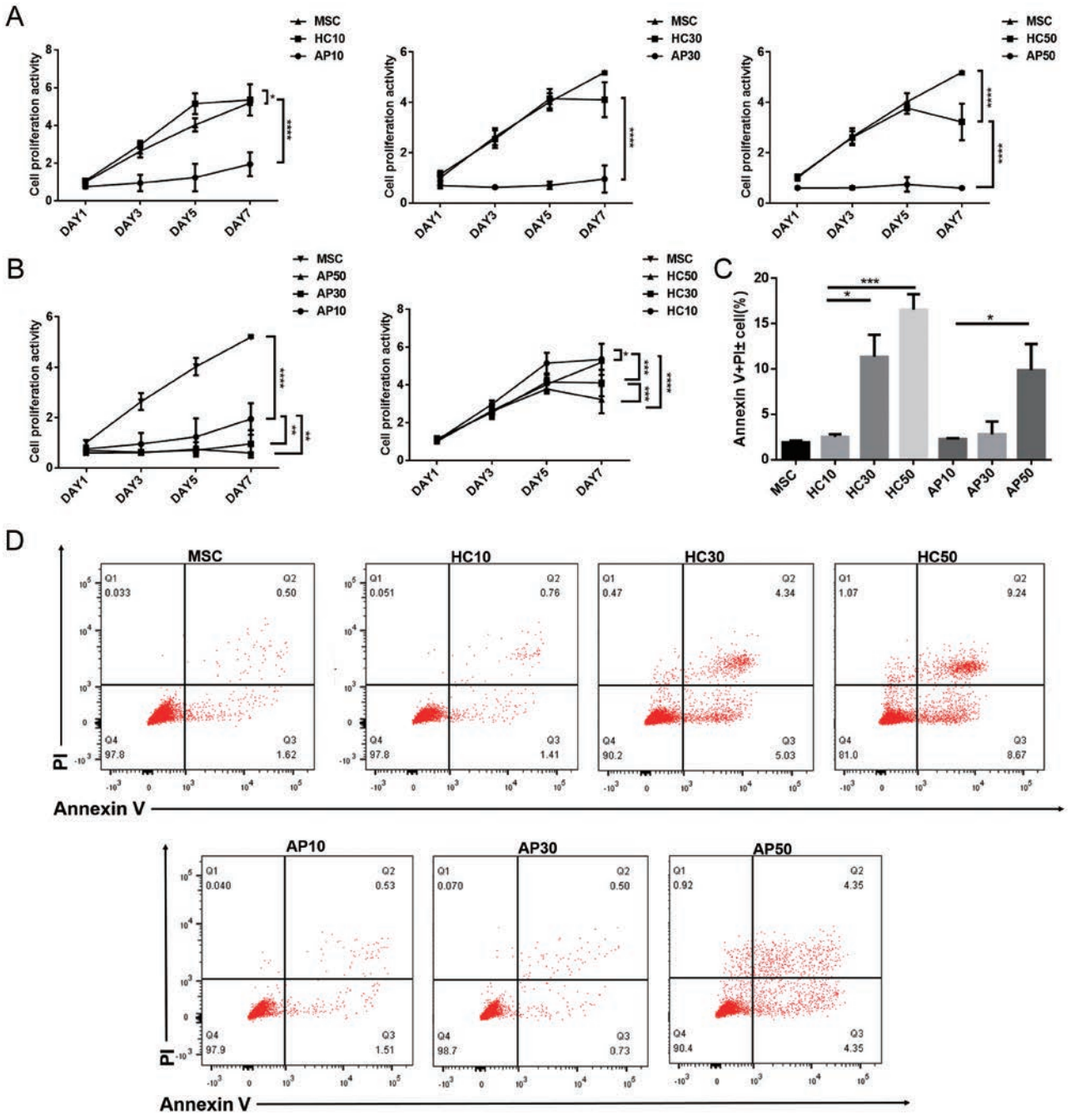

Fig. 2. Serum pretreatment obviously decreased MSC proliferation in the AP group and increased apoptosis only in the AP50 group. (A) MSC proliferation in the AP group compared with the MSC group and HC group at the same serum pretreatment concentration. (B) In the AP group or HC group, MSC proliferation activity decreased compared with that of the MSC group after pretreatment with different serum concentrations. The data were normalized to MSCs plated on day 1 without serum treatment. (C) Percentage of annexin V+ apoptotic MSCs in each group. (D) Proportion of early and late apoptotic cells in different groups. The graphs indicate the means±standard deviations, with statistically significant differences indicated as follows: *$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$; $n=5$. AP, ACLF patient; HC, healthy control; MSCs, mesenchymal stem cells.

and induce apoptosis when accumulated to a certain degree.

ACLF serum dose-dependently regulated the immunosuppressive effects of MSCs

In cell transplantation therapy, the immunomodulatory effect of MSCs is extremely important for the improvement of liver failure. Thus, we investigated the immunosuppressive potential of MSCs after serum pretreatment in each group (Fig. 3). The sorted CD3+ T lymphocytes cultured alone proliferated after adding stimulants, such as phytohemagglutinin, while MSCs in normal culture inhibited the proliferation of these activated T cells to exert an immuno-

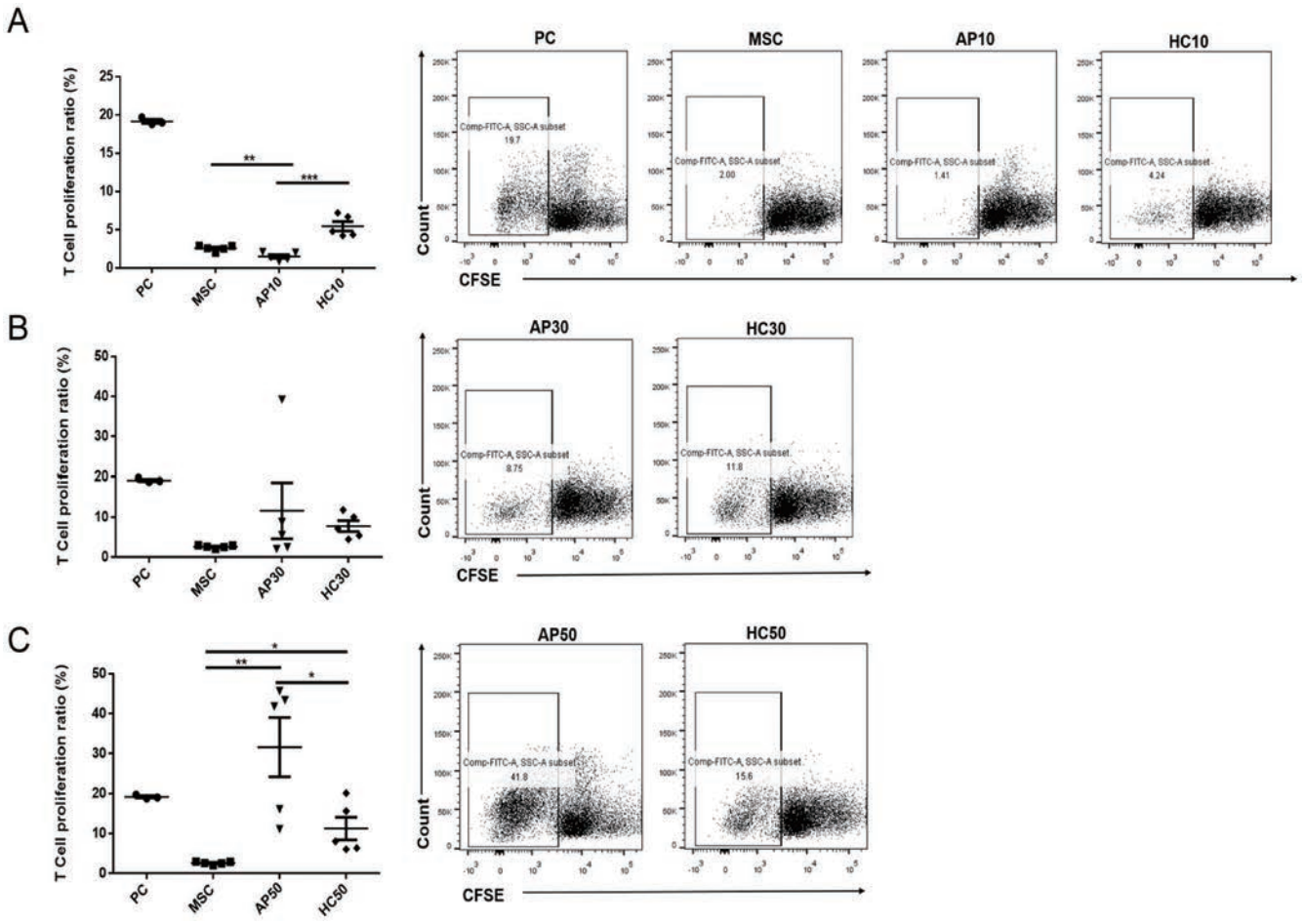

Fig. 3. MSCs exerted a stronger anti-inflammatory effect under ACLF serum pretreatment at 10% concentration but transformed into proinflammatory function at 50% concentration. The percentage of T lymphocyte proliferation after culture alone as the positive control (PC) or culturing at a 1:10 MSC:T lymphocyte ratio with MSCs that were pretreated for 48 h with human serum from each group. (A) MSCs from the AP10 group markedly inhibited the proliferation and activation of naive CD3+ T cells compared with those from the MSC group and HC group. (B–C) MSCs from the AP group increased the proliferation and activation of naive CD3+ T cells compared with those from the MSC group and HC group when the serum concentration was increased to 30% and 50%. The bar graphs indicate the means±standard deviations, statistically significant differences are indicated as follows: *p<0.05, **p<0.01, ***p<0.001; n=5. AP, ACLF patient; HC, healthy control; MSCs, mesenchymal stem cells.

suppressive effect. When the serum pretreatment concentration was 10%, the proliferation rate of T cells in the AP10 group was the least, which indicated MSCs in this group exhibited significantly enhanced anti-inflammatory effects, while the effect on MSCs in the HC group was not significantly different (Fig. 3A). When the serum concentration was 30%, MSCs did not exhibit stronger immunosuppressive potential, even though the anti-inflammatory effect of MSCs shifted to a proinflammatory effect when the concentration was 50%; although, there may have been individual differences (Fig. 3B–C). Overall, MSCs that were pretreated with 50% ACLF serum promoted the proliferation of activated T lymphocytes. All the data suggest that the pathological ACLF microenvironment may have an adverse effect on the infused MSCs. After all, the 50% serum concentration *in vitro* is closer to the environment in the human body.

Molecular pathways by which ACLF serum enhanced MSC immunosuppressive functions in the AP10 group

In order to investigate the mechanism by which ACLF serum pretreatment at 10% concentration enhanced the immunosuppressive function of MSCs and the key driver genes, thereby suggesting some upstream molecules, we collected samples of serum-treated MSCs and performed mRNA sequencing analysis according to the experiment workflow (Fig. 4). The volcano maps show the differentially expressed genes (DEGs) between the AP10 group and HC10 group (Fig. 5A). Then, 1,221 up-regulated genes and 1,641 down-regulated genes were analyzed by KEGG, GO and GSEA to identify the pathways of interest (the screening conditions were log2-fold change (referred to herein as log2FC) >1 and Q value <0.05). Therefore, in the KEGG analysis of down-regulated genes (Fig. 5B), we selected immune-related pathways (shown in red boxes) and performed PPI network analysis on the DEGs involved in these pathways (Fig. 5C). PPI analysis indicated that the proteins expressed by the genes in the blue circle affected each other, which were more likely to be in the same pathway. Moreover, KDA was used to screen the key genes (denoted by the black arrow) that affected these pathways (Fig. 5D). In addition, the expression cluster heatmap clearly showed the differences in the expression of these core genes in each group (Fig. 5E). Furthermore, we performed re-enrichment of these KDA

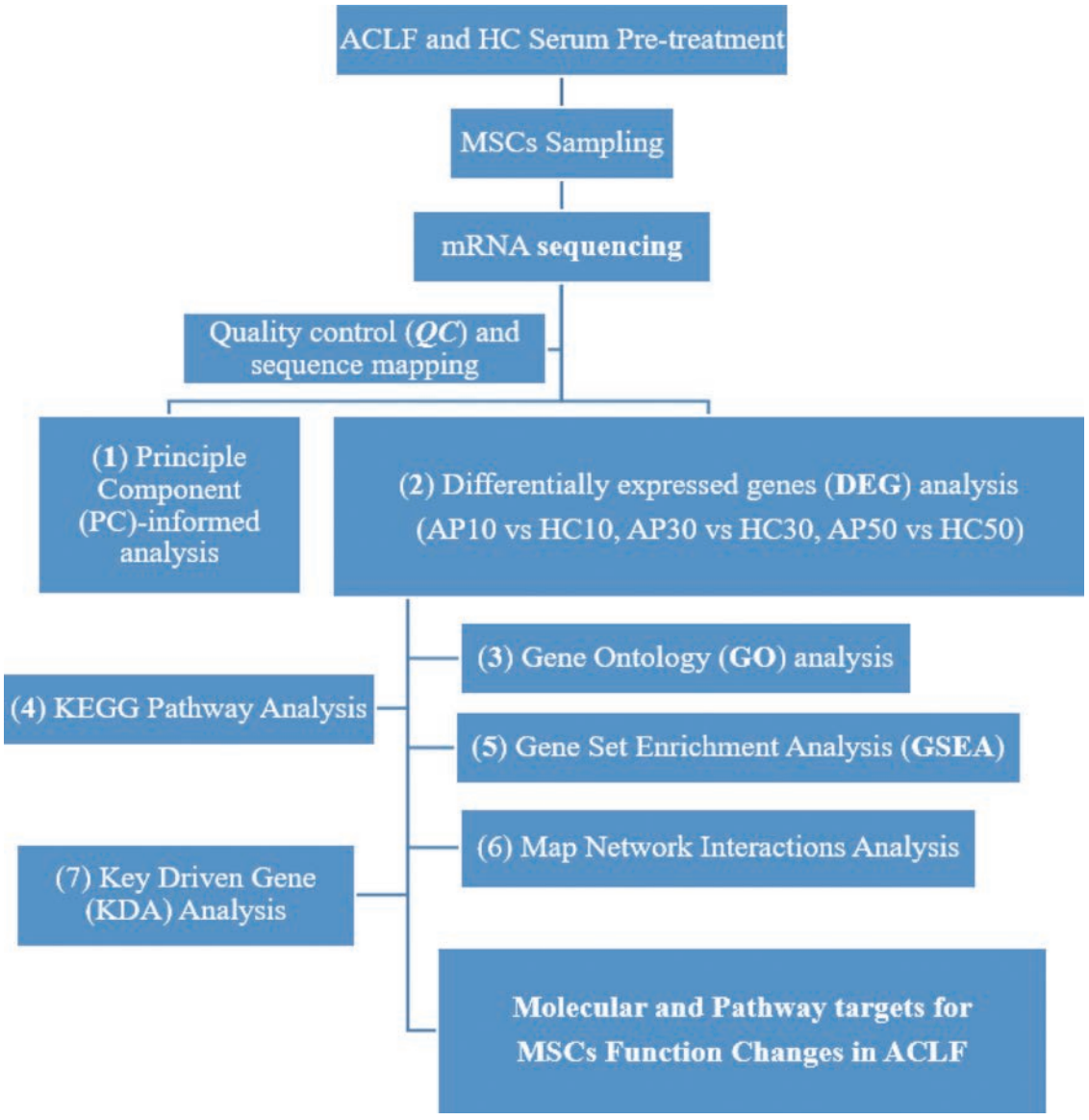

Fig. 4. RNA sequencing experiment workflow. The workflow of serum pretreatment, MSC sample collection, mRNA sequencing data analysis and interpretation is presented. More information on the detailed methods is provided in the Materials and Methods section. Since the AP10 group and AP50 group exhibited significantly different effects on the immunoregulatory function of MSCs, subsequent sequencing analysis should focus on these two groups. AP, ACLF patient; MSCs, mesenchymal stem cells.

genes and found that the PI3K-Akt signaling pathway might play a major role in the serum-mediated MSCs' exerting stronger immunosuppressive function.

Molecular pathways by which serum transformed MSCs into proinflammatory cells in the AP50 group

According to the same analysis workflow, we performed GO enrichment analysis of up-regulated DEGs between the AP50 group and HC50 group (Fig. 6A). In fact, we found that immune-related pathways were rarely enriched in various enrichment analyses. However, the pathways related to cell cycle, cell division, cell proliferation and apoptotic process were significantly enriched. For the DEGs involved in these pathways, we also performed PPI analysis and KDA. Similarly, the expression cluster heatmap (Fig. 6B) and the histogram based on Fragments per kilobase of exon model per million mapped fragments (FPKM) (Fig. 6C) clearly showed the differences in the expression of these core genes between the AP50 group and HC50 group. The re-enrichment results of the KDA genes (Fig. 6D) suggested that ACLF serum at 50% concentration might affect the cell cycle and threaten the basic metabolic activities, leading to the transformation of MSCs into a proinflammatory function.

Discussion

Our research idea was originally derived from the longitudinal comparison of two clinical studies of the use of MSCs in the treatment of ACLF, which were performed in our depart-

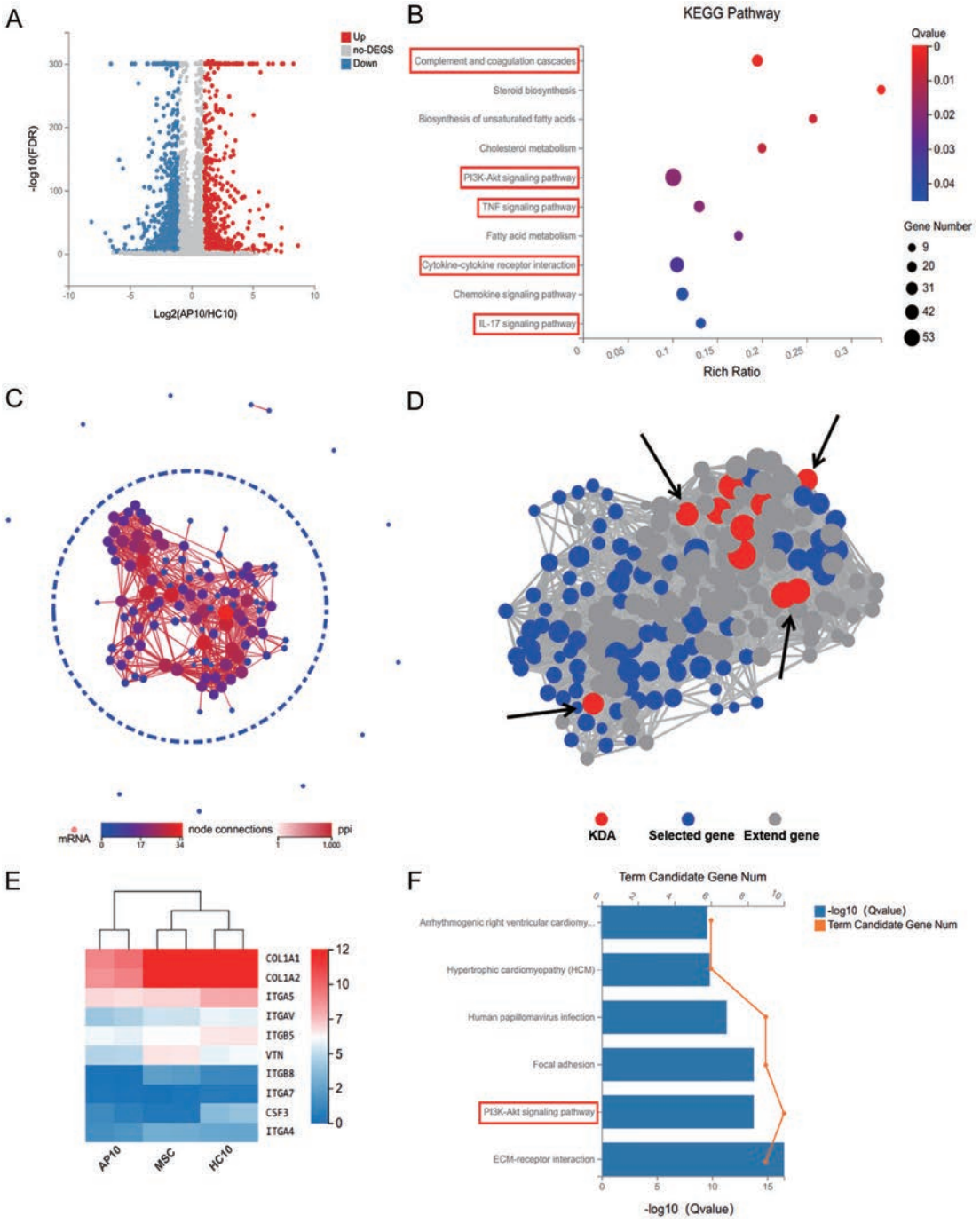

Fig. 5. Molecular pathways by which ACLF serum enhanced the immunosuppressive effects of MSCs in the AP10 group compared with the HC10 group. (A) The DEGs between the AP10 group and HC10 group are shown by volcano plots. The X-axis represents the difference multiplied after the log2 conversion, and the Y-axis represents the significance value after the log10 conversion. The red dots represent up-regulated DEGs, the blue dots represent down-regulated DEGs, and the gray dots represents non-DEGs. log2FC≥1 and Q value < 0.05. The Q value is the calibration value of the P value. (B) Bubble diagram showing KEGG pathway enrichment analysis of down-regulated DEGs. The X-axis is the enrichment ratio and the Y-axis is the KEGG pathway. The size of each bubble represents the number of genes annotated to the KEGG pathway, while the color represents the enrichment Q value and darker color represents smaller Q values. The red box encloses the pathways of interest. (C) PPI map showing how genes in our pathway of interest interact, are coexpressed or regulate relationships. Each circle represents a gene; the larger the circle, the more highly the gene is connected to other genes. The line represents the interaction among genes. (D) KDA showed which genes are major regulators in the PPI map (denoted by the black arrow). (E) Expression cluster analysis was conducted on the FPKM value among the AP10 group, HC10 group and MSC group, to show 10 KDA genes. The thermogram showing the log2 (FPKM+1) of the sample, which is represented by the horizontal axis, and the gene is represented by the vertical axis. Under default color-matching, the redder the color of the block, the higher the expression level, and the bluer the color, the lower the expression level. (F) Histogram diagram showing the KEGG re-enrichment of KDA genes between AP10 group and HC10 group. Y-axis represents the KEGG enrichment pathway; the bar chart length represents the number of genes annotated to a KEGG pathway. ACLF, acute-on-chronic liver failure; AP, ACLF patient; DEGs, differentially expressed genes; KDA, Key driver analysis; KEGG, Kyoto Encyclopedia of Genes and Genomes; MSCs, mesenchymal stem cells; PPI, protein-protein interaction.

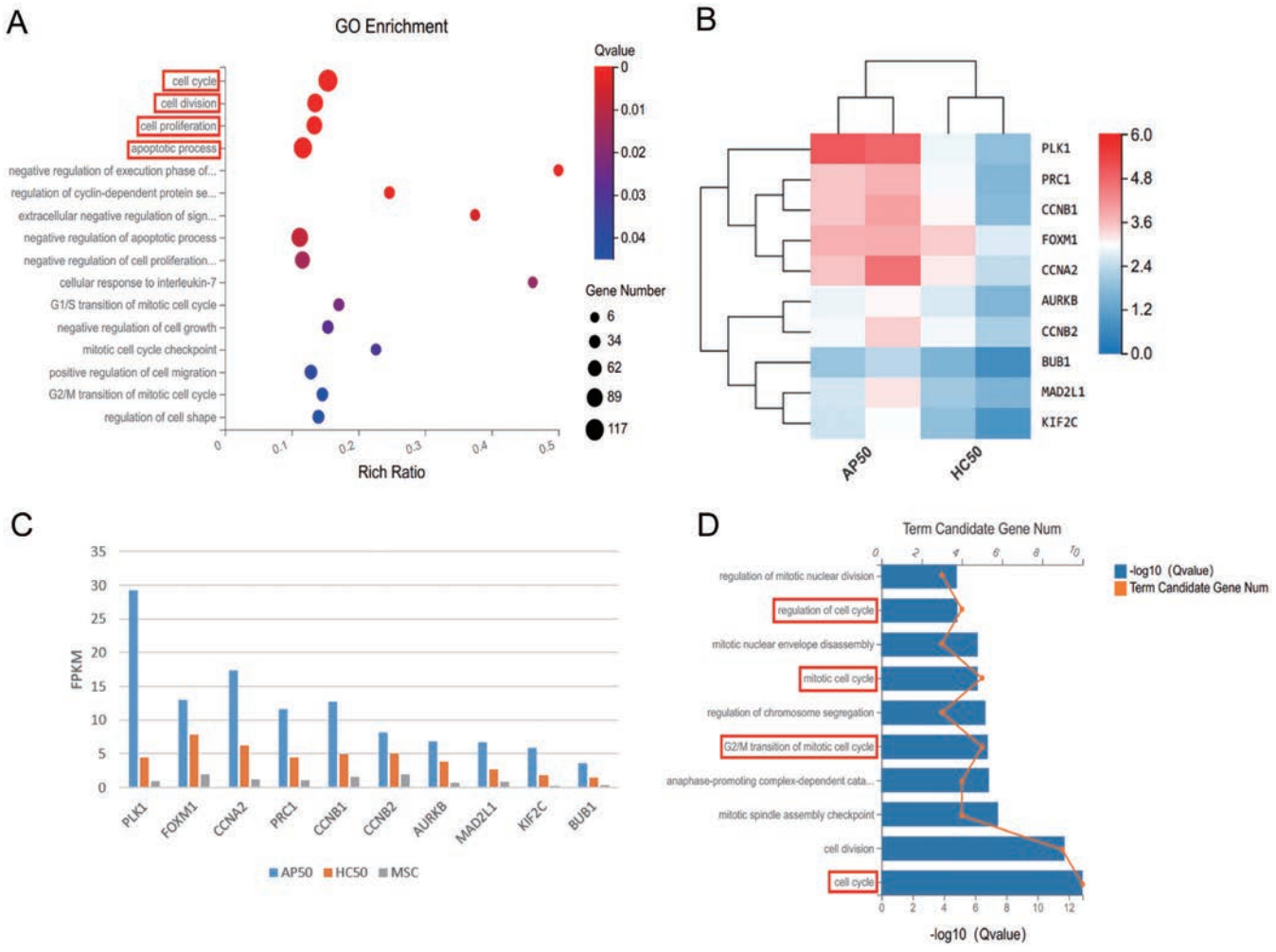

Fig. 6. Molecular pathways target how ACLF serum transformed MSCs into a proinflammatory effect in the AP50 group compared with the HC50 group, following the same sequencing analysis workflow. (A) Bubble diagram showing GO enrichment analysis of up-regulated DEGs. The red boxes enclose pathways of interest that are significantly enriched. (B–C) The expression cluster heatmap and the histogram based on FPKM of 10 KDA genes after PPI analysis and KDA. (D) The GO re-enrichment results of KDA genes between AP50 group and HC50 group. ACLF, acute-on-chronic liver failure; AP, ACLF patient; DEGs, differentially expressed genes; GO, Gene Ontology; HC, healthy control; KDA, Key driver analysis; MSCs, mesenchymal stem cells.

ment in 2011 and 2017 respectively.[3,4] There were many differences, such as the cell source, generation, infusion volume and infusion methods, between the two studies. Nonetheless, we were concerned about why a small group of patients did not respond well to MSC treatment. Moreover, it was reported that the infusion of MSCs via a peripheral vein did not rescue acute liver failure pigs, while most of the acute liver failure pigs survived more than 6 months after the transplantation of MSCs via the portal vein.[16,17] We wondered whether MSCs were more susceptible to the adverse effects of the pathological microenvironment in the body when they were administered via intravenous infusion compared with *in situ* infusion, rendering these cells unable to exert a beneficial therapeutic effect. Thus, this study aimed to investigate MSC properties in the specific context of allogeneic transplantation and the molecular and mechanism pathways that affect the plasticity of MSCs.

In this study, we observed that pretreatment of MSCs with ACLF serum reduced proliferation but did not obviously increase the level of apoptosis at 10% concentration. Besides, the immunosuppressive function of these cells was significantly enhanced at 10% concentration, while becoming shifted to a proinflammatory state at 50% concentration. In another study, Fonteneau *et al*.[11] found that in the oxidative environment of systemic sclerosis patient serum, MSCs retained their proliferative potential, with increased apoptosis rate occurring at day 10. In addition, the immunosuppressive function of these cells was slightly decreased. Although systemic sclerosis and ACLF have different disease backgrounds, the phenomena we observed in terms of proliferation and apoptosis were the same, while the immunosuppressive functions were not exactly the same. Moreover, several studies have reported that apoptotic cells could modulate immune responses.[18–20] Galleu *et al*.[21] used a murine model of graft-versus-host disease to demonstrate that MSCs were actively induced to undergo perforin-independent apoptosis by recipient cytotoxic cells and that this process was essential for initiating MSC-induced immunosuppression. In addition, it was reported that MSCs could be shifted from a suppressive to supportive phenotype when exposed to defective immune cells, since MSCs are very sensitive to their environment. This immune activating effect may be due to MSC prestimulation.[22]

In this study, although ACLF serum pretreatment concen-

tration of 50% caused obvious apoptosis, the MSCs did not exert a stronger immunosuppressive effect. According to the mRNA sequencing results of the AP50 group, the pathways related to cell cycle, cell division, cell proliferation and apoptotic process were significantly enriched. We have to suspect that some unfavorable factors in the serum seriously affect the basic metabolism of MSCs, and even exceed the role of some inflammatory factors that can empower MSCs, causing MSCs to exhibit proinflammatory effects. Interestingly, the proliferative activity of MSCs pretreated with HC serum was not significantly weakened, whereas the level of apoptosis was significantly increased and the immunosuppressive function was not significantly enhanced. We wondered whether when the apoptosis rate exceeded a certain threshold or if the lack of some inflammatory factors that stimulate MSCs in HC serum prevented pretreatment with healthy donor serum from enhancing the immunosuppressive function of MSCs.

Human serum accounts for approximately 50% of the total blood volume. Compared with ACLF serum pretreatment of 10% concentration, the 50% concentration may be more similar to the environment in the human body. Thus, MSCs enter the pathological ACLF microenvironment and may be negatively affected and unable to exert a beneficial therapeutic effect. However, combining treatments of plasma exchange or double plasma molecular adsorption system to reduce harmful substances in serum may promote MSCs to exert a stronger anti-inflammatory effect. According to our mRNA sequencing results of the MSCs pretreated with ACLF serum at 10% concentration, the PI3K-Akt signaling pathway might play a major role in the serum-mediated MSCs exerting stronger immunosuppressive function. As we know, the PI3K-Akt pathway is indispensable in immunologic defense mechanisms and acts in part as a compensatory mechanism in response to the activation of intracellular proinflammatory signaling pathways.[23–25] We suspected that ACLF serum pretreatment may down-regulate the PI3K-Akt pathway, thereby stimulating cascade reactions and driving MSCs to exert stronger immunoregulatory effects.

Our study also has many shortcomings. First, our experimental design cannot fully simulate the internal environment of ACLF, since it is an extremely complex disease condition. Indeed, no specific factor can play a completely different role in the immunomodulation of MSCs at different concentrations. Second, we did not verify the mRNA sequencing results by measuring transcription or protein levels. Considering that simple verification can only show that the corresponding pathway was affected, it cannot indicate whether the affected pathway is the main reason for the alteration in MSC functional characteristics. Therefore, we plan to explore the importance of the KDA genes in the involved pathways and their influence on MSCs in subsequent experiments.

Presently, most clinical studies on MSC treatment of diseases generally use intravenous infusion due to safety considerations. However, this also means that compared to the short and direct infusion route of *in situ* infusion, MSCs are likely to be affected by the environment during the lengthy internal circulation. To determine which substances in the serum would adversely affect the MSCs and filter out these harmful substances by plasma exchange or double plasma molecular adsorption system may further improve the efficiency of MSCs transplantation as well as suggest the reasons for the poor response of some patients to treatment. The above is also the content of our subsequent research.

Acknowledgments

The authors would like to thank Professor Zhang Qi and her team for helpful discussions on topics related to this work.

Funding

This study was supported by grants from the Natural Science Foundation of China (No. 82070611 and 81873572), National Major Science and Technology Project for the Prevention and Treatment of AIDS and Viral Hepatitis (No. 2018ZX10302204-002 and 2018ZX10302205-002), Guangzhou Science and Technology Plan Projects (No. 201904010442), Sun Yat-sen University Clinical Research 5010 Program (No. 2018009), Research Project on Degree and Postgraduate Education Reform in Guangdong Province (No. 2018JGXM04), and Young Teacher Training Program of Sun Yat-sen University (No. 16ykpy40).

Conflict of interest

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

Author contributions

Design of the study (YZ, CX, LP), acquisition of data (YZ, SZ, XZ, WX, XL, JL), data analysis (YZ, SZ), manuscript preparation (YZ, SZ, CX, LP). All authors reviewed the manuscript and gave final approval for the work.

Data sharing statement

No additional data are available.

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Journal of Clinical and Translational Hepatology **2021** vol. 9(4) | 514–520
DOI: 10.14218/JCTH.2021.00028

Original Article

Novel Prognostic Models for Predicting the 180-day Outcome for Patients with Hepatitis-B Virus-related Acute-on-chronic Liver Failure

Ran Xue[1,2#], Jun Yang[3#], Jing Wu[1], Zhongying Wang[4] and Qinghua Meng[1*]

[1]Department of Medical Oncology, Beijing You'an Hospital, Capital Medical University, Beijing, China; [2]Key Laboratory of Carcinogenesis & Translational Research (Ministry of Education/Beijing), Early Drug Development Center, Peking University Cancer Hospital & Institute, Beijing, China; [3]Department of Integrated Traditional and Western Liver Disease, Beijing You'an Hospital, Capital Medical University, Beijing, China; [4]Department of Infection Center, Beijing You'an Hospital, Capital Medical University, Beijing, China

Received: 16 January 2021 | **Revised:** 22 March 2021 | **Accepted:** 18 April 2021 | **Published:** 17 May 2021

Abstract

Background and Aims: It remains difficult to forecast the 180-day prognosis of patients with hepatitis B virus-acute-on-chronic liver failure (HBV-ACLF) using existing prognostic models. The present study aimed to derive novel-innovative models to enhance the predictive effectiveness of the 180-day mortality in HBV-ACLF. ***Methods:*** The present cohort study examined 171 HBV-ACLF patients (non-survivors, n=62; survivors, n=109). The 27 retrospectively collected parameters included the basic demographic characteristics, clinical comorbidities, and laboratory values. Backward stepwise logistic regression (LR) and the classification and regression tree (CART) analysis were used to derive two predictive models. Meanwhile, a nomogram was created based on the LR analysis. The accuracy of the LR and CART model was detected through the area under the receiver operating characteristic curve (AUROC), compared with model of end-stage liver disease (MELD) scores. ***Results:*** Among 171 HBV-ACLF patients, the mean age was 45.17 years-old, and 11.7% of the patients were female. The LR model was constructed with six independent factors, which included age, total bilirubin, prothrombin activity, lymphocytes, monocytes and hepatic encephalopathy. The following seven variables were the prognostic factors for HBV-ACLF in the CART model: age, total bilirubin, prothrombin time, lymphocytes, neutrophils, monocytes, and blood urea nitrogen. The AUROC for the CART model (0.878) was similar to that for the LR model (0.878, p=0.898), and this exceeded that for the MELD scores (0.728, p<0.0001). ***Conclusions:*** The LR and CART model are both superior to the MELD scores in predicting the 180-day mortality of patients with HBV-ACLF. Both the LR and CART model can be used as medical decision-making tools by clinicians.

Citation of this article: Xue R, Yang J, Wu J, Wang Z, Meng Q. Novel prognostic models for predicting the 180-day outcome for patients with hepatitis-B virus-related acute-on-chronic liver failure. J Clin Transl Hepatol 2021;9(4):514–520. doi: 10.14218/JCTH.2021.00028.

Introduction

Acute-on-chronic liver failure (ACLF) is a common type of clinical syndrome with rapid deterioration of liver function, organ failure(s) and high short-term mortality.[1] Hepatitis B virus (HBV) poses a serious threat to human health, due to its devastating effect on liver function.[2] In the Asia-Pacific region, HBV is the leading cause of chronic liver disease.[3]

At present, liver transplantation (LT) is still the most beneficial and feasible therapy for patients with ACLF.[4–5] However, 20–30% of patients remain at risk to be delisted from the transplant list, and wait-list mortality is high due to patients being too sick for LT and succumbing to the condition. Hence, it is a significant unmet need to accurately distinguish ACLF patients who are suitable for LT therapy, and seize the best chance for LT.[6] Therefore, an accurate prognostic scoring system is needed to guide and optimize the therapeutic strategy for patients with ACLF.[7] At present, the model of end-stage liver disease (MELD) score is the most commonly used tool for designating patients to the wait-list for LT.[8] However, among the candidates listed for LT, the MELD score may not capture the ACLF severity and adequately evaluate the outcome in the ACLF. Meanwhile, due to differences in patient background queues, the MELD score may not be reasonably applied for HBV-ACLF. Furthermore, although some prognostic scoring systems have been developed to predict the HBV-ACLF short-term (such as 30-day and 90-day) mortality, including the 30-day HBV-ACLFD model previously developed by the inves-

Keywords: Classification and regression tree; Acute-on-chronic hepatitis B liver failure; MELD scores; Logistic regression model.

Abbreviations: ACLF, acute-on-chronic liver failure; ALT, alanine transaminase; AST, aspartate transaminase; AUROC, area under the receiver operating characteristic curve; BUN, urea nitrogen; CART, classification and regression tree; CI, confidence interval; HBsAg, hepatitis B virus surface antigen; HBV, hepatitis B virus; HE, hepatic encephalopathy; HGB, hemoglobin; INR, international normalized ratio; L, lymphocyte; LT, liver transplantation; LR, logistic regression; M, monocyte; MELD, model for end-stage liver disease; N, neutrophil; OR, odds ratio; PLT, platelet; PTA, prothrombin activity; RBC, red blood cell; SD, standard deviation; TBIL, total bilirubin; WBC, white blood cell.

[#]Both authors contributed equally to this work.

[*]**Correspondence to:** Qinghua Meng, Department of Medical Oncology, Beijing You'an Hospital, Capital Medical University. No. 8 Xi Tou Tiao, You An Men Wai Street, Fengtai District, Beijing 100069, China. ORCID: https://orcid.org/0000-0001-9967-6403. Tel: +86-10-8399-7160, Fax: +86-10-6329-3371, E-mail: meng_qh0805@ccmu.edu.cn

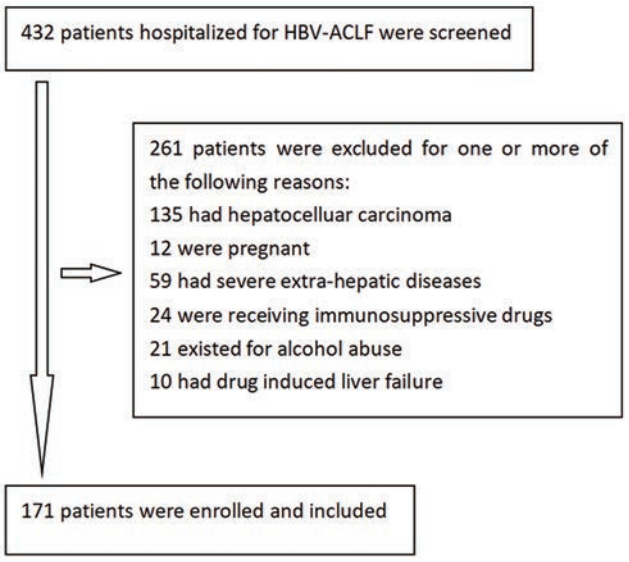

Fig. 1. Flow diagram of inclusion of study participants in the study.

tigators[9], the efficacy is still scanty to predict the mid-term (such as 180-day) mortality of patients with HBV-ACLF.

The present study aimed to derive novel predictive models to evaluate the 180-day mortality of patients with HBV-ACLF based on the backward stepwise logistic regression (LR) and classification and regression tree (CART) analysis, and to evaluate whether these new models are superior to the MELD scores, providing guidance for clinical treatment decision making.

Methods

Study design

A total of 445 patients, who were diagnosed with HBV-ACLF at Beijing You'an Hospital, Capital Medical University, from June 2014 and December 2018, were selected for the present study. Among these patients, merely 171 entered the final selection. The selection process for HBV-ACLF patients inclusion in the present study is presented in Figure 1.

The enrolment criteria for these patients corresponded to the Asian Pacific Association for ACLF.[10] The inclusion criteria were as follows: (a) patients who were at least 16 years-old; (b) patients who were HBV surface antigen (HBsAg)-positive for at least 6 months; (c) patients with a total bilirubin (TBIL) of >171 μmol/L and a sudden exacerbation of liver disease; (d) patients with an international normalized ratio (INR) of >1.5; (e) patients who had ascites within 4 weeks and/or had an onset of hepatic encephalopathy (HE). The exclusion criteria were as follows: (a) pregnant or lactating patients; (b) patients co-infected with human immunodeficiency virus; (c) patients with severe diseases, such as heart dysfunction, previous renal failure, cancer, etc.; (d) patients with infection upon admission to the hospital; (e) patients compounded by other causes of liver damage, such as hepatitis A, C, or E, autoimmune hepatitis, alcohol consumption, or hereditary liver diseases.

The study protocol was approved by the Ethics Committee on Clinical Trials of Beijing You'an Hospital, Capital Medical University. All methods and procedures related to the present study were morally accorded with the laws of the Declaration of Helsinki.

A total of 27 parameters were retrospectively collected as potential risk factors. The parameters included sex, age, serum creatinine level, blood urea nitrogen (BUN) level, aspartate transaminase (AST) level, aspartate alanine transaminase (ALT) level, albumin level, TBIL level [normal reference range: 5–21 μmol/L], serum sodium level, serum potassium level, ammonia level, prothrombin activity (PTA), INR, white blood cell (WBC), hemoglobin (HGB), red blood cell (RBC) count, platelet (PLT) count, lymphocytes (L), neutrophils (N), monocytes (M), time begin, HBV DNA, HBsAg, and complications such as hepatorenal syndrome, ascites, infection, pleural effusion, cirrhosis, and HE. The result (survival or death) for each subject with HBV-ACLF was recorded. The MELD equation was applied to calculate the score for severity as: 9.57 × ln (creatinine, mg/dL) + 3.78 × ln (bilirubin, mg/dL) + 11.20 × ln (INR) + 6.43. The minimal values were forced to 1.0 for calculation purposes.[11]

LR analysis and nomogram generation

A multivariable LR analysis was performed for the prediction of HBV-ACLF. The candidate predictors were as follows: sex, age, creatinine, BUN, AST, ALT, albumin, TBIL, serum sodium level, serum potassium level, ammonia level, PTA, INR, WBC, RBC, HGB, PLT, L, N, M, time begin, HBV DNA, HBsAg, hepatorenal syndrome, ascites, infection, pleural effusion, cirrhosis, and HE.

In order to identify the significant predictors, 1,000 random samples were generated from the 171 patients through bootstrap resampling with replacement, and backward stepwise LR was conducted for each patient. Then, the predictors selected by the backward stepwise regression were included in the final model. Next, a 10-fold cross-validation was used to calculate the C-index and generate the calibrated statistics. Finally, the parameters for the final model were generated. Based on the results of the logistic regression, the 95% confidence interval (CI) and odds ratio (OR) were calculated. The performance of the model was assessed by sensitivity, and by evaluating the discriminative capacity via the area under the receiver operating characteristic curve (AUROC). A nomogram was created based on the LR analysis, and the nomogram was constructed using the rms package.

Analysis of the CART

A CART analysis was performed for the 171 patients, and it was verified whether this method could calculate more useful clinical results, when compared to the LR model. The candidate predictors were the same as those used in the LR model. The CART analysis divided the data (parent node) into two subsets (child nodes) through the function of the predictor variables. These two subsets were the new parent nodes, which were further split into two child nodes. This process was continued until all patients were classified. After finding the best split for each variable, the CART algorithm used the best overall split to divide the data, and assigned a prediction category for each subgroup. The CART recursively proceeded in this manner, until a predetermined stopping criterion was reached. The algorithm was allowed to go on indefinitely, enabling the model to identify the entirely or almost entirely homogeneous splits.

In the present study, the CART analysis was used to predict the 180-day mortality of patients with HBV-ACLF. The mortality rate, 95% CI and OR were determined. The 10-fold cross-validation was used to trim and optimize the tree, and minimize the relative misclassification. The C-index and the receiver operating characteristic curve were

Table 1. Baseline characteristics of the patients, stratified by mortality

Variable	Overall, *n*=171	Non-survivors, *n*=62	Survivors, *n*=109	*p*
Age in years	45.17 (12.49)	48.74 (12.54)	43.14 (12.05)	<0.0001
Men, *n*(%)	151(88.3)	51(82.3)	100(91.7)	0.064
Ascites, *n*(%)	103(60.2)	46(74.2)	57(52.3)	0.005
HE, *n*(%)	20(11.7)	13(20.9)	7(6.4)	0.004
Infection, *n*(%)	92(53.8)	38(61.3)	54(49.5)	0.138
K/Na, *n*(%)	17(9.9)	5(8.1)	12(11)	0.536
HBeAg, *n*(%)	91(53.2)	32(51.6)	59(54.1)	0.751
HRS, *n*(%)	5(2.9)	4(6.4)	1(0.9)	0.111
Pleural effusion, *n*(%)	7(4.1)	5(8.1)	2(1.8)	0.115
Cirrhosis, *n*(%)	137(80.1)	49(79)	88(80.7)	0.789
lgHBV DNA	4.76(1.93)	4.61(2.06)	4.84(1.87)	0.491
HBsAg	3,948.19 (5,194.35)	4,541.64 (7,356.64)	3,610.64 (3,403.74)	0.944
ALT	464.02 (577.17)	325.65 (305.42)	542.73 (674.12)	0.047
AST	377.83 (413.63)	342.99 (286.41)	397.66 (471.05)	0.393
TBIL	353.83 (138.49)	408.7 (146.06)	322.62 (124.20)	<0.0001
BUN	4.86 (2.49)	5.56 (2.78)	4.46 (2.22)	0.002
Cr	75.16 (36.97)	81.50 (42.99)	71.55 (32.72)	0.174
WBC	7.34 (3.52)	7.89 (4.28)	7.022 (2.99)	0.422
L	20.59 (8.58)	16.77 (6.74)	22.76 (8.78)	<0.0001
M	9.35 (3.78)	10.20 (4.38)	8.86 (3.32)	0.025
N	93.6 (65.55)	69.2 (18.06)	63.47 (7.38)	0.002
PTA	35.97 (9.3)	31.50 (8.58)	38.51 (9.62)	<0.0001
INR	2.11 (0.56)	2.36 (0.65)	1.97 (0.45)	<0.0001
RBC	3.88 (0.85)	3.82 (0.90)	3.91 (.82)	0.336
HGB	124.3 (21.0)	122.33 (21.95)	125.42 (20.46)	0.402
PLT	104.77 (51.73)	96.12 (52.58)	109.68 (50.83)	0.036
Time begin	22.72 (19.01)	23.44 (16.22)	22.31 (0.49)	0.081
ALB	31.06 (4.13)	30.85 (4.31)	31.18 (4.04)	0.615

ALB,albumin;ALT, alanine transaminase; AST, aspartate transaminase; BUN, urea nitrogen; HBsAg, hepatitis B virus surface antigen; HBV, hepatitis B virus; HE, hepatic encephalopathy; HGB, hemoglobin; HRS, hepatorenal syndrome;INR, international normalized ratio; L, lymphocyte; M, monocyte; N, neutrophil; PLT, platelet; PTA, prothrombin activity; RBC, red blood cell; TBIL, total bilirubin; WBC, white blood cell.

generated to evaluate the performance of the final decision tree.

Statistical analysis

Continuous variables were presented as mean ± standard deviation (SD), and compared using the Mann-Whitney test, and unpaired or two-tailed *t*-test. Categorical variables were compared using the chi-square test. The predictive accuracy of the LR model was calculated with the concordance statistic, which ranged from 0.5 (no discrimination) to 1.0 (perfect discrimination). The calibration was assessed using the calibration plot, which was implied by a 45° diagonal line with the 1,000 bootstrap samples, in order to decrease the overfit bias.[12] The ROC curve analysis was performed using the MedCalc 17.0 software (Mariakerke, Belgium). The nomogram and CART analysis were performed using the R statistical software, version 4.0.2 (http://www.Rproject.org). The additional statistical analysis was analyzed using the SPSS 25 software (IBM Corp., Armonk, NY, USA). The reported statistical significance levels were all two-sided, and the statistical significance was set at 0.05.

Results

Baseline characteristics

A total of 171 patients who were diagnosed with HBV-ACLF were involved in the present study. The comparison of the clinical characteristics of HBV-ACLF patients stratified by mortality are presented in Table 1. There were no significant differences in sex distribution, potassium/sodium, cirrhosis, HBV DNA, and time begin between the non-survivor (death) group and survivor group ($p>0.05$). However, the differences in age, PTA, INR, TBIL and L were statistically

Table 2. Multivariable predictors of mortality of HBV-ACLF

Variable	β-coefficient	OR(95% CI)	*p*
HE	1.635	5.13 (1.282,20.512)	0.021
TBIL	0.006	1.006 (1.002,1.009)	0.001
PTA	−0.115	0.892 (0.845,0.941)	0.0001
L	−0.130	0.878 (0.825,0.935)	0.0001
M	0.215	1.240 (1.087,1.414)	0.001
Age	0.049	1.050 (1.014,1.087)	0.006

HE, hepatic encephalopathy; L, lymphocyte; M, monocyte; PTA, prothrombin activity; TBIL, total bilirubin.

significant between these two groups ($p<0.0001$).

LR analysis and nomogram

In order to deeply identify the independent predictors of mortality in the present study, multivariate backward stepwise LR analysis was performed. It was found that age, TBIL, PTA, L, M and HE were significantly associated with the 180-day mortality (Table 2). The C-index for the LR model with these predictors was 0.878. In the 1,000 bootstrap data, the calibration plot for the prediction indicated a good fit (Fig. 2), and the Brier score was 0.1898. Based on the results of the LR analysis, a nomogram was drawn to predict the patient's mortality rate (Fig. 3). A higher score calculated based on the sum of assigned points of each predictor in the nomogram corresponded to a higher probability of death.

CART analysis

In the CART model, TBIL was identified as the variable for the initial split, with an optimal value of 381.10 μmol/L, and L was selected as the variable for the second split, with a dis-

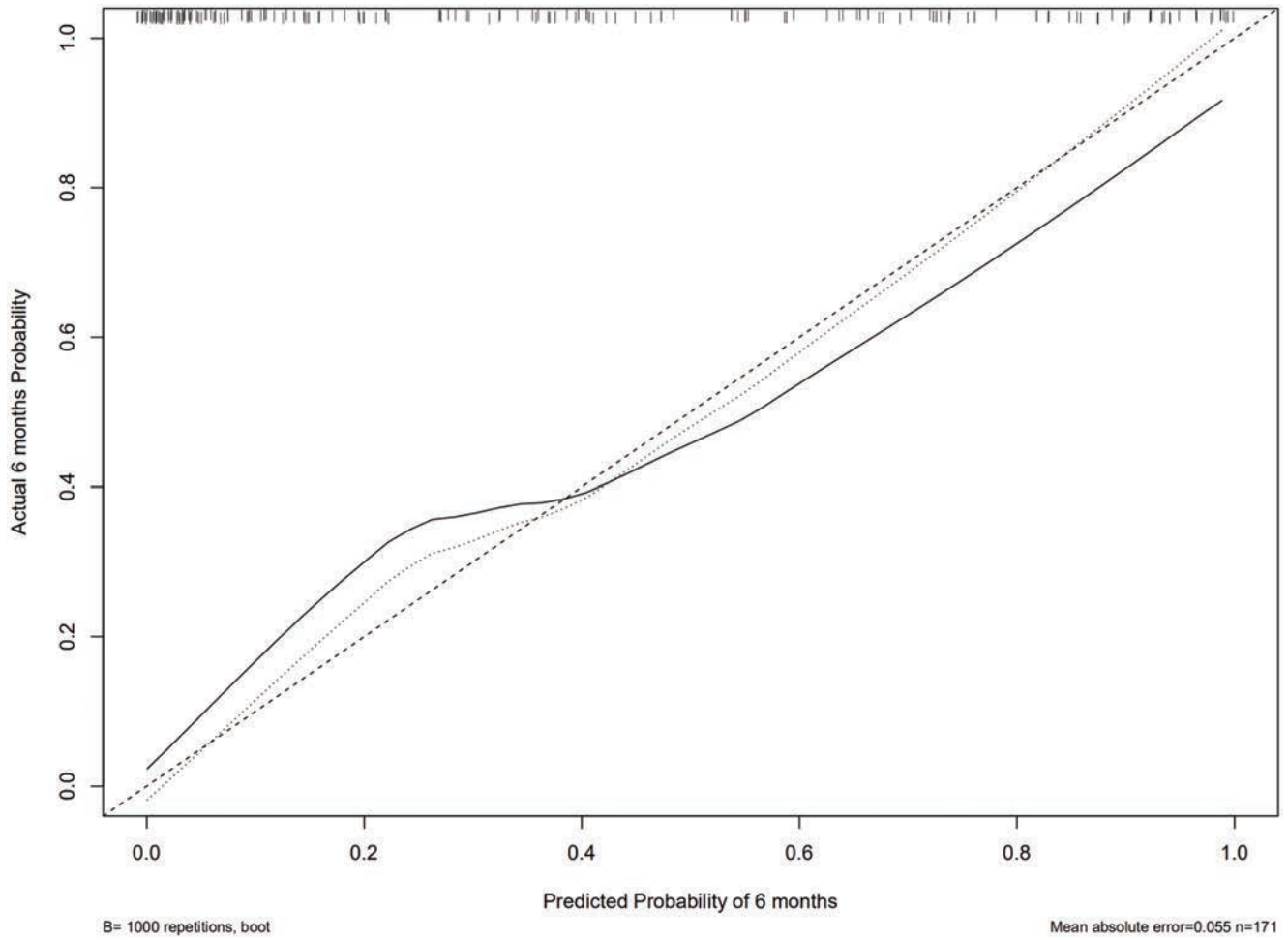

Fig. 2. Calibration plots for predicted using bootstraps.

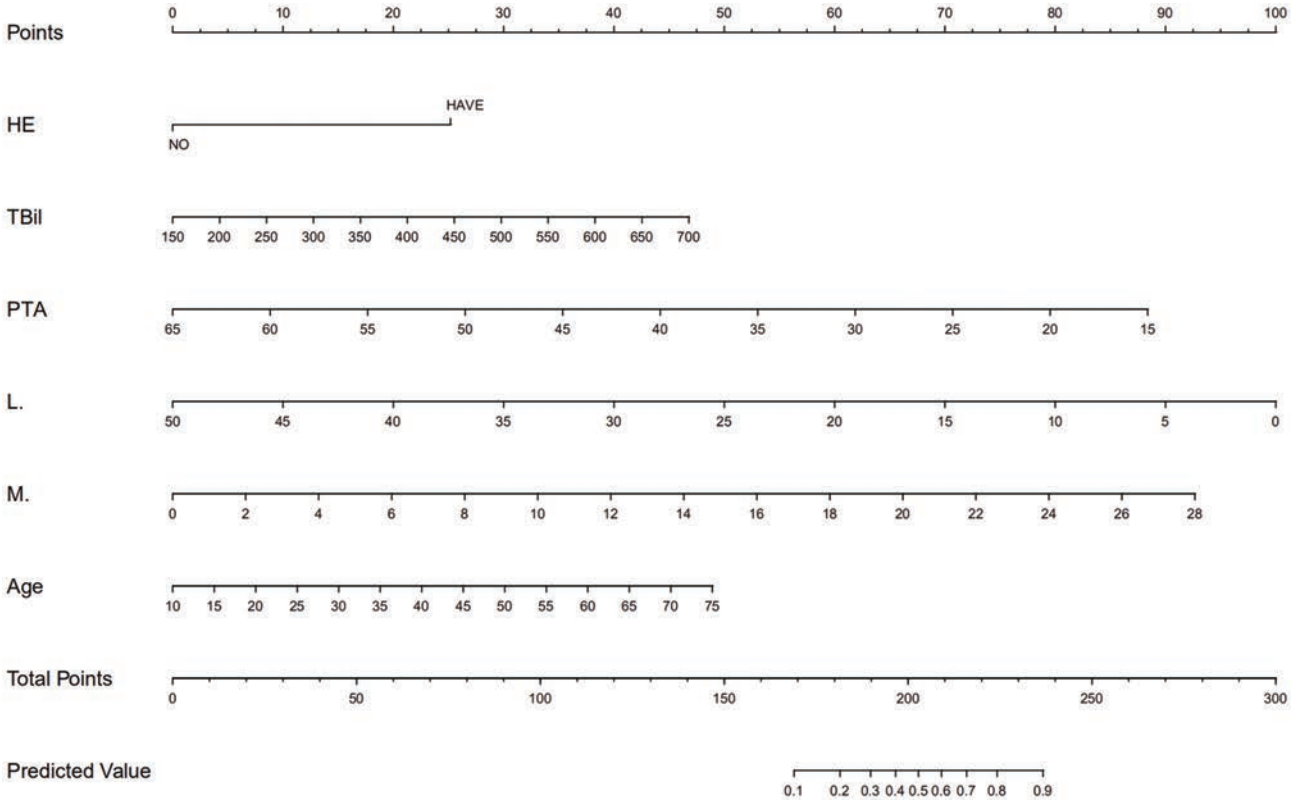

Fig. 3. The nomogram was developed by incorporating the following six parameters: age (years), total bilirubin (μmol/L), prothrombin activity, lymphocyte (%), monocyte (%), and HE. For example, a Hepatitis-B virus-related acute-on-chronic liver failure (HBV-ACLF) patient was 65 years-old, with total bilirubin (TBIL) of 400 μmol/L, L% of 40%, M% of 12%, prothrombin activity (PTA) of 35, and having hepatic encephalopathy (HE). The corresponding total points were: 40+20+20+40+55+25=200. The predicted value of death risk in the nomogram was about 50%.

crimination level of 13.78%. When L was >13.78%, the next best predictor for HBV-ACLF was PTA, with an optimal cut-off value of 33.2. For the node of patients who have a TBIL level of >381.1 μmol/L, an L of >13.78% and a PTA level higher than 33.2, M was selected as the additional significant variable, and this was dichotomized at a level of 10.96%.

Finally, a total of nine subgroups of patients were generated through the seven predictive variables chosen via the CART analysis: subgroup 1 (TBIL ≥381.10 μmol/L and L <13.78%), subgroup 2 (TBIL <381.10 μmol/L and BUN ≥7.915 mmol/L), subgroup 3 (TBIL <381.10 μmol/L, BUN <7.915 mmol/L, and age <56.00 years-old), subgroup 4 (TBIL ≥381.10 μmol/L, L ≥13.78%, PTA <33.20, and age <43.50 years-old), subgroup 5 (TBIL ≥381.10 μmol/L, L ≥13.78%, PTA <33.20, and age ≥ 43.50 years-old), subgroup 6 (TBIL ≥381.10 μmol/L, L ≥13.78%, PTA ≥33.20, and M ≥10.96%), subgroup 7 (TBIL ≥381.10 μmol/L, L ≥13.78%, PTA ≥33.20, and M <10.96%), subgroup 8 (TBIL <381.10 μmol/L, BUN <7.915 mmol/L, age ≥56.00 years-old, and N ≥65.10%), and subgroup 9 (TBIL <381.10 μmol/L, BUN <7.915 mmol/L, age ≥56.00 years-old, and N <65.1%) (Fig. 3). Each patient was sorted to subgroups based on flow chart of the derived CART. The mortality rates for each subgroup are presented in Figure 4. The C-index for the CART model with these predictors was 0.878.

Comparison among the LR, CART and MELD score

As shown in the Figure 5, the predictive power for the 180-day mortality for HBV-ACLF among the LR, CART and MELD score was determined. The CART analysis had an AUROC of 0.878 (95% CI: 0.810–0.923). The performance of the LR analysis was high, with an AUROC of 0.878 (95% CI: 0.820–0.923). However, there was no significant difference between the CART and LR model (p=0.9659). In Table 3, the MELD score had an AUROC of 0.728 (95% CI: 0.655–0.793), which was significantly lower than that for the LR and CART model (p<0.0001).

Discussion

HBV-ACLF is defined as a hazardous syndrome with multiorgan failure.[3–5] Worldwide, it has been demonstrated that LT brings survival profit for selected patients with ACLF. Due to the rapid progression and unpredictable results, accurate prognostic scoring systems are the precondition for optimizing the clinical therapeutic strategy for HBV-ACLF patients. Although the MELD score has been verified to promote the allocation of donor livers, this is still not an ideal indicator for HBV-ACLF patients.[13] Although several prognostic models have been developed to predict the HBV-ACLF short-term (such as 30-day and 90-day) mortality,[14–17] including the 30-day HBV-ACLFD model previously developed by the investigators,[9] there is still a lack of a prognostic model to predict the mid-term (such as 180-day) mortality of patients with HBV-ACLF. In the present study, the LR and CART models were developed to predict the 180-day mortality of patients with HBV-ACLF. Both the LR and CART models could be used as medical decision-managing tools by clinicians.

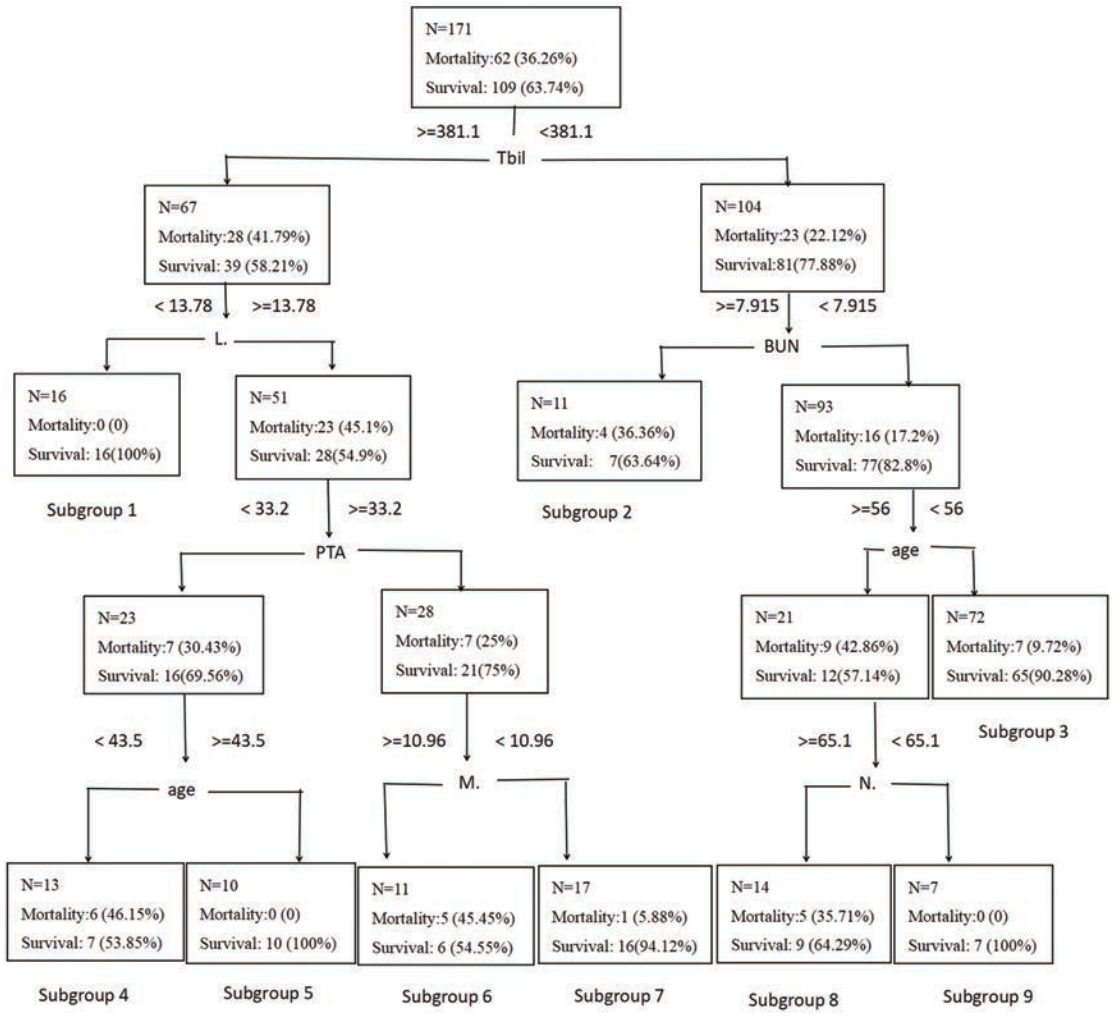

Fig. 4. Predictors from classification and regression tree (CART). Terminal subgroups of patients discriminated by the analysis were numbered from 1 to 9.

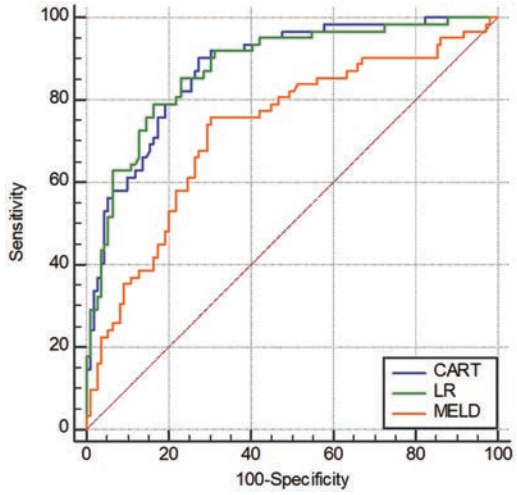

Fig. 5. ROC analysis of the predictive accuracy of the classification and regression tree (CART) model, logistic regression (LR) and model for end-stage liver disease (MELD) score to predict 180-day mortality of hepatitis-B virus-related acute-on-chronic liver failure (HBV-ACLF).

In the present study a new LR model was established, which included age, TBIL, L, M, HE and PTA as prognostic factors for the 180-day mortality. The AUROC for this prognostic model was significantly higher than that for the MELD score. Except for LR, a novel CART model was also developed to predict the 180-day outcome of HBV-ACLF patients. In the present study, the CART model included age, TBIL, PTA, L, M, N and BUN. These seven potential variables were the important predictors for the survival of HBV-ACLF patients. Both the LR and CART models appeared to perform better than the MELD score. Meanwhile, the investigators also made the LR models easier to use in clinic by drawing a nomogram.

Compared to traditional models, the CART model has many advantages. First, the CART can conduct highly biased clinical data, and reveal the complicated relationships among different variables. This generates a clearly visible decision tree that contains many binary splits, which are more accessible and convenient for clinical applications. Second, in the present study, the CART model had better predictive accuracy, when compared to the MELD score. At present, some organ function-based scoring systems, including the chronic liver failure-sequential organ failure assessment score, the CLIF Association ACLF score,[18] the chronic liver failure-sequential organ failure assessment score (, the Acute Physiology and Chronic Health Assessment II score,[19] and the sequential or-

Table 3. The predictive value of mortality of the CART score and other models

Models	AUROC	95% CI	*p*	Youden's index	Sensitivity, %	Specificity, %
CART	0.878	0.819–0.923	0.0001	0.6280	90.32	72.48
LR	0.878	0.820–0.923	0.0001	0.6255	85.48	77.06
MELD	0.728	0.655–0.793		0.4553	75.81	69.72

AUROC, area under the receiver operating characteristic curve; CART, classification and regression tree; CI, confidence interval; LR, logistic regression; MELD, model for end-stage liver disease.

gan failure assessment score, have also been used to make predictions for the mortality of ACLF. Compared to these scoring systems, the CART model is much easier to apply. Third, the CART model is more convenient for LT patients, in terms of estimating the risk stratification. Shi et al.[20] used a CART model to validate the 3-month mortality of patients with HBV-ACLF. This revealed the profit of the CART model to predict the HBV-ACLF risk stratification.

However, there were some limitations in the present study. The present study was a single-center retrospective study that mostly involved male patients. However, it was not easy to collect more data of the mid-term outcome of HBV-ACLF patients. Hence, further validation is needed through a larger study.

Conclusions

The LR and CART model was derived to predict the 180-day clinical outcomes in HBV-ACLF patients. These models can be helpful for doctors who need to make vital clinical decisions for patients with HBV-ACLF. However, larger multicenter studies and further evaluations are needed.

Funding

The study was supported by the National Natural Science Foundation of China (No. 81470888) and (No. 82002461), the Medjaden Academy and Research Foundation for Young Scientists (No. MJR20211110), and the Fund for Fostering Young Scholars of Peking University Health Science Center (No. BMU2021PY010).

Conflict of interest

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

Author contributions

Conception and design of the study (QM), collection of the data (JW, ZW), analysis of the data (RX, JY), and writing of the paper (RX, JY).

Data sharing statement

No additional data are available.

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Journal of Clinical and Translational Hepatology **2021** vol. 9(4) | 521–527
DOI: 10.14218/JCTH.2021.00049

Original Article

Re-evaluating Transarterial Chemoembolization Failure/Refractoriness: A Survey by Chinese College of Interventionalists

Bin-Yan Zhong[1#], Wan-Sheng Wang[1#], Shen Zhang[1], Hai-Dong Zhu[2], Lei Zhang[1], Jian Shen[1], Xiao-Li Zhu[1], Gao-Jun Teng[2*] and Cai-Fang Ni[1*]

[1]*Department of Interventional Radiology, the First Affiliated Hospital of Soochow University, Suzhou, Jiangsu, China;* [2]*Center of Interventional Radiology & Vascular Surgery, Department of Radiology, Zhongda Hospital, Medical School, Southeast University, Nanjing, Jiangsu, China*

Received: 3 February 2021 | **Revised:** 2 March 2021 | **Accepted:** 8 March 2021 | **Published:** 8 April 2021

Abstract

Background and Aims: The recognition of transarterial chemoembolization (TACE) failure/refractoriness among Chinese clinicians remains unclear. Using an online survey conducted by the Chinese College of Interventionalists (CCI), the aim of this study was to explore the recognition of TACE failure/refractoriness and review TACE application for hepatocellular carcinoma (HCC) treatment in clinical practice. ***Methods:*** From 27 August 2020 to 30 August 2020 during the CCI 2020 annual meeting, a survey with 34 questions was sent by email to 264 CCI clinicians in China with more than 10 years of experience using TACE for HCC treatment. ***Results:*** A total of 257 clinicians participated and responded to the survey. Most participants agreed that the concept of "TACE failure/refractoriness" has scientific and clinical significance (n=191, 74.3%). Nearly half of these participants chose TACE-based combination treatment as subsequent therapy after so-called TACE failure/refractoriness (n=88, 46.1%). None of the existing TACE failure/refractoriness definitions were widely accepted by the participants; thus, it is necessary to re-define this concept for the treatment of HCC in China (n=235, 91.4%). Most participants agreed that continuing TACE should be performed for patients with preserved liver function, presenting portal vein tumor thrombosis (n=242, 94.2%) or extrahepatic spread (n=253, 98.4%), after the previous TACE treatment to control intrahepatic lesion(s). ***Conclusions:*** There is an obvious difference in the recognition of TACE failure/refractoriness among Chinese clinicians based on existing definitions. Further work should be carried out to re-define TACE failure/refractoriness.

Keywords: Hepatocellular carcinoma; TACE; Failure; Refractoriness; Survey.
Abbreviations: CCI, Chinese College of Interventionalists; CNLC, China liver cancer; HCC, hepatocellular carcinoma; JSH, Japan Society of Hepatology; LCSGJ, Liver Cancer Study Group of Japan; mRECIST, modified Response Evaluation Criteria in Solid Tumors; PVTT, portal vein tumor thrombosis; TACE, transarterial chemoembolization.

[#]Both authors contributed equally as joint first authors.

[*]**Correspondence to:** Cai-Fang Ni, Department of Interventional Radiology, First Affiliated Hospital of Soochow University, 188 Shizi St, Suzhou, Jiangsu 215006, China. ORCID: https://orcid.org/0000-0002-5953-781X. Tel/Fax: +86-512-6797-2173, E-mail: cjr.nicaifang@vip.163.com; Gao-Jun Teng, Department of Radiology, Zhongda Hospital, Medical School, Southeast University, 87 DingjiaqiaoRoad, Nanjing Jiangsu 210009, China. ORCID: https://orcid.org/0000-0003-1145-0798. Tel/Fax: +86-25-8379-2103, E-mail: gjteng@vip.sina.com

Citation of this article: Zhong BY, Wang WS, Zhang S, Zhu HD, Zhang L, Shen J, *et al*. Re-evaluating transarterial chemoembolization failure/refractoriness: a survey by Chinese college of interventionalists. J Clin Transl Hepatol 2021;9(4):521–527. doi: 10.14218/JCTH.2021.00049.

Introduction

Transarterial chemoembolization (TACE) plays a key role in the management of unresectable hepatocellular carcinoma (HCC).[1–4] According to the global BRIDGE study, TACE is the most widely applied approach in both intermediate and advanced stages of HCC, as recommended by several guidelines.[5] Considering the epidemiological differences between countries, HCC patients in China treated with TACE are often reported to have a higher tumor burden compared to those in Western countries.[6] The purpose of TACE for HCC is to control or shrink the lesion(s) locally. Due to the high heterogeneity of HCC, which varies according to the number, size, location, and growth pattern of tumors, it is difficult to achieve a satisfactory tumor response from a single session of TACE.[7,8] However, repeated TACE could damage liver function and increase treatment-related side effects.[9] Therefore, a delicate balance between the necessity and benefits of repeated TACE treatment should be considered, where benefits are also balanced against treatment side effects.

To assess such balance in clinical practice and clinical trials, several organizations and panels, including the Japan Society of Hepatology (JSH) (Kyoto, Japan), the International Association for the Study of the Liver (Shanghai, China), and a European expert panel, introduced various definitions of TACE failure/refractoriness.[10–12] Among them, the 2014 definition by the JSH-Liver Cancer Study Group of Japan (LCSGJ) is most widely applied in clinical practice and trials. According to JSH-LGSGJ 2014 criteria, the incidence of TACE failure/refractoriness ranges from 37.0% to 49.3%.[13,14]

Nevertheless, by emphasizing retrospective studies and consensus rather than high-level evidence, these definitions and subsequent treatment recommendations for TACE failure/refractoriness remain somewhat ambiguous and controversial. In addition, the epidemiological difference in research between Japan/Western countries and China reveals discrepancies in the extent of disease burden, whereby a

relatively higher burden of HCC is reported in China. Under these circumstances, three questions remain to be answered before the definitions and subsequent treatment recommendations can be applied in China. (1) Is TACE failure/refractoriness widely accepted and applied in real-world clinical practice in China? (2) Is the definition-recommended subsequent treatment after TACE failure/refractoriness accepted and applied in real-world clinical practice in China? (3) What are the ideal definition and subsequent treatment recommendations of TACE failure/refractoriness in China?

The Chinese College of Interventionalists (CCI) conducted an online survey to identify the trends in real-world clinical practice of TACE, recognition of TACE failure/refractoriness, and subsequent treatment strategies in China.

Methods

Study population and questionnaire

The present study did not require an approval from an institutional review board, because it was solely based on reported statistics and did not involve humans or animals as subjects. The TACE procedure mentioned in this survey was conventional TACE. During the CCI 2020 annual meeting from 27 August 2020 to 30 August 2020, the questionnaires were sent by email to 264 clinicians with more than 10 years of experience in using TACE for HCC treatment in China. On 28 August 2020 and 30 August 2020, follow-up telephone calls were made to the nonresponders and to the responders who did not fill out the questionnaires completely, respectively.

The questionnaire was designed and formulated with four major parts: (1) the overall understanding of TACE in real-world clinical practice; (2) factors influencing the treatment response of TACE; (3) understanding and expectations of TACE failure/refractoriness and subsequent treatment patterns; and (4) perspectives on TACE.

Completed questionnaires returned before 31 August 2020 were collected for analysis. Questionnaires returned after 30 August 2020 and incomplete questionnaires were excluded.

Statistical analysis

The data, including number and proportion of every question, were collected and calculated with the SPSS version 22.0 software for Windows (IBM Corporation, Somers, New York).

Results

Participants

Three participants did not respond, and four participants sent back incomplete questionnaires and did not revise them even after our telephone calls. A total of 257 clinicians from 184 hospitals participated and responded correctly to the survey, with a response rate of 97.3%. The participating clinicians included 196 interventional radiologists, 37 oncologists, 16 gastroenterologists, and 8 surgeons. More than half of the included clinicians (n=156, 61%) were chief physicians/professors, and the remaining 101 (39%) were associate chief physicians/associate professors. All of the participating physicians routinely discuss HCC treatment in the local tumor board of their hospitals. The locations of the participating clinicians' hospitals covered all 31 provinces in China. A total of 34 questions were included in the survey (supplementary Table 1).

Overall understanding of TACE in real-world clinical practice

In this part, the survey included the eight single-choice questions (Figs. 1 and 2). Most clinicians (n=229, 89.1%) agreed that TACE acts as a palliative treatment but can achieve curative effects under certain conditions. Despite various treatment outcomes of TACE, clinicians still choose TACE as the first choice for intermediate stage HCC treatment. TACE combined with other approaches might achieve better treatment outcomes (n=251, 97.7%). The guidelines of the China Liver Cancer (CNLC) were followed by most participants (n=147, 57.2%) for TACE application in clinical practice, and none of the current scoring systems are effective in guiding TACE treatment.[15] Therefore, participants agreed that there is a need to subgroup the intermediate stage HCC in the current guidelines, since none of the existing subclassification systems are widely accepted.

Factors influencing treatment response of TACE

In this part, the survey included six single- or multiple-choice questions (Fig. 3). Most clinicians agreed that multiple factors, including the tumor burden, tumor morphology, and liver function, are associated with treatment response to TACE. More than half of the participants (n=139, 54.1%) reported that it is difficult to achieve a satisfactory response after TACE for tumor lesion(s) larger than 7 cm in diameters. Similarly, more than half of the participants (n=141, 54.9%) reported that a good tumor response after TACE is hard to achieve for patients with more than three tumor lesions. Most participants (n=224, 87.2%) agreed that the modified Response Evaluation Criteria in Solid Tumors (mRECIST) is the best criteria to assess tumor response after TACE, and at least two or three sessions of TACE should be performed before assessing comprehensive treatment outcome.

Understanding and expectations of TACE failure/refractoriness and subsequent treatment pattern

In this part, the survey included 17 single- or multiple-choice questions (Supplementary Figs. 1–6). Most participants (n=221, 86.0%) agreed that repeated TACE should be performed even if incomplete tumor necrosis was not achieved after the previous super-selective TACE. Of the 221 participants, most (n=166, 75.1%) believed that repeated TACE should be performed only if new tumor arteries appear and super-selective TACE could be provided. A proportion of participants (n=106, 41.2%) disagreed that the "occurrence of two consecutive insufficient responses of the target tumor" should be defined as TACE failure/refractoriness. For these participants, TACE-based combination therapy ranked first (n=84, 79.2%) as the ideal subsequent therapy. Moreover, nearly one third of participants (n=75, 29.2%) chose three consecutive treatments of insufficient TACE sessions as the most ideal number to define TACE failure/refractoriness. Nearly half of the participants (n=121, 47.1%) disagreed that "new intrahepatic lesion(s)" should be considered as TACE failure/refractoriness, while only 16.3% of the participants chose the opposite answer. The majority of the above-mentioned participants (n=93, 76.9%) who answered "No" to the "new intrahepatic lesion(s)" question considered combination

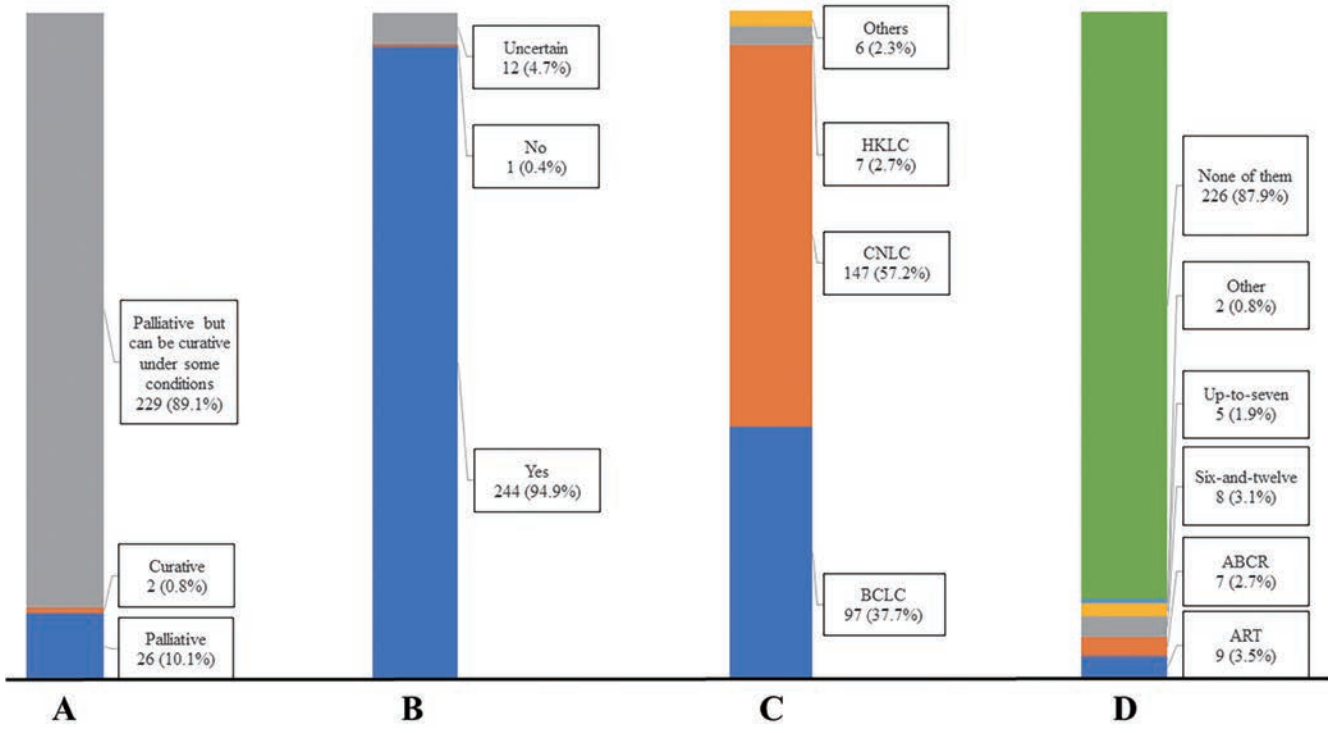

Fig. 1. Answers to questions 1–4 about the overall understanding of transarterial chemoembolization (TACE) in the real-world clinical practice. (A) Q1, most participants (n=229, 89.1%) agreed that TACE acts as a palliative method, but can achieve curative outcomes under some conditions. (B) Q2, most participants (n=244, 94.9%) agreed that treatment outcomes of TACE have a high variation. (C) Q3, more than half of the participants (n=147, 57.2%) followed the CNLC staging system for TACE application. (D) Q4, most participants (n=226, 87.9%) agreed that none of the scoring systems are suitable to assess and predict treatment benefits for initial or repeated TACE. HKLC, Hong Kong Liver Cancer; CNLC, China National Liver Cancer; BCLC, Barcelona Clinic Liver Cancer.

therapy, including TACE, as the ideal subsequent therapy. Of the participants who answered "Yes", half of them (n=21, 50.0%) considered "3 consecutive times of new intrahepatic lesion(s) should be defined as TACE failure/refractoriness."

Most participants agreed that repeated TACE should be performed to control intrahepatic lesion(s) for patients with preserved liver function, who developed portal vein tumor thrombosis (PVTT) (n=242, 94.2%) or extrahepatic spread (n=253, 98.4%) following TACE. Multiple treatments are also recommended as a combination approach with TACE to control PVTT or extrahepatic spread. More than half of the participants (n=165, 64.2%) agreed that continuous elevation of tumor markers, such as alpha fetoprotein and Protein Induced by Vitamin K Absence or Antagonist-II immediately after TACE, should be considered as TACE failure/refractoriness.

Most participants (n=191, 74.3%) agreed that the concept of TACE failure/refractoriness has scientific and clinical significance. However, current existing definitions are not suitable for clinical practice in the real-world and need to be re-defined, especially for the treatment of HCC patients in China (n=235, 91.4%). For participants who accepted the concept of TACE failure/refractoriness, "combination treatment including TACE" ranked first (n=88, 46.1%) as the ideal subsequent treatment after TACE failure/refractoriness.

Perspectives on TACE

In this part, the survey included the three single- or multiple-choice questions (Figs. 4 and 5). More than half of the participants (n=166, 64.6%) did not think that the number of TACE sessions would decrease in clinical practice in the future. Most of the participants (n=252, 98.1%) believed that the TACE technique would be improved in the future with more advanced embolic agents, chemotherapeutic drugs, embolization technique, and micro-catheters.

Discussion

In clinical practice, it is critical to establish a balance between the potential treatment benefits and liver function impairment of repeated TACE. To do so, the concept of "TACE failure/refractoriness" should be considered carefully, especially since the real-world clinical applicability of the existing definitions and subsequent recommended therapies is under debate in China. Therefore, the CCI survey was conducted to identify how clinicians specialized in HCC treatment in China apply TACE, and their opinions about the concept of "TACE failure/refractoriness". Results reveal that the majority of the participating clinicians accept the concept of TACE failure/refractoriness, which has scientific and clinical significance. Moreover, the participants believe that the current existing definitions are not suitable and need to be re-defined, especially for HCC treatment in real-world clinical practice in China.

Because of the high heterogeneity of HCC, the prognosis of patients treated with TACE varies from a median survival of 19.4 months to around 49.1 months.[16,17] Therefore, several subclassifications and predictive scoring systems have been established to subclassify ideal candidates receiving initial or repeated TACE.[7,8,18-21] Among them, the criteria proposed by Bolondi and Kinki is based on the tumor burden (up-to-seven criteria) and liver function to stratify patients

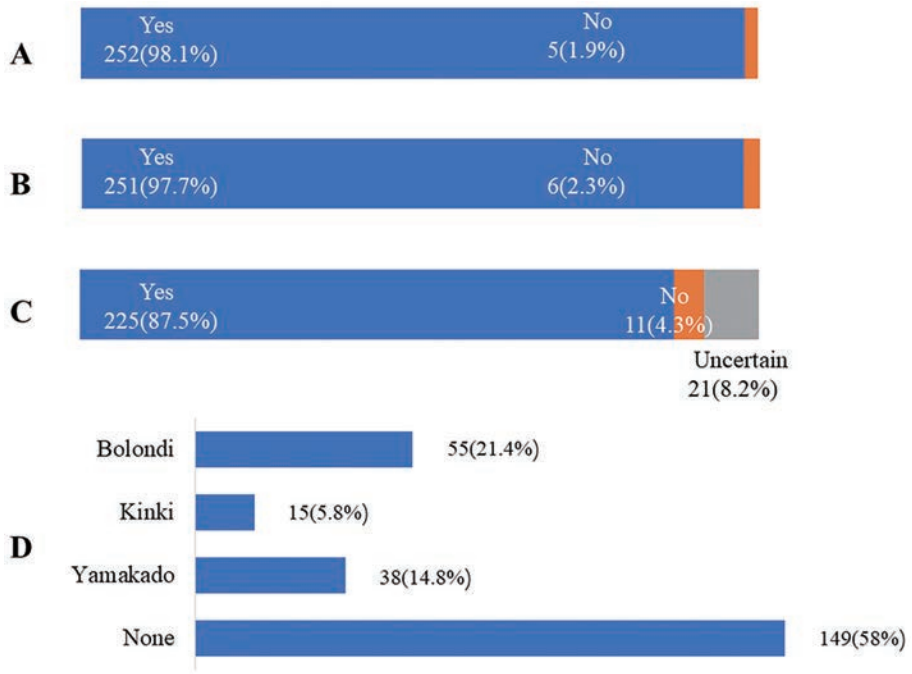

Fig. 2. Answers to questions 5–8 about the overall understanding of transarterial chemoembolization (TACE) in the real-world clinical practice. (A) Q5, 252 participants (98.11%) agreed that TACE is still the first choice for intermediate stage hepatocellular carcinoma (HCC). (B) Q6, 251 participants (97.7%) agreed that TACE combined with other approaches could achieve a better treatment outcome. (C) Q7, 225 participants (87.5%) agreed that there is a need to subgroup intermediate stage HCC in the current guidelines. (D) Q8, 149 participants (58.0%) agreed that none of the current subgroups are suitable for intermediate stage HCC.

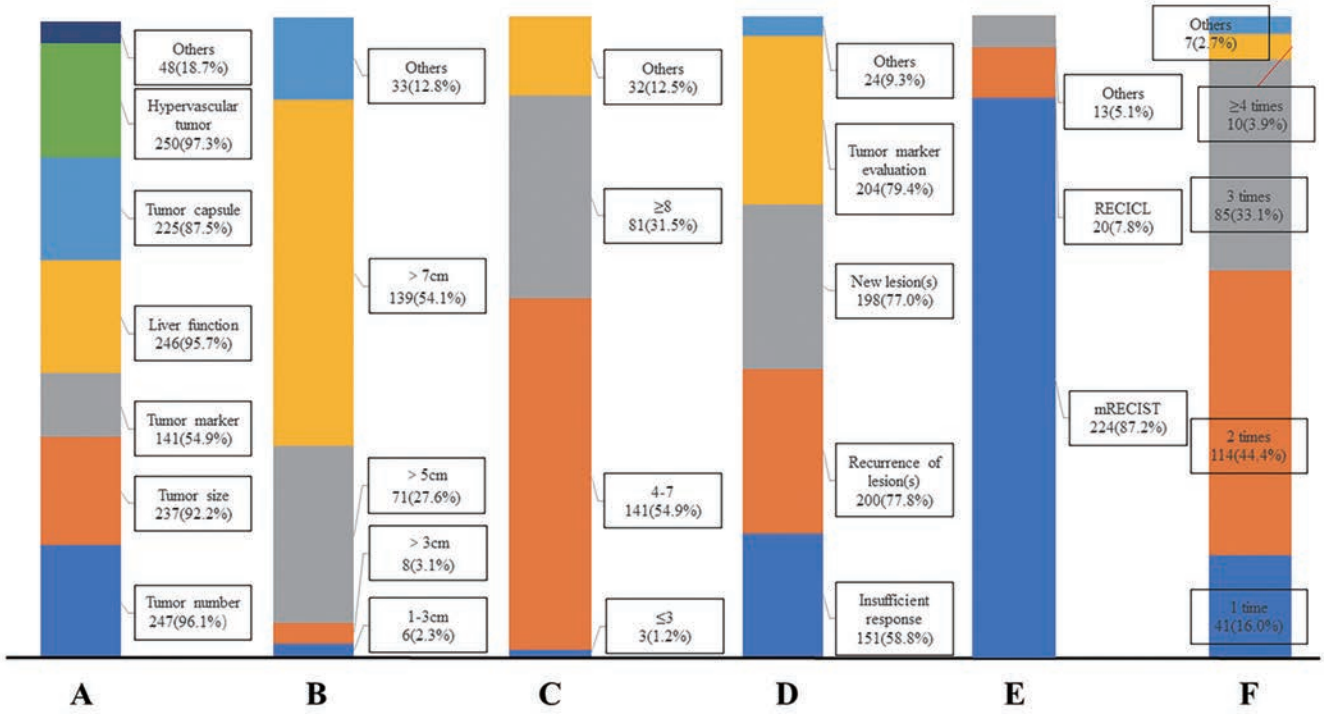

Fig. 3. Answers to questions 9–14 about factors influencing treatment response of transarterial chemoembolization (TACE). (A) Q9, multiple variables affect the treatment outcome of TACE. (B) Q10, the majority of participants (n=139, 54.1%) agreed that it is difficult to achieve a satisfied tumor response after TACE for lesion(s) with diameters larger than 7.00 cm. (C) Q11, most participants (n=141, 54.9%) agreed that it is difficult to achieve a satisfied tumor response after TACE for 4–7 target lesion(s) (D) Q12, multiple variables predict an unsatisfied treatment outcome of TACE. (E) Q13, most participants (n=224, 87.2%) agreed that mRECIST is the most suitable tool to assess tumor response after TACE. (F) Q14, 114 participants (44.4%) agreed that at least two sessions of TACE should be performed before assessing the comprehensive treatment outcome. RECICL, Response Evaluation Criteria in Cancer of the Liver; mRECIST, Modified Response Evaluation Criteria in Solid Tumors.

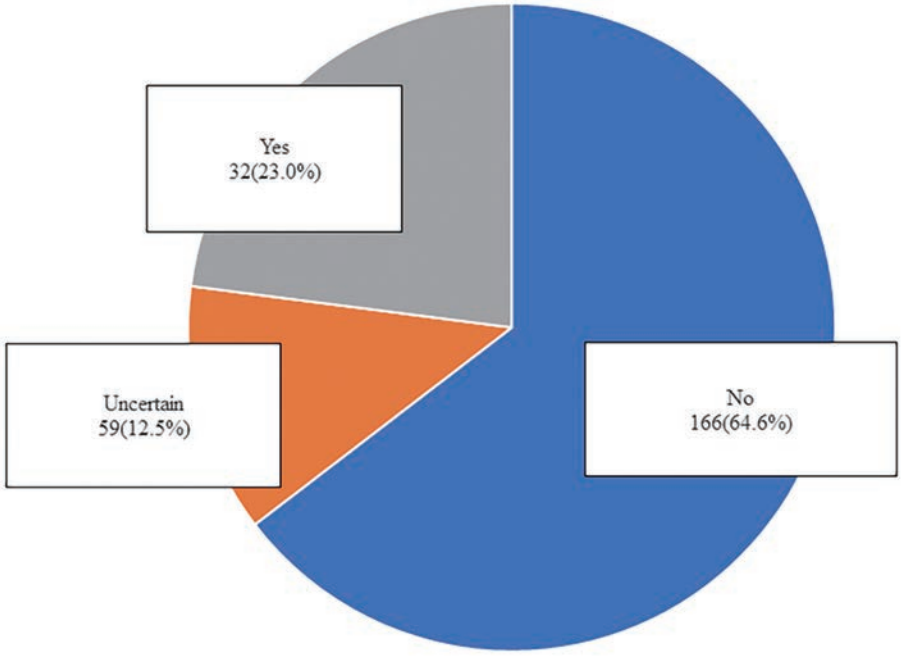

Fig. 4. Answers to questions 32 about predictions for future transarterial chemoembolization the number of (TACE). More than half of the participants (n=166, 64.6%) agreed that the number of TACE sessions would not decrease in clinical practice in the future.

who would benefit from initial TACE.[7,8] The Assessment for Retreatment with TACE (ART) score is based on pre-procedural liver function, including the Child-Pugh score and serum aspartate aminotransperase, and tumor response evaluation after initial TACE to determine whether repeated TACE would still be beneficial.[20] Nevertheless, none of these subclassifications or scoring systems have been widely accepted or applied in clinical practice, which is further confirmed by the results of this survey . The existing definitions consider the concept of TACE failure/refractoriness as consecutive insufficient responses of the target tumor and new intrahepatic lesion(s); thus, it is used to better assess the benefit of repeated TACE. While the JSH-LCSGJ 2014 criteria define two consecutive insufficient responses or two consecutive new intrahepatic lesion(s) as TACE failure/refractoriness, the present survey revealed different opinions. A larger proportion of participants (n=106, 41.2%) did not think that "two consecutive insufficient responses of the target tumor occurs" should be defined as TACE failure/refractoriness, while a smaller proportion (n=85, 33.1%) agreed with such definition. In addition, a larger proportion of participants (n=75, 29.2%) believed that three consecutive insufficient responses should be considered as TACE failure/refractoriness, while a smaller proportion (n=74, 28.8%) agreed with two consecutive insufficient responses. Similar responses were also observed for the definition regarding new intrahepatic lesions that occur after TACE. The majority of participants disagreed that new intrahepatic lesion(s) after TACE should be considered as TACE failure/refractoriness compared to one-third of that majority who

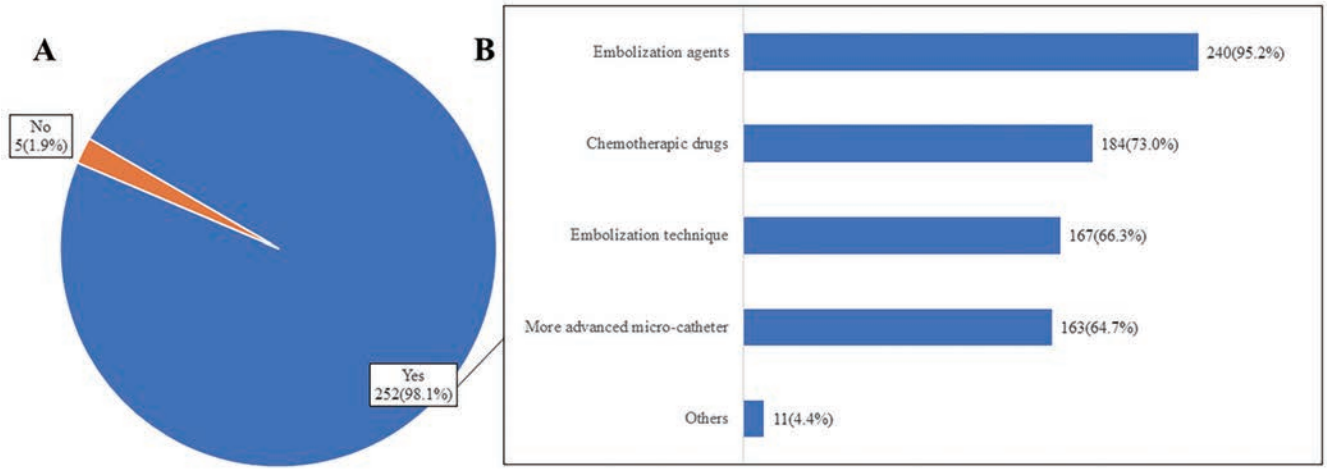

Fig. 5. Answers to questions 33–34 about perspectives on transarterial chemoembolization (TACE). (A) Q33, almost all participants (n=252, 98.1%) agreed that the TACE technique would be improved in the future. (B) Q34, participants agreed that multiple aspects of the TACE technique would be improved.

agreed with such definition. Instead of sorafenib that is recommended by the existing TACE failure/refractoriness definitions, TACE-based combination therapy ranked first as the ideal subsequent therapy after two consecutive insufficient responses of the target tumor or new intrahepatic lesion(s).

All existing definitions regard the presence of PVTT or extrahepatic spread after TACE as TACE failure/refractoriness, and recommend witching to sorafenib. In contrast, the current survey showed that most participants believe continuing TACE is necessary to control intrahepatic lesion(s) for HCC patients with preserved liver function who presented PVTT or extrahepatic spread after the previous TACE. Certainly, combination therapies, including molecular targeted therapy, immune checkpoint inhibitors, I^{125} seeds implantation, and ablation, with TACE are recommended by the participants to control PVTT/extrahepatic spread. Considering the fatality of more than two-thirds of patients with advanced HCC due to intrahepatic tumor progression or liver failure instead of metastatic disease progression, TACE targeting the intrahepatic lesion(s) would be a reasonable and beneficial treatment for advanced HCC. Many previous studies have demonstrated the treatment efficacy and safety of TACE monotherapy or TACE combined with sorafenib in advanced HCC patients with PVTT or extrahepatic spread.[22–26]

Apart from the topic on TACE failure/refractoriness, the survey was also conducted to determine the understanding of TACE in real-world clinical practice, factors influencing treatment response, and perspectives on TACE. Most of the participants agreed that tumor burden, tumor morphology, and liver function are the major factors associated with tumor response. They also agreed that a subclassification of the intermediate stage is needed. This might be the reason that the existing subclassification systems or prognostic score systems for HCC are not widely accepted in clinical practice, especially in China.

Limitations

The study has several limitations, although it reveals the present recognition of TACE failure/refractoriness and could promote a more standardized application of TACE in clinical practice in China. First, more than half of the participants are interventional radiologists. More participants from the department of oncology, gastroenterology, surgery, *et al.* should be included to avoid selection bias. Second, the study did not introduce a new definition of TACE failure/refractoriness. Further meetings and study should be carried out to introduce the modified criteria of TACE failure/refractoriness. Third, the survey was carried out in the mainland of China and did not include participants from other countries, which might limit the readership interest around the world.

Conclusions

In conclusion, the survey conducted by CCI demonstrates an obvious difference in the recognition of TACE failure/refractoriness in HCC treatment between Chinese experts when compared to the existing definitions. Re-defining the criteria for TACE failure/refractoriness and introducing the subclassification for intermediate stage HCC are warranted to better select HCC patients who will benefit most from TACE and to optimize treatment strategies for HCC.

Funding

This study was supported by the National Natural Science Foundation of China (No. 81901847, 81520108015 and 81827805), the Clinical Innovation Center of Medical Imaging and Interventional Radiology (No. YXZXA2016005), the Natural Science Foundation of Jiangsu Province (No. BK20190177), and the Suzhou Science and Technology Youth Plan (No. KJXW2018003). Funding sources had no involvement in the financial support for the conduct of the research and preparation of the article.

Conflict of interest

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

Author contributions

Concept and design of the study (CFN, GJT), acquisition of data (BYZ, WSW, SZ, HDZ), analysis and interpretation of data (BYZ, LZ, JS), drafting of the manuscript (BYZ, HDZ), critical revision of the manuscript for important intellectual content (CFN, GJT, XLZ), administrative, technical, or material support, study supervision (CFN, GJT).

Data sharing statement

No additional data are available.

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Journal of Clinical and Translational Hepatology **2021** vol. 9(4) | 528–536
DOI: 10.14218/JCTH.2021.00111

Original Article

Loss of ARID1A Promotes Hepatocellular Carcinoma Progression via Up-regulation of MYC Transcription

Yao Xiao[1,2,3], Guodong Liu[1,2], Xiwu Ouyang[1], Denggao Zai[1], Jixiang Zhou[1], Xiaoli Li[1], Qi Zhang[1] and Jie Zhao[4*]

[1]*Department of General Surgery, Xiangya Hospital, Central South University, Changsha, Hunan, China;* [2]*National Clinical Research Center for Geriatric Disorders, Xiangya Hospital, Central South University, Changsha, Hunan, China;* [3]*International Joint Research Center of Minimally Invasive Endoscopic Technology Equipment & Standards, Changsha, Hunan, China;* [4]*Department of General Surgery, Sir Run Run Shaw Hospital, School of Medicine, Zhejiang University, Hangzhou, Zhejiang, China*

Received: 25 March 2021 | **Revised:** 14 May 2021 | **Accepted:** 6 July 2021 | **Published:** 23 July 2021

Abstract

Background and Aims: AT-rich interactive domain-containing protein 1A (ARID1A) is frequently mutated or deficient in hepatocellular carcinoma (HCC). However, the role of ARID1A in HCC remains unclear. Therefore, the biological role of ARID1A in HCC was evaluated and a potential mechanism was investigated. ***Methods:*** *Arid1a* was knocked out in the livers of mice using the CRISPR/Cas9 system delivered by hydrodynamic tail vein injection. The development of HCC was observed in different mouse models. The correlation of ARID1A and prognosis in patients with HCC was analyzed using cBioPortal. The effect of ARID1A on cell proliferation was assessed by MTT assay following the manipulation of candidate genes. ***Results:*** ARID1A deficiency alone did not cause HCC in mice, but knockout of *ARID1A* accelerated liver tumorigenesis in response to diethylnitrosamine (DEN) or when a combination knockout of phosphatase and tensin homolog (*Pten*) plus tumor protein P53 (*p53*) was introduced. *ARID1A* mutations were associated with a poorer prognosis in HCC patients. The mRNA level of MYC was significantly higher in patients with an *ARID1A* mutation compared to those without a mutation. Ectopic expression of ARID1A inhibited HCC cell proliferation. ARID1A knockout increased HCC cell growth and resulted in disruptions to DNA damage repair and apoptosis following radiation stress. Furthermore, mechanistic studies revealed that ARID1A inhibited the proliferation of HCC cells via transcriptional down-regulation of MYC. ***Conclusions:*** These results describe ARID1A as a tumor suppressor in the liver. A deficiency in ARID1A predicts worse survival in HCC patients and promotes HCC progression via up-regulation of MYC transcription.

Citation of this article: Xiao Y, Liu G, Ouyang X, Zai D, Zhou J, Li X, *et al*. Loss of ARID1A promotes hepatocellular carcinoma progression via up-regulation of MYC transcription. J Clin Transl Hepatol 2021;9(4):528–536. doi: 10.14218/JCTH.2021.00111.

Keywords: ARID1A; Hepatocellular carcinoma; MYC.

Abbreviations: γ-H2AX, gamma histone 2 A variant X; ARID1A, AT-rich interactive domain-containing protein 1A; c-PARP, cleaved poly (ADP-ribose) polymerase; CRISPR/Cas9, clustered regularly interspaced short palindrome repeats/CRISPR-associated protein 9; DAPI, 4′,6-diamidino-2-phenylindole; DEN, diethylnitrosamine; DMEM, Dulbecco's modified Eagle's medium; eGFP, enhanced green fluorescent protein; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GDAC, Genome Data Analysis Centre; HCC, hepatocellular carcinoma; IF, immunofluorescence; IHC, immunohistochemical; IR, ionizing radiation; KO, knockout; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide; p53, tumor protein P53; PBS, phosphate-buffered saline; PFA, paraformaldehyde; PI3K/AKT, phosphoinositide 3-kinase/protein kinase B; PTEN, phosphatase and tensin homolog; qPCR, quantitative reverse-transcription polymerase chain reaction; RPMI, Roswell Park Memorial Institute; SD, standard deviation; SEM, standard error of the mean; sg, single guide; TBST, Tris-buffered saline with Tween-20; TCGA, The Cancer Genome Atlas; WT, wild type.

[*]**Correspondence to:** Jie Zhao, Department of General Surgery, Sir Run Run Shaw Hospital, 3 Qingchun East Road, Hangzhou, Zhejiang 310058, China. ORCID: https://orcid.org/0000-0002-8795-2770. Tel: +86-13805787418, Fax: +86-571-8788-7081, E-mail: 05lcyxzhaojie@zju.edu.cn

Introduction

Hepatocellular carcinoma (HCC) is one of the most common types of malignant digestive system tumors and is associated with a high mortality rate.[1] Occurrence and development of HCC involves the alteration of many genes and signaling pathways, but its pathogenic mechanism has not been fully elucidated.[2] To gain a comprehensive understanding of the genetic alterations that occur during HCC initiation, many researchers have analyzed the HCC genome using whole-genome sequencing strategies.[3,4] To date, several genes, including *telomerase reverse transcriptase* (*TERT*), *tumor protein P53* (*p53*), *AT-rich interactive domain 1A* (*ARID1A*), *cyclin dependent kinase inhibitor 2A* (*CDKN2A*), *catenin beta 1* (*CTNNB1*), *axin 1* (*AXIN1*), and *cyclin D1* (*CCND1*), among others, have been shown to be related to HCC.[5]

ARID1A, its encoding gene located on chromosome 1p36.11, represents a subunit of the switch/sucrose nonfermentable (SWI/SNF) chromatin remodeling complex.[6] Chromatin remodeling complexes modify chromatin structures and regulate the transcription of genes to control various cellular processes.[7] Inactivating mutations in ARID1A have been identified in a wide variety of cancers, suggesting that it functions as a tumor suppressor.[8] However, its anticancer mechanisms of action in HCC are not fully understood.

MYC is a transcription factor encoded by the *c-MYC* gene that regulates an estimated 15% of genes in the human genome.[9] MYC is an oncoprotein that contributes to the ma-

lignancy of many aggressive cancers.[10] The *c-MYC* locus is the most frequently amplified locus across all human cancers, leading to MYC overexpression.[11] MYC is frequently overexpressed in patients with HCC[12–14] and experimental overexpression of MYC in the livers of mice can lead to the development of HCC.[15]

In this study, the role of ARID1A in HCC progression was investigated. Using *in vitro* cell models and *in vivo* mouse models, ARID1A deficiency was shown to accelerate the development and progression of liver cancer. Furthermore, mechanistic studies revealed that ARID1A inhibits proliferation in HCC cells via the down-regulation of *c-MYC* transcription.

Methods

Cell culture

The Bel7404 cell line was a gift from Professor Cang (Zhejiang University, Hangzhou, China). The Huh7 and HepG2 cell lines were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). Huh7 and Bel7404 cells were grown in Roswell Park Memorial Institute (RPMI) 1640 media (Invitrogen, Grand Island, NY, USA) supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin and 1% glutamine in an incubator maintained at 37°C with 5% CO_2. HepG2 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Corning Life Science, Corning, NY, USA).

Plasmid and lentivirus

The clustered regularly interspaced short palindrome repeats/CRISPR-associated protein 9 (CRISPR/Cas9) system (PSpCas9(BB)-2A-Puro; PX459) was purchased from Addgene (Watertown, MA, USA). Knockout cells were generated by transfecting the cells with CRISPR/Cas9 using Lipofectamine™ 2000 (Invitrogen), according to a previous report.[16] Individual cells were selected to generate monoclonal cell lines (Bel7404 ARID1A KO-1 and KO-2). LentiCRISPR v2-sgARID1A (Addgene) and pLenti-puro-ARID1A (Addgene) lentiviral vectors were used to knockout and overexpress ARID1A, respectively. To generate stable transfectants, the lentiviral vector, the psPAX2 packaging plasmid (10 μg), and the pMD2.G envelope plasmid (10 μg) were transfected into 293T cells using the standard calcium phosphate transfection method. Lentivirus soups were collected and concentrated by density gradient after 48 h for immediate use, or were frozen at −80°C for later use.

In vivo experiment and hydrodynamic tail vein injection

The animal experiment closely adhered to the Zhejiang University guide for the care and use of laboratory animals. For DEN treatment, 14 day-old male C57BL/6 mice were administered with a single intraperitoneal injection of DEN (25 μg/g body weight). A pX459 vector co-expressing an sgRNA targeting *Arid1a*, *Pten* or *p53* was cloned. Vectors for hydrodynamic tail vein injections were prepared using the EndoFreeMaxi Kit (Qiagen, Hilden, Germany). For hydrodynamic liver injection, plasmid DNA suspended in 2 mL saline was injected into 8 week-old male C57BL/6 mice via the tail vein within 6–7 sec. The amount of injected DNA was 60 μg for sg*Arid1a*, and 60 μg each for sg*Arid1a*+sg*Pten*+sg*p53*. An equal amount of pX459 was used as a control for each experiment.

Western blot

Cells were lysed in NETN lysis buffer and 30 μg total protein was run on a gel using sodium dodecyl sulfate polyacrylamide gel electrophoresis. After electrophoresis, proteins were transferred onto a polyvinylidene fluoride membrane (Millipore, Burlington, MA, USA). After blocking for 1 h, membranes were rinsed with Tris-buffered saline with Tween-20 (TBST) three times and incubated in the corresponding primary antibody at 4°C overnight (antibodies listed in Supplementary Table 2). The membranes were then rinsed three times with TBST and incubated with secondary antibodies. Finally, membranes were incubated with an enhanced chemiluminescence system (ThermoFisher Scientific, Waltham, MA, USA). The bands were detected by ChemiDoc XRS Image System (Bio-Rad Laboratories, Hercules, CA, USA).

Immunohistochemistry

Tissues were fixed in 4% paraformaldehyde (PFA) and embedded in paraffin. Paraffin sections (4 μm) were dewaxed by xylene and rehydrated in decreasing concentrations of ethanol. Epitope retrieval was performed in 10 mM citrate buffer (pH 6.0) at 95°C for 20 m in a microwave oven. Endogenous peroxidase activity was blocked for 10 min by 0.3% H_2O_2 in phosphate-buffered saline (PBS). Tissue sections were incubated with antibodies overnight (shown in Supplementary Table 2). Next, the sections were incubated with a diluted biotinylated goat anti-rabbit IgG antibody (Vector Laboratories, Burlingame, CA, USA) for 30 m at room temperature. Chromogenic reactions were carried out according to the protocols provided in the ImmPACT™ DAB kit (Vector Laboratories).

Immunofluorescence (IF)

Cells were plated and grown on glass slides, washed with PBS, and fixed with 4% PFA for 15 m. Cells were then washed again with PBS and treated with 0.2% Triton X-100 (ThermoFisher Scientific) in PBS for 10 m to permeabilize the cells. After washing with PBS again, the cells were blocked with 2% bovine serum albumin (ThermoFisher Scientific) in PBS at room temperature for 1 h, then incubated with primary antibodies (Supplementary Table 2) at 4°C overnight. Cells were washed once with PBS, then incubated with secondary IF-specific antibodies at room temperature for 1 h. The cells were observed under a laser scanning confocal microscope (LSM710; Zeiss, Oberkochen, Germany). 4′,6-diamidino-2-phenylindole (DAPI) and F-actin were used as staining controls.

Quantitative reverse-transcription polymerase chain reaction (qPCR)

TRIzol reagent (Invitrogen) was used to extract the total RNA from cells, and 2 μg of total RNA was used for reverse transcription. The Bio-Rad CFX96 system was used to conduct the qPCR and calculate the expression of mRNA. Data were normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and relative expression was assessed using the ΔΔCt method. All primers used are shown

in Supplementary Table 3. Experiments were performed in triplicate.

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) cell viability assay

HCC cells were plated in 24-well plates at a density between 2×10^3 and 5×10^3 cells per well. After treatment, culture media was removed and 500 μL of MTT (0.5 mg/mL) per well was added and cells were incubated at 37°C with 5% CO_2 for 1 h. The absorbance at 570nm was detected. Cell viability was calculated using the formula: [optical density (OD; sample) – OD (blank)] / [OD (control) – OD (blank)]. Experiments were repeated at least three times.

Analysis of The Cancer Genome Atlas (TCGA) data

The subset of data from TCGA Liver Hepatocellular Carcinoma (source data from Genome Data Analysis Centre [GDAC] Firehose) of the cbioportal.org website was analyzed. Specifically, on the home page of the website, "liver" was selected, then "Liver Hepatocellular Carcinoma (TCGA, Firehose Legacy)". "Explore Selected Studies" was chosen, "hepatocellular carcinoma" in cancer type was detailed, and "ARID1A" was entered as the gene. The cBioPortal source code is freely available under the GNU Lesser GPL open-source license and is hosted by Google code (http://code.google.com/p/cbio-cancergenomics-portal/).[17]

Statistical analysis

Data are presented as mean±standard deviation (SD) or standard error of the mean (SEM) from independent experiments. Statistical analyses included the Student's *t*-test and chi-squared test using GraphPad Prism 6 (GraphPad Software, Inc., La Jolla, CA, USA). A *p*-value <0.05 was considered statistically significant.

Results

ARID1A deficiency accelerates liver tumorigenesis in mice

To explore the roles of ARID1A in HCC initiation, *Arid1a* was knocked out in mice using the CRISPR/Cas9 system, as previously described.[18] A pX459 vector co-expressing a single guide (sg)RNA targeting *Arid1a* (*Arid1a* target sequence presented in Supplementary Table 1, termed sg*Arid1a*) and Cas9 was cloned. *In vitro*, sg*Arid1a* caused the loss of ARID1A in murine Hep1-6 cells (Fig. S1A). A hydrodynamic tail vein injection was used to deliver CRISPR to the livers in mice, which can affect a large proportion of hepatocytes. As shown in Figure S1B, the hydrodynamic injection of an enhanced green fluorescent protein (eGFP) plasmid DNA resulted in liver-specific expression of eGFP in mice.

First, a cohort of wild type (WT) C57BL/6 mice were administered sg*Arid1a* to determine if knockout of *Arid1a* could induce the development of tumors in the liver. Immunohistochemical (IHC) staining of liver sections using an ARID1A-specific antibody revealed that approximately 10% of hepatocytes were negative for ARID1A, but these cells were surrounded by ARID1A-positive cells (Fig. S1C). Ten months later, five sg*Arid1a*-treated mice were examined. At necropsy, zero hepatic neoplasms were noted in any of the mice (Fig. 1A).

Considering ARID1A may not directly drive liver tumorigenesis, liver damage was induced using a single intraperitoneal injection of diethylnitrosamine (DEN) at 2 weeks of age, followed by sg*Arid1a* or pX459 as control at 6 weeks of age. At 6 months, the liver phenotypes were assessed. With sg*Arid1a* treatment, four of the five mice developed hepatic tumors, whereas no tumors were found in the control group ($p=0.048$; Fig. 1B).

Considering liver tumorigenesis may result from the accumulation of multiple mutations, mice were treated simultaneously with sg*Arid1a,* sg*Pten and* sg*p53* (target sequences listed in Supplementary Table 1). Liver-specific knockout of *Pten* in mice has been shown to induce lipid accumulation and the incidence of liver cancer.[19,20] As shown in Figure S1D, knockout of *Pten* was successful, as some hepatocytes were negative for PTEN and showed signs of lipid degeneration. At 3 months, the livers were harvested from sg*Arid1a*+sg*Pten*+sg*p53* and control pX459+sg*Pten*+sg*p53* mice. There were more nodules in the livers of sg*Arid1a*+sg*Pten*+sg*p53* mice compared to the control group, although this difference did not reach statistical significance ($n=5$/group, $p=0.067$; Fig. 1C).

The above *in vivo* data suggested that ARID1A deficiency alone cannot cause liver cancer, but ARID1A may play a tumor suppressive role and its loss can accelerate liver tumorigenesis when other pro-oncogenic factors are introduced.

ARID1A mutations are associated with a poorer prognosis in HCC patients

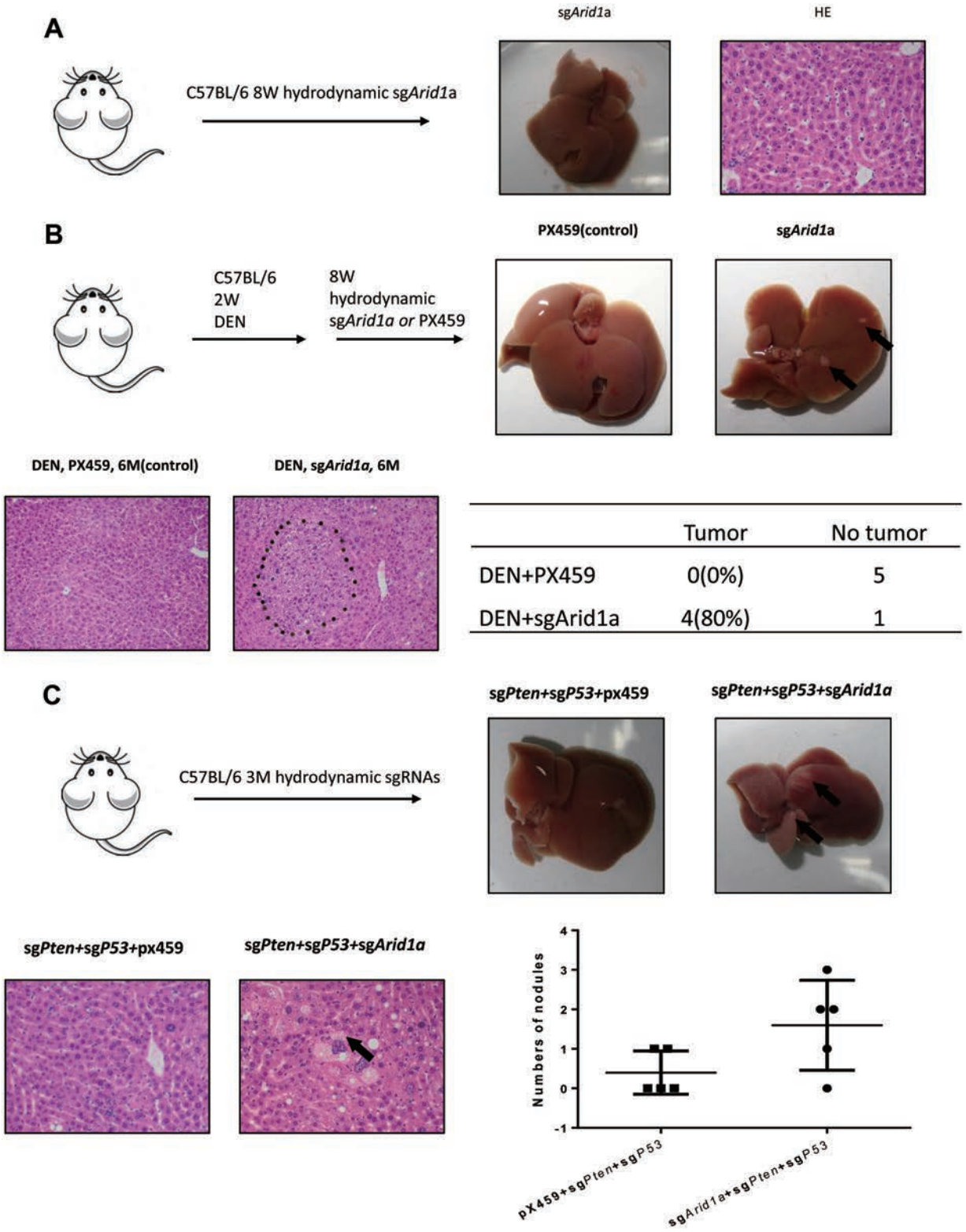
To dissect the function of *ARID1A* in HCC patients, a human survival analysis was conducted using cBioPortal (https://www.cbioportal.org/)[17,21] with data from TCGA Liver Hepatocellular Carcinoma (source data from GDAC Firehose). Thirty-four mutations of *ARID1A* were observed in 32 HCC patients (specific mutations listed in Supplementary Table 4). The results of the Kaplan-Meier survival analysis suggested that patients with an *ARID1A* mutation had a poor prognosis in terms of overall survival ($n=365$, $p=0.008093$); however, there was no significant difference in disease-free survival ($n=315$, $p=0.0719$; Fig. 2A). Interestingly, the human clinical survey showed that patients with an *ARID1A* mutation had more adjacent hepatic tissue inflammation compared to those with WT *ARID1A* ($p=0.002194$, $q=0.0757$; Fig. 2B).

Next, expression profiling analysis was performed based on the subset data of TCGA Liver Hepatocellular Carcinoma. Expression profiles of mRNA were displayed using a volcano plot (Fig. 2C). A total of 216 differentially expressed genes are listed in Supplementary Table 5. The mRNA levels of *MYC*, a known oncogene, were significantly higher in the *ARID1A* mutation group compared to the WT *ARID1A* group ($p=9.28\ e{-8}$, $q=1.332\ e{-4}$; Fig. 2D).

In summary, these human clinical results revealed that an *ARID1A* mutation was associated with poorer prognosis in HCC patients. *MYC* was a candidate gene that is regulated by ARID1A, which may exert its tumor suppressive functions.

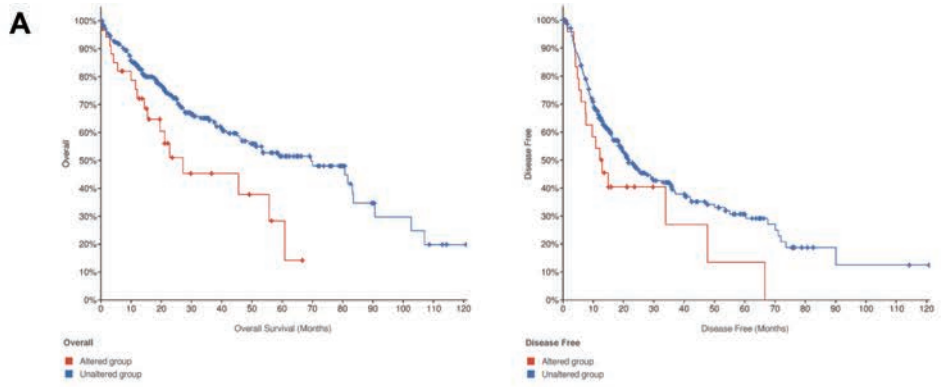
ARID1A inhibits HCC cell proliferation and is required for DNA damage repair and apoptosis

To study the function of ARID1A *in vitro*, the expression levels of ARID1A were first detected in a variety of HCC cell lines. Using western blot analysis, Bel7404 and HepG2 cells were found to be "ARID1A-positive". In contrast, Huh7 cells were "ARID1A-negative" (Fig. 3A). To test whether ARID1A

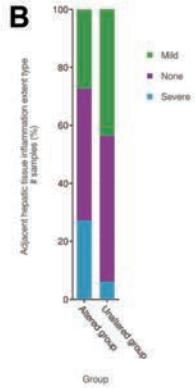


	Tumor	No tumor
DEN+PX459	0(0%)	5
DEN+sgArid1a	4(80%)	1

Fig. 1. ARID1A deficiency accelerates liver tumorigenesis in mice. (A) Knockout of *Arid1a* alone in C57BL/6 mice using the CRISPR/Cas9 system did not cause liver cancer (n=5). (B) Knockout of *Arid1a* in C57BL/6 mice previously exposed to DEN can accelerate liver tumorigenesis (n=5/group, p=0.048). Arrows indicate liver tumors. (C) C57BL/6 mice were injected with sg*Pten*+sg*p53*+sg*Arid1a* or sg*Pten*+sg*p53* (control). Nodules formed in the sg*Pten*+sg*P53*+sg*Arid1a* group and the sg*Pten*+sg*P53* group (n=5/group, p=0.067). Arrows indicate liver nodules. ARID1A, AT-rich interactive domain-containing protein 1A; CRISPR/Cas9, clustered regularly interspaced short palindrome repeats/CRISPR-associated protein 9; DEN, diethylnitrosamine.

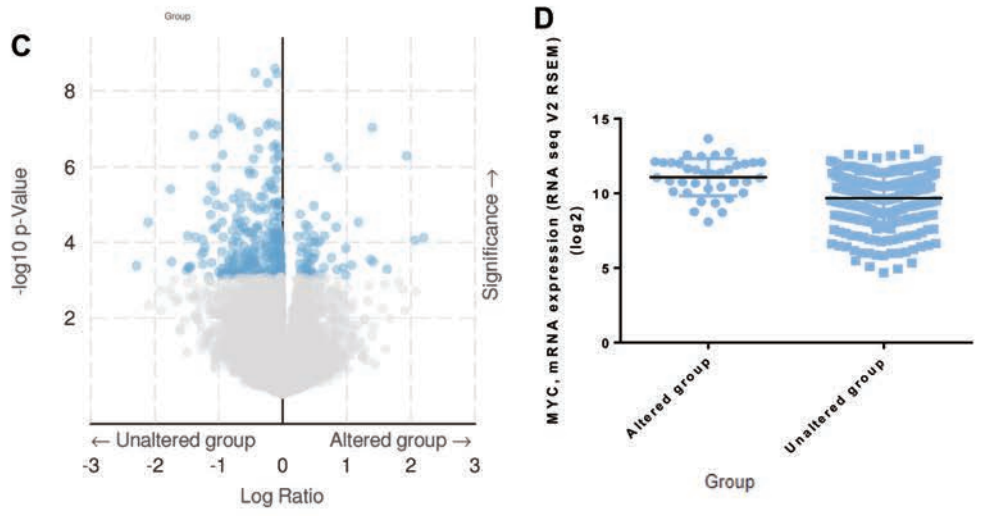


Survival Type	Number of Patients	# in Altered group	# in Unaltered group	Median months survival in Altered group (95% CI)	Median months survival in Unaltered group (95% CI)	p-Value	q-Value
Overall	365	36	329	27.17 (15.41 - NA)	69.51 (48.95 - 90.64)	8.093E-03	0.0162
Disease Free	315	27	288	12.91 (7.59 - NA)	21.55 (18.43 - 29.66)	0.0719	0.0719



Clinical Attribute	Attribute Type	Statistical Test	p-Value	q-Value
Adjacent hepatic tissue inflammation extent type	Patient	Chi-squared Test	2.194E-03	0.0757

Inflammation Type	# in Altered group	# in Unaltered group
Mild	6(27.27%)	92(43.6%)
None	10(45.45%)	106(50.24%)
severe	6(27.27%)	13(6.16%)



Gene	μ in Altered group	μ in Unaltered group	σ in Altered group	σ in Unaltered group	Log Ratio	p-Value	q-Value	Higher expression in
MYC	11.08	9.68	1.25	1.62	1.4	9.28E-08	1.33E-04	Altered group

Fig. 2. An *ARID1A* mutation was associated with a poorer prognosis in HCC patients. (A) Kaplan-Meier survival analysis (data from TCGA, Firehose Legacy) using cBioPortal suggested that patients with an *ARID1A* mutation had a poor prognosis regarding overall survival (n=365, p=8.093 e−03) but no significant difference in disease-free survival (n=315, p=0.0719). (B) HCC patients with an *ARID1A* mutation had more adjacent hepatic tissue inflammation (p=2.194e−03). (C) Volcano plot of the differential expression of mRNAs between HCC patients with an *ARID1A* mutation and those with WT *ARID1A*. (D) mRNA expression of MYC was significantly higher in HCC patients with an *ARID1A* mutation (p=9.28 e−8, q=1.332 e−4). ARID1A, AT-rich interactive domain-containing protein 1A; HCC, hepatocellular carcinoma; TCGA, The Cancer Genome Atlas.

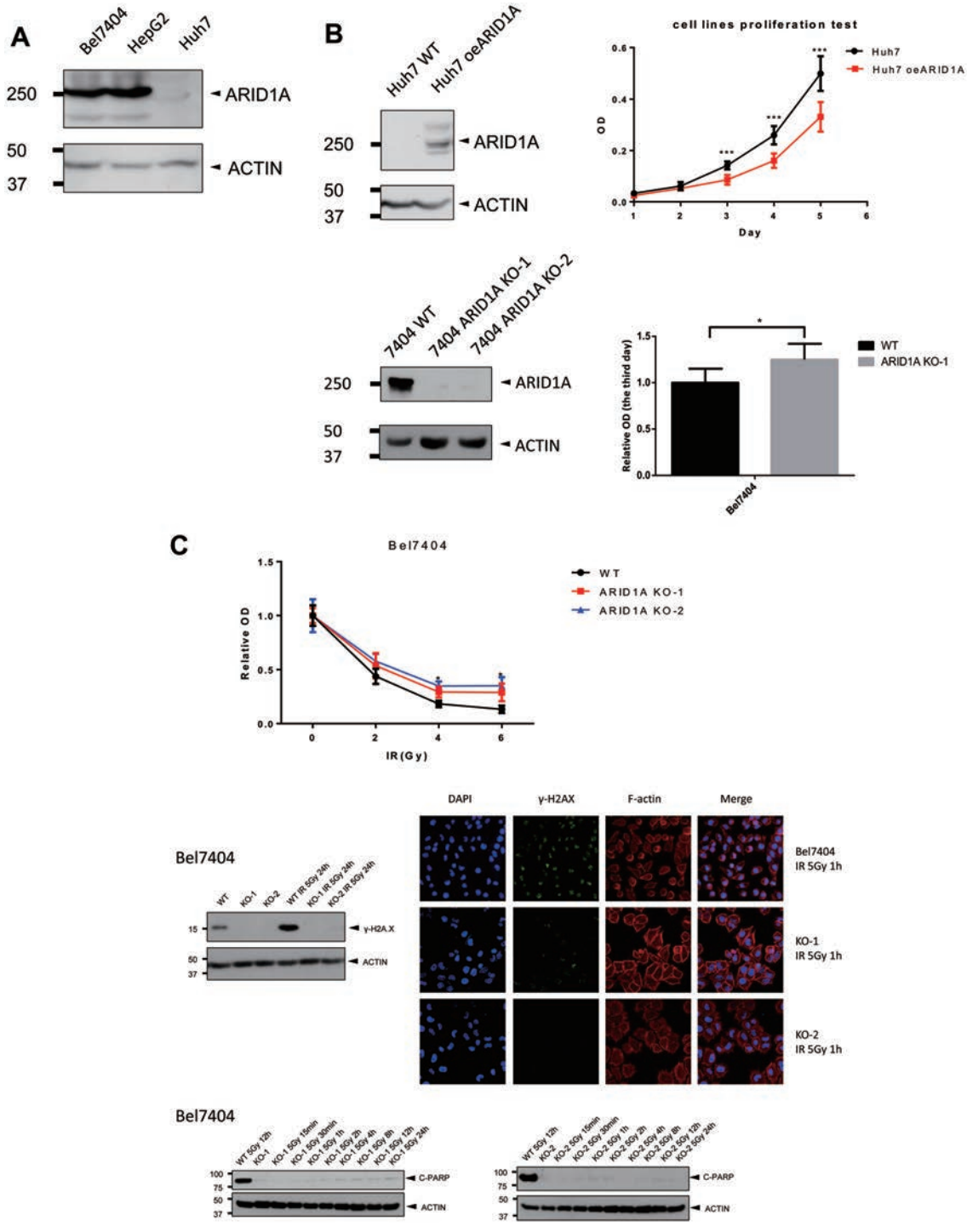


Fig. 3. ARID1A inhibits HCC cell proliferation, DNA damage repair and apoptosis. (A) Western blotting revealed protein expression level of ARID1A and actin in different HCC cell lines. (B) Huh7 cells were stably infected with a lentivirus expressing ARID1A cDNA for overexpression or ARID1A (above left). Bel7404 cells were transiently transfected with Cas9 and sg*ARID1A* and two randomly chosen monoclonal *ARID1A* knockout cell lines (KO-1, KO-2; lower left) were used for subsequent experiments. The MTT assay was performed to determine cell proliferation capacity (above right, below right). Experiments were performed in triplicate. Quantification data are presented as mean±SD. *$p<0.05$, ***$p<0.001$. (C) WT and ARID1A knockout Bel7404 cells were treated with radiation (0, 2, 4, 6 Gy, respectively). The MTT assay was performed to determine cell viability (above). Experiments were performed in triplicate. Quantification data are presented as mean±SD. *$p<0.05$. WT and ARID1A knockout Bel7404 cells were treated with 5 Gy IR. Twenty-four hours after radiation, γ-H2AX and actin were detected by western blot (middle left). One hour after radiation, γ-H2AX, DAPI and F-actin were detected by IF (middle right). WT and *ARID1A* knockout Bel7404 cells were treated with 5 Gy IR. The protein levels of c-PARP and actin at various time points were detected using western blotting. ARID1A, AT-rich interactive domain-containing protein 1A; c-PARP, cleaved poly (ADP-ribose) polymerase; DAPI, 4′,6-diamidino-2-phenylindole; HCC, hepatocellular carcinoma; KO, knockout; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide; SD, standard deviation; WT, wild type; γ-H2AX, gamma histone 2 A variant X.

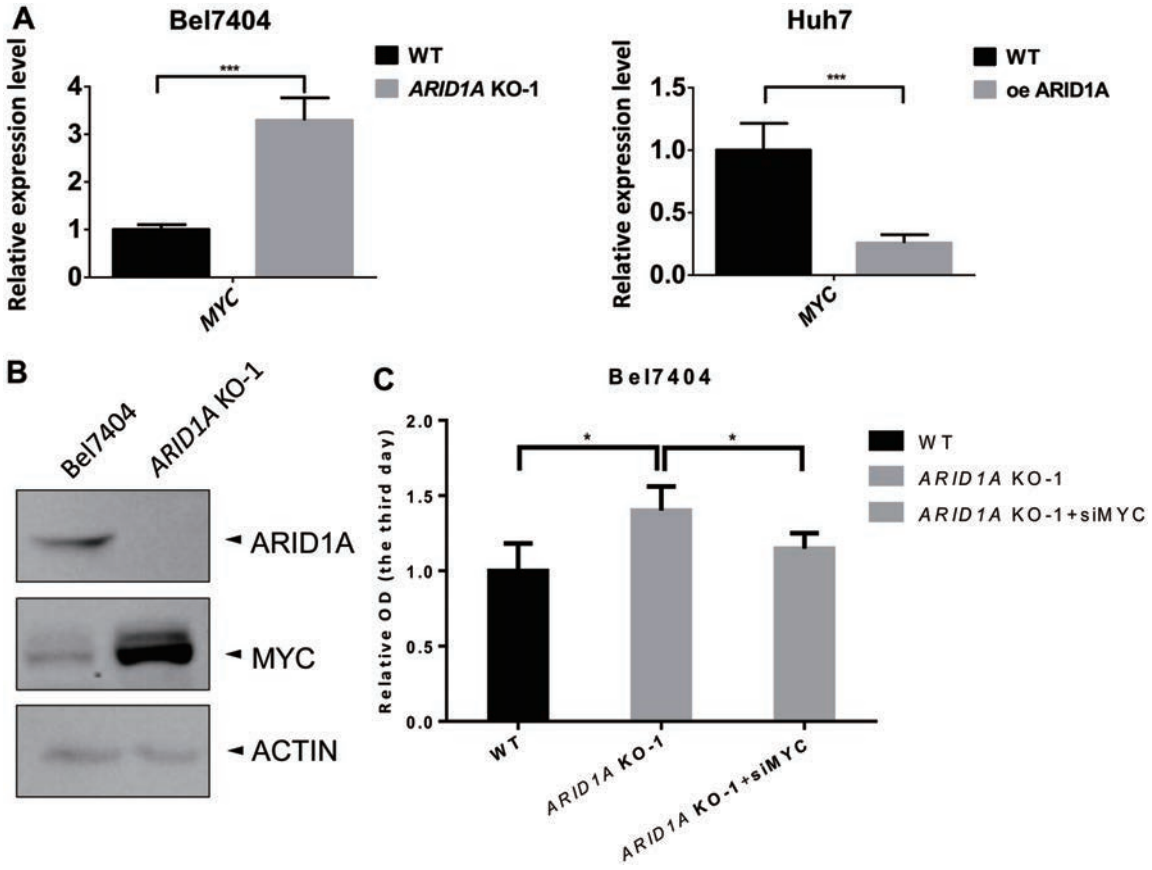


Fig. 4. ARID1A inhibits HCC cell proliferation via down-regulation of MYC transcription. (A) In WT and *ARID1A* knockout Bel7404 cells (left), WT Huh7 cells and those with over expressed ARID1A (right), the differential mRNA levels of MYC were detected using qPCR. Experiments were performed in triplicate. Quantification data are presented as mean±SD. ***$p<0.001$. (B) In WT and ARID1A knockout Bel7404 cells, the protein levels of MYC were detected by western blot. (C) ARID1A knockout Bel7404 cells were transfected with siMYC. The proliferation capacity of WT, ARID1A knockout and ARID1A knockout plus siMYC Bel7404 cells was detected using an MTT assay. Experiments were performed in triplicate. Quantitation data are presented as mean±SD. *$p<0.05$. si, small interfering RNA. ARID1A, AT-rich interactive domain-containing protein 1A; HCC, hepatocellular carcinoma; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide; qPCR, quantitative reverse-transcription polymerase chain reaction; SD, standard deviation; WT, wild type.

is crucial for HCC cell proliferation, ARID1A was further manipulated and the proliferation of HCC cells was examined using a MTT assay. The results revealed that overexpression of ARID1A in Huh7 cells inhibited their proliferation and knockout of ARID1A in Bel7404 cells (ARID1A KO cells) resulted in enhanced proliferation (Fig. 3B).

Next, the cellular responses to DNA damage were investigated. An MTT assay revealed better cell viability in ARID1A KO cells after ionizing radiation (IR) treatment, suggesting that the knockout of ARID1A in HCC cells caused the cells to become more resistant to IR stress (Fig. 3C, top). Results from western blot and immunofluorescence (IF) assays revealed that ARID1A KO cells could not accumulate the same level of gamma histone 2 A variant X after radiation treatment compared to WT Bel7404 cells (Fig. 3C, middle). Results from the apoptosis assay, where cleaved poly (ADP-ribose) polymerase (c-PARP) was detected by western blotting, also revealed that knockout of ARID1A could dramatically decrease the levels of c-PARP after IR treatment (Fig. 3C, bottom), suggesting that the tumor suppressive role of ARID1A could be the result of inducing apoptosis in damaged cells.

Taken together, the above data suggest that ARID1A inhibits HCC cell proliferation and is required for DNA damage repair and apoptosis.

ARID1A inhibits cell proliferation of HCC cells via downregulation of MYC transcription

Expression profiles of mRNA indicated that MYC might contribute to the ARID1A-dependent regulation of cell proliferation. To test this hypothesis concerning the molecular mechanism, transcript levels of MYC were detected using qPCR. Consistent with the gene expression profiling analysis, knockout of ARID1A increased the level of MYC mRNA in Bel7404 cells, while overexpression of ARID1A resulted in a decrease of MYC mRNA in Huh7 cells (Fig. 4A). Using western blotting, knockout of ARID1A increased the level of MYC protein in Bel7404 cells (Fig. 4B). In addition, knockdown of MYC in Bel7404 cells could reverse the proproliferative effect caused by knockout of ARID1A (Fig. 4C).

Taken together, these data presented in Figure 4 suggest that MYC can be regulated by ARID1A and this contributes to the regulation of cell proliferation.

Discussion

HCC is one of the most common types of liver cancer, and accounts for 90% of all primary liver cancers.[22] However, effective treatments for HCC are lacking due to its het-

erogeneity.[23] HCC patients carry mutations in numerous genes, including *ARID1A*. In the TCGA data set, 8.22% (30/365) of HCC patients carry an *ARID1A* mutation, and most of these are inactivating mutations. The clinicopathologic significance of ARID1A expression in HCC has been investigated previously, and it was revealed that 12.17% of HCC tumors (14/115) were ARID1A-negative and that loss of ARID1A was significantly associated with larger tumors.[24]

In this study, the knockout of *Arid1a* alone could not initiate liver cancer in mice, suggesting that it is not a cancer-driver gene. However, knockout of *Arid1a* accelerated liver tumorigenesis when DEN or a combination knockout of *Pten* and *p53* were introduced, suggesting that liver tumorigenesis is a multistep process that requires various other factors. Similar results have been reported by others, such as the finding of mice with homozygous or heterozygous deletions in *Arid1a* not developing ovarian lesions but mice with an *Arid1a* and *Pten* double-knockout developing ovarian endometrioid cancer.[25]

Further TCGA data analysis showed that *ARID1A* mutations are associated with a poorer prognosis in HCC patients, indicating that ARID1A may have prognostic value. Expression profiles of mRNA showed that *MYC* transcription was significantly higher in patients with an *ARID1A* mutation. *ARID1A* mutations resulted in abnormal chromatin remodeling that diverted gene transcription. Previously, others have also reported that mutant ARID1A is able to promote cell proliferation by triggering the phosphoinositide 3-kinase/protein kinase B (PI3K/AKT) signaling pathway,[26,27] which affects the expression of other cell cycle regulators, such as the *MYC* gene.[28] Consistent with previous reports, the contribution of MYC to the antiproliferative effect of ARID1A in HCC was definitively shown in the present study.

The role of ARID1A in DNA damage repair and apoptosis was also investigated. ARID1A knockout resulted in disruptions of DNA damage repair and apoptosis following IR exposure. Loss of ARID1A led to disrupted SWI/SNF function, which caused enhanced mutagenesis due to the defective DNA repair and aberrant apoptosis evasion.[29,30] Thus, tumorigenesis of HCC with an *ARID1A* mutation is complex and involves an intricate network of mechanisms, including cell proliferation, DNA damage repair and apoptosis signaling pathways.

Undoubtedly, the full decoding of the ARID1A tumor suppressive mechanism may have future therapeutic implications. These data support the role of ARID1A in protection against HCC progression. Several targeted therapy drugs should also be considered in future studies, including inhibitors of MYC or PI3K/AKT signaling, PARP inhibitors targeting the DNA damage signaling pathway, and synthetic lethal therapies targeting epigenetic changes in *ARID1A* mutation-based cancers. Interestingly, it was also shown that patients with an *ARID1A* mutation had more severe adjacent hepatic tissue inflammation, suggesting that ARID1A is involved in tumor immunity and may be targeted by immunotherapy.

In summary, the current findings further elucidate the tumor-suppressive mechanism of ARID1A in HCC. A loss-of-function *ARID1A* mutation promotes cell proliferation and disrupts DNA damage repair and apoptosis pathways. Loss of ARID1A may promote HCC progression via the transcriptional up-regulation of MYC.

Acknowledgments

We thank Yong Cang, Tong Ji, and Lidan Hou in the Life Sciences Institute of Zhejiang University for expert technical assistance.

Funding

This work was supported by Zhejiang Provincial Natural Science Foundation (LQ18H160010, LQ20C010005) and National Natural Science Foundation of China (82003062, 81903004).

Conflict of interest

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

Author contributions

Study concept and design (JZ, YX), acquisition of data (JZ, YX, GL, XO, DZ), analysis and interpretation of data (JZ, YX, GL, JxZ, XL, QZ), drafting of the manuscript (JZ, YX), critical revision of the manuscript for important intellectual content (JZ, YX), administrative, technical, or material support, study supervision (JZ).

Data sharing statement

No additional data are available.

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Journal of Clinical and Translational Hepatology **2021** vol. 9(4) | 537–544
DOI: 10.14218/JCTH.2021.00180

Original Article

Systematic Training of Liver Imaging Reporting and Data System Magnetic Resonance Imaging v2018 can Improve the Diagnosis of Hepatocellular Carcinoma for Different Radiologists

A-Hong Ren[1], Hui Xu[1], Da-Wei Yang[1], Nan Zhang[1], Te Ba[2], Zhen-Chang Wang[1] and Zheng-Han Yang[1*]

[1]*Department of Radiology, Beijing Friendship Hospital, Capital Medical University, Beijing, China;* [2]*Department of Radiology, The First Hospital of Beijing Fangshan District, Beijing, China*

Received: 13 May 2021 | **Revised:** 20 June 2021 | **Accepted:** 1 July 2021 | **Published:** 16 July 2021

Abstract

Background and Aims: Liver imaging reporting and data system (LI-RADS) provides standardized lexicon and categorization for diagnosing hepatocellular carcinoma (HCC). However, there is limited knowledge about the effect of LI-RADS training. We prospectively explored whether the systematic training of LI-RADS v2018 on magnetic resonance imaging (MRI) can effectively improve the diagnostic performances of different radiologists for HCC. ***Methods:*** A total of 20 visiting radiologists and the multiparametric MRI of 70 hepatic observations in 61 patients with high risk of HCC were included in this study. The LI-RADS v2018 training procedure included three times of thematic lectures (each lasting for 2.5 h) given by a professor specialized in imaging diagnosis of liver, with an interval of a month. After each seminar, the radiologists had a month to adopt the algorithm into their daily work. The diagnostic performances and interobserver agreements of these radiologists adopting the algorithm for HCC diagnosis before and after training were compared. ***Results:*** A total of 20 radiologists (male/female, 12/8; with an average age of 36.75±4.99 years) were enrolled. After training, the interobserver agreements for the LI-RADS category for all radiologists ($p=0.005$) were increased. The sensitivity, specificity, positive predictive value, negative predictive value, and coincidence rate of all radiologists for HCC diagnosis before and after training were 43% vs. 54%, 86% vs. 88%, 74% vs. 81%, 62% vs. 67%, and 65% vs. 71%, respectively. The diagnostic performances of all radiologists ($p<0.001$) showed improvement after training. ***Conclusions:*** The systematic training of LI-RADS can effectively improve the diagnostic performances of radiologists with different experiences for HCC.

Citation of this article: Ren AH, Xu H, Yang DW, Zhang N, Ba T, Wang ZC, *et al*. Systematic training of liver imaging reporting and data system magnetic resonance imaging v2018 can improve the diagnosis of hepatocellular carcinoma for different radiologists. J Clin Transl Hepatol 2021;9(4):537–544. doi: 10.14218/JCTH.2021.00180.

Introduction

Primary liver cancer is currently the seventh most frequently occurring cancer and the second most common cause of cancer mortality in the world.[1,2] Hepatocellular carcinoma (HCC) accounts for >80% of primary liver cancers worldwide.[3] Early diagnosis of HCC can significantly improve survival, with liver imaging playing a critical role in detecting and diagnosing HCC early, especially the contrast-enhanced magnetic resonance imaging (MRI).[4] There are several clinical practice guidelines for HCC, such as guidelines endorsed by the American Association for the Study of Liver Diseases (AASLD), European Association for the Study of the Liver (EASL), and National Comprehensive Cancer Network (NCCN).[4–6]

The Liver Imaging Reporting and Data System (LI-RADS) is a comprehensive system endorsed by the American College of Radiology (ACR) for standardizing the terminology, interpretation and reporting of liver imaging in patients at risk for or with HCC.[7] In the LI-RADS v2018 computed tomography (CT)/MRI manual, the entire spectrum of hepatic lesions and pseudolesions that may occur in patients at high-risk of HCC, each LI-RADS category, and the major and ancillary features visible on CT and MRI are addressed in detail, with basic concepts, systematic descriptions, and numerous schematic diagrams and examples.[8] Therefore, LI-RADS can be used for radiologist education and training in addition to clinical care, as it is designed to increase the knowledge of radiologists, improve radiologists' diagnostic skills and reduce imaging interpretation variability and errors.[8] Consequently, the dissemination and application of LI-RADS are very important for the diagnosis of HCC. However, there are few studies concerning the value of systematic LI-RADS training for HCC diagnosis, with very limited knowledge about the necessity and effect of LI-RADS training.

Therefore, the goal of this study was to explore whether the systematic LI-RADS MRI v2018 training can effectively improve the diagnostic performance of radiologists with different experiences for HCC in high-risk patients. In addition, we assessed the interobserver agreements of the LR category for all participants before and after systematic LI-RADS training.

Keywords: Magnetic resonance imaging; Liver neoplasm; Diagnostic imaging; Training program; Liver Imaging Reporting and Data System.

Abbreviations: AASLD, American Association for the Study of Liver Diseases; CT, computed tomography; HCC, hepatocellular carcinoma; LI-RADS, liver imaging reporting and data system; MRI, magnetic resonance imaging.

[*]**Correspondence to:** Zheng-Han Yang, Department of Radiology, Beijing Friendship Hospital, Capital Medical University, 95 YongAn Road, Beijing 100050, China. ORCID: https://orcid.org/0000-0003-3986-1732. Tel: +86-10-6313-8490, Fax: +86-10-6313-8625, E-mail: yangzhenghan@vip.163.com

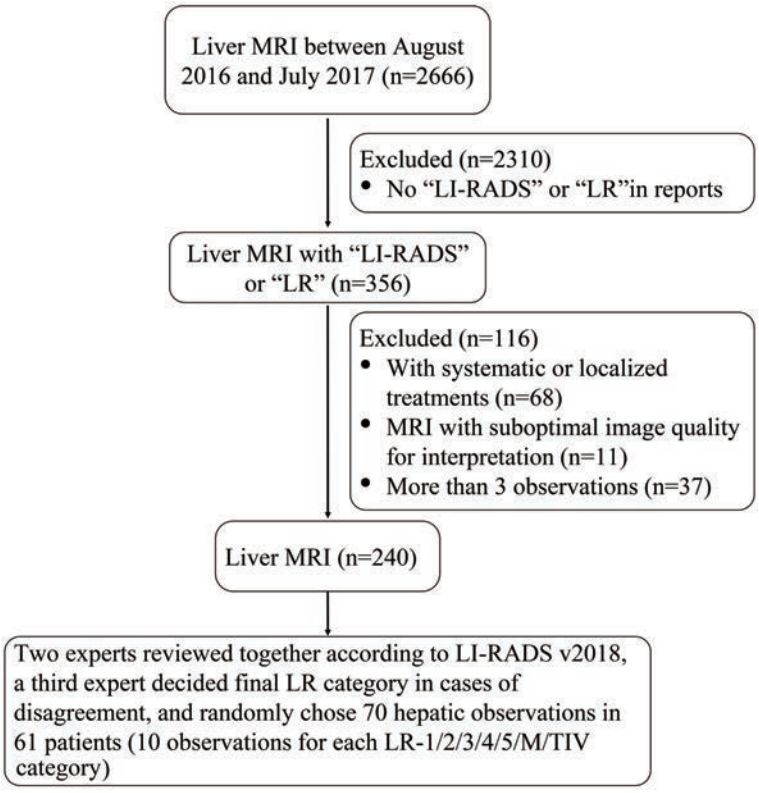

Fig. 1. Flowchart of the patients enrolled in this study.

Methods

Ethics statement

This prospective single-center study was approved by the Institutional Review Board of our hospital (2020-P2-220-01), and informed consent was obtained from all enrolled radiologists. The requirement for informed consent from patients was waived, as they were retrospectively reviewed and enrolled. This study was performed within 6 months at the hospital of the lead author, from August 2019 to January 2020.

Patient selection

Consecutive liver MRI reports from August 2016 to July 2017 were reviewed and filtered using the terms "LI-RADS" or "LR" in our picture archiving and communication system (PACS) (DJ Health Union Systems Corporation, Shanghai, China). The inclusion criteria were as follows: 1) patients with a high risk of HCC, including those with cirrhosis or chronic hepatitis B viral infection; and 2) patients with at least one hepatic observation in the LR category. The exclusion criteria were as follows: 1) patients without the above risk factors, those <18 years-old, and those with cirrhosis due to congenital hepatic fibrosis or vascular disorder; 2) patients who had accepted any locoregional or systemic treatment concerning hepatic observations; 3) patients with more than three hepatic observations; and 4) MR examinations that did not satisfy the technical recommendation of LI-RADS v2018 or those with poor image quality as assessed by three experienced radiologists (with 11 [AHR], 15 [HX] and 32 [ZHY] years of experience in abdominal imaging).[8] As these consecutive cases were reported according to LI-RADS v2014 or v2017, all hepatic observations were firstly recategorized by two experienced radiologists working together (with 11 [AHR] and 15 [HX] years of experience in abdominal imaging) and according to LI-RADS v2018. In cases of disagreement on LI-RADS category, a third radiologist with 32 years of experience (ZHY) decided the final LI-RADS category. Finally, 70 hepatic MRI observations from 61 patients with a high risk of HCC were enrolled in this study, with 10 observations per LR category (LR-1/2/3/4/5/LR-M/LR-TIV) (Fig. 1). All these three radiologists were specialists of the LI-RADS CT/MRI algorithm, had adopted the LI-RADS algorithm in routine work for more than 5 years and were very familiar with the update and revisions of v2018.

Subjects

A total of 30 Residents or Fellows with different levels of experience in abdominal MRI diagnosis coming from other hospitals/institutions in China to our department as visiting scholars for at least 6 months were included in this study. All participants were asked to complete a questionnaire to collect baseline demographic information at the beginning of this study. The contents of the questionnaire included the classification and category of their hospitals/institutions, experience in abdominal MRI (years), number of abdominal MRI reports reviewed per day, and extent of knowledge about LI-RADS before training. A total of 10 participants who failed to complete the entire training procedure were excluded. Finally, 20 participants with different experiences were enrolled in this study (Fig. 2).

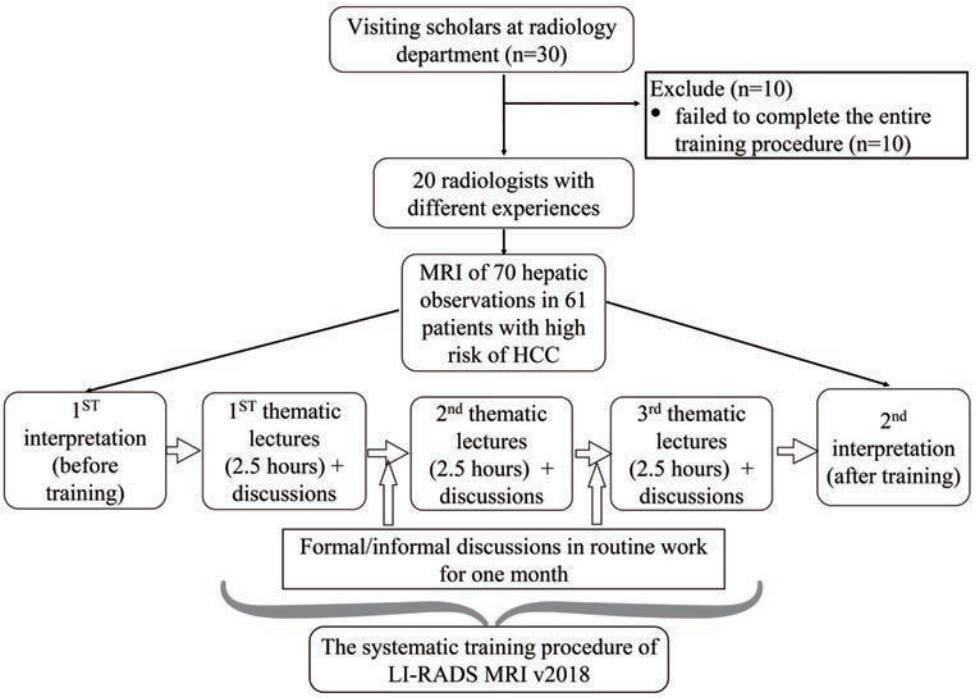

Fig. 2. Flowchart of the systematic LI-RADS training procedure. LI-RADS, liver imaging reporting and data system.

MRI protocol

All patients underwent MR examinations with 1.5-T (Signa HDxt 1.5T; GE Healthcare, Chicago, IL, USA) or 3.0-T (Discovery 750w from GE Healthcare; MAGNETOM Prisma from Siemens AG, Munich, Germany; Ingenia from Philips Healthcare, Amsterdam, The Netherlands) MRI scanners with an 8/16-element phased array coil. The liver MRI technique is summarized in Supplemental Table 1 (online). All patients underwent MRI using gadobenate dimeglumine (Magnevist; Bayer Schering Pharma AG, Berlin, Germany), which was intravenously injected at a dose of 0.1 mmol/kg and a rate of 2 mL/s followed by a normal saline flush. After the administration of contrast agent, dynamic T1-weighted imaging (T1WI) was obtained in the late arterial phase (30–40 s after injection), portal venous phase (60–70 s after injection), equilibrium phase (3–4 m after injection), and delayed phase (5–8 m after injection).

Systematic LI-RADS MRI v2018 training procedure

The CT/MRI LI-RADS algorithm has been adopted daily at the Radiology Department of our institution since October 2015, from v2014 to v2018. The LI-RADS CT/MRI v2018 training procedure included three thematic lectures given by a professor (ZHY, PhD, MD) with 32 years of experience, who specialized in imaging diagnosis of liver neoplasms and was well versed in the application of the LI-RADS CT/MRI algorithm. The major topics of the lectures included an introduction of the LI-RADS categories and explanations of the major and ancillary features, and the typical manifestations of each category and feature, with plenty of cases (Supplementary File 1). Of note, the three lectures were almost the same, except a few subtle changes according to reader feedback. Electronic instructional materials, including slideshows, journal articles, and recorded lectures, were shared with the participants to facilitate the training process. Each seminar lasted for 2.5 h, with an interval of a month. After the former two seminars, the participants had a month to learn, practice, and adopt the LI-RADS MRI v2018 algorithm in daily work. During these 2 months, they reported the MR of routine patients, including LI-RADS practice in proper patients, and this was also a part of the training. Moreover, formal discussions concerning LI-RADS in specific cases proceeded twice per week, and each discussion lasted for ≥30 m during the 2 months. In addition, informal discussions were carried out whenever necessary during the training procedure. The flow chart of the systematic LI-RADS training procedure is displayed in Figure 2.

Imaging interpretations

All MRI data were transferred to the workstations, and imaging analyses were anonymously performed on PACS. All MR images were interpreted separately by 20 participants twice according to the LI-RADS v2018 algorithm, once before the training and once after the 3rd systematic LI-RADS training.[8] The participants were informed about the localization and size of hepatic observations, which was preliminarily provided by one of our radiologists with 11 years of experience (AHR). All image interpretations, both before and after training, were recorded as structured LI-RADS template reports (Supplemental Table 2, online), which were designed before training. All participants were blinded to any clinical information, the number of each LR category, the imaging reports, and the pathological results. The order of MRI exams to be reviewed was randomized for each participant. However, for the assessment of threshold growth, a prior examination (CT or MRI) was used when available. All hepatic observations were interpreted based on major and ancillary features in combination according to LI-RADS v2018.[8] The ancillary feature of ultrasound visibility as a discrete nodule was not used, while the tiebreaking rules

Table 1. Characteristics of hepatic observations and risk factors of HCC

Characteristic		Total of 61 patients and 70 observations
Age in years	37–84, average 59.5±10.1	–
Sex	Male	47 (77.1%)
	Female	14 (22.9%)
Risk factors	HBV	45 (73.8%)
	HCV	3 (4.9%)
	HBV+HCV	2 (3.4%)
	Alcoholic liver cirrhosis	3 (4.9%)
	HBV+alcoholic liver cirrhosis	3 (4.9%)
	NAFLD/NASH	1 (1.6%)
	PBC	1 (1.6%)
	Cryptogenic cirrhosis	3 (4.9%)
Observation characteristic	HCC	36 (51.4%)
	iCCA	5 (7.2%)
	HChC	1 (1.4%)
	Epithelioid hemangioendothelioma	1 (1.4%)
	Benign lesions	27 (38.6%)

HBV, hepatitis B virus; HChC, combined hepatocellular-cholangiocarcinoma; HCV, hepatitis C virus; iCCA, intrahepatic cholangiocarcinoma; NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; PBC, primary biliary cirrhosis; HCC, hepatocellular carcinoma.

were used at the participants' discretion if needed.

Reference standard

The high risk for HCC and final clinical diagnoses of 61 patients with 70 liver observations are displayed in Table 1. Of these, 52 patients underwent a single observation, while the other nine patients underwent two observations. For observations with histopathological diagnoses, pathological diagnoses were used as the gold standard. For those who were diagnosed with HCC without histopathology, follow-up imaging demonstrated substantial growth associated with arterial phase hyperenhancement and washout or enhancement of the capsule.[9] The reference standards for LR-1/2/3 observations were based on typical imaging findings or the absence of progression to a malignant category (LR-4, LR-5, LR-M or LR-TIV) during the follow-up period.[10,11] These patients were followed-up for at least 2 years. The LR category and diagnostic methods of all hepatic observations are displayed in Table 2.

Table 2. LR category and diagnostic method of enrolled hepatic observations

LR category	Diagnostic method	Diagnosis	Number	Total
LR-1	Imaging+clinical	Cyst/perfusion alteration/hemangioma	8/1/1	10
	Pathology	–	–	
LR-2	Imaging+clinical	RN/DN/cyst/hemangioma	5/2/1/2	10
	Pathology	–	–	
LR-3	Imaging+clinical	RN/DN/HCC/coagulative necrosis/chronic fibrosis	1/3/2/1/1	10
	Pathology	DN/HCC	1/1	
LR-4	Imaging+clinical	HCC	7	10
	Pathology	HCC	3	
LR-5	Imaging+clinical	HCC	1	10
	Pathology	HCC	9	
LR-M	Imaging+clinical	HCC/iCCA	1/1	10
	Pathology	HCC/iCCA/HChC	3/3/2	
LR-TIV	Imaging+clinical	HCC	5	10
	Pathology	HCC/iCCA/epithelioid hemangioendothelioma	1/3/1	

TIV, tumor in vein; HChC, combined hepatocellular-cholangiocarcinoma; RN, regenerative nodule; DN, dysplastic nodule; iCCA, intrahepatic cholangiocarcinoma; HCC, hepatocellular carcinoma.

Table 3. Basic characteristics in related experience of enrolled 20 participants

Characteristics	Total
Sex	
Male	12 (60%)
Female	8 (40%)
Age in years	36.75 ± 4.99
≤35	10 (50%)
35–50	10 (50%)
Post-graduate year	
≤5	7 (35%)
5–10	7 (35%)
>10	6 (30%)
Classification of hospitals/institutions	
3A	13 (65%)
3B	3 (15%)
2A	4 (20%)
Experience of abdominal MRI in years	
<5	9 (45%)
5–10	9 (45%)
≥10	2 (10%)
Number of abdominal MRI reports per day	
<5	12 (60%)
5–10	6 (30%)
≥10	2 (10%)
Extent of knowledge about LI-RADS before training	
Very familiar, adopt in MRI reports	0 (0%)
General understanding, did not use in MRI reports	8 (40%)
Heard of, did not use in MRI reports	10 (50%)
Not familiar at all	2 (10%)

LI-RADS, liver imaging reporting and data system; MRI, magnetic resonance imaging.

Statistical analysis

Raw data and cleaned data were stored in Excel, and statistical analysis was performed with Stata statistical software version 13.1 (https://www.stata.com/). The distribution of ordinal categorical data between groups was compared by the Wilcoxon rank-sum (Mann-Whitney) test after rank transformation. Proportions were compared using the chi-squared test. Indicators of diagnostic accuracy were calculated for each participant before and after training using the formulas as follows.[12] In this study, LR-5 was used as a predictor of HCC.[8,13] Compared to the final diagnosis of the sampled MRIs, the number of true positive (TP), false positive (FP), true negative (TN), and false negative (FN) findings were extracted, and a 2×2 table was constructed. The sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), coincidence rate, positive likelihood ratio (+LR) and negative likelihood ratio (−LR) were calculated (Supplementary File 1). The means with 95% confidence intervals (95% CIs) of these indicators were calculated separately for all participants. The hierarchical summary receiver operating characteristic package was used to calculate the pooled estimates of the different operators. The inter-observer agreement among radiologists with different experience levels was calculated directly. If all radiologists classified the same LR category for one hepatic lesion, then it was considered as consensus. As long as there was a different LR category assessed by any one of these radiologists, it was considered as inconsistent. Then, the percentage and 95% CI were calculated. A *p*-value of less than 0.05 was regarded as statistically significant.

Results

Characteristics of all participants

The demographic information of the participants enrolled in this training program is described in Table 3. In China, hospitals are classified into three tiers, each with three sub-levels (A, B and C), and the highest ranking is 3A. All 20 participants were general radiologists, and none of them

Table 4. Comparison of diagnostic performance of all participants for HCC before and after systematic LI-RADS training

	Before training	After training
Sensitivity	0.43 (0.37–0.50)	0.54 (0.51–0.56)
Specificity	0.86 (0.82–0.89)	0.88 (0.86–0.90)
PPV	0.74 (0.70–0.78)	0.81 (0.79–0.84)
NPV	0.62 (0.60–0.64)	0.67 (0.66–0.68)
Coincidence rate	0.65 (0.62–0.67)	0.71 (0.70–0.73)
+LR	3.60 (2.62–4.58)	5.14 (4.28–6.01)
−LR	0.66 (0.60–0.72)	0.53 (0.50–0.55)
AUC	0.64 (0.62–0.67)	0.71 (0.70–0.72)
p-value	<0.001	

LI-RADS, liver imaging reporting and data system; HCC, hepatocellular carcinoma; +LR, positive likelihood ratio; −LR, negative likelihood ratio; PPV, positive predictive value; NPV, negative predictive value; AUC, area under the curve.

were primarily liver specialists. These participants were further classified into junior and senior subgroups according to their seniorities (Supplemental Table 3).

Interobserver agreements of the LR category before and after systematic training

The comparison results of interobserver agreement of LR category for overall, junior and senior radiologists before and after systematic LI-RADS training are demonstrated in Supplemental Table 4. Before LI-RADS training, the participants had a relatively low level of agreement on the diagnosis of 70 hepatic observations on MRI. The diagnosis of only 17 hepatic observations was agreed upon by all 20

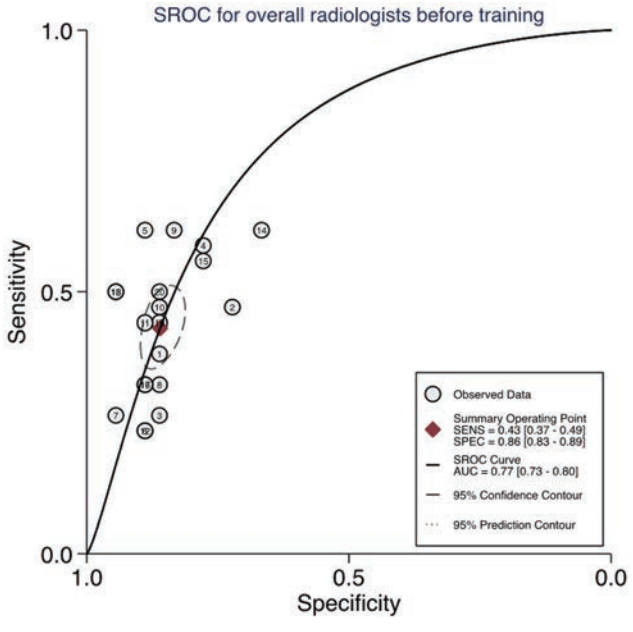

Fig. 3. Hierarchical summary receiver operating characteristic curves for the MRI diagnosis of all the enrolled participants before LI-RADS training. Circles with numbers represent each participant, and dotted lines represent the credible interval. SROC, summary receiver operating characteristic curve; LI-RADS, liver imaging reporting and data system.

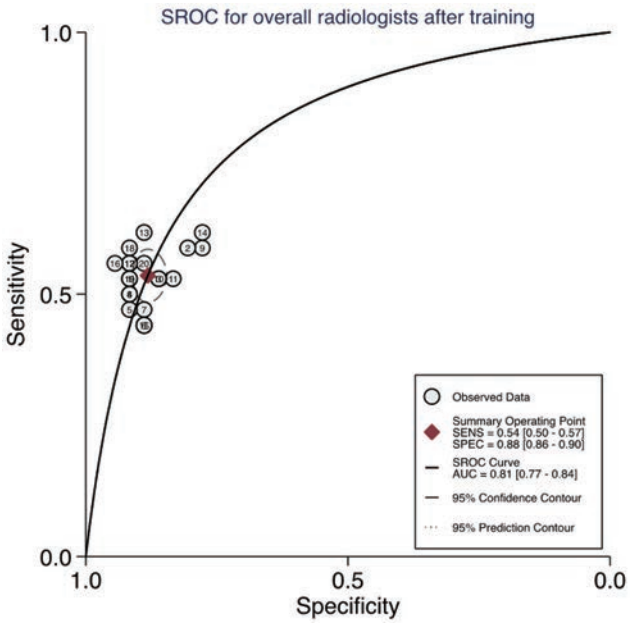

Fig. 4. Hierarchical SROC curves for the MRI diagnosis of all the enrolled participants after LI-RADS training. Circles with numbers represent each participant, and dotted lines represent the credible interval. SROC, summary receiver operating characteristic curve; LI-RADS, liver imaging reporting and data system; MRI, magnetic resonance imaging.

radiologists, making their interobserver agreement 0.243 (0.148–0.360). After systematic LI-RADS training, a total of 33 hepatic observations reached a consensus with an interobserver agreement of 0.471 (0.351–0.594) for all participants, including 24 observations they did not regard as HCC and 9 observations they agreed on as an HCC diagnosis. After systematic LI-RADS training, the interobserver agreements of the LR category for overall, junior and senior participants are significantly increased ($p<0.001$).

Diagnostic performance for HCC before and after systematic training

The comparison results of the diagnostic performance of the overall, junior and senior participants for HCC before and after systematic LI-RADS training are shown in Table 4 and Supplemental Table 5. The sensitivity of their diagnosis of HCC improved from 0.43 (0.37–0.50) to 0.54 (0.51–0.56), and the PPV improved from 0.74 (0.70–0.78) to 0.81 (0.79–0.84) ($p<0.001$). The diagnostic performances of both junior and senior radiologists were all increased after systematic training of LI-RADS (junior, $p=0.037$; senior, $p=0.004$). The area under the curve also improved with statistical significance among overall, junior and senior participants after training ($p<0.001$) (Figs. 3–5; Supplemental Figs. 1, 2).

Discussion

In this study, 20 participants with different abdominal imaging experiences, serving as visiting scholars in our department, underwent systematic training with the newest version of the LI-RADS algorithm, and their interobserver agreements and diagnostic performance outcomes for diagnosing HCC on MRI before and after training were compared. Our results showed that the interobserver agreement for the LR category for all

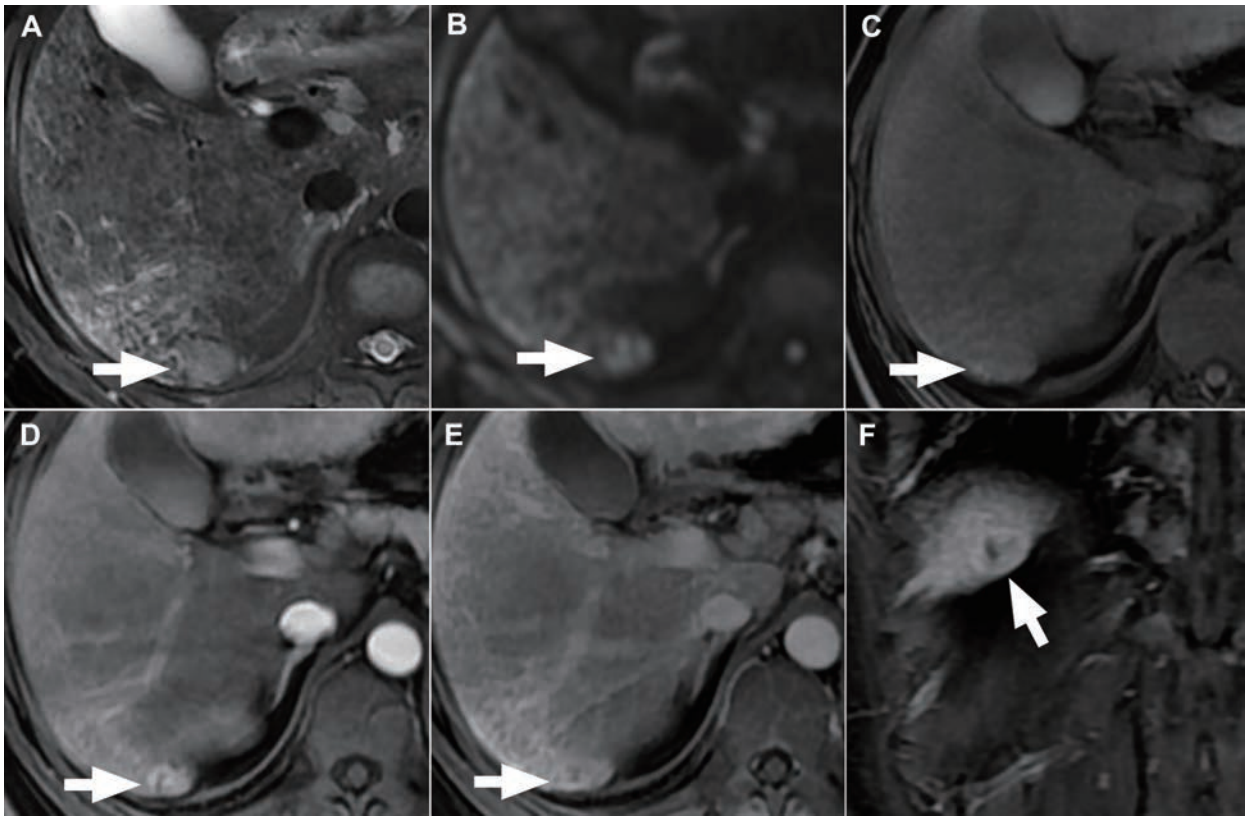

Fig. 5. A 46 year-old man with alcoholic liver cirrhosis. The 1.8-cm observation at segment VI displayed moderate T_2WI hyperintensity (A), with restricted diffusion on DWI (B), with nonrim APHE on the late arterial phase image (D) compared with the precontrast enhanced T_1WI (C), with nonperipheral washout on portal venous phase image (E) and enhancing capsule on the coronal image of delayed phase (F). The observation was preliminarily categorized as LR-5 by three experts according to LI-RADS v2018, and the diagnosis of HCC was confirmed by pathology after partial hepatectomy. However, the observation was misclassified as LR-4 for unfamiliar with washout appearance or enhancing capsule by participants before systematic training. APHE, arterial phase hyperenhancement; DWI, diffusion-weighted image; T_1WI, T_1-weighted image; T_2WI, T_2-weighted image; LI-RADS, liver imaging reporting and data system.; HCC, hepatocellular carcinoma.

participants was significantly increased after systematic training. The diagnostic performance of all participants for HCC was significantly increased after systematic training.

In this study, we performed systematic LI-RADS training v2018 with 20 participants at an academic radiology department. Our institution has a national key cultivation discipline of gastroenterology and hepatology and a liver transplant center, with sufficient patients with focal liver lesions undergoing MR examinations. In addition, LI-RADS has been introduced and adopted in daily work in our radiology department for 5 years, with updates to the newest version of the algorithm.[8,14–16] Therefore, our lead radiologists have considerable experience with LI-RADS and have devoted much effort to disseminating LI-RADS in China. In addition, our institution is a teaching hospital, so we attach great importance to the training of residents and the continuing medical education of visiting scholars.

Davenport *et al*.[17] compared the repeatability of diagnostic features and different scoring systems for HCC on MRI between five fellowship-trained radiologists and five novice radiology residents at a liver transplantation center. They reported a fair overall inter-reader agreement (0.35 [95% CI: 0.34, 0.37]) for LI-RADS v2013.1, which was slightly lower than our results after training. However, they did not perform systematic training for radiologists, and the participants were given only 1 h of lecture-based and hands-on instructions concerning each liver observation scoring system.[17] They did not compare the diagnostic performance outcomes of the experts and novice radiologists. LI-RADS is currently consistent with the AASLD and NCCN guidelines and fully integrated into AASLD clinical practice guidance.[14] AASLD does not have an official definite scoring system, and the Organ Procurement and Transplantation Network (commonly referred to as OPTN) is a unique system for transplantation adopted in the USA.[4,18] Therefore, we only evaluated the systematic training effect of LI-RADS in this study. In our study, the interobserver agreements of all radiologists for the LR category were increased after systematic training. Our results of the inter-reader agreement for the LR category are slightly greater than those of Kang *et al*.[9] and Fowler *et al*.[19]

There are relatively few studies concerning the dissemination of HCC diagnosis guidelines. Elmohr *et al*.[20] discussed the feasibility and efficacy of the concept of teaching teachers in disseminating and motivating the application of the LI-RADS v2018 clinical practice guideline. They used different teaching methods for different continents and countries, with a total of 8,342 attendees participating in their study. We implemented a systematic training program with 20 participants with different experience levels using the hybrid method of classroom training combined with a one-on-one model. Our results reveal that the systematic training model can effectively improve the diagnostic performance of the attendees for HCC. All these visiting scholars came from different provinces and districts in China, and they may subsequently disseminate the LI-RADS v2018 clinical practice guideline in their own hospitals/institutions. Unfortunately, we did not study how much of this training was retained at 6 months or 1 year after the training.

In this study, the improvement in the interobserver agreement and diagnostic performance for HCC after training for all participants was real and expected. The possible reasons for the improvements of the interobserver agreement and diagnostic performance in doctors are based on learning and training. However, it is rather modest. Less than half of the observations were correctly classified, possibly because all participants were general radiologists and not liver specialists, although LI-RADS is supposed to be a standard and straightforward tool. Another reason may be that all participants came from different classifications of hospitals and reported an average number of abdominal MRIs of only 3.9±3.31.

Limitations

This study had several limitations. First, the number of lesions assessed was too small. We included only 10 hepatic observations per LR category, which is not enough for robust analysis. We would include more cases to verify and improve the reliability of the result in a future investigation. Second, this was a single-center retrospective study, and selection bias of patients inevitably exists. Third, the three expert radiologists did not review the enrolled cases independently, and the inter-reader agreement between them was not assessed. However, our previous study displayed a good intraclass correlation coefficient (0.965 [95% CI: 0.956–0.972]) for the LR category among these three radiologists adopting LI-RADS v2018. Fourth, 85.2% (52/61) of the patients had a single observation in this study. This may not represent the daily routine in other hospitals; perhaps this is a bias related to the local recruitment of a transplantation center. Fifth, in terms of real-life applicability, a limitation may be that most radiology departments do not have 7.5 h available to devote to formal LI-RADS training didactics.

Conclusions

In conclusion, the systematic LI-RADS training can effectively improve the diagnostic performance and the interobserver agreements of radiologists with different experience levels for HCC, both for junior and senior radiologists.

Funding

This work was supported by funds from the National Natural Science Foundation of China (Nos. 61871276 and 82071876), Beijing Municipal Administration of Hospitals Clinical Medicine Development of Special Funding Support (No. ZYLX202101), Research Foundation of Beijing Friendship Hospital (No. yyqdkt2019-30), and Cultivation Scientific Research Foundation of Capital Medical University (No. 1210020247).

Conflict of interest

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

Author contributions

Study concept and design (AHR, ZHY, ZCW), acquisition of data (AHR, NZ, TB), analysis and interpretation of data (AHR, HX, DWY), drafting of the manuscript (AHR), critical revision of the manuscript for important intellectual content (ZHY, ZCW), and administrative, technical, or material support, study supervision (ZHY).

Data sharing statement

The data concerning LI-RADS v2018 training used in support of the findings of this study are included within the supplementary information file(s) accompanying this publication in the [Xia & He Publishing Inc. Journal of Clinical and Translational Hepatology].

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Journal of Clinical and Translational Hepatology **2021** vol. 9(4) | 545–550
DOI: 10.14218/JCTH.2020.00098

Original Article

COVID-19 in Liver Transplant Recipients

Ravina Kullar[1], Ankur Prakash Patel[2] and Sammy Saab[2*]

[1]Expert Stewardship, Inc., Newport Beach, CA, USA; [2]David Geffen School of Medicine at the University of California at Los Angeles, Los Angeles, CA, USA

Received: 28 October 2020 | **Revised:** 11 December 2020 | **Accepted:** 4 February 2021 | **Published:** 22 February 2021

Abstract

Background and Aims: Coronavirus disease 2019 (COVID-19) has infected over 93 million people worldwide as of January 14, 2021. Various studies have gathered data on liver transplant patients infected with COVID-19. Here, we discuss the presentation of COVID-19 in immunosuppressed patients with prior liver transplants. We also evaluate patient outcomes after infection. ***Methods:*** We searched the PubMed database for all studies focused on liver transplant patients with COVID-19. ***Results:*** We identified eight studies that evaluated COVID-19 infection in liver transplant patients (n=494). Hypertension was the most prevalent comorbidity in our cohort. Calcineurin inhibitors were the most common immunosuppressant medications in the entire cohort. The average time from liver transplant to COVID-19 infection in our cohort was 74.1 months. Fever and cough, at 70% and 62% respectively, were the most common symptoms in our review. In total, 50% of the patients received hydroxychloroquine as treatment for COVID-19. The next most prevalent treatment was azithromycin, given to 30% of patients in our cohort. In total, 80% of the patients were admitted to a hospital and 17% required intensive care unit-level care, with 21% having required mechanical ventilation. Overall mortality was 17% in our review. ***Conclusions:*** Given the immunocompromised status of liver transplant patients, more intensive surveillance is necessary for severe cases of COVID-19 infection. As liver transplantations have been restricted during the COVID-19 pandemic, further investigation is warranted for studying the risk of COVID-19 infection in liver transplant patients.

Citation of this article: Kullar R, Patel AP, Saab S. COVID-19 in liver transplant recipients. J Clin Transl Hepatol 2021;9(4):545–550. doi: 10.14218/JCTH.2020.00098.

Keywords: COVID-19; Liver transplantation; Immunosuppression.

Abbreviations: AASLD, American Association for Study on Liver Diseases; ACE2, angiotensin-converting enzyme 2; AZM, azithromycin; CDC, Center for Disease Control and Prevention; CNI, calcineurin inhibitor; COVID-19, coronavirus disease 2019; DM, diabetes mellitus; HCQ, hydroxychloroquine; HCV, hepatitis C virus; HTN, hypertension; ICU, intensive care unit; IFN-B, interferon-beta; MMF, mycophenolate mofetil; SARS-CoV-2, severe acute respiratory syndrome-coronavirus-2; SOT, solid organ transplantation; TOZ, tocilizumab; WHO, World Health Organization.

***Correspondence to:** Sammy Saab, Pfleger Liver Institute, UCLA Medical Center, 200 Medical Plaza, Suite 214, Los Angeles, CA 90095, USA. Tel: +1-310-206-6705, Fax: +1-310-206-4197, E-mail: SSaab@mednet.ucla.edu

Introduction

The coronavirus disease (COVID-19) caused by the severe acute respiratory syndrome-coronavirus-2 (SARS-CoV-2) has resulted in over 107 million documented cases globally and close to 2.5 million deaths, as of February 10, 2021.[1] On March 11, 2020, the World Health Organization (WHO) classified COVID-19 as a pandemic.[2] The severity of COVID-19 in the general population ranges from asymptomatic/mild symptoms to critically ill in a proportion of patients.[3] The Centers for Disease Control and Prevention (CDC) has recognized that those with certain underlying medical conditions are at increased risk for severe illness from COVID-19, including cancer, chronic kidney disease, liver disease, chronic obstructive pulmonary disease, obesity, type 2 diabetes mellitus, serious heart conditions, respiratory diseases, and immunocompromised status, including those requiring immunosuppression following solid organ transplantation (SOT).[3]

Accordingly, liver transplantation is the second most common SOT globally after kidney transplantation, with the overall rate reported at 3.7 per million population.[4] In developed countries, hepatitis C virus (commonly known as HCV) is the primary reason for liver transplant; however, HCV is now being replaced by alcoholic liver disease, non-alcoholic liver disease, and hepatocellular carcinoma.[5] Based on familiarity with viral respiratory infections in patients with SOT, the clinical presentation of COVID-19 would likely be more severe in these liver transplant recipients, as they are immunosuppressed and, therefore, have a weaker immune system. To date, no donor-derived COVID-19 cases have been reported. Angiotensin-converting enzyme 2 (often referred to as ACE2), which is a receptor for SARS-CoV-2, is present in almost all organs, including the lung, heart, kidney, liver, and intestine.[6] Therefore, SARS-CoV-2 viremia could potentially infect any transplant organ and suppress itself until the immunosuppressed status exists. In a survey conducted in >80 major transplant centers in the USA between March 24 and March 31, 2020, 31 (35.2%) centers reported 148 patients with SOT with COVID-19 overall.[7] Of these patients, 80 (54.1%) were mildly symptomatic, 31 (20.9%) were moderately symptomatic with pneumonia, and 37 (25.0%) were critically ill. These findings suggest a greater disease severity compared with patients without SOT with COVID-19. Further, SOT recipients who have COVID-19 may shed greater amounts of virus for longer durations compared to non-immunosuppressed patients.[8] This has been shown in other viruses as well, such as influenza. In immunocompetent adults, the majority of patients shed influenza for a maximum of 5 days.[9] Conversely, a study of allogeneic hematopoietic stem cell transplant) recipients with influenza revealed that the mean duration of viral shedding was 7 days (range: 2–37 days); in those patients

who received no therapy, the mean duration of shedding was 11.3 days.[10]

Transplantations have been restricted due to the high risk of serious COVID-19 infection in this population as well as risk of transmission in health care workers. In fact, there has been a steep decline in organ donations and SOT (for kidney, liver, heart, and lung) procedures since the beginning of the COVID-19 pandemic in the USA, decreasing by 51.1%.[11] The American Association for Study on Liver Diseases (commonly known as the AASLD) recommends that liver transplantation should be limited to emergency cases (e.g., patients with high model for end-stage liver disease scores) or hepatocellular carcinoma patients who are at risk of disease progression and removal from the waiting list.[12]

Limited data exist from evaluations of the impact of COVID-19 on liver transplant recipients. In this paper, we review real-world studies evaluating the effect of COVID-19 in liver transplant recipients.

Methods

Search strategy

We searched the PubMed database for all studies focused on liver transplant patients with COVID-19. We used a combination of the keywords 'COVID-19', 'SARS-CoV-2', and 'liver transplant' in our literature search. The PubMed search was limited to articles published on 1/1/2020 or later to ensure the search was more specific to COVID-19 articles only. The search was run on 1/14/2021.

Inclusion and exclusion criteria

We included all studies published in scientific journals that provided information on COVID-19 infection in liver transplant patients. Only studies focused on COVID-19 were included in data collection, and studies discussing SARS, MERS, or other infections were excluded from data collection. Only studies with liver transplant patients were included; papers including non-transplant patients, pediatric papers, and papers without patient data were excluded. Case reports were excluded, as well as papers that did not exclusively focus on liver transplant patients. Only papers with data on five or more patients in their cohort were included. We collected data on country of origin, size of cohort, patient comorbidities, time from liver transplant to COVID-19 infection, immunosuppressant medications, COVID-19 symptoms, COVID-19-specific treatments, hospitalization rate, intensive care unit (ICU) admission rate, need for mechanical ventilation, and mortality rate.

Results

The results of our review of the literature yielded eight studies containing data on liver transplant patients infected with COVID-19 (Fig. 1 and Table 1).[13–20] Patients from our review were from Europe, North America, South America, and Asia. Six studies with patients from Europe were included in our analysis.[13,15–18,20] The total number of patients in our cohort was 494. The largest study we included had 151 patients.[20] The majority of patients were men. The mean patient age was 62.7. Fifty-one percent of patients in our cohort had hypertension as a comorbidity. The next most prevalent comorbidity was diabetes mellitus. Calcineurin inhibitors (CNIs) were the most prevalent immunosuppressant medications among the entire cohort. The next most common immunosuppressant was mycophenolate mofetil (MMF), followed by steroids.

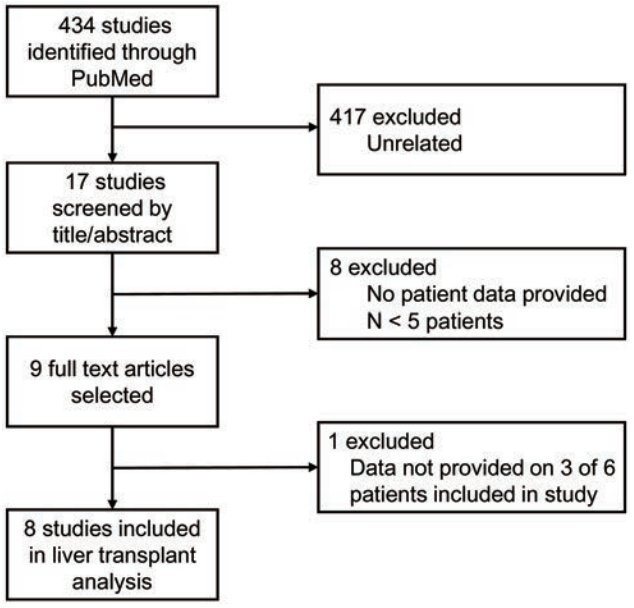

Fig. 1. Flowchart for literatures review of the liver transplant patients with COVID-19 from PubMed.

All studies provided data on symptoms, treatments, and outcomes of COVID-19-infected liver transplant patients (Table 2).[13–20] The average time from liver transplant to COVID-19 infection in our cohort was 74.1 months. Fever and cough, at 70% and 62% respectively, were the most common symptoms in our review. In total, 50% of patients received hydroxychloroquine (HCQ) as treatment for COVID-19. The next most prevalent treatment was azithromycin, given to 30% of patients in our cohort. Eighty percent of the entire cohort was admitted to a hospital. Seventeen percent of patients required ICU-level care. Twenty-one percent of patients required mechanical ventilation. Overall mortality was 17% in our review.[13–20]

Webb *et al*[20] reported the largest cohort in our review, with 151 liver transplant patients. Of these patients, 82% were admitted, with 28% requiring ICU-level care. Thirty patients required mechanical ventilation, and twenty-eight patients died. Waisberg *et al*[14] reported data on five patients from Brazil with an average time from liver transplant to COVID-19 infection of 0.56 months, which was the shortest in our cohort. All five of those patients had been admitted and two died. One study reported data on 38 patients from the USA,[19] with 71% of that cohort having been admitted, 21% requiring ICU-level care, and 18% having died.

Discussion

As the COVID-19 pandemic continues to spread and impact the entire world, SOT recipients are at high risk of infection and poor outcomes due to high rates of pre-existing conditions in addition to chronic immunosuppression. Here, we provide one of the largest reviews of COVID-19 in liver transplant recipients. With a median age of 64 years, over 50% and 40% of patients had hypertension and diabetes, respectively, as a comorbidity. The most common immunosuppressive agents used were CNIs (82%), MMF (39%), and steroids (26%). There are inadequate data on the relationship between immunosuppressive therapy and COVID-19

Table 1. Demographic data of COVID-19-infected liver transplant patients

	Study								Summary
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	
Author[Ref]	Colmenero *et al*[13]	Waisberg *et al*[14]	Becchetti *et al*[15]	Loinaz *et al*[16]	Patrono *et al*[17]	Belli *et al*[18]	Lee *et al*[19]	Webb *et al*[20]	
Country	Spain	Brazil	Europe[c]	Spain	Italy[d]	Europe (Italy, Spain, France)	US	International[e]	
Cohort Size	111	5	57	19	10	103	38	151	Total (*n*) 494; Average (*n*) per study 61.8
Age	Mean 65.3	Mean 59.6	Median 65	Median 58	Mean 65.6	Median 65	Median 63	Median 60	Mean 62.7 Median 64
Sex, M/F	79/32 (71%/29%)	4/1 (80%/20%)	40/17 (70%/30%)	14/5 (74%/26%)	8/2 (80%/20%)	76/27 (74%/26%)	26/12 (68%/32%)	102/49 (68%/32%)	349/145 (71%/29%)
Comorbidities									
HTN	64 (58%)	3 (60%)	32 (56%)	10 (53%)	Unknown	52 (50%)	24 (63%)	63 (42%)	248 (51%)
DM	53 (48%)	1 (20%)	21 (37%)	6 (32%)	Unknown	41 (40%)	18 (47%)	65 (43%)	205 (42%)
Cardiovascular	22 (20%)	0 (0%)	21 (37%)	0 (0%)	Unknown	0 (0%)	11 (29%)	22 (15%)	76 (16%)
CKD	0 (0%)	0 (0%)	16 (28%)	0 (0%)	Unknown	15 (15%)	24 (63%)	0 (0%)	55 (11%)
Other[b]	13 (12%)	1 (20%)	18 (32%)	4 (21%)	Unknown	0 (0%)	2 (5%)	19 (13%)	47 (10%)
Immunosuppressant regimen[a]									
CNI	72 (65%)	5 (100%)	49 (86%)	8 (42%)	10 (100%)	86 (83%)	38 (100%)	135 (89%)	403 (82%)
mTORi	23 (21%)	0 (0%)	6 (11%)	4 (21%)	2 (20%)	0 (0%)	1 (3%)	7 (5%)	43 (9%)
MMF	57 (51%)	4 (80%)	25 (44%)	7 (37%)	6 (60%)	0 (0%)	19 (50%)	77 (51%)	195 (39%)
Steroids	24 (22%)	5 (100%)	10 (18%)	3 (16%)	3 (30%)	0 (0%)	15 (39%)	67 (44%)	127 (26%)
Other	0 (0%)	2 (40%)	1 (2%)	1 (5%)	0 (0%)	0 (0%)	0 (0%)	13 (9%)	17 (3%)

Abbreviations: HTN, hypertension; DM, diabetes mellitus; CKD, chronic kidney disease; mTORi, mammalian target of rapamycin inhibitors.

[a]Immunosuppressant regimens were grouped as follows: CNI included tacrolimus and cyclosporine; mTORi included everolimus and sirolimus; MMF included MMF and mycophenolic acid; steroids included steroids; and other included basiliximab, anti-thymocyte globulin, and azathioprine.

[b]"Other" included "pulmonary", pulmonary arterial hypertension, "bronchopulmonary", "respiratory", hyperlipidemia, and malignancy.

[c]Becchetti *et al* did not specify which countries in Europe their data came from.

[d]Patrono *et al* did not provide any data on comorbidities.

[e]Webb *et al* collected data from 18 countries, namely the USA, UK, an unspecified Middle East country, Italy, Mexico, Canada, Sweden, Belgium, Netherlands, Brazil, Switzerland, Germany, Egypt, Spain, Greece, India, Philippines, Portugal, and Turkey.

Table 2. Symptoms, treatment, and outcome of COVID-19-infected liver transplant patients

	Study								Summary
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	
Author[Ref]	Colmenero *et al*[13]	Waisberg *et al*[14]	Becchetti *et al*[15]	Loinaz *et al*[16]	Patrono *et al*[17]	Belli *et al*[18]	Lee *et al*[19]	Webb *et al*[20]	
Country	Spain	Brazil	Europe	Spain	Italy	Europe (Italy, Spain, France)	US	International	
Cohort Size	111	5	57	19	10	103	38	151	Total (*n*) 494; Average (*n*) per study 61.8
Average time from transplant to COVID-19 infection	105 months	0.56 months	72 months	83 months	85 months	Unknown[c]	45.6 months	60 months	Average: 74.1 months
Symptoms									
Fever	83 (75%)	4 (80%)	44 (77%)	8 (42%)	6 (60%)	71 (69%)	23 (61%)	Unknown[d]	239 (70%)
Cough	78 (70%)	2 (40%)	31 (54%)	16 (84%)	3 (30%)	60 (58%)	21 (55%)	Unknown[d]	211 (62%)
Dyspnea	46 (41%)	4 (80%)	26 (46%)	9 (47%)	1 (10%)	35 (34%)	13 (34%)	Unknown[d]	134 (39%)
GI	38 (34%)	1 (20%)	18 (32%)	6 (32%)	1 (10%)	24 (23%)	16 (42%)	45 (30%)	149 (30%)
Treatment									
HCQ	88 (79%)	1 (20%)	24 (42%)	11 (58%)	6 (60%)	63 (61%)	18 (47%)	38 (25%)	249 (50%)
Anti-viral therapy[a]	41 (37%)	0 (0%)	3 (5%)	2 (11%)	2 (20%)	16 (16%)	0 (0%)	19 (13%)	83 (17%)
AZM	60 (54%)	2 (40%)	35 (61%)	0 (0%)	0 (0%)	31 (30%)	18 (47%)	1 (1%)	147 (30%)
Steroids	12 (11%)	0 (0%)	19 (33%)	0 (0%)	3 (30%)	17 (17%)	5 (13%)	0 (0%)	56 (11%)
IFN-B	3 (3%)	0 (0%)	5 (9%)	2 (11%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	10 (2%)
TOZ	15 (14%)	0 (0%)	0 (0%)	2 (11%)	0 (0%)	7 (7%)	0 (0%)	2 (1%)	26 (5%)
Hospital course									
Admitted (*n*)	96 (86%)	5 (100%)	41 (72%)	12 (63%)	9 (90%)	83 (81%)	27 (71%)	124 (82%)	397 (80%)
ICU (*n*)	12 (11%)	Unknown[b]	4 (7%)	1 (5%)	0 (0%)	15 (15%)	8 (21%)	43 (28%)	83 (17%)
Ventilation (*n*)	22 (20%)	2 (40%)	12 (21%)	2 (11%)	2 (20%)	25 (24%)	8 (21%)	30 (20%)	103 (21%)
Death (*n*)	20 (18%)	2 (40%)	7 (12%)	2 (11%)	2 (20%)	16 (16%)	7 (18%)	28 (19%)	84 (17%)

Abbreviations: AZM, azithromycin; GI, gastrointestinal; IFN-B, interferon-beta; TOZ, tocilizumab.

[a]Antiviral therapy included remdesivir, lopinavir/ritonavir, sofosbuvir, and darunavir/cobicistat.

[b]Waisberg *et al* did not provide any data on ICU admissions.

[c]Belli *et al* did not provide any data on average time from transplant to COVID-19 infection.

[d]Webb *et al* did not provide any data on fever, cough, and dyspnea.

in liver transplant recipients with COVID-19. Various studies have shown that CNIs display antiviral effects *in vitro* against coronaviruses and may also ameliorate the cytokine storm.[21,22] Similar to SARS-CoV-2, MMF yields a cytostatic effect on activated lymphocytes; therefore, MMF and SARS-CoV-2 may result in a synergic and damaging outcome on reducing peripheral lymphocytes.[23,24] Although SOT recipients may be at greater risk for COVID-19 due to immunosuppressive therapy, there are still no definitive data to suggest that the immunosuppressive protocol be altered. For patients with mild to moderate COVID-19, it is advised that immunosuppression be continued;[25] however, if there is fever, lymphopenia, or worsening of the patient's pneumonia, reducing the dose of azathioprine or MMF should be considered.[26] Patients with severe COVID-19 may need their dose of CNI reduced. Further research evaluating the role of immunosuppressive agents and COVID-19 is warranted.

As in the general population, the most common symptoms patients had were fever and cough. However, there were also other presentations, including digestive symptoms and dyspnea.[27] The most common treatment for COVID-19 was HCQ. The paradigm of treatment has evolved rapidly, with HCQ now not being recommended for the treatment of COVID-19. We revealed that the overall mortality rate in liver transplant recipients was 17%, which is in alignment with the general population (15–22%).[28,29] However, pooled data from various SOT studies report worse outcomes, with the in-hospital case fatality rate ranging from 24% to 27%.[30,31]

Several limitations of this study should be noted. The majority of patients in our analysis were from European centers; therefore, generalizability to people from other countries may be limited. Moreover, many of the studies included patients treated with HCQ, which is not currently recommended per the Infectious Diseases Society of America COVID-19 guidelines.[32] The use of HCQ, as it was applied in earlier studies, is considered less efficacious than currently available treatments. Furthermore, the cohorts included in this study varied in time from liver transplant to COVID-19 infection (ranging from 0.56 to 105 months), leading to variability in study outcomes. Finally, over or under reporting of symptoms in our various cohorts may have contributed to reporting bias.

In conclusion, patients with liver disease and transplant candidates are at risk from COVID-19. Unfortunately, SOT transplant recipients are a highly susceptible population; therefore, clinicians should have an understanding of the disease and take the essential precautions to ensure the safety of liver transplant recipients.

Funding

None to declare.

Conflict of interest

Ravina Kullar was a former employee of Gilead Sciences. Sammy Saab is on the speaker bureau and honoraria recipient of AbbVie, Bristol Myers Squibb, Bayer, Eisai, Exelixis, Gilead, Intercept, and Salix; Sammy Saab is also an advisor/consultant for AbbVie, Bayer, Eisai, Exelixis, Gilead, Intercept, Mallinckrodt, and Salix. The other author has no conflict of interests related to this publication.

Author contributions

Study concept and design (RK, SS), acquisition of data (RK, APP), analysis and interpretation of data (RK, APP, SS), drafting of the manuscript (RK, APP, SS), critical revision of the manuscript for important intellectual content (RK, APP, SS), statistical analysis (APP), administrative, technical, or material support; study supervision (SS).

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Journal of Clinical and Translational Hepatology **2021** vol. 9(4) | 551–558
DOI: 10.14218/JCTH.2020.00171

Original Article

Coronavirus Disease 2019 and Liver Injury: A Retrospective Analysis of Hospitalized Patients in New York City

Joshua M. Bender[1,2] and Howard J. Worman[1,2*]

[1]*Department of Medicine, Vagelos College of Physicians & Surgeons, Columbia University, New York, NY, USA;* [2]*Department of Pathology and Cell Biology, Vagelos College of Physicians & Surgeons, Columbia University, New York, NY, USA*

Received: 18 December 2020 | **Revised:** 16 February 2021 | **Accepted:** 22 March 2021 | **Published:** 30 April 2021

Abstract

Background and Aims: Coronavirus disease 2019 (COVID-19) is a global threat, affecting more than 100 million people and causing over 2 million deaths. Liver laboratory test abnormalities are an extrapulmonary manifestation of COVID-19, yet characterization of hepatic injury is incomplete. Our objective was to further characterize and identify causes of liver injury in patients with COVID-19. ***Methods:*** We conducted a retrospective cohort study of 551 patients hospitalized with COVID-19 at NewYork-Presbyterian Hospital/Columbia University Irving Medical Center between March 1, 2020 and May 31, 2020. We analyzed patient demographics, liver laboratory test results, vital signs, other relevant test results, and clinical outcomes (mortality and intensive care unit admission). ***Results:*** Abnormal liver laboratory tests were common on hospital admission for COVID-19 and the incidence increased during hospitalization. Of those with elevated serum alanine aminotransferase and/or alkaline phosphatase activities on admission, 58.2% had a cholestatic injury pattern, 35.2% mixed, and 6.6% hepatocellular. Comorbid liver disease was not associated with outcome; however, abnormal direct bilirubin or albumin on admission were associated with intensive care unit stay and mortality. On average, patients who died had greater magnitudes of abnormalities in all liver laboratory tests than those who survived. Ischemic hepatitis was a mechanism of severe hepatocellular injury in some patients. ***Conclusions:*** Liver laboratory test abnormalities are common in hospitalized patients with COVID-19, and some are associated with increased odds of intensive care unit stay or death. Severe hepatocellular injury is likely attributable to secondary effects such as systemic inflammatory response syndrome, sepsis, and ischemic hepatitis.

Citation of this article: Bender JM, Worman HJ. Coronavirus disease 2019 and liver injury: A retrospective analysis of hospitalized patients in New York City. J Clin Transl Hepatol 2021;9(4):551–558. doi: 10.14218/JCTH.2020.00171.

Keywords: Aminotransferase; Bilirubin; Ischemic hepatitis; SARS-CoV-2.

Abbreviations: ACE2, angiotensin-converting enzyme 2; ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; COVID-19, coronavirus disease 2019; CUIMC, Columbia University Irving Medical Center; DBIL, direct bilirubin; ICD, International Classification of Diseases; ICU, intensive care unit; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; TBIL, total bilirubin; ULN, upper limit of normal.

[*]**Correspondence to:** Howard J. Worman, Department of Medicine, Vagelos College of Physicians and Surgeons, Columbia University, New York, NY 10032, USA. ORCID: https://orcid.org/0000-0002-7063-7889. Tel: +1-212-305-1306, Fax: +1-212-342-5759, E-mail: hjw14@columbia.edu

Introduction

In December 2019, the first cases of coronavirus disease 2019 (COVID-19), the illness caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), were identified in Wuhan, China.[1–3] SARS-CoV-2 has since spread rapidly, infecting more than 100 million people and causing over 2 million deaths worldwide (as of February 3, 2021).[4] Common symptoms of COVID-19 include fever, cough, dyspnea, and fatigue; multiorgan dysfunction and death can occur in severe cases.[5] Although several studies have examined hepatic abnormalities in patients with COVID-19, the types and causes of liver injury and the influence of pre-existing liver disease on outcome remain poorly characterized.[6–10] There are also reported differences in the prevalence of liver laboratory test abnormalities in patients with COVID-19 from different parts of the world.[6]

SARS-CoV-2 binds to the angiotensin-converting enzyme 2 (ACE2) receptor to enter target cells, where it replicates and infects nearby cells.[1,11–13] Preliminary reports suggest that ACE2 receptor is expressed in cholangiocytes at a level comparable to alveolar type 2 cells, but is only minimally expressed in hepatocytes, revealing a potential mechanism for direct infection and damage of bile ductules by SARS-CoV-2.[14] While SARS-CoV-2 has been detected in post-mortem liver samples from patients with COVID-19, histopathologic features do not show significant hepatocyte or cholangiocyte damage but rather nonspecific hepatitis and macrovesicular steatosis.[15–17] This suggests that COVID-19-related liver injury may result from secondary causes.

Previous data from a New York City cohort shows that elevated serum alanine aminotransferase (ALT) activity is common in patients who test positive for SARS-CoV-2.[18] The injury is most often considered mild, although patients with serum ALT more than 5 times the upper limit of normal (ULN) have worse outcomes. In the current study, we characterize abnormalities in ALT, aspartate aminotransferase (AST), alkaline phosphatase (ALP), bilirubin, and albumin in hospitalized patients with COVID-19. We correlate abnormalities in these parameters at admission and subsequently during hospitalization with clinical outcomes. Finally, we establish a likely etiology of severe hepatocellular injury observed in a subset of patients hospitalized with COVID-19.

Methods

Inclusion criteria and data collection

Study participants were admitted to NewYork-Presbyterian Hospital/Columbia University Irving Medical Center (referred to as CUIMC) between March 1 and May 31, 2020 with an encounter diagnosis of COVID-19 (International Classification of Diseases, Tenth Revision [ICD-10] code U07.1 documented in the problem list), resulting in the inclusion of 551 patients. ICD-10 code U07.1 is only used for a confirmed diagnosis of COVID-19 as documented by the provider. We used this criterium, rather than including all patients with a positive SARS-CoV-2 test result, to exclude patients who may have tested positive while admitted for other reasons but did not experience symptoms of COVID-19. All subjects had a positive reverse transcription-PCR nasal swab for SARS-CoV-2 RNA.

The Columbia University Institutional Review Board approved the protocol with a waiver of informed consent. Patient demographics, laboratory values, vital signs, clinical outcomes, and medical histories were obtained by query of the Epic Systems electronic health record. Outcomes were assessed at the time of data collection on July 21, 2020. Race and ethnicity data were self-reported in prespecified categories. Liver laboratory test abnormalities were defined as: AST >37 U/L, ALT >50 U/L, ALP >129 U/L, direct bilirubin (DBIL) > 0.3 mg/dL, total bilirubin (TBIL) >1.3 mg/dL, and serum albumin <3.9 g/dL, per CUIMC laboratory reference ranges.

Admission laboratory values were defined as results documented closest to and within 60 hours of admission. Admission ALT, AST, ALP, TBIL, and albumin were available for 533 (96.7%) patients, whereas admission DBIL was available for 531 (96.4%). Peak laboratory values were defined as the highest ALT, AST, ALP, DBIL, and TBIL, and the lowest albumin recorded during hospitalization. Peak values for each liver laboratory test were available for 539 (97.8%) patients.

Characterization of liver injury

Liver injury for patients with abnormal ALT and/or ALP was characterized as cholestatic, mixed, or hepatocellular at the time of admission by calculating the R factor, computed as serum ALT/ULN divided by serum ALP/ULN. R≥5 is considered hepatocellular liver injury, R≤2 cholestatic, and 2<R<5 is interpreted as a mixed type of liver injury.[19,20]

Statistical analyses

All analyses were performed using MATLAB R2020a (version 9.8.0.1396136; The MathWorks, Inc., Natick, MA, USA). A *p*-value ≤0.05 was considered statistically significant. Comorbidities and laboratory test results were correlated with mortality and intensive care unit (ICU) admission (primary and secondary outcomes, respectively) using Fisher's exact test for nonrandom association between two categorical variables. Laboratory test result trends were stratified by outcome and plotted against time as the mean of each patient's individual change from their admission level, with error bars representing the 95% confidence interval of each point estimate. Outliers were defined as elements more than three standard deviations from the mean and were removed from these point estimates to prevent large fluctuations caused by a few extreme values.

Table 1. Characteristics of patients hospitalized with COVID-19

Characteristic	No. (%)
Total no.	551
Deaths	115 (20.9)
Age in years	
<25	31 (5.6)
25–49	86 (15.6)
50–64	149 (27)
65–79	194 (35.2)
>80	91 (14.5)
Sex	
Female	235 (42.6)
Male	316 (57.4)
Race	
Asian	9 (1.6)
African American	102 (18.5)
White	136 (24.7)
Other/Multiracial	199 (36.1)
Declined	113 (20.5)
Ethnicity	
Hispanic or Latino	284 (51.5)
Not Hispanic or Latino	159 (28.9)
Declined	108 (19.6)
Body Mass Index in kg/m^2	
Underweight, <18.5	20 (3.6)
Normal, 18.5–24.9	127 (23.0)
Overweight, 25.0–29.9	153 (27.8)
Obese, 30.0–39.9	150 (27.2)
Extremely obese, >40	40 (7.3)
Unknown	61 (11.1)
Comorbidities	
Liver disease	32 (5.8)
Kidney disease	96 (17.4)
Cardiovascular disease	307 (55.1)
Diabetes	177 (32.1)

Results

Study cohort characteristics

Clinical characteristics and demographics of the patient cohort are summarized in Table 1. A total of 551 patients met inclusion criteria, of which 170 (30.9%) were admitted to the ICU and 115 (20.9%) died during hospitalization. Mean age was 63 years (range: 1–102 years), 57.4% were male, and 34.5% were obese with a body mass index (BMI) ≥30.0. Only 5.8% of patients suffered from comorbid liver disease.

Mean and median length of hospital stay were 16 days

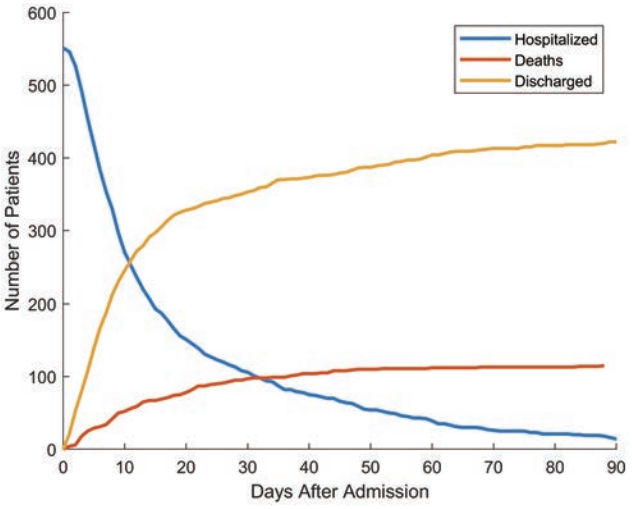

Fig. 1. Hospital course of the patient cohort. A total of 551 patients were studied. At the time of data collection, 115 patients had died, 424 patients had been discharged, and 12 patients remained hospitalized. The mean and median length of hospital stay was 16 days and 9 days, respectively, with a range of 0-104 days.

and 9 days, respectively, with a range of 0–104 days. Over half the patients were discharged or died after 10 days (Fig. 1). At the time of data collection, 12 patients remained hospitalized.

Liver laboratory test abnormalities

Abnormal admission liver laboratory tests were common in patients with COVID-19 (Fig. 2A–F). ALT was abnormally elevated in 28.1%, AST in 61.0%, ALP in 19.1%, DBIL in 18.5%, and TBIL in 7.9%; albumin was below normal in 65.9%. Peak ALT was abnormal in 55.7% of patients, AST in 79.2%, ALP in 39.7%, DBIL in 44.3%, and TBIL in 21.5%; albumin was below normal in 93.0% of patients during their illness (Fig. 2G–L).

For patients with abnormal ALT, ALP, or both at time of admission, we calculated each patient's R factor to determine if the pattern of liver injury was mostly likely cholestatic, hepatocellular, or mixed. The pattern of laboratory test abnormalities suggested that liver injury was most often cholestatic. In 213 patients, 58.2% had a cholestatic injury pattern, 35.2% mixed, and 6.6% hepatocellular (Fig. 3). Given that the rate of abnormal AST elevation (61.0%) was notably higher than the rate of abnormal ALT (28.1%) elevation in our cohort, we computed the R factor for each patient using the admission AST value rather than ALT. In this instance, we found that in the 352 patients with an abnormal AST and/or ALP on admission, 36.1% had a cholestatic injury pattern, 45.4% mixed, and 18.5% hepatocellular.

Association of liver abnormalities with patient outcomes

Certain admission laboratory test abnormalities were associated with ICU admission or death; however, pre-existing liver disease was not (Table 2). Pre-existing cardiovascular disease was associated with increased odds of death. While there was not a significantly increased mortality rate in patients that presented with a history of pre-existing liver disease, they presented with a significantly higher prevalence of abnormalities in ALP (34.4% vs. 19.1%, p=0.035) and TBIL (21.9% vs. 7.9%, p=0.0086), but not aminotransferases, DBIL, or albumin. There were no significant differences in the prevalence of abnormal peak liver tests; however, the mean peak DBIL (2.97 vs. 0.77, p=1.61e−6) and TBIL (3.74 vs. 1.18, p=4.29e−7) were significantly higher in the subcohort of patients with pre-existing liver disease than those with no history of liver disease. Abnormal admission DBIL and albumin were associated with ICU admission and mortality, elevated AST was associated with ICU admission but not mortality, and elevated TBIL was associated with death but not ICU admission. Elevated admission ALT and ALP were not associated with mortality or ICU admission. Mortality risk was increased in patients who presented with normal liver laboratory tests (ALT, AST, ALP, DBIL, TBIL, and albumin) on admission but subsequently had an abnormal ALP, DBIL, or TBIL. A subsequent abnormal ALT, AST, or albumin was not associated with mortality in these patients. The risk of ICU admission was increased in patients who presented with normal liver laboratory tests on admission but had an abnormal ALP or DBIL later during their hospital course. Subsequently abnormal ALT, AST, TBIL, or albumin were not associated with ICU admission in these patients.

Laboratory test result trends showed a rise in mean ALT, AST, ALP, TBIL, and DBIL, and a decrease in mean albumin during the first 14 days of hospitalization (Fig. 4). In patients who died, a spike in mean serum aminotransferase activities occurred around day 8 (Fig. 4A, B), followed by a corresponding increase in ALP (Fig. 4C), DBIL (Fig. 4D), and TBIL (Fig. 4E) about 2 days later. Of patients who survived, a gradual increase in mean ALT, ALP, and DBIL occurred with a corresponding decrease in average serum albumin concentration (Fig. 4F). On average, patients who died had greater magnitude abnormalities in all liver laboratory tests during hospitalization than those who survived.

Patients with COVID-19 and severe hepatocellular injury

During hospitalization, 21 of 551 (3.81%) patients suffered severe hepatocellular injury, defined as an ALT greater than 10 times the ULN. Of these patients, 19 were admitted to the ICU, 17 were intubated, and 9 died. At the time of peak serum ALT activity, 19 had a hepatocellular pattern of injury (R factor ≥5), 2 a mixed pattern (2<R factor<5), and none had a cholestatic injury pattern (R factor ≤2). The mean±standard deviation R factor in this subcohort at the time of peak ALT activity was 35.3±37.9; the median was 21.1, and range was 3.18–173.

To investigate the etiology of severe hepatocellular liver injury in this subcohort, we plotted the trend of ALT activities along with systemic markers of pathology: mean arterial pressure, body temperature, oxygen saturation, white blood cell count, platelet count, and serum creatinine concentration (Supplementary Fig. 1). One-third of these patients were hypoxic (oxygen saturation <90%), and 38% showed signs of sepsis indicated by fever (temperature >100.4°F), and an elevated white blood cell count (>8.44×10^3/μL).

Data from three representative patients that had consistent documentation of laboratory test results and vital signs revealed a pattern consistent with ischemic hepatitis likely secondary to septic shock (Fig. 5). In these cases, mean arterial pressure dropped prior to a spike in serum aminotransferase activities, with subsequent increases in the serum TBIL concentration in two of the three. Associated

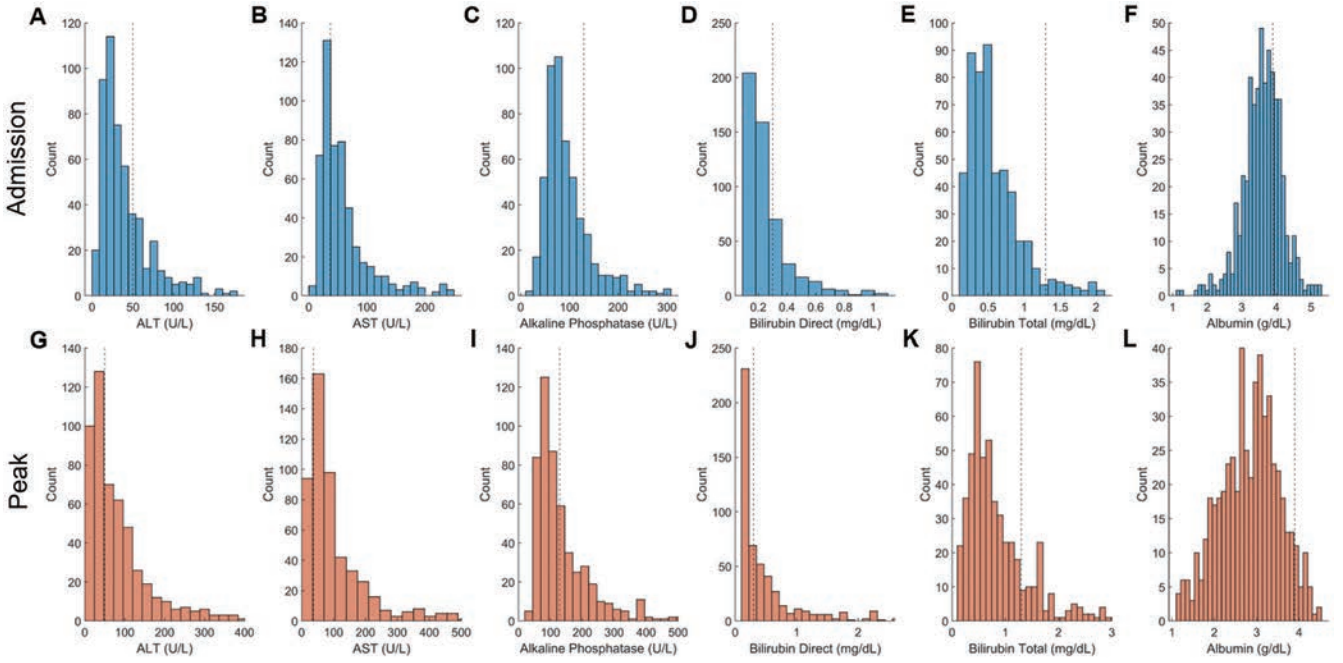

Fig. 2. Histograms of admission and peak liver laboratory test results. ALT (A) was abnormally elevated on admission in 28.1%, AST (B) in 61.0%, ALP (C) in 19.1%, DBIL (D) in 18.5%, and TBIL (E) in 7.9%; serum albumin concentration (F) was below normal on admission in 65.9%. Peak ALT (G) was abnormal in 55.7% of patients, AST (H) in 79.2%, ALP (I) in 39.7%, DBIL (J) in 44.3%, TBIL (K) in 21.5%; serum albumin concentration (L) was below normal in 93.0% of patients. Dashed lines represent the ULN for ALT, AST, ALP, DBIL, and TBIL, and the lower limit of normal for serum albumin. Histograms are scaled to show the bulk of the data; therefore, some outliers are not shown.

increases in the serum creatinine concentrations indicated concurrent kidney dysfunction. Elevated white blood cell counts in all three, and fever in two out of three suggested concurrent infection; decreasing platelet counts suggested possible disseminated intravascular coagulation (Supplementary Fig. 2).

Discussion

Our results show that liver laboratory test abnormalities are common in hospitalized patients with COVID-19. The numbers of patients with abnormalities in these laboratory tests increase during hospitalization. For serum aminotransferase activities, a higher prevalence of elevations in AST compared to ALT may be attributable to non-hepatic sources, as AST is expressed to a great degree in heart, skeletal muscle, and erythrocytes.[21] Among hospitalized patients with COVID-19, a subset of about 4% develop severe hepatocellular injury often associated with hypoxia, signs of sepsis, and systemic hypotension.

Our cohort was restricted to patients admitted to a tertiary care academic medical center and, as such, was likely significantly sicker than most patients with COVID-19. We included only patients with an encounter diagnosis of COVID-19, which eliminated subjects who may have been hospitalized for other reasons and subsequently tested positive for SARS-CoV-2. Almost a third of our patients were transferred to the ICU during the course of hospitalization and 20.9% died, resulting in a case fatality rate higher than generally reported previously in most studies.[22–25] However, our cohort's case fatality rate was similar to that reported in 5,700 patients hospitalized with COVID-19 in the New York City area.[26] Similar to our study, 39.0% and 58.4% of subjects in that cohort had elevated ALT and AST, respectively; however, data on ALP, DBIL and TBIL were not provided.

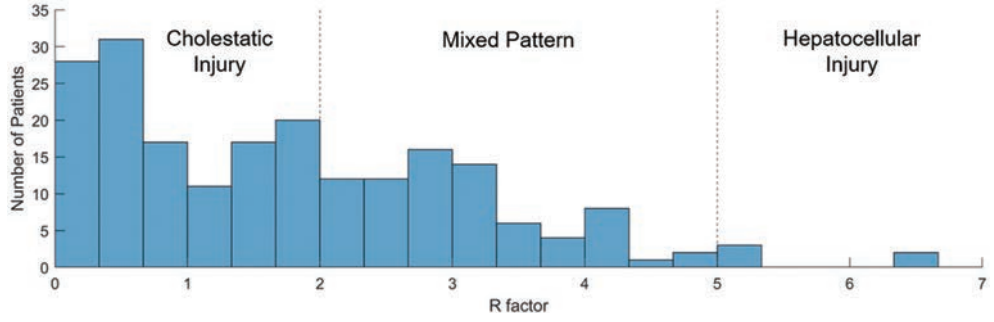

Fig. 3. Liver injury at time of admission in patients with abnormal ALT or ALP results characterized by R factor. In 213 patients with abnormal ALT and/or ALP activities on admission, 58.2% had a cholestatic injury pattern, 35.2% mixed, and 6.6% hepatocellular. Dashed lines at R=2 and R=5 represent the borders between cholestatic, mixed, and hepatocellular liver injury. The plot is scaled to show the bulk of the data; therefore, some outliers are not shown.

Table 2. Correlation of comorbidities and liver-related laboratory tests with outcome

Comorbidity	Odds of death			Odds of ICU admission		
	OR*	95% CI**	*p*	OR	95% CI	*p*
Liver disease	0.689	(0.259, 1.829)	0.6534	0.617	(0.262, 1.456)	0.326
Kidney disease	1.333	(0.795, 2.233)	0.2716	0.623	(0.372, 1.042)	0.877
Cardiovascular disease	1.649	(1.075, 2.528)	0.0264	0.866	(0.602, 1.246)	0.458
Diabetes	1.346	(0.876, 2.067)	0.1794	1.069	(0.727, 1.573)	0.767
Obesity: BMI ≥30	0.904	(0.577, 1.416)	0.733	1.072	(0.730, 1.575)	0. 768

Patients with abnormal liver laboratory tests on admission						
Laboratory test	Odds of death			Odds of ICU admission		
	OR	95% CI	*p*	OR	95% CI	*p*
ALT	0.817	(0.507, 0.316)	0.478	1.460	(0.981, 2.173)	0.077
AST	1.458	(0.938, 2.268)	0.1025	1.778	(1.203, 2.627)	0.0041
ALP	1.372	(0.829, 2.272)	0.225	1.183	(0.749, 1.869)	0.4780
DBIL	2.275	(1.017, 3.953)	0.0014	2.077	(1.325, 3.257)	0.0017
TBIL	2.005	(1.017, 3.953)	0.0488	1.239	(0.641, 2.396)	0.6033
Albumin	1.947	(1.204, 3.151)	0.0069	1.926	(1.278 2.903)	0.0016

Patients with normal admission liver laboratory tests but abnormal peak values						
Laboratory test	Odds of death			Odds of ICU admission		
	OR	95% CI	*p*	OR	95% CI	*p*
ALT	1.041	(0.348, 3.119)	1.00	1.923	(0.633, 5.846)	0.290
AST	1.719	(0.429, 6.898)	0.533	2.439	(0.267, 22.291)	0.657
ALP	8.017	(1.235, 12.17)	0.022	15.30	(4.19, 55.86)	7.326e−6
DBIL	2.941	(2.249, 28.58)	0.001	8.123	(2.467, 26.748)	0.0004
TBIL	6.58	(1.731, 25.01)	0.007	3.429	(1.04, 11.302)	0.0571
Albumin	Inf***	(Inf, Inf)	0.181	Inf	(Inf, Inf)	0.173

*OR, odds ratio; **CI, confidence interval; ***Inf, infinity. *P*-values were calculated using Fisher's exact test for nonrandom association between two categorical variables.

We found no significant association between pre-existing liver disease and clinical outcome, consistent with the findings in a small cohort of 60 patients studied at Massachusetts General Hospital, another academic tertiary care center.[27] In contrast, in a study of 363 patients in a single healthcare system in Massachusetts with two tertiary care centers and seven community hospitals, 69 patients with chronic liver disease had worse outcomes, and cirrhosis was an independent predictor of mortality.[28] In a USA multicenter study of 2,798 patients, there was also an increased relative risk of mortality in a subset of 250 with pre-existing liver disease.[29] The overall severity of illness, high mortality rate, and small number of patients with cirrhosis in our cohort may explain the difference. Similar factors may explain why we did not find a correlation between diabetes mellitus or obesity with poor outcomes.

Certain liver laboratory test results increased the odds of a poor clinical outcome. Evidence of liver dysfunction rather than simply injury, as manifested by an abnormally elevated DBIL either at the time of admission or during hospitalization in patients who initially had normal liver laboratory tests, correlated with an increased risk of both ICU admission and death. At the time of admission, an elevated serum AST, but not ALT, correlated with ICU admission, while neither correlated with mortality, consistent with a study of patients in the Yale New Haven Health System.[30] Elevated AST, more so than elevated ALT, may reflect extrahepatic organ involvement, such as COVID-19-related myocarditis or other myocardial injury.[31]

Elevations in serum liver enzyme activities and bilirubin concentration can occur secondary to systemic infection, systemic inflammatory response syndrome, or sepsis.[32,33] These are likely causes of serum liver laboratory test abnormalities in our cohort. This is supported by the fact that the serum albumin was below normal in 65.9% of patients on admission and in 93.0% sometime during hospitalization. The half-life of albumin in adult plasma is approximately 3 weeks.[34] Therefore, in acute inflammatory states, the decreasing serum albumin concentration is not due to defective hepatic synthesis or secretion, but rather capillary leak, possible kidney dysfunction, or other systemic factors. While some patients in our cohort may have had pre-existing chronically low serum albumin, hypoalbuminemia is strongly associated with systemic inflammatory response syndrome and sepsis.[35,36] The finding that an abnormal ALP and or DBIL during hospitalization increased the risk of death in patients who had normal liver laboratory test results on admission could also reflect the development of

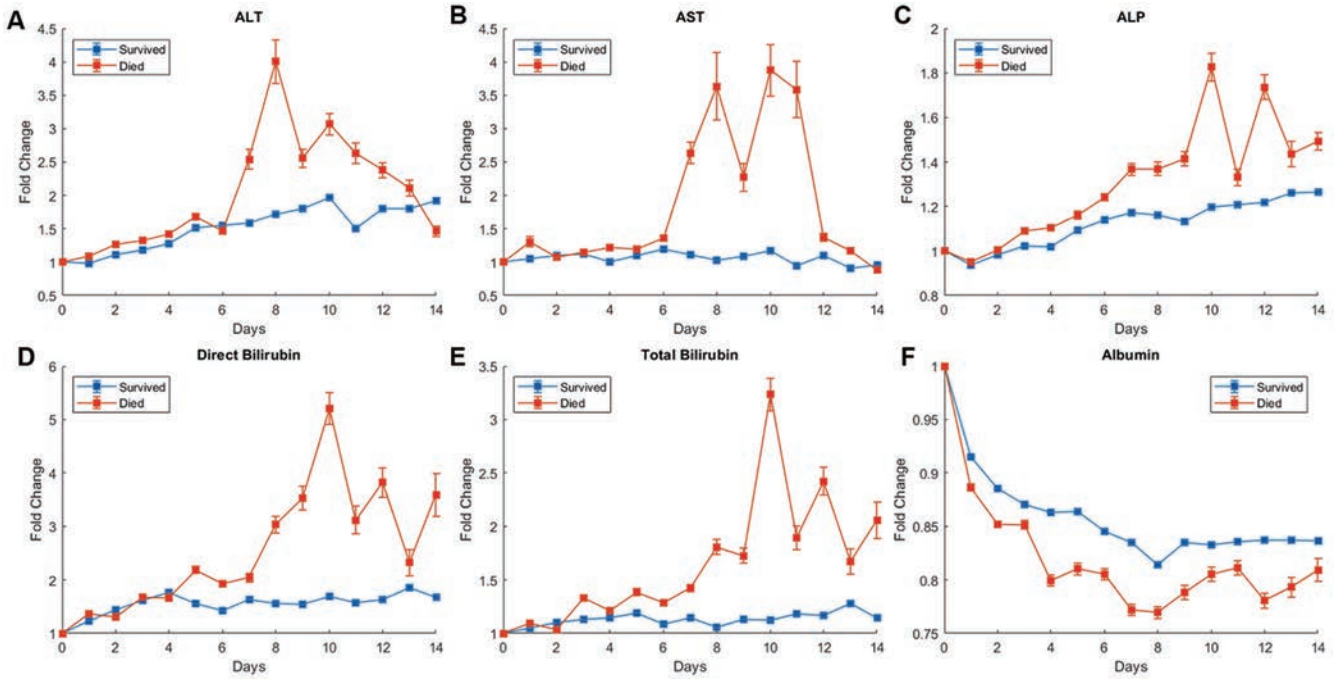

Fig. 4. Trends for ALT (A), AST (B), ALP (C), DBIL (D), TBIL (E) and albumin (F) stratified by outcome and plotted against time. Values are the mean of every patient's individual change from their admission value, with error bars representing the 95% confidence interval of each point estimate. Outliers were defined as elements more than three standard deviations from the mean and were removed from these point estimates in order to prevent large fluctuations caused by a few extreme values.

sepsis, given its association with cholestasis.[32]

The most common pattern of ALT and ALP elevations on hospital admission suggested cholestatic or mixed liver injury. Only 6.6% of patients had a pattern suggestive of purely hepatocellular injury on admission. We characterized the injury pattern using the R factor, a metric originally developed for drug-induced liver injury and not widely validated in other situations. However, an American College of Gastroenterology Clinical Guideline has recommended that it be used more broadly to characterize abnormal liver chemistries.[37] When computing the R factor with AST instead of ALT, we found that in 352 patients with an abnormal AST and/or ALP on admission, 36.1% had a cholestatic injury pattern, 45.4% mixed, and 18.5% hepatocellular. However, the R factor has only been validated for use with ALT and would thus require further study to validate its use with AST in place of ALT. AST is also more likely than ALT to arise from non-hepatic sources such as striated muscle. Cholestatic or mixed injury raises the suspicion that SARS-CoV-2 could infect cholangiocytes, as suggested in preliminary studies,[14] and supported by data from the mouse Gene Expression Database[38] showing that *Ace2* is expressed in the biliary system. Nonetheless, this theoretical mechanism of cholestatic liver injury in COVID-19 remains unproven. Another potential cause of cholestatic and hepatocellular injury in hospitalized patients is drug toxicity. However, we were unable to establish associations of liver laboratory test abnormalities to specific drugs given the myriad agents administered at different times. In contrast to our findings of primarily cholestatic liver injury, a study from Italy reported that the predominant liver injury in patients with COVID-19 is hepatocellular, using vague criterium of "predominantly raised" ALT and AST.[39]

We identified a subset of 21 patients with COVID-19 who developed severe hepatocellular injury, defined as an ALT >10 times the ULN. We realize that this cutoff is subjective and selected it in order to isolate and study the patients suffering from a severe acute liver injury, indicated by massive amounts of hepatocyte death, as measured by a highly elevated serum ALT. Other authors have also suggested ALT >10 times the ULN as "severe" or "marked."[40,41] Of the patients that suffered severe hepatocellular injury, 81% were intubated and 43% died. In a few of these cases, we identified hypotension along with evidence of sepsis and acute renal failure, suggesting ischemic hepatitis secondary to shock as the etiology of liver injury. However, hypotension is documented in only approximately half of patients with ischemic hepatitis, with cardiac failure, sepsis, and respiratory failure accounting for most cases.[40–42] Indeed, the term hypoxic hepatitis is often used alternatively to emphasize that the liver injury may be due to decreased oxygen delivery to hepatocytes rather than solely low blood perfusion.[9,42–44]

There has been considerable heterogeneity in geographic location, sample sizes, and patient populations among previous studies of the liver in patients infected with SARS-CoV-2 (Supplementary Table 1). Some included only hospitalized patients, while others also included outpatients and those discharged from emergency rooms. Our results on the prevalence of liver laboratory abnormalities with COVID-19 are similar to a study of 1,827 patients in the Yale New Haven Health System,[30] and another study of 2,780 patients across 34 health care organizations in the United States.[29] They surprisingly differ, however, from those reported in another study of inpatients and outpatients in New York City, which did not find TBIL or ALP elevations to be common and did not observe any clinically significant acute liver injury.[45] This may be because approximately 27% of the patients in that study were not hospitalized. In a cohort of 60 patients in Boston, ALP and TBIL elevations were also reported to be rare; however, 17% of patients developed serum aminotransferase activities more than 5 times the ULN.[27] A meta-analysis of international data showed that the pooled prevalence of elevated serum aminotransferase

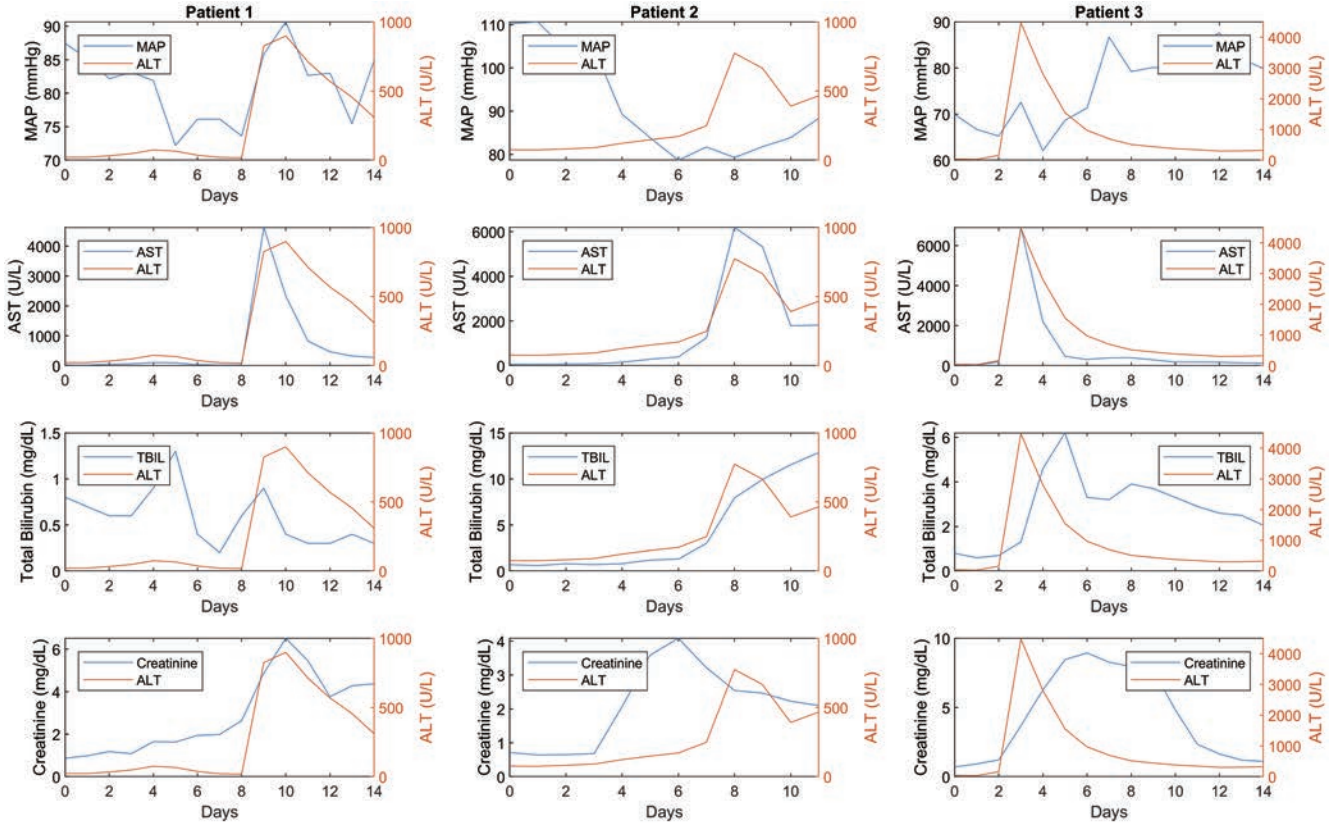

Fig. 5. Trends of mean arterial pressure, aminotransferase activities, TBIL, and serum creatinine over the first 14 days of hospitalization in three representative patients that suffered severe hepatocellular injury. Severe hepatocellular injury was defined as an ALT activity greater than 10 times the upper limit of normal. Day 0 represents the date of admission, and each value was computed as the mean of all laboratory test results documented on that day of hospitalization.

activities in patients with COVID-19 was approximately 15.0%, with higher percentages reported from countries outside of China.[6] In one cohort of 115 hospitalized patients with COVID-19 in Wuhan, China, only 9.57% had an abnormally elevated ALT, 14.8% an elevated AST, and 5.2% an elevated ALP on admission; however, closer to our findings, 9.69% had an elevated TBIL and 54.8% a low albumin.[46] In 329 patients hospitalized with COVID-19 in Italy, 58% had abnormalities in liver function tests and this correlated with a higher risk transfer to an ICU or death.[39]

Our study, as others like it, had limitations. We used a retrospective observational cohort design with inclusion restricted to patients hospitalized at a single medical center with an encounter diagnosis of COVID-19. This excluded some patients that may have tested positive for SARS-CoV-2 but did not have any symptoms of COVID-19. Further study of liver injury in a broader group of all patients that test positive for SARS-CoV-2 is warranted. Our study also included only a small number of patients with pre-existing liver disease. Daily laboratory tests were not obtained in many patients, hindering our ability to trend results in some over their entire hospital course. We had minimal past medical history for many patients who accessed our healthcare system for the first time. Finally, although we restricted our inclusion criteria to patients with an encounter diagnosis of COVID-19, factors such as comorbidities, simultaneous illnesses, and medications could have contributed to laboratory test results and outcomes.

Our results confirm that liver laboratory test abnormalities are common in hospitalized patients with COVID-19, some of which are associated with ICU stay or mortality. While our data cannot exclude direct SARS-CoV-2 infection of the liver as a cause of injury, they are consistent with secondary hepatic involvement from systemic inflammatory response syndrome, sepsis, or ischemic hepatitis. The mechanisms of liver injury in patients with COVID-19 are therefore most likely similar to what occurs in many other critically-ill patients.[32,33,47,48]

Acknowledgments

We thank Jianhua Li for his contributions to data collection.

Funding

National Institute of Diabetes and Digestive and Kidney Disease (No. T35DK93430) to JMB and the National Center for Advancing Translational Sciences (No. UL1TR001873) of the National Institutes of Health to support the Irving Center for Clinical and Translational Research at Columbia University. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

Conflict of interest

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

Author contributions

Study concept and design (JMB, HJW), acquisition of data (JMB), analysis and interpretation of data (JMB, HJW), statistical analysis (JMB), writing of the manuscript (JMB, HJW), and obtaining of funding (JMB).

Data sharing statement

All data are available upon request.

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Journal of Clinical and Translational Hepatology **2021** vol. 9(4) | 559–567
DOI: 10.14218/JCTH.2021.00007

Review Article

Horizons on the Therapy of Biliary Tract Cancers: A State-of-the-art Review

Ran Xue[1#], Rong Li[2#], Jianxin Wang[3], Weiping Tong[2] and Jianyu Hao[3*]

[1]*Key Laboratory of Carcinogenesis & Translational Research (Ministry of Education/Beijing), Early Drug Development Center, Peking University Cancer Hospital & Institute, Beijing, China;* [2]*Department of Gastroenterology, Beijing Shuang-Qiao Hospital, Beijing, China;* [3]*Department of Gastroenterology, Beijing Chao-Yang Hospital, Capital Medical University, Beijing, China*

Received: 2 January 2021 | **Revised:** 24 March 2021 | **Accepted:** 14 April 2021 | **Published:** 12 May 2021

Abstract

Biliary tract cancers (BTCs) comprise a group of heterogeneous poor prognosis cancers with increasing incidence recent years. The combination chemotherapy with cisplatin and gemcitabine is the first-line therapy for advanced BTC. There remains no accepted standard treatment in the second-line setting. Nowadays, more and more novel treatment strategies have entered development, with some encouraging results being seen. Here, we review the current treatment status and clinical characteristics of BTC, the role of immunotherapy in BTC as well as the design of clinical trials for oncology drugs for BTC which aim to focus on the future profiles of clinical care and resolution of BTC.

Citation of this article: Xue R, Li R, Wang J, Tong W, Hao J. Horizons on the therapy of biliary tract cancers: A state-of-the-art review. J Clin Transl Hepatol 2021;9(4):559–567. doi: 10.14218/JCTH.2021.00007.

Introduction

Biliary tract cancer (BTC) is a kind of malignant tumor arising from epithelial cells of the biliary system. According to different origins, it is divided into intrahepatic cholangiocarcinoma (ICC), perchilar/hilar cholangiocarcinoma (PCC), extrahepatic cholangiocarcinoma (ECC) and gallbladder cancer (GBC).[1] The histology of BTC is mainly adenocarcinoma. Surgery is the only curable technique available for BTC. However, more than 65% of patients with BTC are unable to undergo radical surgical resection when they are discovered, with a 5-year survival rate of about 5–15% and a recurrence rate of 67% in 1 year after operation.[2,3] In the absence of surgery, BTC is not sensitive to traditional chemotherapy. Gemcitabine plus cisplatin (GC) is the first-line standard chemotherapy for advanced BTC.[3] Morizane *et al*. confirmed that gemcitabine plus S-1 (GS) is not inferior to GC in terms of overall survival rate, and recommended GS as a new choice for first-line treatment of BTC.[4] However, the survival benefits of chemotherapy with either GS or GC are still limited, and the median survival time is only about 12 months. Therefore, it is urgent to develop new clinical strategies for the treatment of BTC.

Keywords: Biliary tract cancers; Immunotherapy; Clinical trials.

Abbreviations: ACT, adoptive cell transfer therapy; BTC, biliary tract cancer; CAR-T, chimeric antigen receptor T lymphocyte; CIK, cytokine-induced killer; ECC, extrahepatic cholangiocarcinoma; ECOG, Eastern Cooperative Oncology Group; EMEA, European Medicines Agency; EORTC, European Organization for Cancer Therapy Research; ERCP, endoscopic retrograde cholangiopancreatography; FDA, Food and Drug Administration; JCOG, Japanese Clinical Oncology Cooperative Organization; GBC, gallbladder cancer; GC, gemcitabine plus cisplatin; GS, gemcitabine plus S-1; HR, hazard ratio; ICC, intrahepatic cholangiocarcinoma; LAK, lymphokine-activated killer cells; MMR, mismatch repair; MRCP, magnetic resonance cholangiography; MSI, microsatellite instability; PCC, perchilar/hilar cholangiocarcinoma; PD-1, programmed death-1; PD-L1, programmed death-ligand 1; PTC, percutaneous transhepatic cholangiography; SWOG, Southwest Oncology Cooperative Group; TCR-T, T lymphocyte receptor chimeric T lymphocyte; TIL, tumor infiltrating lymphocytes.

[#]These authors contributed equally to this study.

[*]**Correspondence to:** Jianyu Hao, Department of Gastroenterology, Beijing Chao-yang Hospital, Capital Medical University, Chao yang Area, Beijing 100020, China. ORCID: https://orcid.org/0000-0001-7881-9380. Tel: +86-10-85231000, E-mail: hao_jianyu@126.com

Current treatment status and clinical characteristics of BTC

GBC

GBC is the most aggressive and most common type of BTC, and the majority of cases represent adenocarcinomas. Its incidence increases with age, and the incidence in women is higher than that in men, especially for white women. GBC generally occurs locally, easily invades blood vessels, and is prone to local or extensive lymph node metastasis and distant metastasis. The clinical manifestations are similar to biliary colic or chronic cholelithiasis, so it is usually discovered at an advanced stage when it is diagnosed. Based on the data from 177 patients who underwent potentially curative resection (GBC: n=97; PCC: n=80), the median time to disease recurrence was shorter for patients with GBC compared with patients with PCC (11.5 vs. 20.3 months; p=0.007). In total, 52 (68%) of the patients with PCC and 53 (66%) of the patients with GBC had disease recurrence at a median follow-up of 24 months. It was indicated that compared with PCC, patients with GBC have a shorter median survival time and are prone to recurrence; the survival time after recurrence is shorter as well.[5]

For patients with jaundice, if GBC is suspected, surgery must be done for the purpose of treatment. It is recommended that multidisciplinary consultations evaluate the possibility of surgery first. The assessment should include

Table 1. The summary the surgical/non-surgical treatment plans for different types of BTC

BTC type	Resectable	Unresectable
GC	Cholecystectomy + en bloc hepatic resection + lymphadenectomy ± bile duct excision for malignant involvement	GC combination therapy Fluoropyrimidine-based or other gemcitabine-based chemotherapy regimen EBRT with concurrent fluoropyrimidine Radiation therapy Clinical trial Best supportive care Pembrolizumab (only for MSI-high/MMR defect tumors)
ICC	Consider staging laparoscopy Resection Consider lymphadenectomy for accurate staging	GC combination therapy Clinical trial Fluoropyrimidine-based or other gemcitabine-based chemotherapy regimen EBRT with concurrent fluoropyrimidine Consider locoregional therapy Radiation therapy Arterially directed therapies Best supportive care Pembrolizumab (only for MSI-high/MMR defect tumors)
ECC	Surgical exploration Consider laparoscopic staging Consider preoperative biliary drainage Multidisciplinary review	GC combination therapy Clinical trial Fluoropyrimidine-based or other gemcitabine-based chemotherapy regimen EBRT with concurrent fluoropyrimidine Radiation therapy Pembrolizumab (only for MSI-high/MMR defect tumors) Best supportive care

cholangiography to determine the degree of tumor invasion to the hepatobiliary system, with non-invasive magnetic resonance cholangiography (MRCP) being preferred and the second choice being endoscopic retrograde cholangiopancreatography (ERCP) or percutaneous transhepatic cholangiography (PTC).[6]

For operable patients, biliary drainage should be considered before surgery. Cholecystectomy, hepatectomy and lymph node dissection with or without bile duct resection are performed, combined with adjuvant treatment and monitoring after surgery. It is worth noting that GBC with jaundice usually indicates a poor prognosis, so the possibility of surgery needs to be carefully evaluated.[7] For inoperable patients, biliary drainage should be performed before chemotherapy. The chemotherapy regimen can involve GS, fluorouracil or gemcitabine-based chemotherapy, combined with radiotherapy, clinical trials and supportive care.

ICC

Patients with ICC usually have no specific clinical manifestations and generally do not have symptoms of bile duct obstruction. They are often found incidentally due to a solitary mass on the liver being found upon imaging examination. Although most patients are diagnosed with advanced disease and are not suitable for surgery, complete resection is still the only curative method for patients with ICC.

For isolated intrahepatic masses, if the imaging examination findings are consistent with adenocarcinoma, it is recommended to conduct a multidisciplinary assessment immediately to determine the possibility of surgery. For operable patients, the presence of multiple liver lesions, lymph node metastasis or distant metastasis should be evaluated before surgery, since lymph node metastasis and distant metastasis beyond the hepatic hilar are contraindications to surgical resection. Partial hepatectomy is the surgical option, and while hepatectomy is usually performed, as long as the margin is negative, liver wedge resection, segmentectomy and extended resection can also be considered. It is worth noting that hilar lymph node dissection is reasonable, because it can not only provide staging information of cholangiocarcinoma but also assess the prognosis to a certain extent. However, lymph node metastasis to the hilar is usually related to a poor prognosis, and resection must be performed on highly specific patients. Patients should receive adjuvant treatment and monitor changes in their condition after surgery. For inoperable patients, GC chemotherapy, clinical trials, fluorouracil or gemcitabine-based chemotherapy, fluorouracil chemotherapy and radiotherapy, local treatment and supportive treatment could be used.

The tumor size of ICC has no significant effect on the survival rate after surgery. The influential factors include the number of tumors, vascular invasion and the status of lymph nodes. Furthermore, the number of tumors and vascular invasion only have guiding significance at N0.[8]

ECC

Patients with ECC often have symptoms of bile duct obstruction, such as jaundice, pain, and abnormal liver function, followed by abnormal lesions on imaging examination. The radical treatment for ECC is to completely remove the lesion and ensure that the margin is negative. The 5-year survival rates for hilar cholangiocarcinoma and distal cholangiocarcinoma undergoing radical resection are 20–40% and 16–52%, respectively.[9] When the above-mentioned clinical manifestations occur, it is recommended to conduct a multidisciplinary assessment immediately to determine whether there is a possibility of surgery.

The radical treatment for extrahepatic cholangiocarcinoma involves complete removal of the lesion and provision of negative resection margins. The 5-year survival rates

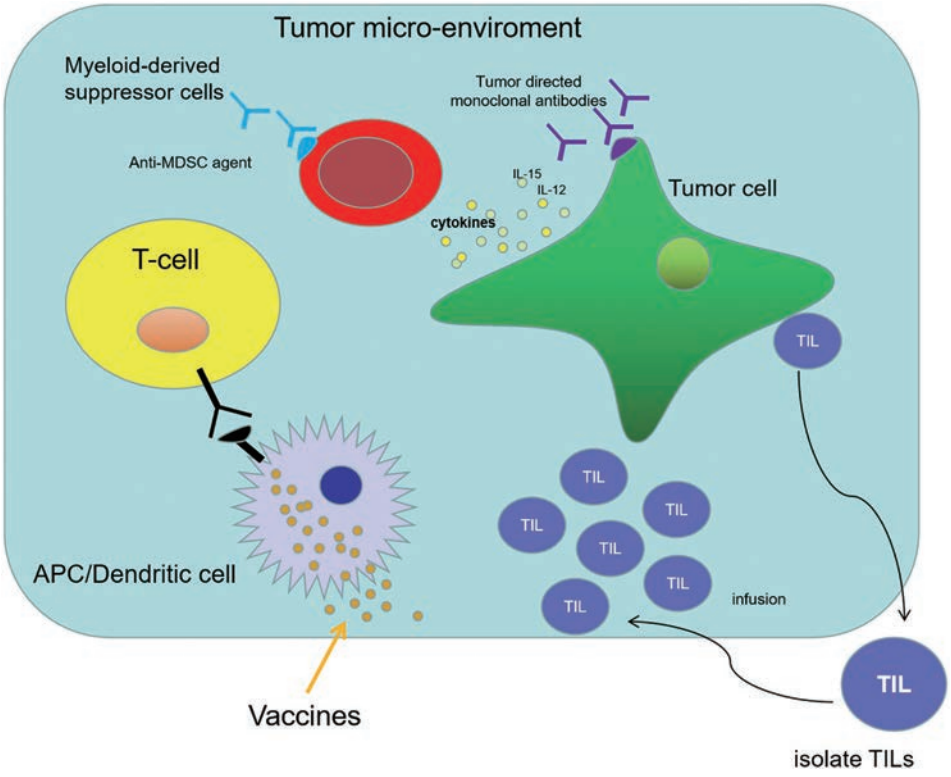

Fig. 1. Cancer immunotherapy approaches in BTC.

of hilar cholangiocarcinoma and distal cholangiocarcinoma undergoing radical resection are 20–40% and 16–52%, respectively.[9] In the presence of the above clinical manifestations, a multidisciplinary assessment is recommended immediately to determine the possibility of surgery.

For nonoperative patients, biliary drainage is recommended, referral to a transplant center if suitable for transplantation, or needle biopsy if not, followed by GC chemotherapy, clinical trials, fluorouracil or gemcitabine-based chemotherapy, fluorouracil radiotherapy, and supportive care. For operable patients, preoperative laparoscopic determination of staging and biliary drainage can be considered. For nonresectable patients found after intraoperative exploration, the treatment is the same as above. For resectable patients, surgical treatment can be performed, and postoperative adjuvant treatment and monitoring can be performed. For patients with metastases, it is recommended to use surgical bypass or endoscopy (such as ERCP) or percutaneous methods (such as PTC) for biliary drainage. Most patients often receive biliary stent implantation and biopsy at the same time. After the diagnosis of cholangiocarcinoma, the treatments are GC combined with chemotherapy, clinical trials, fluorouracil or gemcitabine-based chemotherapy and supportive care.[10] Table 1 summarizes the surgical/nonsurgical treatment plans of different types of BTCs.[11]

Will immunotherapy become a "savior" for BTC?

With the rapid development and cross-penetration of oncology, immunology, molecular biology and other related disciplines, immunotherapy has become an emerging research focusing on cancer treatment. Tumor immunotherapy began about 100 years ago, when Coley *et al*.[12] discovered that the application of streptococcus and *Staphylococcus aureus* toxins, later called Coley toxins, could control the growth of certain cancers. In the late 1980s, with the mature application of *in vitro* cell culture technology, lymphokine activated killer cells (LAKs) and tumor infiltrating lymphocytes (TILs) in clinical application, combined with chemotherapy and radiation treatment, obviously improve the curative effect of patients with cancer.

In the 21st century, medical science has continued to advance, and new cellular immunotherapy technologies have been developed rapidly. On April 29, 2010, the USA Food and Drug Administration (FDA) approved dendritic cells to treat advanced prostate cancer. This historic breakthrough enabled this treatment technology, that had undergone 15 years of lengthy clinical research, to enter into the clinical application stage.[13] Immunotherapy has become another important antitumor treatment after surgery, radiotherapy and chemotherapy, and it has been the hope of conquering malignant tumors (Fig. 1).

Potential benefit mechanisms of immunotherapy in BTC

Tumor cells survive and grow in the process of the body's antitumor immune response through an immune escape mechanism. Moreover, immunotherapy can kill tumor cells by activating and enhancing the body's antitumor immune response. At present, immunotherapy has been demonstrated to have definite effects in the treatment of various cancers, including melanoma, renal cell carcinoma and non-small cell lung cancer.[14–16] Chronic inflammation is known to promote tumor development in a number of ways and ultimately lead to immunosuppressive status in the tumor microenvironment. Inflammation also plays a key role in the occurrence and development of BTC, such as viral hepatitis,

primary sclerosing cholangitis, biliary inflammation caused by parasites or stones, etc., which are all the risk factors for BTC.[17] Therefore, it is speculated that chronic inflammation, antitumor immune response and immunosuppressive state in tumor microenvironment may have an interaction relationship in BTC, and immunotherapy could be a potential choice for the treatment of BTC (Fig. 1).[18]

In addition, a large number of studies have confirmed that infiltration of different immune cell subsets, including lymphocytes, macrophages, dendritic cells and granulocytes, can promote or inhibit tumor progression and/or metastasis in the tumor microenvironment of BTC.[19,20] Studies showed that the survival time of patients with high expression of immune-activating factors (CD4+, CD8+, Foxp3+T cells, MHC-I presenting cells, and NKG2D cells) was significantly higher than that of patients with low expression (hazard ratio [HR]: 0.52, $p<0.001$). In contrast, high expression of immunosuppressive factors (CD66b+ neutrophils, neutrophil-lymphocyte ratio, intratumoral IL-17+ cells, and PD-1+/CD8+TILs) was significantly associated with poor prognosis (HR: 1.79, $p<0.001$).[21] A study of ECC also found some similar conclusions; high expression of CD66b+ tumor-associated neutrophils ($p=0.01$), low expression of CD8+T cells ($p=0.02$), and high expression of Foxp3+ regulatory T cells ($p=0.04$) were all significantly associated with poor prognosis.[22] These studies further provide a theoretical basis for immunotherapy as a novel treatment for BTC. However, tumor-associated neutrophils and tumor-associated macrophages in the immune microenvironment have not yet become therapeutic targets in clinical trials of cholangiocarcinoma.

Immunocheckpoint inhibitors

Immunocheckpoint is an inhibitory signaling pathway that inhibits excessive inflammation in the body by modulating the autoimmune response. When a tumor appears, activation of the immune checkpoint can inhibit the activation and proliferation of T lymphocytes and induce the apoptosis of T lymphocytes, so that tumor cells can escape the immune response and increasingly reproduce. Blocking immune checkpoints can promote the activation of T lymphocytes and trigger antitumor immune response, so as to achieve the purpose of treating tumors.[23] The main targets of the present study are programmed cell death-1 (PD-1)/programmed death-ligand 1 (PD-L1) and cytotoxic T-lymphocyte-associated antigen 4. Others include lymphocyte activation gene 3 and T lymphocyte immunoglobulin myxin-3. PD-1 is an immunosuppressive transmembrane protein expressed on the surface of T lymphocytes, and PD-L1 is a PD-1 ligand induced by pro-inflammatory cytokines in tumor cells. In the tumor microenvironment, PD-L1 expressed by tumor cells can induce T lymphocyte failure through binding to PD-1, thus inhibiting the immune response of the body. Protein antibodies designed for PD-1/PD-L1 can block the recognition process of PD-1 and PD-L1 and restore the immune response of the body to achieve the therapeutic purpose. At present, PD-1/PD-L1 antibody has been used in the first-line treatment of non-small cell lung cancer, Hodgkin's lymphoma and other cancers.[24]

Potential benefit groups

Tumor mutation burden (TMB)

Studies have shown that PD-1 antibody has better immune response and antitumor effect in BTC patients with high TMB.[25,26] Lenvatinib is a multikinase inhibitor, mainly targeting vascular endothelial cell growth factor receptor, while pembrolizumab and nivolumab are both FDA-approved PD-1 inhibitors for a variety of advanced tumors. The antitumor activity of these three drugs alone has been evidenced in clinical trials. In the 2018 ASCO-GI, there was a single-center phase 2 study of lenvatinib combined with PD-1 monoclonal antibody (pembrolizumab or nivolumab) in the treatment of advanced ICC, which included a total of 14 patients who failed advanced multi-line therapy. The median progression-free survival was 5.0 months. Through the further stratified analysis of the sequencing results, a high TMB value (≥12) was found to be strongly correlated with better treatment response and longer progression-free survival time, suggesting that TMB may be used as a characteristic marker for judging prognosis.[27]

Mismatch repair (MMR) function

Microsatellite instability (MSI) refers to the phenomenon of changes in the length of tandem repeat DNA sequences caused by insertion or deletion mutations during DNA replication, often caused by MMR defects. MMR function is an important DNA repair mechanism that can accurately identify and repair base mismatches generated during DNA replication or recombination, and plays an important role in maintaining genome stability. MMR dysfunction is an abnormality in the MMR repair mechanism, which is generally highly consistent with MSI. It has been well demonstrated that MMR defects can cause immune cells to respond to cancer and that they can be used as a biomarker for PD-1 immunotherapy. However, most patients with cholangiocarcinoma do not have any mutations that can be used as therapeutic targets, which means that this is a typical highly immune-resistant cancer.

Studies have shown that patients with DNA MMR/MSI-high may represent the dominant-benefit population for BTC immunotherapy, and the incidence of MSI-high in BTC is 3%. Le *et al*.[28] reported the efficacy of pembrolizumab in solid tumors of DNA MMR in 86 patients with 12 tumor types and achieved an objective response rate of 53%. That study included four cases of cholangiocarcinoma, one case of complete remission, three cases of stable disease, and 100% disease control rate.

In May 2017, the USA FDA accelerated the approval of pembrolizumab for the treatment of MSI-high or DNA MMR refractory unresectable or metastatic solid tumors. It was the first drug that relied solely on specific genetic characteristics for treatment. The National Comprehensive Cancer Network guidelines also recommend PD-1 monoclonal antibody for BTC patients with MSI-H.

PD-1/PD-L1-positive expression

According to the immunohistochemical analysis of BTC patients, 32.3% of tumor cells and 74.2% of tumor-related macrophages can be observed to have positive expression of PD-L1, and the expression of PD-L1 is related to infiltrating lymphocytes, TILs) and human leukocyte antigen class I, and up-regulated PD-1/PD-L1 in BTC patients usually means worse overall survival.[29] In addition, the high expression of PD-L1 and the loss of human leukocyte antigen expression in BTC provide the basis for immune escape of tumor cells, which leads to worse prognosis and faster disease progression.[30] In the multicohort Ib study of KEYNOTE028 reported by the "ESMO" in 2019, pembromizumab (PD-1 monoclonal antibody) was used to treat advanced BTC with positive PD-L1 (>1%), and 42% (37/89) patients were

found to have positive PD-L1 expression (>1%). Among the 23 patients evaluated for curative effect, 4 cases were partially relieved, objective response rate was 17% (4/23), and 4 cases were stable. The results showed that the effective rate of immunotherapy for cholangiocarcinoma was similar to other solid tumors, close to the average, and had good tolerance.[31] In 2019, the ASCO reported that nivolumab alone or combined with GC was used to treat unresectable or recurrent cholangiocarcinoma. Moreover, 30 patients were enrolled in the single-drug group and combined-drug group respectively. Subgroup analysis showed that the median overall survival of patients with PD-L1 >1% in the single-drug group was better than that of patients with PD-L1 <1%. However, the relationship between the expression of PD-L1 and overall survival in the combined-drug group is still unclear.[32] In the 2020 ESMO, an open-label, one-arm, phase II clinical trial evaluated the survival benefits of chemotherapy with treprazolam, lamivudine combined with oxaliplatin and Gemox for unresectable advanced ICC patients. A total of 30 patients were included, and the results showed that PD-L1 protein expression was significantly positively correlated with objective response rate. Specifically, PD-L1+ vs. PD-L1- showed objective response rates of 100% vs. 68.8% (p=0.048) (NCT 03951597; Abstract No. 56P). It is noteworthy that the KEYNOTE-158 study reported in the 2019 ASCO, a phase 2 study, evaluated the antitumor activity and safety of pembrolizumab against advanced cholangiocarcinoma. A total of 104 patients were involved, and 6 patients were partially relieved, with objective response rate of 5.8%, median progression-free survival of 2 months and median overall survival of 9.1 months. This study found that pembrolizumab showed certain antitumor activity and controllable toxicity in patients with advanced BTC, regardless of the combined positive score of PD-L1.[33]

A clinical meta-analysis of 16,176 tumor patients, including those with BTC, showed that PD-L1 expression levels varied greatly in different tumor types; overall PD-L1 expression was associated with poor disease-free survival and overall survival was significantly positively correlated.[34] From this, we can speculate that the antitumor effects of PD-1/PD-L1 antibodies in different subtypes of BTC may also be significantly different. Therefore, in the future, more studies should be carried out with different types of BTCs to further clarify the relationship between the positive expression of PD-1/PD-L1 and the efficacy of BTC immunotherapy.

Insertion deletion variation

Studies have reported that two BTC patients with insertion deletion variation that was significantly higher than the median level (48% and 66.84% respectively, with a median level of 12.77%) were completely relieved after receiving PD-1 antibody combined with chemotherapy. Therefore, it is speculated that high-level insertion deletion variation can produce more tumor-specific antigens, and then express higher affinity with MHC class I. The high level of insertion deletion variation is a predictive factor for the good response of PD-1 treatment of BTC patients.[35]

Safety assessment

There are few reports on the adverse reactions of PD-1 antibody during BTC treatment. In the 2019 ASCO, there was a phase 2 study of nivolumab in the treatment of patients with advanced refractory BTC, in which nivolumab was used after at least first-line but no more than third-line systematic treatment. The most common treatment-related adverse events were elevated alkaline phosphatase (24.5%), and the common grade 3 to 4 adverse reactions were hyponatremia (3 cases) and elevated alkaline phosphatase (2 cases).[32]

Combination therapy-future development direction

At present, clinical trials using PD-1/PD-L1 antibody to activate the antitumor immune response to treat BTC has been carried out gradually. Combination therapy will be the main trend in the future. However, the efficacy of combination therapy remains controversial. A phase 1 study of the safety and efficacy of ramucirumab combined with pembrolizumab in patients with advanced BTC showed no significant improvement in overall survival with only 4% objective response rate, 1.6 months for median PFS, and 6.4 months for overall survival. However, the study found that PD-L1-positive patients had better overall survival than PD-L1-negative patients, which suggested that the baseline characteristics of patients may affect treatment efficacy. It is key to select the group reasonably.

In April 2020, the American Cancer Society online meeting announced a new study. That study is a multicenter, randomized phase II trial to explore the combination of PD-L1 monoclonal antibody (atrizizumab) and MEK inhibitor. In addition, the efficacy of cobimetinib is being assessed in the treatment of BTC. A total of 77 patients who had previously undergone one to two lines of treatment were included. For group A (n=37, ICC=21, ECC=7, GBC=11), aterizumab (840 mg, q2w) were injected intravenously. For group B (n=38, ICC=22, ECC=8, GBC=8) daily oral cobitinib (60 mg, taken for 21 days/7 days off) combined with intravenous aterizumab (840mg, q2w) were administered. Initial results of group B vs. group A include median progression-free survival of 3.65 months vs. 1.87 months (0.027), disease control rate of 45.2% vs. 32.4%, including one case of partial response (3.2%) in group B and 13 cases of stable disease (41.9%). As for the adverse reactions, the two groups had similar grade 3–4 treatment-related adverse events, and no treatment-related deaths. Atrizizumab combined with cobitinib reached its primary endpoint and significantly prolonged progression-free survival. The toxicity is controllable and worthy of further study in BTC.

Keynote-966 is a randomized, double-blind, placebo-controlled phase III study designed to investigate the treatment of patients with advanced cholangiocarcinoma with papolizumab combined with GC. This study includes metastatic or non-resectable local BTC patients who have not received systematic treatment. Patients are randomized 1:1 (n=788) to the pembro+GC and placebo+GC groups. The primary endpoints are progression-free survival and overall survival, and the secondary endpoints were objective response rate and duration of response. The final results will be released soon, but it is known that some positive results have been obtained thus far. Combination therapy will become the exploration trend of BTC in the future.

In January 2021, the American Cancer Society online meeting, the multicohort phase II LEAP-005 study showed the data of lenvatinib plus pembrolizumab for patients with previously treated BTC. Thirty-one BTC patients were included in this study (partial response: n=3; stable disease: n=18). objective response rate was 10% (95% confidence interval: 2–26) and DCR was 68% (95% confidence interval: 49–83). The median DOR was 5.3 months. The median PFS was 6.1 months (95% confidence interval: 2.1–6.4). The median OS was 8.6 months (95% confidence interval: not reported-5.6). Lenvastinib combined with pablizumab has shown encouraging efficacy and manageable toxicity in patients with advanced BTC who have previously received first-line treatment.[36]

Advantages of immunotherapy

The treatment effect of "immunoinflammatory" tumor is good, and the long-term survival rate is significantly improved. The treatment initiates the body's immune system to restore immune function and kill tumor cells over a long term. Meanwhile, it can also restore and improve the body's immune function, fully identify, search for and kill tumor cells, and effectively prevent tumor recurrence and metastasis. Moreover, the side effects are less than the traditional treatment. All in all, immunotherapy has a high accuracy, specificity and targeting of immune system.[37]

Existing problems

Although immune checkpoint inhibitors based on PD-1/PD-L1 antibodies have some effectiveness in the treatment of BTC, they are still faced with problems, such as low objective response rate and drug resistance. How to select the target group and control the timing of immunotherapy combination, such as sequential, intermittent, continuous, and the interval between the therapy. All these questions require further exploration. In addition, although current studies have confirmed the partial effectiveness and short-term safety of PD-1/PD-L1 antibody in the treatment of BTC, immune checkpoints are the normal physiological functions of the human body. It is still unclear whether the artificial suppression of immune checkpoints to enhance the body's immune response will cause long-term chronic tissue and organ immune loss and autoimmune diseases. At the same time, the specific mechanism of signal transduction of immunosuppressive pathway including PD-1/PD-L1 and the interaction with the tumor microenvironment are not yet fully clear. Future research directions should also focus on the above aspects.

Adoptive cell transfer (ACT) therapy

ACT refers to the isolation of immunocompetent cells from tumor patients, which are amplified *in vitro* and then returned to the patient's body, so as to achieve the purpose of stimulating the body's immune response or directly killing tumor cells. ACT therapy is currently divided into two categories, namely non-specific cell therapy (including cytokine-induced killer (CIK) therapy, TILs, etc.) and specific cell therapy (including T lymphocyte receptor chimeric T lymphocyte (TCR-T), chimeric antigen receptor T lymphocyte (CAR-T), etc.).[38]

Nonspecific cell therapy

CIK is a class of fast growing high-efficiency immune effector cells that are not restricted by MHC. The combination culture of dendritic cells that recognizes antigens and activates the immune system and CIK with highly effective anticancer activity has been used in clinical trials for tumor therapy.

It has been well demonstrated that CIK can delay tumor progression in a variety of solid tumors, including gastrointestinal malignancies. A clinical study involving 72 patients with advanced BTC who received adoptive treatment with dendritic cell-CIK showed that there were 1 complete response, 25 partial response, 34 stable disease, 12 progressive disease, and disease control rate of 83.3%. Nine cases (12.5%) of low-grade fever occurred, which were relieved after symptomatic treatment, no other adverse reactions were seen, indicating high safety. In addition, IL-6 and serum CA199 levels decreased significantly after receiving treatment. The percentages of CD8+CD38+T, CD8+DRT cells and CD3-CDL5+CD56+T and CD3+CDl6+CD56+T cells were significantly increased.[39]

TIL is a heterogeneous lymphocyte population in tumor stroma, including T lymphocytes and natural killer cells, which directly kills tumor cells by regulating the immune function of the body and releasing cytotoxins. Through immunohistochemical analysis of 375 cases of BTC patients, some studies found that TIL infiltration of different degrees could be observed in about half of the patients. The level of TIL infiltration was closely related to tumor grade and overall survival. A high level of TIL infiltration often predicted better overall survival.[40] A number of studies have shown the potential prospects of TIL adoptive therapy for BTC. A randomized controlled study showed that the 5-year progression-free survival and overall survival of the experimental group combined with TIL adoptive therapy and dendritic cell vaccine treatment were significantly higher than those of the control group that only underwent surgical resection (the experimental group progression-free survival and overall survival were respectively 18.3 and 31.9 months, while the control group showed 7.7 and 17.4 months respectively).[41]

Specific cell therapy

CAR-T and TCR-T used genetic engineering technology to genetically modify ordinary T lymphocytes in tumor patients. The modified T lymphocytes can express specific receptors and recognize specific tumor cells without MHC presentation, which can induce strong antitumor immune response without toxicity to normal cells.

Although there have been no reports of effective treatment of BTC using CAR-T and TCR-T, studies have shown that CD19 antigen-specific CAR-T technology produces sustained disease remission in clinical trials for the treatment of adult and childhood B lymphocytic leukemia and lymphoma. In addition, CAR-T and TCR-T technologies have also achieved certain results in the treatment of malignant melanoma, breast cancer, liver cancer, prostate cancer, lung cancer, and colorectal cancer.

Compared with non-specific cell therapy, CAR-T and TCR-T have the characteristics of specific killing of tumor cells and stronger immune effect, which have become hot spots in the field of ACT therapy. However, there is still a lack of breakthrough progress. Chinese researchers used CAR-T therapy targeting EGFR and CD133 for patients with metastatic cholangiocarcinoma and achieved partial remissions, lasting 8.5 months and 4.5 months respectively. However, the damage caused by CAR-T cell infusion cannot be ignored.[42]

Most of the existing studies believe that T lymphocytes injected by CAR-T and other exogenous agents failed and impaired effector function after entering the body, which may be due to adaptability of T lymphocytes and immunosuppressive state of the tumor microenvironment. How to ensure the accurate homing of T lymphocytes from peripheral blood infusion to the local solid tumor, break through the immunosuppression of tumor microenvironment, infiltrate into the tumor and ensure the continuous expansion of T lymphocytes so as to play a killing role are still difficulties currently. Furthermore, so far, studies of CAR-T cells have focused more on enhancing its function, but in almost all clinical trials there have been adverse reactions (such as cytokine release syndrome and neurotoxicity), and some may be fatal. With the transformation of CART cells, adverse reactions may increase, so the toxicity control of CART cells is

a problem that cannot be ignored.

Prospective considerations

The application of immunotherapy in the treatment of BTC has achieved initial results. Existing studies have shown that immunotherapy can improve the immune function and quality of life of patients with advanced BTC, and have some survival benefits to a certain extent. However, current research is mostly limited to small samples and lack of large sample, high-quality prospective randomized controlled trials. With the advent of the era of precision medicine and the in-depth understanding of BTC from the molecular level, the selection of specific treatment options for BTC patients in different populations and subtypes is the key to immunotherapy in the future. The combined application of multiple immunotherapies or immunotherapy combined with chemotherapy, targeted therapy and other treatment methods are also the focus of future research.

Design of clinical trials for oncology drugs in BTC

Drug therapy is an important means of tumor treatment, and the development of new antitumor drugs is an urgent clinical requirement in the world. Among them, clinical trials are the fastest, safest and most effective way to find new antitumor drugs and provide the optimal treatment for a cancer patient. However, since there is no human data and experience before the new drug enters the clinical trial, the clinical evaluation is full of unknown risks and challenges.[43]

In recent years, the level of clinical trials on new antitumor drugs has significantly improved. We reviewed the anti-cancer drug clinical trials registered on the USA clinical trial website in 2019. There were 238 ongoing oncology phase I clinical trials in mainland China. Among them, there were 160 solid tumor trials and 78 hematological malignant tumor trials. In terms of the total number of phase I clinical trials in oncology, there were 44 in Japan and 28 in South Korea in Asia. There were 327 ongoing oncology-related phase I clinical trials in Europe in 2019, of which 62 were from Spain (ranking first), followed by 50 from France, 41 from the UK, 25 from Italy, and 19 from Germany. There were 675 tumor-related phase I clinical trials in the USA in 2019. In the context of global accelerated research and development of innovative drugs, how the design of clinical trials of BTC is a topic worthy of attention.

Application of phase zero clinical trials in clinical research of antitumor drugs

In order to guide the rapid development of innovative drugs and control the clinical risks in the development of new drugs, the European Medicines Agency (EMEA) and the FDA issued respectively in 2004 and 2006 “new exploratory research guiding principle”, put forward before the traditional I stage of clinical trials in the concept of zero phase of clinical trial, and a series of meaningful results are obtained.

The phase zero clinical trial refers to a drug trial conducted by the developer using a micro-dose on a small number of healthy volunteers or patients (usually 6–15 people) before the active compound is formally entered into the clinical trial after the pre-clinical trial is completed, and the necessary relevant information is collected. The test data of drug safety and pharmacokinetics to evaluate whether the research and development drug has the possibility of further development as a new drug or biological agent. It is the intermediate link in the transition from pre-clinical trials to phase I clinical trials.[44]

The purpose of phase zero clinical trial is to obtain human pharmacokinetic data, containing protein binding, enzyme inhibition rate and the combination of target, and to adopt various means of imaging studies of human tissue distribution, so that early identification of the most valuable lead compound from a set of candidates of phase I clinical trials can be facilitated. In addition, understanding the metabolic characteristics of lead compounds in humans as early as possible is also of great significance for the selection of animals for non-clinical safety studies and improving the predictive value of animal test results.[45–47]

Analysis of the mechanism of innovative drugs

The in-depth research of translational medicine has put forward new topics for the clinical research of antitumor drugs. It is necessary to develop new clinical trial methods and effective detection technology of related targets, attach importance to the construction of clinical trial-related laboratories, and actively carry out translational medicine research, so as to draw correct conclusions on the clinical application value of these new drugs with different mechanisms of action. Therefore, only a more in-depth exploration of the molecular mechanism and pharmacological mechanism of drugs in the laboratory stage can lay the foundation for the success rate of its translational research.

Individualized clinical research design

The classification of tumors of the biliary system is complex and heterogeneous, and the sensitivity of different tumors to drugs is bound to be different. When designing a clinical trial, a specific target population should be selected. Individualized molecular therapy programs and technologies based on the expression status of multiple genes or markers and the changing laws of related proteins and metabolites are the future development direction.

Multicenter collaborative research

The establishment of a multicenter collaborative organization can accelerate the process of drug development and marketing, and ensure the quality of clinical trials.[48] Many anticancer drug clinical trial multicenter collaborative organizations have been established internationally, such as the European Organization for Cancer Therapy Research (EORTC), the Eastern Cooperative Oncology Group (ECOG), the Japanese Clinical Oncology Cooperative Organization (JCOG), the Southwest Oncology Cooperative Group (SWOG) and so on, and have achieved a series of results. These research results have significantly promoted the development of clinical oncology and have become the basis for the current clinical diagnosis and treatment guidelines. Based on multicenter collaborative research, it is bound to accelerate the research and development of innovative drugs.

Funding

The study was supported by the National Natural Science Foundation of China (No. 82002461), and Medjaden Academy and Research Foundation for Young Scientists (MJR20211110), as well as the Fund for Fostering Young Scholars of Peking University Health Science Center (BMU2021PY010)

Conflict of interest

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

Author contributions

Writing of the manuscript (RX, RL), and conception of the idea for the study (JW, JH, WT). All authors reviewed and approved the final version of the manuscript.

Data sharing statement

All data are available upon request.

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Journal of Clinical and Translational Hepatology **2021** vol. 9(4) | 568–575
DOI: 10.14218/JCTH.2021.00001

Review Article

Special Considerations in the Management of Autoimmune Hepatitis in COVID-19 Hotspots: A Review

Deepak Madhu[1,2], Sanchit Sharma[3], Ashish Agarwal[3,4] and Anoop Saraya[3*]

[1]Department of Gastroenterology, Aster MIMS Calicut, Kerala, India; [2]Department of Gastroenterology, Caritas Hospital, Kottayam, Kerala, India; [3]Department of Gastroenterology and Human Nutrition, All India Institute of Medical Sciences, New Delhi, India; [4]Department of Gastroenterology, All India Institute of Medical Sciences, Jodhpur, Rajasthan, India

Received: 31 December 2020 | **Revised:** 22 April 2021 | **Accepted:** 27 April 2021 | **Published:** 14 May 2021

Abstract

The ongoing coronavirus disease-2019 (COVID-19) pandemic has necessitated special considerations in the management of diseases. The way presence of pre-existing diseases or treatment for it predisposes to, alters course of, and changes the management of COVID-19, is of relevance and is being extensively studied. Autoimmune hepatitis (AIH) is unique in that it is an autoimmune disease mandating treatment with immunosuppressive drugs, as well as a liver disease with potential for varying degrees of underlying fibrosis. The use of immunosuppressive drugs could alter the risk of acquiring COVID-19, the clinical course and severity of COVID-19 and the degree of underlying liver fibrosis could alter the clinical outcomes of patients with COVID-19. In this review, we try to summarize key areas relevant in understanding and improving the clinical care of patients with AIH in the current pandemic. Special considerations required in the management of patients with AIH in COVID-19 hotspots have been outlined based on the current evidence.

Citation of this article: Madhu D, Sharma S, Agarwal A, Saraya A. Special considerations in the management of autoimmune hepatitis in COVID-19 hotspots: A review. J Clin Transl Hepatol 2021;9(4):568–575. doi: 10.14218/JCTH.2021.00001.

Introduction

First noticed as a cluster of viral pneumonia among people known to have visited a market in the Wuhan City of Hubei province in China,[1] and later on investigated by the China Center for Disease Control and Prevention and found to be due to infection with a new beta coronavirus,[2] the disease was later named Coronavirus disease-2019 (COVID-19),[3] and the virus causing it was christened severe acute respiratory syndrome-coronavirus-2 (SARS-CoV-2).[4] The disease spread throughout the world over a period of a few months, to be declared as a pandemic by the World Health Organization (WHO) on the 11th of March 2020.[5] By the 30th of December 2020, it had infected over 80 million people and resulted in the death of over 1.7 million people.[6] While predominantly a respiratory pathogen, SARS-CoV-2 has also been shown to cause significant neurologic, cardiac, gastrointestinal, hepatic, renal, hematologic, obstetric, gynecologic, and rheumatologic abnormalities as well.[7]

The focus of this review is on special considerations for the management of autoimmune hepatitis (AIH) in areas with widespread community transmission of COVID-19. Factors that need to be considered include the risk of acquiring COVID-19 and the risk of poor outcomes with COVID-19. Outcomes in patients with COVID-19 could be altered due to AIH itself, because of the immunosuppressive medicines used to treat AIH, or by virtue of liver impairment that AIH has caused. We will be discussing aspects that are of specific relevance to a practitioner caring for AIH in COVID-19 hotspots. Relevant aspects of COVID-19-induced liver injury, aspects of COVID-19 prevention (including vaccination), and special considerations required in the management of AIH have been discussed herein. We have also discussed possible approaches that a clinician can adopt in various case-scenarios that may be encountered.

Liver injury in COVID-19

Liver abnormalities noted to be present in patients having COVID-19 include transaminitis, hyperbilirubinemia and hypoalbuminemia.[8–11] These abnormalities are thought to occur by one or more of the following several mechanisms (Fig. 1): direct cytopathy;[12] immune-mediated;[13] 3. hypoxia-related;[14] drug-induced;[14] and, microvascular thrombosis.[15,16] Patients with COVID-19 and liver injury,[17] as well as those with prior hepatic comorbidities, have been shown to have poor outcomes with COVID-19.[18] Patients with cirrhosis are thought to be at moderate risk, whereas patients with decompensated cirrhosis are at high risk of poor outcomes with COVID-19.

Keywords: Hepatitis; Autoimmune; Liver cirrhosis; COVID-19; SARS-CoV-2 infection; Immunosuppressive agents.

Abbreviations: AASLD, American Association for the Study of Liver Diseases; ACLF, acute on chronic liver failure; AD, acute decompensation; AIH, autoimmune hepatitis; ALF, acute liver failure; APASL, Asia Pacific Association for Study of Liver; CNIs, calcineurin inhibitors; COVID-19, coronavirus disease-2019; DILI, drug-induced liver injury; EASL, European Association for Study of Liver; IBD, inflammatory bowel diseases; MMF, mycophenolate mofetil; SARS-CoV-2, severe acute respiratory syndrome-coronavirus-2; WHO, World Health Organization.

[*]**Correspondence to:** Anoop Saraya, Department of Gastroenterology and Human Nutrition, All India Institute of Medical Sciences, Ansari Nagar; New Delhi 110029, India. ORCID: https://orcid.org/0000-0002-3921-6752. Tel: +91-9868397203, E-mail: ansaraya@yahoo.com

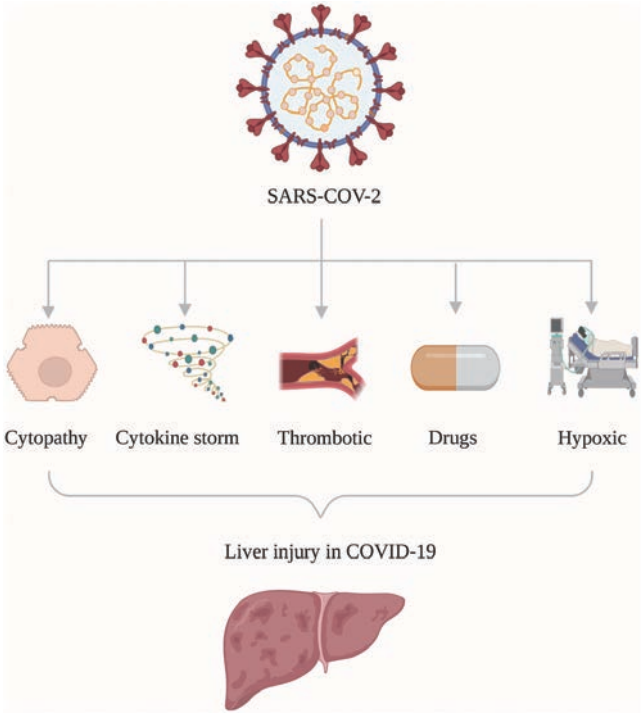

Fig. 1. Pathogenesis of liver injury in COVID-19.

Direct cytopathy

ACE2 receptors, which the SARS-CoV-2 virus utilizes for entering cells have been shown to be expressed in cholangiocytes[19] and probably hepatocytes[20] as well. It has also been shown that SARS-CoV-2 invades liver cells and causes cytopathy.[12] This may, at least partially, be responsible for the hepatic dysfunction seen in COVID-19 patients.[12]

Immune-mediated

SARS-CoV-2 infection results in a disordered inflammatory response, i.e. the cytokine storm,[21] with increase in pro-inflammatory cytokines. This has been shown to be responsible for severe pulmonary and extrapulmonary dysfunction, including liver injury. Liver dysfunction has been shown to be particularly more in patients with increased levels of inflammatory markers, such as CRP, TNF and IL-6.[13]

Hypoxic/ischemic

In severe COVID-19, multiorgan dysfunction can lead to hypoxia-related to acute-respiratory distress syndrome,[22] hypotension,[23] or congestive cardiac failure. All of these can result in liver dysfunction.[24]

Drug-induced liver injury (DILI)

In addition to supportive therapy, antivirals, immunomodulators and antithrombotic drugs are used in the management of COVID-19. Several of these drugs, including antivirals such as lopinavir-ritonavir, remdesivir, favipiravir etc., and immunomodulators such as tocilizumab, baricitinib, etc. may cause liver dysfunction.[25,26]

Microvascular thrombosis

Endothelial dysfunction along with inflammation in patients with COVID-19 produces vascular thrombosis in multiple organs.[27] Elevated D-dimer levels were found to be independently associated with liver dysfunction in one study,[15] which could point to an association of thrombosis with liver dysfunction. Studies of liver biopsies from patients with COVID-19 and liver dysfunction have shown significant microvascular thrombosis which could lead to liver dysfunction.[16] This could point to a contribution by microvascular thrombosis to the liver dysfunction seen in patients with COVID-19.

Pre-existing liver diseases and COVID-19

Patients with AIH have varying degrees of underlying fibrosis and as much as 40% of these patients develop cirrhosis.[28] The degree of underlying fibrosis in patients with AIH has the potential to have an effect on the risk of acquiring COVID-19, as well as on clinical outcomes in patients with COVID-19.

It can be assumed that, despite a reduction in the immunity of patients with cirrhosis, the risk of acquiring COVID-19 does not seem to be higher in patients with cirrhosis, as evidenced by the results of a meta-analysis which demonstrated that the prevalence of cirrhosis in patients with COVID-19 is similar to that in the COVID-19-negative population.[18]

There seems to be an upregulation of ACE2 receptors in the liver, which probably makes patients with cirrhosis more vulnerable to COVID-19-related liver injury.[29] Patients with pre-existing liver disease have been shown to have increased mortality and morbidity, with COVID-19.[18] Multiple studies have found deterioration of liver functions and decompensation in cirrhotic patients with COVID-19.[30–32] Patients were found to have significantly higher risk of mortality with worsening Child-Pugh status.[31,32] Cirrhosis was also found to be an independent predictor of severe COVID-19, in patients with AIH in recent multicenter studies.[33,34] With such poor outcomes, it becomes pertinent that patients with cirrhosis be considered high risk.

Immunosuppressants and COVID-19

Patients on immunosuppressants have a complex interplay of factors in favor of and against SARS-CoV-2. On one hand, immunomodulators such as mycophenolate mofetil (MMF)[35,36] and calcineurin inhibitors (CNIs) like tacrolimus[37] and cyclosporine[38,39] have been demonstrated to have antiviral activity against coronaviruses, and glucocorticoids administered for COVID-19 have been shown to prevent the disordered immune response that is responsible for poor outcomes in COVID-19.[40] On the other hand, the immunosuppression attributable to these drugs may cause increased susceptibility to SARS-CoV-2 infection,[41] secondary bacterial or fungal infections, and prolongation of viral clearance.[42] There have been studies that demonstrated increased risk[41] as well as others that demonstrated average risk[43] of acquiring SARS-CoV-2 infection for patients on immunosuppressants, and the question largely remains unresolved to date. Retrospective studies have demonstrated a risk of bacterial superinfection[44] or increased use of antibiotics[45] in patients managed with steroids for COVID-19, whereas randomized controlled trials have negated this[46–48] as an adverse event

Table 1. Published data of AIH patients with COVID

Study	Region	Number of COVID-19-positive AIH	COVID-19 requiring hospitalization	Survived
Verhelst 2021[50]	Flanders, Belgium	1	100% (1/1)	100% (1/1)
Rigamonti 2020[51]	Northern Italy	4	50% (2/4)	100% (4/4)
Di Giorgio 2020[52]	Northern Italy	4	50% (2/4)	75% (3/4)
Gerussi 2020[53]	Italy	10	60% (6/10)	90% (9/10)
Marjot 2021[34]	Multinational	70	76% (53/70)	77% (54/70)
Efe 2021[33]	Multinational	110	46% (51/110)	90% (99/110)

of steroids. The data on the effect of steroids on viral shedding is mixed; although, the wealth of evidence suggests that low-dose steroids are not associated with any increase in viral shedding.[42] Studies have also been performed to assess how these seemingly opposing actions translate to clinical outcomes in COVID-19. The risk of acquiring SARS-CoV-2 infection seems to be higher in patients with autoimmune diseases on steroids[49] and clinical outcomes seem to be worse in patients on steroids as well as in patients on immunomodulators, in patients with autoimmune diseases.[49] Early data available specifically in the context of AIH seem to show that continued immunosuppression does not lead to poor outcomes with COVID-19.[33,34]

AIH and COVID-19

Two large multicenter studies have addressed the impact of COVID-19 in patients with AIH.[33,34] In both the studies, the outcomes of patients with AIH were compared with a cohort of non-AIH patients with chronic liver disease. The consistent findings across both the studies was that there is no increase in severity of COVID-19 infection across patients with AIH compared to other etiologies of chronic liver disease.[33,34] Presence of cirrhosis, particularly Child-C disease, was the most significant factor of poor outcomes in these patients.[34] New-onset liver injury was seen in one-third of the patients with AIH after COVID-19 in one study.[33] However, the use of immunosuppressants was not associated with poor outcomes in patients with AIH and COVID-19.[33] In fact, one study showed that continuation of immunosuppression was associated with lower risk of new-onset liver injury.[33] This suggests that immunosuppression needs to be continued in patients with AIH and COVID-19. Apart from these two studies, however, data on AIH and COVID-19 are limited to a few small case series (Table 1).[50–53]

Diagnostic approach

The diagnostic approach for AIH in COVID-19 hotspots can be the same as elsewhere, broadly speaking. Selected patients who are asymptomatic and being evaluated for abnormal transaminases or only mildly symptomatic for AIH can be, in the initial part of the diagnostic workup, evaluated by a telemedicine-based approach. Liver biopsy is necessary for a diagnosis of AIH to be made, as per American Association for the Study of Liver Diseases (AASLD) guidelines[54] and can be performed in a COVID-minimal pathway, safely, for COVID-19-negative patients. Liver biopsy in COVID-19-positive patients need to be decided on a case-by-case basis, depending on the urgency to treat AIH, the severity of COVID-19, and other factors like the presence of coagulopathy, sepsis, logistics, chance of cross-infection, etc. The Asia Pacific Association for Study of Liver (APASL) recommends liver biopsy in COVID-19-negative patients when autoimmune flare is suspected and advises against liver biopsy in COVID-19-positive patients.[55]

Prevention of COVID-19

General measures

Owing to the high risk of poor outcomes that can be expected with the degree of pre-existing liver dysfunction and immunosuppression, prevention of SARS-CoV-2 infection is extremely important in patients with AIH. The preventive strategy against COVID-19 should comprise general measures as well as vaccination. General measures should involve measures to be adopted by the patient,[56] hospital-designed infrastructure measures,[57] and hospital operational measures,[57] which include a COVID-minimal pathway,[58] as well as measures to be adopted by health-care personnel.[59] Telemedicine becomes particularly relevant during the current COVID-19 epidemic as it has the potential to reduce the need for hospital visits, which in turn reduces the chance of crowding in hospital outpatient clinics, thereby reducing the risk of spreading SARS-CoV-2.[60] Telemedicine has been evaluated specifically in the context of management of AIH during the COVID-19 pandemic and has been shown to improve patient adherence to therapy thereby minimizing the chances of relapse, as compared to the standard care group.[60]

Vaccination

As on December 23rd, 2020, there have been at least seven COVID-19 vaccines[61] licensed in different parts of the world and over 200 vaccines in different stages of development.[62] In the context of patients with AIH, four specific aspects need to be addressed; these include: 1) safety of the vaccine in patients on immunosuppressants; 2) safety of the vaccine in patients with liver diseases; 3) efficacy of the vaccine in patients on immunosuppressants; and, 4) efficacy of the vaccine in patients with liver diseases.

As far as the licensed vaccines are concerned, patients on immunosuppression were excluded from vaccine licensing trials. While some trials included a small number of patients with pre-existing liver diseases, patients with advanced liver diseases were still excluded and no subgroup analysis was performed to assess outcomes or adverse events specifically in patients with liver diseases.[63] Thus, it is not clear at this moment how safe and effective these vaccines would be, in standard doses, for patients with AIH, especially while on immunosuppressive medications. Extrapolating from the experience with vaccination for other diseases in patients with liver diseases,[64–66] in patients on immunosuppression,[67] and specifically in AIH,[68] the likely efficacy of the

Table 2. Special considerations in the management of AIH in COVID-19 hotspots: A suggested approach

COVID-19 status	AIH status	Special considerations
COVID-19-negative	Diagnosis of AIH	Diagnostic algorithm same as otherwise. Liver biopsy to be planned in COVID–minimal pathway
	Newly diagnosed patients with activity	Steroids and azathioprine can be given as indicated otherwise. Budesonide to be preferred over prednisolone in appropriate situations, in noncirrhotic patients, and in patients without acute severe AIH
	Patients in remission	Continue immunosuppressant at lowest recommended dose required to maintain remission. Decision to stop immunosuppression to be made in patients who have had long-term remission, as per latest guidelines for AIH. Telemedicine-based follow-up in appropriate cases
	Patients who require start of second-line agent	CNIs (tacrolimus) may be preferred over mycophenolate in patients with no other contraindications[a]
	Patients who require start of third-line agent	Infliximab may be preferred over rituximab in patients with no other contraindications[a]
	Decompensated cirrhosis	Treatment algorithm same as otherwise. Living donor liver transplant to be considered for urgent/emergency indications only
	Acute severe AIH	Diagnostic and treatment algorithm same as otherwise
	ALF due to AIH	Diagnostic and treatment algorithm same as otherwise. May require urgent liver transplantation
COVID-19-positive	Diagnosis of AIH	Evaluation by serology, imaging same as otherwise. Decisions regarding liver biopsy to be taken on case-by-case basis
	Newly diagnosed patients with activity/patients in remission/ patients who require start of second-line agents/patients who require start of third-line agents/patients with decompensated cirrhosis	Decisions regarding management to be taken on an individualized, case-by-case basis. Patients with AIH in remission may continue immunosuppressants as before, unless other contraindications or considerations are present. Treatment decisions in patients requiring induction or escalation of therapy for AIH needs to be taken on a multidisciplinary, case-by-case basis
	Acute severe AIH	Need for aggressive immunosuppression likely to override all other considerations, final decision to be taken on a multidisciplinary, case-by-case basis
	ALF due to AIH	Decision to be taken on a multidisciplinary, case-by-case basis

[a]Weak suggestion, based on data extrapolated from other conditions.

COVID-19 vaccine in this subgroup of patients is likely to be lower compared to the normal healthy adult.

Considering the high risk of poor outcomes due to COVID-19 infection in patients with liver diseases[18] and patients with immunosuppression,[69] the benefits of vaccination may outweigh the risks. COVID-19 vaccination is being suggested in patients with chronic liver disease and solid organ transplant recipients on immunosuppression by various international societies, including AASLD[63] and European Association for Study of Liver (EASL).[70] Till further data on safety and efficacy are available, COVID-19 vaccines need to be administered at standard doses, unless other contraindications are present. Due to the high risk of adverse events, live vaccines[71] and replicating viral vector vaccines[72] are best avoided in patients on immunosuppressive medications. Further, household members and care providers of these patients should also receive vaccination while continuing appropriate use of masks, sanitizers and social distancing — the keystone of protection against COVID-19. EASL and APASL recommend patients with AIH to be also vaccinated against influenza and *Streptococcus pneumoniae*.[55,73] The formation of neutralizing antibodies in liver transplant recipients (especially those receiving immunosuppressants) have been suboptimal, as shown by a recent study.[74] The clinical impact of this suboptimal response remains to be seen. However, this should not deter any clinician from prescribing the vaccine in these patients.

Treatment in COVID-19 negative patients

Principles of treatment

It is clear that the presence of pre-existing liver disease has a significant bearing on the outcomes of patients with COVID-19,[18] and given the fact that the present pandemic has been ongoing for the past several months and will continue to do so for some time, it seems prudent that the patients not having active COVID-19 but requiring induction or maintenance therapy for AIH be given immunosuppression as required because withdrawing, delaying or denying it may result in worsening fibrosis or cirrhosis.[75–77] Recommendations by APASL[55] and the EASL[73] seem to support the view that immunosuppression needs to be continued. Strategies for treatment and follow-up should incorporate aspects of prevention as elaborated, including general measures and vaccination. We have discussed special considerations required in the treatment of COVID-19-negative patients with AIH below, and summarized them in Table 2.

Decisions for patients with active COVID-19 requiring immunosuppression for AIH induction or maintenance need to be considered on an individualized, case-by-case basis after assessing risks and benefits.

First-line agents

Patients on systemic steroids have been found to have poor COVID-19-related outcomes.[49] Prednisolone/prednisone or budesonide in combination with azathioprine is used for first-line management of AIH.[54] Owing to high first pass metabolism of budesonide, it is known to have less systemic toxicity and less chance of infections.[78] It seems reasonable that patients with new diagnosis of AIH, no cirrhosis and no acute severe AIH be considered for budesonide over predniso(lo)ne especially, as it has been proven to have a higher efficacy,[78] and it is biologically plausible that patients on budesonide may fare better than patients on predniso(lo)ne, if infected with SARS-CoV-2.

Data from inflammatory bowel diseases (IBD)[79] show that thiopurine monotherapy is associated with poor COVID-19 outcomes. Data regarding the safety of azathioprine specifically in AIH in the context of COVID-19, however, is not available, even though two studies had shown that continuation of immunosuppressive medicines in AIH is not associated with poor outcomes with COVID-19.[33,34] Azathioprine still remains the first-line agent of choice as an immunomodulator, till conclusive evidence regarding an alternate first-line agent with better overall outcomes is available.

Second-line agents

Second-line agents for the management of AIH include MMF as well as CNIs, such as tacrolimus and cyclosporine.[54] As far as AIH-related outcomes are concerned, MMF and tacrolimus are equivalent.[54] Although specific data on AIH patients treated with these medicines and COVID-19-related outcomes are not available, data of safety of these same drugs used in other diseases, in terms of COVID-19-related outcomes, may be cautiously extrapolated to AIH, till specific data are available. In solid organ transplant patients, treatment with mycophenolate has been shown to be risky in a dose-dependent manner,[80] but that with CNIs appears to not be.[80,81] It would therefore seem appropriate that tacrolimus should be preferred over MMF when no other contraindications are present.

Third-line/salvage agents

Salvage options or third-line agents described by the AASLD guideline for AIH include infliximab and rituximab.[54] Being an anti-TNF agent, infliximab has a potential to mitigate the cytokine storm, which is a crucial part of the pathogenesis of COVID-19.[82] Studies in IBD[83] and in rheumatology[84] have shown that anti-TNF drugs are not associated with worse outcomes in COVID-19. Concerns have been expressed regarding risk of poor outcomes for patients on rituximab,[85] and early evidence suggests this as well.[86] Till conclusive evidence that supports safety in this regard is available, it is better to consider other third-line/salvage agents, such as infliximab, over rituximab, whenever possible.

Overlap syndromes

In addition to the immunosuppressants described above, patients with overlap syndromes may also require ursodeoxycholic acid.[54] There is no reason to have concerns regarding the use of this agent in the context of the current COVID-19 pandemic. In fact, ursodeoxycholic acid is an agent with potential to have benefits in the treatment of COVID-19 as well.[87] Till evidence to the contrary is available, treatment with this agent can be initiated or continued as indicated.

Liver transplant

Liver transplant is indicated in patients with AIH who present with acute liver failure (commonly referred to as ALF) or as salvage in acute severe AIH or in cirrhosis with decompensation.[54] In the presence of widespread community transmission of COVID-19, considerations for liver transplant should consider appropriate local guidelines which specifically discuss this aspect. In view of the risk to donors, living donor liver transplantation is best restricted to urgent indications.[88]

Management of AIH in COVID-19 patients

The management of AIH in COVID-19-positive patients would require decisions to be taken on a multidisciplinary, case-by-case basis. Decisions should be based on a multitude of factors, such as urgency to treat AIH, severity of COVID-19, presence of co-existing sepsis, requirement of drugs for COVID-19 that may have interactions with drugs given for AIH, etc. APASL recommends continuing immunosuppression in patients with mild COVID-19.[55] Data from two multinational studies are available which show no benefit of withdrawing immunosuppression in patients with AIH and active COVID-19.[33,34] One study even showed that continuation of immunosuppression lowered the risk of new-onset liver injury.[33] The exact reasons for stopping immunosuppression in these studies are not known. The numbers of patients on high-dose steroids and MMF were low in these studies, for meaningful conclusions to be made. However, these studies do support the continuation of immunosuppression in patients with AIH and COVID-19. Some special considerations required while managing patients with AIH who also have COVID-19 are summarized in Table 2.

Drug interactions are particularly important while managing patients with COVID-19 and AIH. Management of COVID-19 is constantly being revised with a variety of drugs being tried for its treatment, with varying efficacy. New data are emerging every day and with the introduction of new drugs, one needs to be aware of the side effect profile of the drugs being used and their potential interaction with the concomitant medications being used for the management of comorbid conditions. Interactions between drugs used for immunosuppression, such as CNIs, may have significant drug interactions, which, if not paid attention to, can be deleterious.[89] We have listed the interactions between the drugs used for the management of COVID-19 and the drugs used in AIH in the table below (Table 3). As to the optimal treatment and drugs for COVID-19, the data are still evolving; however, it is suggested to check the side effect profile and drug interactions of the drug being used. One such useful updated resource to check for drug interactions is https://www.covid19-druginteractions.org/checker.

Conclusions

Management of auto-immune hepatitis in areas with wide-

Table 3. Drug interactions between immunosuppressants used for management of AIH and drugs used for management of COVID-19

Drug type	Azathioprine	MMF	Tacrolimus	Cyclosporine
Interferons				
Interferon-alpha	Potential additive hematological toxicity	No interaction	No interaction	No interaction
Interferon0beta	Potential additive hematological toxicity	No interaction	No interaction	No interaction
Antivirals				
Favipiravir	No interaction	No interaction	No interaction	No interaction
Lopinavir-ritonavir	No interaction	Potential altered drug levels of mycophenolate; Drug level monitoring recommended	Increased levels of tacrolimus; Risk of QT prolongation	Increased plasma levels of cyclosporine; Drug monitoring recommended
Remdesivir	No interaction	No interaction	No interaction	No interaction
Ribavirin	Potential additive hematological toxicity	No interaction	No interaction	No interaction
Nitazoxanide	No interaction	No interaction	No interaction	No interaction
Antimalarials				
Chloroquine	Potential additive hematological toxicity	No interaction	Increased levels of tacrolimus; Risk of QT prolongation	Increased plasma levels of cyclosporine; Drug monitoring recommended
Hydroxychloroquine	Potential additive hematological toxicity	No interaction	Increased levels of tacrolimus; Risk of QT prolongation	Increased plasma levels of cyclosporine; Drug monitoring recommended
Monoclonal antibody				
Tocilizumab	Potential additive hematological toxicity	No interaction	Monitoring of tacrolimus drug levels recommended	Monitoring of cyclosporine drug levels recommended
Bamlanivimab	No interaction	No interaction	No interaction	No interaction
Canakinumab	Potential additive hematological toxicity	No interaction	Monitoring of tacrolimus drug levels recommended	Monitoring of cyclosporine drug levels recommended
Sarilumab	Potential additive hematological toxicity	No interaction	Monitoring of tacrolimus drug levels recommended	Monitoring of cyclosporine drug levels recommended

spread community transmission of COVID-19 needs special considerations in the diagnostic approach, preventive aspects and treatment. The management of immunosuppression is particularly complex in this set of patients and specific data in the context of AIH in COVID-19 are lacking. Management of immunosuppression in patients with AIH in COVID-19 hotspots requires a tailored approach based on data from relatively small observational studies and from data extrapolated from other auto-immune diseases till better evidence is available. The management of AIH in patients diagnosed with COVID-19 requires a multidisciplinary approach with decisions considered on a case-by-case basis.

Acknowledgments

We would like to acknowledge the contribution of Madhu Mathew Vennikandam, Advanced Hepatology Fellow, Columbia University Irving Medical Center, New York, USA, in the initial process of the literature search. Figure 1 was created with BioRender.com.

Funding

None to declare.

Conflict of interest

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

Author contributions

Study concept and design (DM, SS, AS), acquisition of data (DM, AA, SS), drafting of the manuscript (DM, SS, AA), critical revision of the manuscript for important intellectual content (DM, AA, SS, AS), administrative support, study

supervision (AS).

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Journal of Clinical and Translational Hepatology **2021** vol. 9(4) | 576–586
DOI: 10.14218/JCTH.2020.00110

Review Article

Critically Ill COVID-19 Patient with Chronic Liver Disease - Insights into a Comprehensive Liver Intensive Care

Cyriac Abby Philips[1*], Kamna Kakkar[2], Moby Joseph[3], Praveen Kumar Yerol[4], Rizwan Ahamed[5], Sasidharan Rajesh[6] and Philip Augustine[5]

[1]Department of Clinical Hepatology, The Liver Institute, Center of Excellence in GI Sciences, Rajagiri Hospital, Chunangamvely, Aluva, Ernakulam, Kerala, India; [2]Department of Pulmonary and Critical Care Medicine, Pt. B. D. Sharma Post Graduate Institute of Medical Sciences, Rohtak, Haryana, India; [3]Great Western Hospitals, NHS Foundation Trust, Swindon, United Kingdom; [4]Department of Gastroenterology, State Government Medicine College and Hospital, Thrissur, Kerala, India; [5]Gastroenterology and Advanced G.I Endoscopy, Center of Excellence in GI Sciences, Rajagiri Hospital, Chunangamvely, Aluva, Ernakulam, Kerala, India; [6]Interventional Hepatobiliary Radiology, The Liver Institute, Center of Excellence in GI Sciences, Rajagiri Hospital, Chunangamvely, Aluva, Ernakulam, Kerala, India

Received: 12 November 2020 | **Revised:** 7 March 2021 | **Accepted:** 7 April 2021 | **Published:** 28 April 2021

Abstract

The novel coronavirus-related coronavirus disease 2019 (COVID-19) pandemic has been relentless in disrupting and overwhelming healthcare the world over. Clinical outcomes of COVID-19 in patients with chronic comorbidities, especially in those with metabolic syndrome, are well documented. Chronic liver disease and cirrhosis patients are a special subgroup, among whom the management of COVID-19 is challenging. Understanding the pathophysiology of COVID-19 in patients with cirrhosis and portal hypertension improves our identification of at-risk patients for disease progression that will further help compartmentalize generalized and specialized treatment options in this special patient group. In this exhaustive review, we critically review the impact of COVID-19 on the liver and in chronic liver disease and cirrhosis patients. We further discuss common features associated with the pathophysiology of COVID-19 and cirrhosis, based on the renin-angiotensin system and deliberate current literature on guidelines for the treatment of COVID-19 and extrapolate the same to the cirrhosis population to provide a concise and stepwise, evidence-based management for cirrhosis patients with severe and critical COVID-19. There are no specific management guidelines for cirrhosis patients with COVID-19 and current recommendations for treatment are as per guidelines for general population. Nevertheless, specific issues like avoiding corticosteroids in decompensated patients with variceal bleeding, suspected sepsis, high grade hepatic encephalopathy and acute kidney injury, use of early mechanical ventilation strategies in those with severe ascites and hepatopulmonary syndrome, avoidance of remdesivir in advanced liver disease, and application of liver-specific severity scores for prognostication and identification of futility need to be highlighted.

Citation of this article: Philips CA, Kakkar K, Joseph M, Yerol PK, Ahamed R, Rajesh S, *et al*. Critically ill COVID-19 patient with chronic liver disease - Insights into a comprehensive liver intensive care. J Clin Transl Hepatol 2021;9(4):576–586. doi: 10.14218/JCTH.2020.00110.

Introduction

The novel severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), an enveloped, positive single-stranded large RNA virus belonging to the beta-CoV family, is the causative agent of the current pandemic, the coronavirus disease 2019 (COVID-19), that has affected developed and developing countries worldwide. Even though the well-described and reported initial clinical sign of COVID-19 is pneumonia (fever, cough, and shortness of breath), further studies have shed light on the variable presentations and clinical outcomes in affected patients. This spectrum was found to include asymptomatic carriers, patients with gastrointestinal system predominant symptoms (nausea, diarrhea), those with anosmia and dysgeusia, and symptomatic hypercoagulable states affecting multiple organs and immune-mediated organ involvement such as vasculitis-like syndromes.[1] The majority of transmissions occur via coughing and sneezing, through respiratory droplets with particle size >5–10 μm, and through the fecal-oral route. Asymptomatic COVID-19 contributes up to 80% of trans-

Keywords: SARS-CoV-2; Coronavirus; Pandemic; ACLF; Decompensation; Portal hypertension; Sepsis.

Abbreviations: ACE2, angiotensin-converting enzyme receptor type 2; ACLF, acute-on-chronic liver failure; ACTT, adaptive covid-19 treatment trial; Ang I, angiotensin I; Ang II, angiotensin II; AT1, angiotensin II type 1 receptor; ARDS, acute respiratory distress syndrome; CLIF-C, chronic liver failure-consortium; CLD, chronic liver disease; COVID-19, novel coronavirus-related coronavirus disease 2019; ECMO, extracorporeal membrane oxygenation; ICU, intensive care unit; IV, intravenously; MELD, model for end stage liver disease; NAFLD, non-alcoholic fatty liver disease; NIH, national institutes of health; NO, nitric oxide; RAS, renin-angiotensin system; RECOVERY, randomised evaluation of covid-19 therapy; ROTEM, rotational thromboelastometry; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; SBECD, sulfobutylether-β-cyclodextrin; TEG, thromboelastography; TNF, tumor necrosis factor; WHO, world health organization.

[*]**Correspondence to:** Cyriac Abby Philips, Department of Clinical Hepatology, The Liver Institute, Center of Excellence in GI Sciences, Rajagiri Hospital, Chunangamvely, Aluva, Ernakulam, Kerala 683112, India. ORCID: https://orcid.org/0000-0002-9587-336X. Tel: +91-484-2905000, Fax: +91-484-7184000, E-mail: abbyphilips@gmail.com

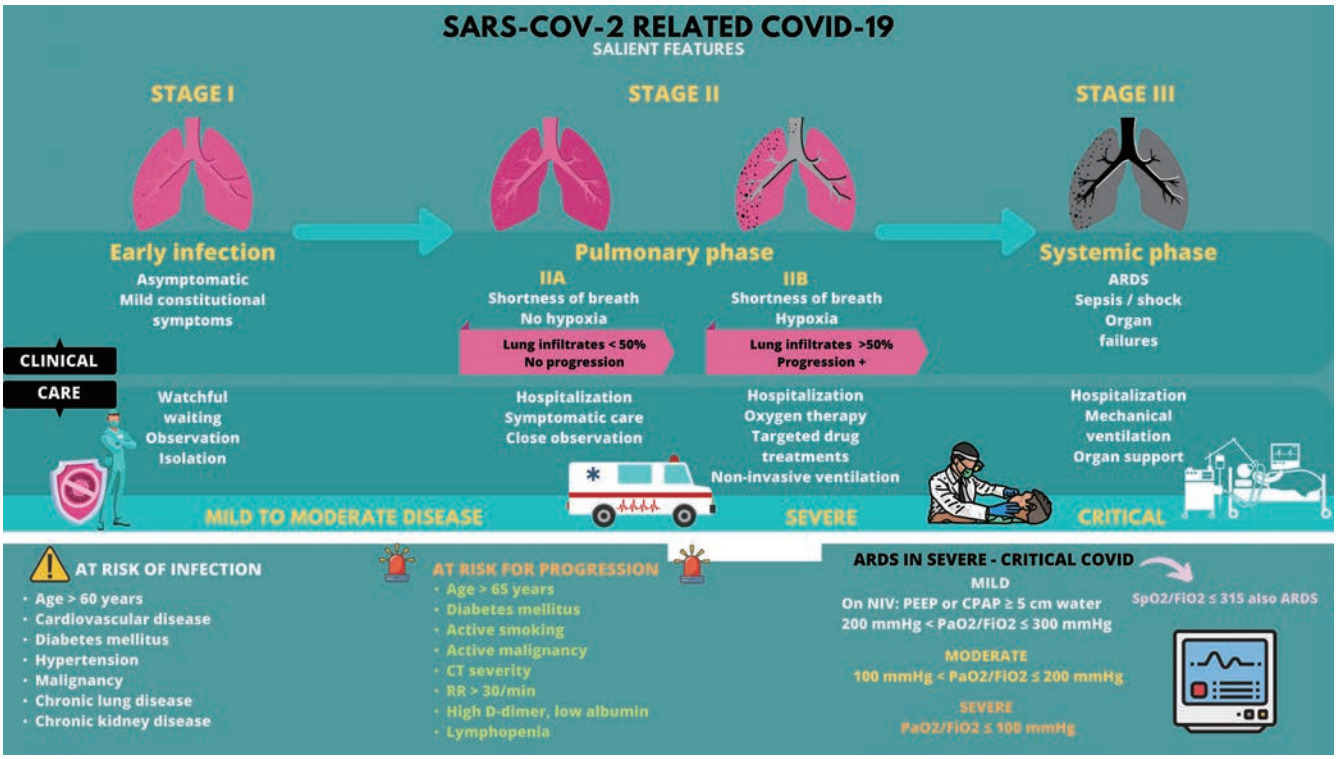

Fig. 1. Salient features of SARS-CoV-2-related COVID-19. CT, computed tomography; CPAP, continuous positive airway pressure; FiO2, fraction of inspired oxygen; NIV, non-invasive ventilation; PaO2, partial pressure of arterial oxygen; PEEP, positive end-expiratory pressure; RR, respiratory rate; SpO2, saturation of peripheral oxygen.

mission, primarily limited to family members, healthcare professionals, and others with close contacts (6 feet, 1.8 meters) within closed-space public gatherings.[2] Initial studies have shown that the incubation time is between 3 to 7 days and the basic reproduction number (R0 or R naught) is 2.2.[3] The viral infection starts with the glycoprotein spike receptor-binding protein, which allows viral attachment to the angiotensin-converting enzyme receptor type 2 (ACE2) in the lungs and other tissues as well. A polybasic amino acid site in the spike protein is functionally processed by the human protease enzyme furin, which further allows exposure of the integrated sequences, resulting in a fusion of the viral and cell membranes and subsequent virus passage into the primary cell.[4]

Around 97.5% of symptomatic presentations will occur within 11.5 days of infection and the median time from symptom onset to hospital admission is 7 days. The median age of hospitalized patients varies between 47 and 73 years, with male preponderance. Overall, a quarter of infected patients have comorbidities but among hospitalized COVID-19 patients, approximately 60% to 90% have comorbidities.[5,6] The most common chronic conditions include hypertension and cardiovascular disease, diabetes, chronic kidney disease, and chronic lung disease. Clinical complications leading to morbidity and mortality include hypoxemic lung failure, myocarditis, cardiomyopathy, ventricular arrhythmias, and hemodynamic instability, stroke and rarely encephalitis, secondary bacterial sepsis, and arterial and venous thromboembolic events, the latter notable in up to 60% of those admitted to the intensive care unit (ICU).[4–7]

In this review, we discuss the impact of COVID-19 on the liver, focus on key aspects of disease pathogenesis and outcomes in patients with pre-existing liver disease, and discuss current evidence-based treatment protocols and exhaustive algorithms for the management of COVID-19 in cirrhosis.

COVID-19 and the liver

The Chinese Centers for Disease Control described three clinical classifications of COVID-19 based on pulmonary symptoms, classified as mild to moderate, severe, and critical disease. Patients with severe pneumonia can develop acute respiratory distress syndrome (ARDS) classified as mild, moderate, and severe depending on clinical and ventilatory criteria. Severe disease is also identified when computed tomography of the chest reveals lung infiltrates >50% within 24 to 48 h, or in the presence of septic shock or multiple organ failure (Fig. 1). ACE2, the host cell receptor for SARS-CoV-2, is present on type 2 alveolar cells and in the gastrointestinal tract and the liver. In the liver, ACE2 is highly expressed in the endothelial layer of small blood vessels and absent in the sinusoidal endothelium and is also expressed greater in cholangiocytes than in hepatocytes.[4]

Multiple studies have shown that index presentation with gastrointestinal symptoms was notable in approximately 19.6% to 73.0% of patients with SARS-CoV. Similarly, 3% to 79% of those with SARS-CoV-2 infection presented gastrointestinal predominant symptoms in various studies.[8–11] Abnormal liver tests have been reported in approximately 19–76% of patients with COVID-19. It is now clear that elevated liver biochemistries are predominantly associated with severe and critical COVID-19 due to multifactorial reasons, such as drug-induced liver test abnormalities, liver involvement in critical illness, and hypoxic insults.[12–15] Even though ACE2 receptors are more greatly expressed in cholangiocytes, the principal pattern of liver test abnormality demonstrated in COVID-19 is of the hepatocellular type, with elevation in aminotransferases rarely above 5-times the upper limit of normal in those with and without pre-existing liver disease.[16,17]

Direct cytopathic effects of SARS-CoV-2 on hepatocytes

remain unconfirmed, and liver test abnormalities are mostly related to multisystem involvement associated with severe disease, multifactorial, and secondary to systemic inflammation, immune-mediated injury, microvascular thrombosis, drug toxicity, hepatic congestion, and intercurrent sepsis.[18,19] Post-mortem liver biopsy studies have shown non-specific features, such as focal or mild to moderate macro/microvesicular steatosis, mild portal and lobular inflammation, and sinusoidal dilatation. The direct viral cytopathic effect, viral nucleic acid, or demonstrable replication has not been demonstrated consistently across studies. Acute liver failure due to COVID-19 is not described. However, concomitant hepatotoxic drug use, including complementary and alternative medications in patients with COVID-19 with a predisposition to acute severe liver injury, could lead to acute liver failure, independent of the primary infection, and should be kept in mind with patients presenting with liver failure.[20,21]

COVID-19 and chronic liver disease (CLD)

To understand the outcomes associated with COVID-19 in patients with CLD, an understanding of the common pathophysiology that plays an important role in the causation and progression of these conditions, attributable to the renin-angiotensin system (RAS), is pertinent. RAS activity starts with the breakdown of angiotensinogen (derived from the liver) by circulating renin (from the juxtaglomerular apparatus of the kidney) to form angiotensin I (Ang I). In the classical pathway, ACE in pulmonary capillaries converts Ang I to angiotensin II (Ang II), which then binds to angiotensin II type 1 receptor (AT1) that, in effect, causes vasoconstriction, is trophic, enhances fibrogenesis, increases sodium reabsorption, and is pro-inflammatory and prothrombotic. In the alternate RAS, ACE2 degrades Ang II to the peptide Ang1-7, which then acts through the *mas* receptor that promotes vasodilation, is anti-trophic and anti-fibrotic, promotes natriuresis, and is anti-inflammatory and anti-thrombotic.[22]

In CLD, the classical pathway and its activation contributes to inflammation and fibrosis, while the alternative pathway is up-regulated to counterbalance the harmful effects. As fibrosis worsens, ACE levels and AT1 gene expressions rise, coinciding with an up-regulation in ACE2 and *mas* expression, increasing in both Ang 1-7 and Ang II. Cirrhotic livers have enhanced capacity to convert Ang II to Ang 1-7, which has been shown to have beneficial effects on liver fibrosis and inflammation. In late-stage cirrhosis, sympathetic nervous system activation, acetylcholine-mediated vasodilation, increased production of dysfunctional nitric oxide (NO), secretion of antidiuretic hormone, central hypovolemic status, and worsening peripheral and splanchnic vasodilation due to high Ang 1-7 renders early beneficial effects of the alternate RAS pathway redundant.[23–25] In chronic inflammation, Ang II expression activates hepatic stellate cells that drive the pathogenesis of portal hypertension. With cirrhosis progression, intrahepatic resistance increases, leading to systemic and splanchnic vasodilation and hypo-responsiveness to vasoconstrictors.[26] Thus, there is a clear change in modus operandi in the RAS-mediated pathophysiology of cirrhosis and its progression.

In patients with cirrhosis and COVID-19, SARS-CoV-2 overwhelms the ACE2 receptors, resulting in the functional inhibition of the alternate RAS pathway, leading to reduced expression of Ang 1-7 (increasing proinflammatory cytokines such as interleukin (IL)-6, IL-1β and tumor necrosis factor-alpha (TNF-α) and paving the way for harmful consequences via the AT1 receptor) within the liver microenvironment and other systems.[27,28] However, in stable cirrhosis, in the presence of highly up-regulated ACE2 receptors and higher expression of Ang 1-7, SARS-CoV-2 infection may not be uncontrollably detrimental and could be associated with better outcomes.[29] In decompensated cirrhosis, chronic activation of RAS and sympathetic nervous system activation and secretion of antidiuretic hormones occur in the presence of persistent arterial hypotension.

COVID-19 can worsen the already perturbed portosystemic hemodynamics through the overwhelming use of ACE2 receptors, increasing Ang 1-7, which leads to worsening systemic and splanchnic vasodilation. Furthermore, cirrhosis is a state of chronic systemic inflammation, immunomodulation, endotoxemia, and hemodynamic alterations that promote subclinical dysfunction in most organ systems that worsen with increasing cirrhosis stages.[30–33] Hence, from our understanding of pathophysiology, it is safe to assume that COVID-19 in cirrhosis is associated with different clinical outcomes, depending on the stages of liver disease and degree of portal hypertension (Fig. 2).

Worsening of pre-existing liver injury is an important clinical aspect of COVID-19. In this regard, new-onset or worsening thrombocytopenia, coagulation tests, and hypoproteinemia or low albumin could be considered part of progression in the multisystem involvement of the primary infection or worsening of the pre-existing liver disease.[34–36] The novel coronavirus itself does not cause acute severe liver injury or trigger liver failure.[20,21] Nonetheless, COVID-19 in patients with pre-existing liver disease, such as alcohol-related or nonalcohol-related fatty liver, early and advanced hepatic fibrosis (CLD), cirrhosis with or without portal hypertension (clinically significant [defined as hepatic venous pressure gradient ≥10 mmHg] or otherwise) can present with acute hepatitis with or without jaundice and rarely cholestatic liver injury, acute decompensation of cirrhosis and acute-on-chronic liver failure (ACLF) due to severity of infection and subsequent treatment interventions.

Impact of COVID-19 in patients with CLD and cirrhosis

A recent meta-analysis on 2,034 adult individuals with a median age of 49 years found that the overall prevalence of CLD at admission was 3%.[37] In a narrative review, Garrido and colleagues,[38] based on published evidence, presumed that patients with CLD were not at greater risk for acquiring the infection. Still, those with compensated advanced and decompensated cirrhosis, hepatocellular carcinoma, nonalcoholic fatty liver disease (due to associated metabolic syndrome), autoimmune liver diseases, or a liver transplant may have a greater risk for severe COVID-19. In a small series of patients with liver disease, Ji *et al*.[39] showed that disease progression was higher in COVID-19 patients with CLD than in those without CLD, with the risk of developing ACLF that portend a worse prognosis. In a retrospective study from Wuhan, China, investigators found that COVID-19 patients with CLD had a longer length of stay in hospital but with mild liver injuries and higher mortality (in the presence of decompensation) than in those without CLD. The neutrophil-to-lymphocyte ratio significantly predicted in-hospital death.[40]

Another systematic review and meta-analysis that included 74 clinical studies demonstrated that the prevalence of CLD among all COVID-19 patients was approximately 3%. This proportion was similar in COVID-19-positive and -negative population, but CLD was significantly associated with more severe COVID-19 infection and overall mortality.[41] In Italian multicenter retrospective studies, Iavarone *et al*.[42,43] showed that COVID-19 was associated with deterioration in liver function and increased mortality in the elderly with cirrhosis compared to a historically-matched group of patients with bacterial sepsis. The severity of lung and liver disease scores according to the Chronic Liver Failure-Consortium

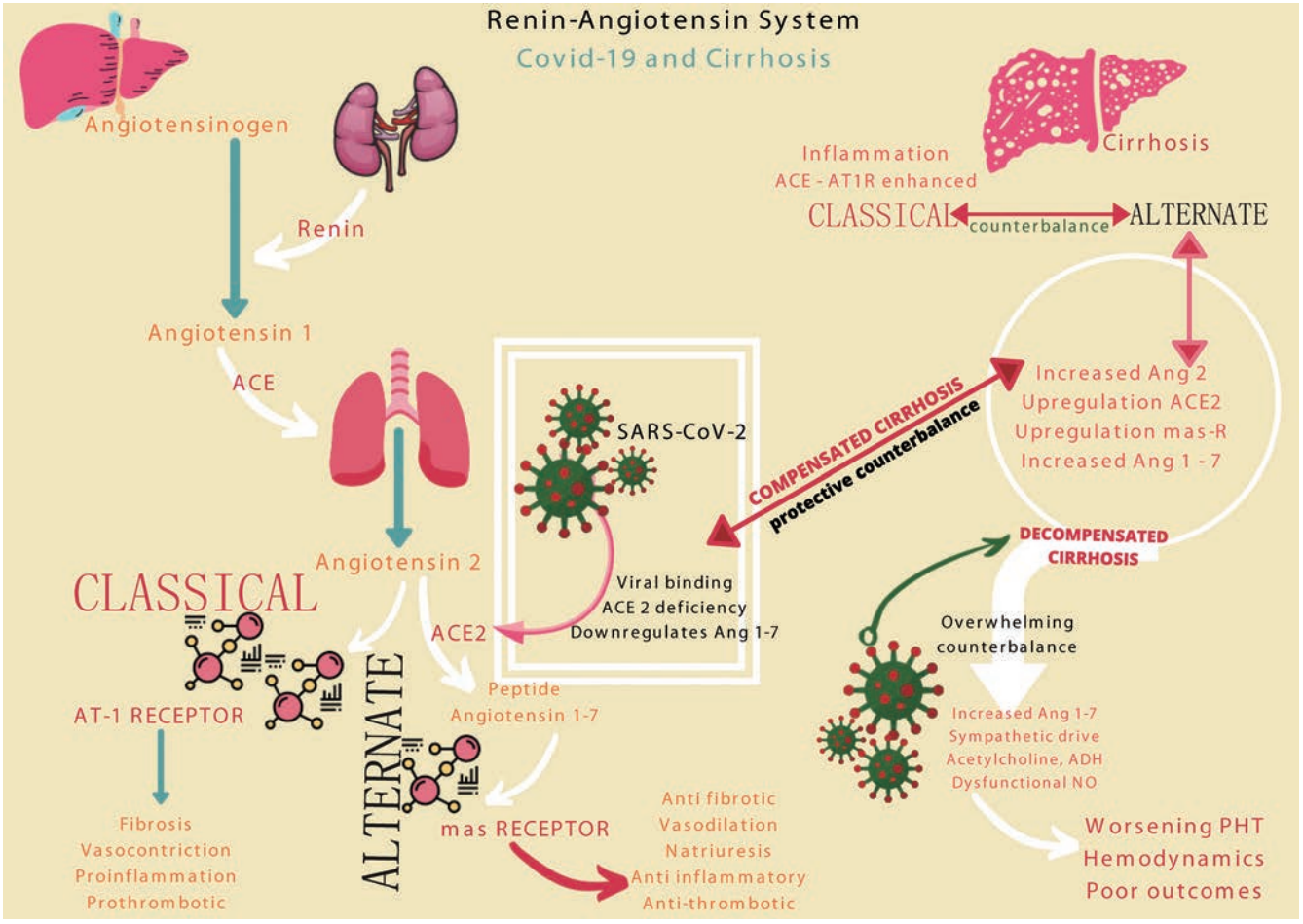

Fig. 2. RAS and its central role in pathophysiology of COVID-19 and cirrhosis. ACE, angiotensin-converting enzyme; ADH, antidiuretic hormone; Ang 1-7, angiotensin peptide 1-7; mas-R, mas receptor; PHT, portal hypertension

(commonly known as CLIF-C)-Organ Failure (>9), CLIF-C-ACLF ≥70 and Model for End-stage Liver Disease (commonly known as MELD; >15) independently predicted mortality. Notably, ACLF was not the cause of death in most patients but respiratory failure was in 71%.[42,43]

An international registry's preliminary results included 103 cirrhosis patients from 21 countries and four continents (60% male, median age 61 years, most common liver etiology being non-alcoholic fatty liver disease or NAFLD). Among patients analyzed from combined registries, 38% decompensated during their disease course (worsening ascites, encephalopathy, or acute kidney injury) due to COVID-19. Mortality in this group was far more than that noted with cirrhosis in the pre-COVID-19 era. However, the cause of death was lung failure in approximately 80% and liver-related in 12% (CLD without cirrhosis 12.2%, <Child class A cirrhosis 23.9%, <Child class B cirrhosis 43.3%, <Child class C 63%). This meant that deaths and liver and portal hypertension-related new onset or worsening of events were seen among those with advanced liver cirrhosis.[44] Marjot *et al*.[45] found that baseline liver disease stage and alcohol-related liver disease were independent risk factors for death from COVID-19. The APCOLIS study from the Asian Pacific region showed that SARS-CoV-2 infection caused a significant liver injury in CLD patients, decompensating one-fifth of cirrhosis, and worsening the clinical status of those already decompensated. The number of cirrhosis patients with symptomatic COVID-19 in that study was less at baseline, showing the lower prevalence of infection associated with early cirrhosis. The authors concluded that CLD patients with diabetes and obesity were more vulnerable to disease risk and progression.[46] A position paper from Europe stated that CLD patients did not *per se* appear to be over-represented in cohorts with COVID-19 and were not at increased risk of contracting SARS-CoV-2. However, the risk of infection and the risk of a severe course of COVID-19 may be different, depending on the nature of the CLD and the presence of advanced fibrosis or cirrhosis and MELD score ≥15.[47]

In a North American multicenter contemporaneously enrolled study, age and sex-matched patients with cirrhosis and COVID-19 had similar mortality compared with cirrhosis patients alone but was higher than among patients with COVID-19 alone. ACLF rates were similar between groups. Nevertheless, Charlson Comorbidity Index scores and lactate levels were worse among cirrhosis patients who were COVID-19-positive. At present, evidence for a strong conclusion that COVID-19 increases the risk for development of ACLF or mortality in patients with cirrhosis (other than in Child class C) more than other etiologies for new-onset or worsening decompensation is lacking.[48] The salient features of pertinent recent studies on patient characteristics and impact of COVID-19 in liver disease are shown in Table 1.[37–45,48]

Current treatments for hospitalized COVID-19 patients and impact in cirrhosis

According to the National Institutes of Health (NIH, which does not consider cirrhosis as a high-risk comorbid condition), in patients with COVID-19 who are hospitalized with moderate disease (clinical or radiographic evidence of lower respiratory tract infection, respiratory rate <24 breaths/m and SpO_2 ≥94% on room air at sea level) but do not require supplemental oxygen, dexamethasone is not recommended, while remdesivir may be considered in those at high risk for clinical deterioration.[49]

Dexamethasone use was associated with the absence of survival benefit in patients who did not require supplemental oxygen at enrolment and lead to a slightly higher 28-day mortality when used in this group of patients, as demonstrated in the Randomised Evaluation of COVID-19 Therapy (RECOVERY) trial, a multicenter, open-label trial in the UK.[50] The use of remdesivir was not associated with clinical benefit in patients with mild to moderate disease in the multinational, randomized controlled Adaptive COVID-19 Treatment Trial (ACTT-1).[51]

For hospitalized patients with COVID-19 who require only supplemental oxygen, the NIH recommends the use of remdesivir at 200 mg intravenously (IV) for 1 day, followed by 100 mg intravenous once daily for 4 days (which can be extended up to 10 days if no clinical improvement is noted on the 5th day) or until hospital discharge, whichever comes first. Alternatively, a combination regimen (yet to be studied in rigorous clinical trials) of remdesivir and dexamethasone at 6 mg intravenous or orally for up to 10 days or until hospital discharge, or if remdesivir cannot be used (especially in resource-poor countries like India), dexamethasone alone is recommended for use. The final analysis of the ACTT-1 trial showed that remdesivir was associated with improved time to recovery in a subgroup. On post hoc analysis of deaths by Day 29, it appeared to provide a substantial survival benefit. The RECOVERY trial showed that COVID-19 patients who required supplemental oxygen, but not those on mechanical ventilation, received survival benefit from using dexamethasone. The use of only dexamethasone would dampen viral clearance by reducing inflammatory responses, and hence concomitant use of an antiviral for improving outcomes has been hypothesized. For hospitalized patients with COVID-19 who require oxygen delivery through a high-flow device or noninvasive ventilation, both remdesivir and steroid use were advocated. In these patients, the ACTT-1 study did not show the clinical benefit of using remdesivir alone. Only early dexamethasone use has been shown to improve outcomes in ventilated patients for those who require invasive mechanical ventilation or extracorporeal membrane oxygenation.[52]

The World Health Organization (commonly known as WHO) recommends the use of systemic corticosteroids for severe and critical cases of COVID-19 but do not recommend the use of remdesivir or other repurposed drugs, such as hydroxychloroquine, lopinavir (fixed-dose combination with ritonavir), and interferon-β1a with or without lopinavir at any stage of the disease due to absence of clinical benefit on early recovery and mortality, as demonstrated in the SOLIDARITY trial. Except for the use of remdesivir, the WHO guidelines are in tune with the NIH guidelines concerning other antiviral use in COVID-19.[53,54] Remdesivir was developed by Gilead® Sciences with collaboration between the USA Centers for Disease Control and the USA Army Medical Research Institute of Infectious Diseases. It is a monophosphate nucleoside analog with broad activity against RNA viruses, targeting the divergent RNA-dependent RNA polymerase through the misintegration of an active nucleoside triphosphate form, which has been shown to reduce viral load in *in vitro* and *in vivo* studies and also in non-human primate models of severe acute respirator syndrome, Middle East respiratory syndrome and Ebola virus infection.[55] Liver toxicity is a rare but potentially severe side effect of remdesivir. A compassionate use study revealed that hepatic enzyme increases were by far the most common adverse event, occurring in 23% of patients, while infusion-related hypotension was seen in 8%.[56] Liver enzyme increases observed in the Gilead®-run SIMPLE trial involved 7% of patients with grade 3 or higher alanine aminotransferase elevations and 3% who stopped the drug over elevated liver enzymes. In a Chinese trial, alanine aminotransferase elevation led to treatment discontinuation in one patient, and acute kidney injury prompted it in another.[57]

In the ACTT-1 study, serious adverse events were reported in 24.6% of patients who received remdesivir. Serious respiratory failure as an adverse event was noted in 8.8% of patients in the remdesivir group, which included acute respiratory failure and the need for endotracheal intubation. Grade 3 or 4 adverse events occurred on or before day 29 in 51.3% in the remdesivir group, of which 41 events were judged by the investigators to be related to remdesivir. The most common nonserious adverse events occurring in at least 5% of all patients included decreased glomerular filtration rate, decreased hemoglobin level, and decreased lymphocyte count. Nonetheless, the incidence of these adverse events was generally similar in the remdesivir and placebo groups.[51] Infusion-related reactions were noted as potential side effects, along with increased liver enzymes in the USA Food and Drug Administration's guideline for remdesivir use. The efficacy and safety of remdesivir have not been studied in patients with liver impairment or CLD. In such clinical situations, current knowledge demands that remdesivir only be used if the potential benefit outweighs the potential risk. The European Medicines Agency summary warned against use in patients with concomitant hepatotoxic drugs and liver enzymes 5 or more times the upper limit of normal. In abnormal liver tests that occur after remdesivir initiation, especially at high levels, adverse drug reactions need to be considered, and drug discontinuation is required. Zampino *et al*.[58] showed that remdesivir might cause hepatocellular injury in those without CLD without progression to severe liver damage or liver failure. In 4/5 patients treated with the antiviral, baseline normal liver tests worsened, suggesting a direct role of remdesivir in hepatocellular toxicity. The authors suggested that remdesivir be used with close monitoring of liver function tests and with caution in subjects with prior liver disease.

Leegwater and colleagues[59] reported the case of a man with COVID-19 who developed acute hepatotoxicity related to remdesivir with potential interaction of P-glycoprotein inhibitors, such as chloroquine or amiodarone, that increased intracellular levels of active drug metabolite within the hepatocytes. Similarly, in those with acute and CLD, potential nephrotoxicity could be due to direct effects or the accumulation of sulfobutylether-β-cyclodextrin (also known as SBECD) carrier, the latter used due to limited water solubility.[60] Animal studies have shown that SBECD accumulation can potentiate liver cell necrosis and renal tubular damage. Remdesivir is not recommended in adults and pediatric patients (>28 days-old) with estimated glomerular filtration rate <30 mL/m or with serum creatinine ≥1 mg/dL, unless the potential benefit outweighs the potential risk.[60,61] Thus, remdesivir has the potential to cause pulmonary, hepatic, and renal toxicity in predisposed individuals.

WHO pharmacovigilance noted a disproportionately high number of reports of liver injuries and renal toxicities among patients receiving remdesivir compared with that among patients receiving other drugs for COVID-19, and the European Medicines Agency initiated a review of patients on remdesivir with acute kidney injuries.[61,62] On the

Table 1. Salient features of pertinent recent studies on impact of COVID-19 in patients with CLD

Author	Type of study	Patients	Outcomes	Comments
Ji *et al.*[39]	Retrospective observational	*n*=22/140; Cirrhosis=3; CLD (HBV, NAFLD)=19	None had decompensation at admission. 13/22 (59%) had COVID-19 disease progression. One patient developed ACLF	Risk of disease progression as high among CLD patients (NAFLD over-represented). Possible that metabolic syndrome was related to poor outcomes
Li *et al.*[40]	Retrospective observational	CLD, *n*=52/104	CLD with COVID-19 had longer hospital stay, but mild liver injury, even though the mortality was higher compared with the non-CLD group	Neutrophil-lymphocyte ratio predictor of mortality in CLD with COVID-19. Liver failure was not a cause for mortality, but progression of disease and lung failure were
Iavarone *et al.*[42,43]	Multicenter retrospective observational	Cirrhosis, *n*=50	Overall, 30-day mortality of 34%. Mortality high in those with respiratory failure	Cirrhosis with presumed underlying risk factors may increase risk of disease progression, rather than cirrhosis alone. Comparison group was cirrhosis with bacterial infection (soft end-points). NAFLD was under-represented; presence of multiple comorbid (obesity) and associated risk factors (smoking) among cirrhosis patients needs further review
Moon *et al.*[44]	Multicenter international observational cohort study	CLD, *n*=152; Cirrhosis=103; CLD non cirrhosis=49; Common – alcohol, NAFLD	Decompensation in 37% during disease course. 24% with new decompensation had no respiratory symptoms. Death in 40%. Cause of death was lung failure in 78.7%; only 12.2% died of liver-related causes	Advanced age, obesity, renal impairment, heart disease, and DM were over-represented among those who died. Child-Pugh B and C were significant predictors of mortality
Marjot *et al.*[45]	Multinational multicenter cohort study	*n*=745; CLD with cirrhosis, 386; CLD non-cirrhosis, 359; NAFLD 43%, ALD 24%; HTN 41%, DM 37%, obesity 28%	Overall death 20%, 8% CLD without cirrhosis and 32% among those with cirrhosis. Acute decompensation in only 1% without cirrhosis, 30% Child-Pugh A; 179 decompensated total, of which 50% met ACLF criteria (EASL, no APASL)	71% died due to COVID-19 lung disease; 19% due to liver disease progression. Case fatality rate lowest in CLD; no cirrhosis and highest in cirrhosis with Child C status at admission. Alcohol-related CLD was an independent risk factor for COVID-19 death
Sarin *et al.*	Multinational multicenter cohort study	*n*=228, CLD without cirrhosis in 185 and cirrhosis in 43; NAFLD in 61%; 43% CLD without cirrhosis had acute liver injury; ACLF in 11% and AD in 9%	Worsening liver disease in those with severe liver disease at baseline. Progression of liver disease in 57% and overall 43% mortality	Higher mortality in this cohort could be due to over-representation of NAFLD, metabolic syndrome, obesity and other well-known associated risk factors and not cirrhosis per say
Bajaj *et al.*[48]	Multicenter in-patient observational cohort	Cirrhosis plus COVID-19 (*n*=37) compared with age/sex-matched patients with COVID-19 alone (*n*=108) and cirrhosis alone (*n*=127)	Risk of mortality in hospitalized patients with cirrhosis plus COVID-19 was not significantly higher than those hospitalized with cirrhosis but without COVID-19. Patients with cirrhosis developed complications related to the viral infection rather than cirrhosis	Active smoking and alcohol use were higher among those with cirrhosis. Charlson Comorbidity Index was the only independent predictor of death among those with cirrhosis. Cirrhosis may not be a risk factor, but advanced cirrhosis may be a risk factor for progression of COVID-19
Mantovani *et al.*[37]	Meta-analysis	CLD: *n*=62/2,034	Abnormal liver tests in only severe COVID-19	Prevalence of CLD in COVID-19 patients was low, at 3%
Kovalic *et al.*[41]	Meta-analysis	CLD: *n*=729/24,299	Prevalence of CLD among COVID-19 was 3%. Severe and critical illness and mortality were greater in the CLD group. No differences in rate of ICU admission and invasive ventilation between the CLD and non-CLD groups	Need prospective case-controlled studies to truly determine outcomes in CLD with COVID-19. Speculation that decompensation precipitated by COVID-19 in those with advanced cirrhosis or perhaps due to comorbidities among CLD patients
Garrido *et al.*[38]	Literature review	Incidence across studies 0.6% to 37.6%	Decompensated cirrhosis patients did not have COVID-19 with precautionary measures. Chronic HBV-related CLD did not show poor outcomes with COVID-19	CLD and cirrhosis are 'expected' to be risk factors for COVID-19

AD, acute decompensation; APASL, Asia-Pacific Association for the Study of Liver; DM, diabetes mellitus; EASL, European Association for the Study of Liver; HBV, hepatitis B virus; HTN, hypertension.

other hand, short courses of corticosteroids, including dexamethasone, are safe and efficacious in acute and chronic hepatocellular or cholestatic inflammatory diseases of the liver, including reactivation of viral hepatitis, severe drug-induced liver injury, ACLF, severe alcohol-related hepatitis and also in advanced decompensation associated with relative adrenal insufficiency.[63–65] However, one must be cautious in patients with decompensated cirrhosis and those with advanced liver failure, such as higher grades of ACLF and CLD patients with uncontrolled metabolic syndrome, in whom steroid use can lead to *de novo* or worsening bacterial or fungal infections. Hydrocortisone use in the non-cirrhosis septic shock population has been shown to improve short outcomes, such as shorter vasopressor therapy, mechanical ventilation, and length of ICU stay.[66] Higher and longer duration of steroid use can also lead to delirium and precipitate hepatic encephalopathy in patients with multiple decompensations at baseline or those who develop decompensation during COVID-19.[67–69]

Current adjuvant treatments for hospitalized COVID-19 patients and impact on cirrhosis

Guidelines at the national level from different regions/countries recommend using multiple other drugs in tandem with a universally-accepted protocol for COVID-19 treatment. However, the evidence to use these medications and adjuvant treatments is currently lacking in literature or has been confirmed to have no benefit and is not recommended for use in the treatment of COVID-19. These include hydroxychloroquine or chloroquine with or without azithromycin, ivermectin, lopinavir/ritonavir, and other protease inhibitors, favipiravir, interferon therapy, interleukin inhibitors (tocilizumab and tocilizumab), kinase inhibitors, COVID-19 convalescent plasma, intravenous immunoglobulin G specific and non-specific to SARS-Cov-2, mesenchymal stem cells, vitamin C and zinc.[70–74]

Chloroquine/hydroxychloroquine have likelihood score D for liver toxicity and are possible rare causes of clinically apparent liver injury. In patients with porphyria cutanea tarda, high doses of hydroxychloroquine can trigger an acute hepatic injury associated with marked serum enzyme elevations resulting from an increased excretion of porphyrins. Both, but more commonly chloroquine, have been associated with life-threatening cardiac events, including arrhythmias and conduction disorders, such as QT prolongation. A majority of cirrhosis patients have subclinical cardiomyopathy.[75–77] Hence, the use of chloroquine derivatives needs caution, especially when combined with another drug, such as fluoroquinolones that may precipitate adverse cardiac events. Azithromycin is well known to cause clinically apparent liver injury in the form of acute, transient, or asymptomatic elevation in serum aminotransferases, which occurs in 1% to 2% of patients treated for short periods.[78] High-dose vitamin C resulting in high circulating concentrations, may affect the accuracy of point-of-care glucometers in assessing glycemic status in the ICU.[79] Interferons are unsafe in advanced cirrhosis, and portal hypertension and interleukin inhibitors are not well studied in cirrhosis, and hence their use should be strictly compartmentalized to research protocols.

Even though zinc supplementation has not been shown to have beneficial disease-modifying properties in COVID-19, a recent meta-analysis demonstrated mild benefits in reducing hepatic encephalopathy. It may be considered an adjuvant to the standard of care in patients with cirrhosis and COVID-19 with hepatic encephalopathy.[80] Plasma and other blood product transfusions have detrimental effects in patients with cirrhosis and portal hypertension. Hence, transfusions must be curtailed in the absence of evidentiary proof for utility.[81] The benefits of vitamin D supplementation in COVID-19 patients remain to be determined. However, in those with insufficient levels (such as cirrhosis), supplementation therapy can be considered as per recommended guidelines. Nonetheless, whether this would benefit clinical outcomes in COVID-19 warrants further study.[82] A large body of published evidence suggests thrombotic events' central role in negative outcomes in patients with severe and critical COVID-19. In this regard, current recommendations for venous thromboembolism prophylaxis should be followed as per the standard of care for hospitalized adults. However, in cirrhosis, in those who have bled from varices or have variceal bleeding or are at high risk for variceal bleeding, anticoagulation use needs caution and must be tailored on a case basis. In this regard, newer diagnostic point-of-care modalities to assess the coagulation state, such as thromboelastography (i.e. TEG™) or rotational thromboelastometry (i.e. ROTEM®), could help guide prophylactic anticoagulation in patients with cirrhosis.[83–86]

Supportive treatment for severe or critically ill COVID-19 patients with cirrhosis

Norepinephrine is the first-choice vasopressor for cirrhosis patients with shock, as per standard recommendations followed in patients with septic shock. Additionally, terlipressin may be considered as it has shown benefits for acute variceal bleeding and kidney injury. The use of dopamine in cirrhosis is not recommended, due to the high risk of inducing arrhythmias, and dobutamine is recommended only in patients with clinically significant myocardial dysfunction. Applications of intravenous human albumin in cirrhosis with sepsis, acute kidney injury, hepatic encephalopathy, and hypotension have been well documented in the literature. Adjuvant use of intravenous human albumin is recommended in severe and critically ill cirrhosis patients, in the absence of absolute contraindications for use. In cirrhosis, profound distributive shock leads to the development of refractoriness towards inotropes and pressors. In such a situation, the use of methylene blue has been shown to reduce the requirement of inotropes and could potentially be used as a salvage option. Methylene blue has also been hypothesized as an inhibitor of NO with antagonistic effects on bradykinin that could improve oxygenation at the pulmonary level in patients with COVID-19. However, with regards to methylene blue, further clinical studies are required to confirm this proposal.[70,71,87–90]

For patients with acute hypoxemic respiratory failure despite conventional oxygen therapy, high-flow nasal cannula oxygen is preferred over noninvasive positive pressure ventilation. However, in patients with severe hepatopulmonary syndrome, the latter may be considered in a well-controlled environment.[91] With high-flow nasal cannula, to optimize the alveolar recruitment, improve dead-space carbon dioxide washout as well as the positive end-expiratory pressure or to reduce airway resistance, it is prudent to initiate flow at 60 L/m, especially in situations of acute respiratory failure. In patients with hypoxemic respiratory failure and those with hypercapnia, targeted oxygen saturation should be in the range of 94–98% in the former and 88–92% in the latter. Cirrhosis patients with overt hepatic encephalopathy with a high risk of aspiration and those with hemodynamic instability should be excluded from high-flow nasal cannula use as risks outweigh benefits, including delay in early intubation.[92] For COVID-19 patients with persistent hypoxemia despite incremental oxygen supplementation, endotracheal intubation is not otherwise indicated; a trial of awake prone positioning to improve oxygenation is recommended. However, this strategy can be difficult to pursue in decompensated cirrho-

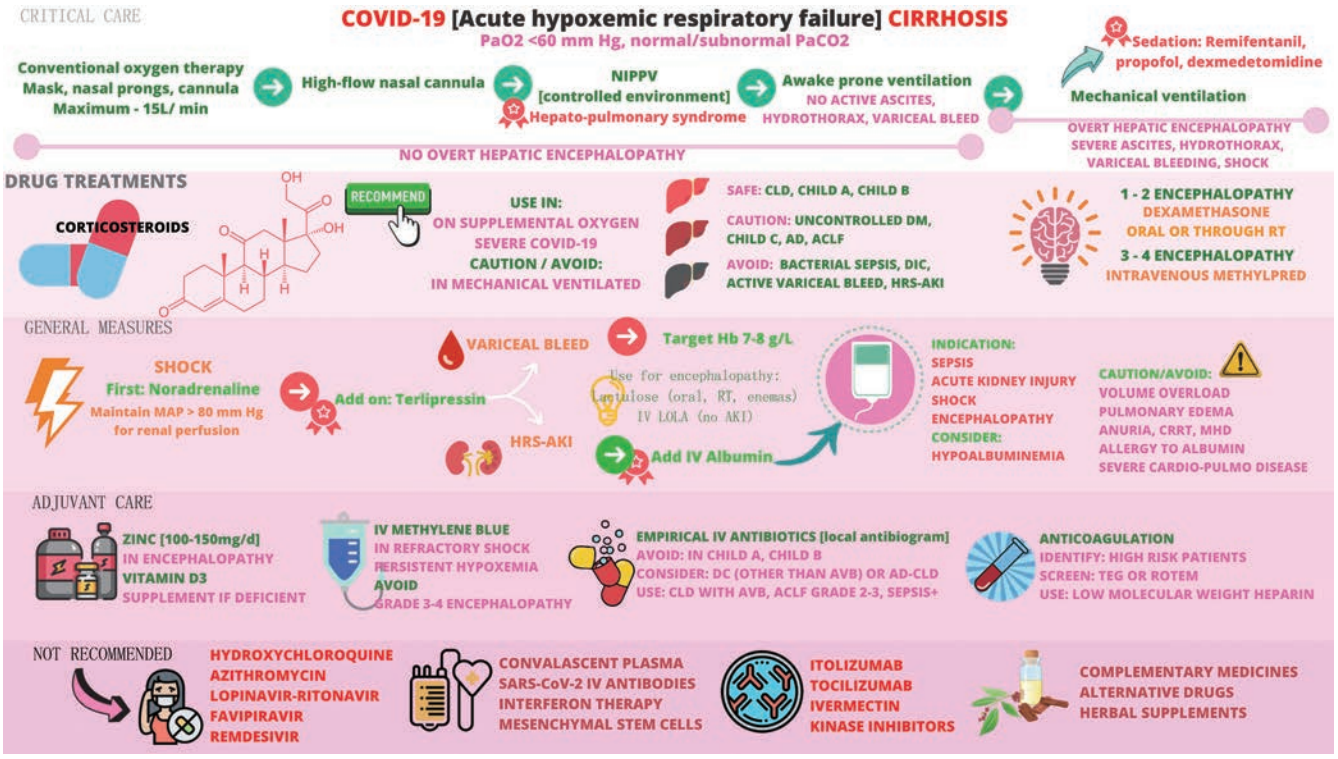

Fig. 3. Summary of proposed management of COVID-19 in patients with cirrhosis. AD, acute decompensation; AKI, acute kidney injury; AVB, acute variceal bleeding; CRRT, continuous renal replacement therapy; DIC, disseminated intravascular coagulation; DM, diabetes mellitus; Hb, hemoglobin; HRS, hepatorenal syndrome; LOLA, L-ornithine L-aspartate; MAP, mean arterial pressure; MHD, maintenance hemodialysis; NIPPV, non-invasive positive pressure ventilation; PaCO2, partial pressure of arterial carbon dioxide; PaO2, partial pressure of arterial oxygen; RT, Ryle's tube; red ribbon logo denotes specific interventions related to cirrhosis.

sis patients who have ascites or hydrothorax. Awake prone positioning as rescue therapy for refractory hypoxemia to avoid intubation is not recommended, and early intubation should be considered in such situations, especially in cirrhosis. In cirrhosis, sedation choice in such circumstances should ideally include medications with short half-lives, such as propofol and remifentanil, with avoidance of benzodiazepines. Dexmedetomidine was well tolerated in patients with liver disease who received the medication for more than 48 h. Nonetheless, patients with liver disease required more time before extubation after drug discontinuation.[93]

For mechanically ventilated adults with COVID-19 and ARDS, low tidal volume ventilation (4–8 mL/kg of predicted body weight) is recommended. In those with refractory hypoxemia, despite optimized ventilation, prone positioning for 12 to 16 h per day is suggested. Inhaled pulmonary vasodilator as rescue therapy if no rapid improvement in oxygenation is suggested if optimized ventilation and other rescue strategies do not resolve hypoxemia. The routine use of extracorporeal membrane oxygenation (ECMO) for patients with COVID-19 and refractory hypoxemia in this situation is controversial, and no recommendation can be made. In patients with cirrhosis and ARDS with life-threatening hypoxemia and hepatopulmonary syndrome, anecdotal reports have shown the benefits of using ECMO, even as a bridge to liver transplantation. Nonetheless, liver tests, especially bilirubin and alkaline phosphatase levels, were found to predict poor outcomes post-ECMO in cardiac surgery patients. Hence, advanced cirrhosis patients may not be ideal candidates for ECMO in critical COVID-19. The use of ECMO also can lead to an elevation in liver enzymes. Further to this, a nationwide population-based cohort study on the outcome of ECMO support in patients with liver cirrhosis showed that its utility for cirrhosis patients, especially when >2 risk factors (age ≥65 years, those with underlying respiratory disease, hypoalbuminemia, and liver transplant receipt) have been identified, was deleterious on clinical outcomes.[94–97] In case staged upgraded medical interventions fail to improve hypoxemic respiratory failure, then ECMO may be considered on a case-by-case basis and following inclusion as per EOLIA trial criteria, which include one of the following: a ratio of the partial pressure of arterial oxygen (Pao2) to the fraction of inspired oxygen (Fio2) of less than 50 mmHg for more than 3 h or Pao2:Fio2 of less than 80 mmHg for more than 6 h or an arterial blood pH of less than 7.25 with a partial pressure of arterial carbon dioxide of at least 60 mmHg for more than 6 h.

Even though the early use of ECMO does not significantly improve mortality at 60 days in patients with severe ARDS, it might help improve short-term survival when used as a rescue modality.[98] For critically ill patients with COVID-19 who have acute kidney injury and develop indications for renal replacement therapy, continuous renal replacement therapy is recommended. In its absence, prolonged intermittent renal replacement therapy holds for those with cirrhosis and advanced liver failure.[99,100] At present, for patients with COVID-19 and severe or critical illness, empiric broad-spectrum antimicrobial therapy in the absence of another indication is not recommended. However, in the cirrhotic population, especially among those with decompensation such as variceal bleeding or those with ACLF, early empirical antibiotics may be considered since the patients are at high risk of developing hospital-acquired infections and secondary bacteremia.[101–103] An exhaustive algorithm for the management of cirrhosis patients with COVID-19 is shown in Figure 3.

Conclusions

The incidence of COVID-19 among patients with cirrhosis may be low but further studies are required to address this topic clearly. The novel coronavirus does not cause direct liver injury or promote liver failure, but severe infections can result in unstable decompensation of cirrhosis and ACLF. COVID-19 in decompensated cirrhosis and in conditions that lead to ACLF are associated with poor clinical outcomes, even though the most common cause for mortality is lung failure and not progressive liver dysfunction. Current recommended treatment guidelines, such as use of corticosteroids appear to be safe in patients with stable cirrhosis, while caution must be exerted towards experimental and nonevidence-based treatments in this special patient population, especially in patients with decompensated cirrhosis with secondary sepsis and those with ACLF. Physician-driven use of experimental methods or therapeutics within clinical research must be guided by its safety in the liver disease population. Critical care management of severe COVID-19 in cirrhosis should be on similar lines as in the general patient population but with the use of specific therapies beneficial in cirrhosis, such as intravenous human albumin and terlipressin. Early recognition of those at risk for worse outcomes is imperative for imparting beneficial critical care management in cirrhosis.

Funding

None to declare.

Conflict of interest

Cyriac Abby Philips is an editorial member, academic editor and guest editor of the special issue on COVID-19 and the Liver in the *Journal of Clinical and Translational Hepatology*. The other authors have no conflict of interests related to this publication.

Author contributions

Study conception and design (CAP, PA, PKY), drafting of the manuscript (CAP, KK, MJ), critical revision of the manuscript for important intellectual content (SR, RA, PA, KK, MJ, PKY), and technical, or material support, study supervision (PA, PKY, MJ).

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Journal of Clinical and Translational Hepatology **2021** vol. 9(4) | 587–591
DOI: 10.14218/JCTH.2021.00095

Review Article

Liver Transplantation Services During the Time of COVID-19

Parita Patel and Anjana Pillai*

Section of Gastroenterology, Hepatology, and Nutrition, Department of Internal Medicine, The University of Chicago Medicine, Chicago, IL, USA

Received: 16 March 2021 | **Revised:** 12 April 2021 | **Accepted:** 8 May 2021 | **Published:** 31 May 2021

Abstract

The coronavirus disease 2019 (COVID-19) is associated with high morbidity and mortality, prompting overwhelmed hospital systems to reallocate resources to those stricken with the disease. In response, many liver transplantation programs unexpectedly came to an abrupt halt, significantly affecting the lives of living donors and recipients around the world. As the risk-benefit scale of liver transplantation has changed in the era of COVID-19, it is prudent to understand the impact of COVID-19 on those with underlying liver disease and those in need of a liver transplant. In this review, we discuss recommendations put forth by hepatology and transplant societies, summarize results from emerging studies, and propose strategies to appropriately risk stratify patients prior to transplantation.

Citation of this article: Patel P, Pillai A. Liver transplantation services during the time of COVID-19. J Clin Transl Hepatol 2021;9(4):587–591. doi: 10.14218/JCTH.2021.00095.

Introduction

The coronavirus disease 2019 (COVID-19) pandemic caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV2) has affected over 72 million patients around the world as of December 2020.[1] Due to the rapid rise of COVID-19, hospitals and policy makers implemented drastic changes to allow for resource reallocation aimed at treating patients stricken by the virus. Surgical operating rooms were transformed into intensive care units (ICUs), specialty providers were deployed to COVID-19 units, and lifesaving ventilators were rationed due to overwhelmed healthcare systems. As a result, organ transplantation centers were brought to an abrupt halt in the wake of a quickly growing pandemic.

Undoubtedly, the COVID-19 pandemic has shifted the risk-benefit scale of liver transplantation.[2] Reliability of diagnostic testing, risk of infection, transmission in the perioperative setting, and the impact of immunosuppression are all major concerns.[3] Additionally, increased perioperative complications in patients infected with COVID-19 at time of surgery have also been reported.[4]

As transplant programs safely reopen, modifications to standard protocols are necessary to provide safe and effective methods of organ transplantation for both deceased and living donors. In this article, we explore how this pandemic has affected and changed liver transplantation and summarize recommendations from multiple health organizations.

COVID-19 and liver disease

Data on the effect of COVID-19 on patients with chronic liver disease is growing exponentially. In a study of over 2,500 patients with COVID-19 in the United States, patients with chronic liver disease and COVID-19 were almost five times more likely to die than those without COVID-19 (relative risk [RR]=4.6, 95% confidence interval [CI]=2.6–8.3, $p<0.001$).[5] Similarly, in a study of over 88,000 patients in the Veterans Affairs national healthcare system, COVID-19 infection was associated with a 3.5-fold increase in mortality in patients with cirrhosis and cirrhosis was associated with a 1.7-fold increase in mortality in patients with COVID-19 infection.[6] Preliminary data suggest mortality attributable to COVID-19 is higher in patients with more advanced liver disease and is strongly correlated with Child-Pugh (CP) class.[7] Mortality rates were 12.2% in patients without cirrhosis compared to 23.9% in patients with CP Class A vs. 43.4% in patients with CP Class B, and 63% in those with CP class C.[7] In addition to baseline liver disease stage, analysis of an international registry of patients with chronic liver disease and COVID-19 found that age (odds ratio [OR] 1.02, 95% CI=1.01–1.04, $p=0.011$) and alcohol-related liver disease (OR 1.79, 95% CI=1.03–3.13, $p=0.040$) were also factors associated with death.[8] Conversely, Bajaj and colleagues[9] observed in a multicenter trial that, when matched for age and gender, patients with cirrhosis and COVID-19 may have similar mortality compared to patients with cirrhosis alone, although higher than in patients with COVID-19 without cirrhosis.

COVID-19 and liver transplantation outcomes

The initial paucity of data left clinicians uncertain about the

Keywords: COVID-19; Liver transplantation; Liver disease.

Abbreviations: AASLD, American Association for the Study of Liver Disease; AST, American Society of Transplantation; CI, confidence interval; COVID-19, coronavirus disease 2019; CP, Child-Pugh; EASL, European Association for the Study of Liver Disease; HCC, hepatocellular carcinoma; ICUs, intensive care units; LDLT, living donor liver transplantation; MELD, model for end-stage liver disease; NAT, nucleic acid testing; NHSBT, National Health Services Blood and Transplant; OPTN, Organ Procurement and Transplant Network; OR, odds ratio; RR, relative risk; SARS-CoV2, severe acute respiratory syndrome coronavirus 2; SOT, solid organ transplant.

***Correspondence to:** Anjana Pillai, University of Chicago Medicine, 5841 S. Maryland Ave MC 7120, Chicago, IL 60637, USA. ORCID: https://orcid.org/0000-0001-6783-2109. Tel: +1-773-795-4985, Fax: +1-773-702-2126, E-mail: apillai1@medicine.bsd.uchicago.edu

impact of COVID-19 in solid organ transplant (SOT) recipients. Early single-center reports, limited by small sample sizes and restricted geographic domains, published variable mortality rates, leaving providers unsure about the safety of transplantation during this time.[10,11] As a result, many transplant centers around the world were suspended during the initial wave of the pandemic. Lombardy, Italy was drastically affected by COVID-19, with hospitals required to expand the total number of ICU beds from 724 to 1,381 to accommodate patients with the virus.[12] Although authorities had not formally halted the transplant programs across Lombardy, there was a temporary decrease in liver transplantation due to several reasons, including an overwhelming influx of COVID-19 patients to ICU beds, redeployment of ICU doctors (leaving a paucity of specialists to care for liver transplant recipients), lack of data regarding the risk of nosocomial COVID-19 in recipients, and concerns regarding the safety of the procurement teams who may be exposed to potentially infected patients.[12] A similar decline in solid organ transplantation procedures was seen in France, with a 90.6% reduction in deceased donor transplantation since the COVID-19 outbreak.[13]

Countries with large living donor programs were similarly affected. In a study conducted in India, the effects of the COVID-19 pandemic on living donor liver transplantation (LDLT) were evaluated from March to June 2020 and compared to a pre-COVID period in 2019. LDLTs in COVID-19 times decreased to 58.9% of the previous year, with no significant difference in age, gender or indication of LDLT. One of twenty-three post-transplant recipients, three of seventy-one recipients and donors during evaluation, and eight of one hundred and twenty-five healthcare workers developed COVID-19 during this time, all with uneventful recovery.[14]

Although transplantation rates initially decreased during the height of the pandemic, growing collaborations among researchers worldwide has since led to multicenter data on outcomes in SOT recipients and has improved risk stratification in this patient population. In a study of 482 SOT recipients from over 50 transplant centers, the 28-day mortality rate after COVID-19 diagnosis was 18.7%. In that study, independent risk factors for mortality included age >65 years (OR 3.0 95% CI=1.7–5.5, $p<0.001$), presence of congestive heart failure (OR 3.2, 95% CI=1.4–7.0, $p=0.004$), chronic lung disease (OR 2.5, 95% CI=1.2–5.2, $p=0.018$) and obesity (OR 1.9, 95%CI 1.0–3.4, $p=0.039$).[10] Immunosuppression was not found to be a risk factor for mortality.[10] Similar mortality rates and risk factors for death were identified in other large-sample studies. In a large, national cohort study performed in England, 597 of the 46,789 SOT (1.3%) recipients who tested positive for COVID-19 had a mortality rate of 25.8%. Increasing recipient age was the only variable independently associated with death after a positive COVID-19 test in that study.[15]

As more data became available, the impact of COVID-19 was specifically evaluated in liver transplant recipients. In a prospective study of 19 European centers, 12 centers had registered 57 cases of liver transplant recipients who contracted COVID-19.[16] The most common symptoms were fever (79%), cough (55%), dyspnea (46%), fatigue or myalgia (56%), and gastrointestinal symptoms (33%). Immunosuppression was reduced in 22 recipients (37%) and discontinued in 4 (7%), but no impact on outcome was observed. The estimated case fatality rate was 12% (95% CI=5% to 24%), and notably five of the seven patients who died had a history of cancer.[16]

Similar data were seen in a prospective nationwide study in Spain, which found fever and cough to be the most common symptoms of COVID-19 in liver transplant recipients.[17] In that study, the mortality rate was 18%, which was lower than in the matched general population (standardized mortality ratio 95.5, 95% CI=94.2–96.8).[17] Clinical predictors of severe COVID-19 among hospitalized patients included higher Carlson co-morbidity index (RR=1.28, 95% CI=1.05–1.56, $p=0.015$), male gender (RR=2.49, 95% CI=1.14–5.41, $p=0.021$), and dyspnea at diagnosis (RR=7.25, 95% CI=2.95–17.82, $p<0.001$).

Webb *et al*.[18] also found that increased age and presence of comorbidities carry more risk than the liver transplantation itself. In a multicenter trial of 151 adult liver transplant recipients from 18 countries and 627 patients who had not undergone liver transplantation, there was no difference in proportion of patients hospitalized (124 [82%] patients in the liver transplant cohort vs. 474 [76%] in the comparison cohort, $p=0.106$), or who required intensive care (47 [31%] vs. 185 [30%], $p=0.837$). ICU admission (43 [28%] vs. 52 [8%], $p<0.0001$) and need for invasive ventilation (30 [20%] vs. 32 [5%], $p<0.0001$), however, were more frequent in the liver transplant cohort. There was no difference in death between the two groups (19% in the liver transplant group vs. 27% in the non-transplant group, $p=0.46$), and in a propensity score matched analysis, liver transplant did not increase the risk of death in patients with COVID-19 (absolute risk difference 1.4%, 95% CI=7.7–10.4). Only age, serum creatinine, and non-liver cancer were associated with increased risk of death in the liver transplantation group.[18] A Swedish study also found older age, male sex, and greater body mass index at presentation to be associated with adverse outcomes.[19]

With new potential treatment options, vaccinations, and emerging data on the safety of liver transplantation, more programs around the world have begun to reopen and increase their transplant volumes. The reopening of transplant programs in England was addressed by the National Health Services Blood and Transplant (NHSBT) unit.[20] Given the challenges faced by units vary by geographical location, organ type and local resource environments, NHSBT felt these considerations were best evaluated at the local level. As these challenges are in constant flux, especially with the new strains of the virus and multiple waves of the pandemic, the NHSBT has proposed guidelines to consider prior to reopening of programs. These include assessing the availability of adequate resources (multidisciplinary team input, number of ward beds, anesthesia availability, personal protective equipment, blood products, etc.) and microbiology and infection control policies meeting national standards. The impact of reopening transplant programs around the world, particularly with new strains of the virus emerging, has yet to be seen.

Liver transplant evaluation in the COVID-19 era

The COVID-19 pandemic has admittedly affected the evaluation and listing process for liver transplantation. During the height of the pandemic, many transplant centers restricted transplants to their sickest patients or completely halted their transplant programs, significantly decreasing organ procurements and thereby adversely affecting transplant wait times and wait list mortality.[21]

While there is no universal policy on how to best utilize resources and ensure patient and healthcare provider safety, it is imperative for centers to critically and carefully develop policies and protocols that best fit their patient population and consider the prevalence of COVID-19 in their area.[21] For patients undergoing liver transplant evaluation, multidisciplinary care teams, such as those involving transplant education, social work, nutrition, and pharmacy and financial consults, should be conducted via telemedicine whenever possible.[21] For patients who are scheduled for in-person visits, precautions should be made in advance to

limit exposure, including staggering patient arrival times, decreasing congregation in patient waiting rooms, ensuring appropriate use of masks, screening for symptoms, and limiting the number of family members/friends that accompany the patient.

When the determination is made to proceed with transplantation efforts, the American Association for the Study of Liver Disease (AASLD) recommends ensuring appropriate resource utilization (ICU beds, ventilators, personal protective equipment, and supply of blood products) and frequent re-evaluation of these resources.[21] In centers with ongoing resource limitations, the European Association for the Study of Liver Disease (EASL) suggests prioritizing liver transplantation in patients with poor short-term prognosis (i.e. those with acute liver failure, acute-on-chronic liver failure, high model for end-stage liver disease [MELD] scores and hepatocellular carcinoma [HCC] at the upper limits of the Milan criteria).[22] Other additional considerations include accepting only grafts with a low risk of delayed graft function, as this can minimize complications and avoid prolonged hospital stays and implementing perioperative management in a specific, designated clean ICU.[21,22] Once listed, patients should be triaged appropriately (as discussed below) and telemedicine visits should be utilized when appropriate.

Waiting on the transplant list during COVID-19

For outpatient management of those on the transplant list, AASLD recommends scheduling specific patients, particularly those with HCC or high MELD scores, for in-person clinic visits while using telemedicine for patients with less urgent issues.[21] Outpatient labs and imaging should be obtained only as clinically necessary. During the height of the pandemic, the Organ Procurement and Transplantation Network (OPTN) temporarily enacted policy changes where centers and patients were no longer required to update labs or imaging as a means to maintain MELD score. During this time, clinical data and imaging from previous exception petitions (i.e. those with HCC) could be maintained if updated data could not be obtained. These policies were implemented by OPTN to help prevent unnecessary exposure to transplant recipients and living donors and to alleviate data burden for transplant centers during the initial wave of the pandemic.[23] However, in patients listed with HCC, Mehta and colleagues[24] recommend obtaining preoperative imaging at time of admission for liver transplant, if not done within 3 months, to ensure tumor characteristics meet standard liver transplant criteria.

Additional measures to reduce transmission during the peri-transplant period include social isolation for waiting list patients, telephone screening for symptoms and exposures before admission, and staggering patient arrival times to avoid congregating in waiting area.[21,22]

COVID-19 diagnosis and testing

The risk of COVID-19 infection from an infected living donor or deceased donor is limited at this time and is evolving as more data becomes available. Furthermore, it is essential to recognize that while testing is helpful, no laboratory test is 100% specific or sensitive, allowing for false positives and false negatives. The positive and negative predictive values are determined by a specific assay performance, taking into account the amount of locally circulating virus and specimen quality.[25] Ultimately, the risks and benefits should be considered on a case-by-case basis prior to performing or denying a transplant.

Testing donors

The American Society of Transplantation (AST) recommends all potential deceased and living donors be screened for suspected COVID-19.[25] Testing by nucleic acid testing (NAT) should occur as close to the time of organ procurement as possible and should be obtained at least once from an upper or lower respiratory sample. Although some centers have serology or antibody testing available, interpretation of these tests is still not fully elucidated. If used, AST recommends to view results as adjunctive data, rather than diagnostic or definitive data (Fig. 1).[25]

Special considerations in living donors

For living donors, if more than 3 days have passed between time of testing and procurement, a repeat sample from the respiratory sample is recommended. Additional recommendations include delaying transplant for asymptomatic living donors with a known exposure history within the previous 14 days. Active COVID-19 infection is considered a contraindication to transplant at this time. If a living donor had a previous COVID-19 infection, consideration for organ acceptance can be made if repeat NAT testing is negative or if the initial infection occurred between 21 and 90 days prior to donor evaluation, irrespective of repeat NAT testing, and symptoms have resolved.[25]

Special considerations in deceased donors

Similar to screening protocols set forth for living donors, AST recommends viral testing of at least one sample from the respiratory tract by NAT within 3 days of procurement. Some experts even recommend a second viral test be performed 24 hours after the initial test and within 24–48 h of procurement, if possible. For deceased donors with previous COVID-19 infection, recommendations similar to those for living donors with previous COVID-19 should be followed.[25]

Post-liver transplant management during the COVID-19 pandemic

Concerns that patients with liver transplants may be at a higher risk from COVID-19 due to use of immunosuppression and underlying comorbidities are still under investigation. Preliminary data suggest a similar or even lower incidence of COVID-19 infection in transplanted patients to that of the general population. In a large Italian survey of 640 patients, the incidence of COVID-19 in liver transplant recipients was only 1.25%, with 75% of patients developing only mild disease.[26]

The impact of immunosuppression in COVID-19 is also not well known. Emerging data suggest that, while post-transplant immunosuppression may prolong viral shedding in patients with COVID-19, mortality from COVID-19 may not be substantially different than in the general population.[21] In a large multicenter cohort study, 151 patients with COVID-19 from 18 countries who had previously received a liver transplant were compared from a contemporaneous cohort of 627 patients with COVID-19 and without a history of liver transplant. After adjustment for age, sex, creatinine, obesity, hypertension, diabetes or ethnicity, liver transplantation did not significantly increase the risk of death in patients with SARS-COV2 (absolute risk difference 1.4%, 95% CI=–7.7 to 10.4, p=0.764).[18] Rather,

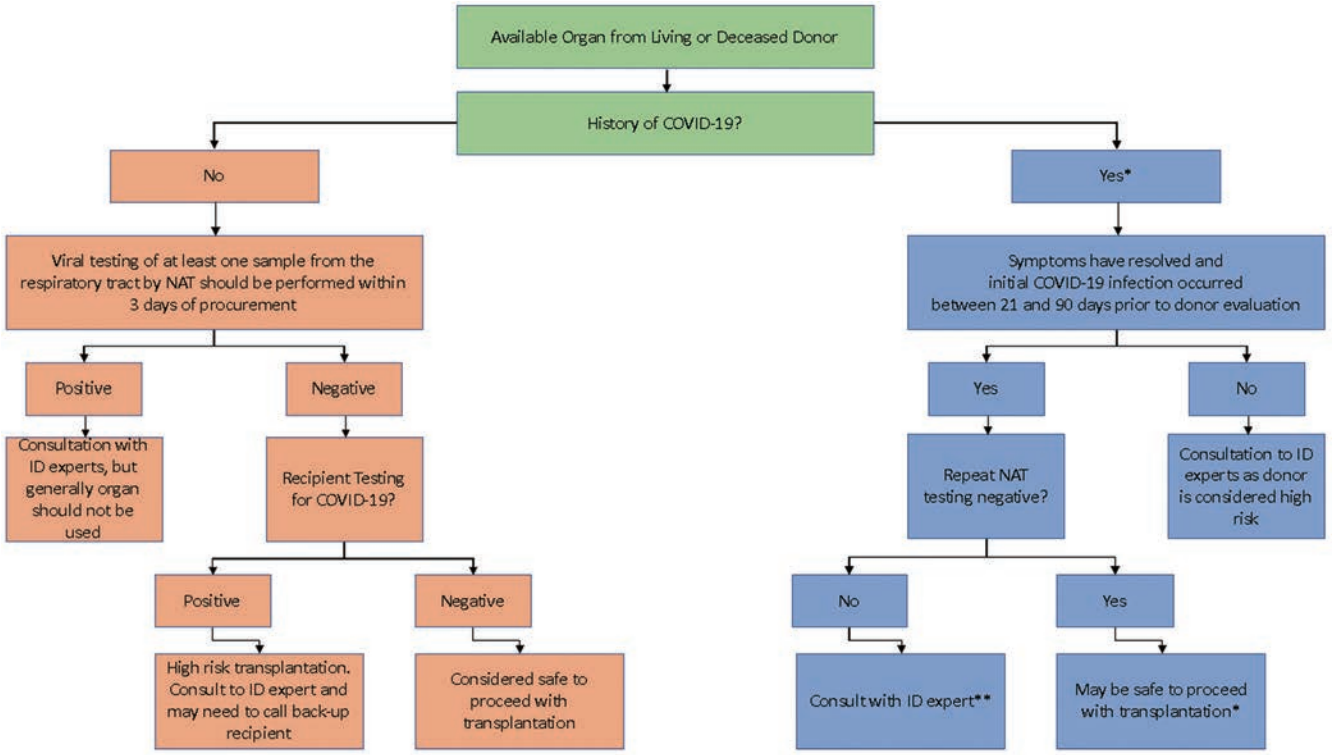

Fig. 1. Proposed approach to COVID-19 testing for liver transplantation. *Data on organ donation from previous COVID-19-infected donors are limited and decision to proceed with transplantation should occur on a case-by-case basis. Discussions with the transplant recipient should be arranged and risks and benefits of transplantation vs. no transplantation should be thoroughly explained.[25] **Repeat positive PCR within 90 days most likely reflects persistent shedding or viral RNA rather than new infection, but organ acceptance should only be considered after consultation with ID experts.[25] Figure adapted from the AST. SARS-COV2: Recommendations and Guidance for Organ Donor Testing. Available at: https://www.myast.org/sites/default/files/Donor%20Testing_100520_revised_ReadyToPostUpdated10-12.pdf.[25] ID, infectious disease; COVID-19, coronavirus disease 2019.

age, serum creatinine and non-liver cancer were associated with death among liver transplant recipients.[18] Another prospective nationwide study of 111 liver transplant recipients in Spain with COVID-19 also demonstrated that chronic exposure to immunosuppressive agents did not increase standardized mortality rates. However, findings did suggest that high doses of mycophenolate could increase the risk of severe COVID-19 among hospitalized liver transplant patients.[17]

With the evidence and data that are available, AASLD and EASL recommend not changing immunosuppressive regimens in post-transplant patients without COVID-19, while emphasizing general precautionary measures such as maintaining social distancing, wearing masks, and avoiding travel.[21,22] In post-transplant patients with COVID-19, adjustment of immunosuppression should be individualized with severity of COVID-19 weighed against risk of graft rejection. Minimizing immunosuppression, particularly antimetabolite medications, should be considered as would be done with contraction of other infections.[21]

Potential treatment options for COVID-19 are under review, but the effect of these medications on liver transplant recipients remains unknown. Given the paucity of data, close monitoring is recommended for possible drug-drug interactions and adverse reactions.[27]

Conclusion

The COVID-19 pandemic has had a dramatic impact on transplant programs, recipients and donors around the world. Resource reallocation during the height of the pandemic brought many transplant programs to a halt. Although data continues to evolve, this review summarizes the available evidence and society recommendations to help liver transplant programs safely perform organ transplantation. Appropriate risk stratification of patients with liver disease, methodological testing of donors and recipients prior to transplantation, and minimizing transmission are key components in providing a safe and effective method to resume deceased donor and living donor liver transplantation around the world.

Funding

None to declare.

Conflict of interest

AP is on the Speaker's Bureau for Simply Speaking Hepatitis (CME) and Medical Advisory Board for Eisai Inc, Exelixis, and Genentech. PP has no disclosures.

Author contributions

Drafted the paper and approved the final version (PP), revised and approved the final version of the manuscript (AP). AP is the guarantor of the article.

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Journal of Clinical and Translational Hepatology **2021** vol. 9(4) | 592–597
DOI: 10.14218/JCTH.2021.00051

Review Article

B Cell-mediated Humoral Immunity in Chronic Hepatitis B Infection

Yang Li[1#], Shengxia Yin[2#], Rahma Issa[3], Xin Tong[2], Guiyang Wang[2], Juan Xia[2], Rui Huang[2], Guangmei Chen[4], Dan Weng[1], Chen Chen[5], Chao Wu[2*] and Yuxin Chen[6*]

[1]School of Environmental and Biological Engineering, Nanjing University of Science & Technology, Nanjing, Jiangsu, China; [2]Department of Infectious Diseases, Nanjing Drum Tower Hospital, Nanjing University Medical School, Nanjing, Jiangsu, China; [3]Department of Infectious Diseases, Nanjing Drum Tower Hospital, Clinical College of Nanjing Medical University, Nanjing, Jiangsu, China; [4]Department of Infectious Diseases, Jiangsu Province Hospital of Chinese Medicine, Nanjing, Jiangsu, China; [5]Department of Clinical Pharmacology, Nanjing First Hospital, Nanjing Medical University, Nanjing, Jiangsu, China; [6]Department of Laboratory Medicine, Nanjing Drum Tower Hospital, Nanjing University Medical School, Nanjing, Jiangsu, China

Received: 3 February 2021 | **Revised:** 24 April 2021 | **Accepted:** 6 May 2021 | **Published:** 27 May 2021

Abstract

B cell-mediated humoral immunity plays a vital role in viral infections, including chronic hepatitis B virus (HBV) infection, which remains a critical global public health issue. Despite hepatitis B surface antigen-specific antibodies are essential to eliminate viral infections, the reduced immune functional capacity of B cells was identified, which was also correlated with chronic hepatitis B (CHB) progression. In addition to B cells, T follicular helper (Tfh) cells, which assist B cells to produce antibodies, might also be involved in the process of anti-HBV-specific antibody production. Here, we provide a comprehensive review of the role of various subsets of B cells and Tfh cells during CHB progression and discuss current novel treatment strategies aimed at restoring humoral immunity. Understanding the mechanism of dysregulated B cells and Tfh cells will facilitate the ultimate functional cure of CHB patients.

Citation of this article: Li Y, Yin S, Issa R, Tong X, Wang G, Xia J, *et al*. B cell-mediated humoral immunity in chronic hepatitis B infection. J Clin Transl Hepatol 2021;9(4):592–597. doi: 10.14218/JCTH.2021.00051.

Introduction

Hepatitis B virus (HBV) infection remains a significant cause of liver cirrhosis and hepatocellular carcinoma globally, especially in developing countries like China. In 2015, the World Health Organization (WHO) estimated that 257 million individuals live with chronic hepatitis B (CHB) worldwide,[1,2] resulting in 887,000 yearly deaths, mostly due to HBV infection-related hepatocellular carcinoma and cirrhosis.[3–5]

The challenge to CHB treatment is the failure to clear covalently closed circular DNA (referred to as cccDNA), which can give the virus the capacity to evade the host immune system, making a complete sterilizing cure unlikely to be feasible.[6] On the other hand, a functional cure is defined as a sustained clearance of hepatitis B surface antigen (HBsAg) with or without seroconversion to anti-HBs antibodies after a finite course of therapy, but with the persistence of residual cccDNA. The functional cure of CHB has been considered as a feasible clinical treatment goal,[7,8] which is correlated with improved clinical outcomes.[9] Nevertheless, only a small proportion of patients reach this milestone.[10,11]

The complex interaction between HBV and the host immune system drives the process of chronic HBV infection, in which the anti-HBV adaptive immune system processes facilitate the clearance of HBV. Despite T cell responses having been well-studied in HBV infection, the beneficial biological function of B cells for functional cure of CHB has been consistently neglected. In addition, T follicular helper (Tfh) cells which regulate the B cell-mediated humoral immune responses have been identified as phenotypically distinct, leading to humoral immunity defection in patients with CHB.[12] Hence, in this review, we will discuss the role of B cell-mediated humoral responses during chronic HBV infection and the current promising treatment strategies to induce robust anti-HBV humoral responses (Fig. 1).

Protective role of antibody in HBV control and clearance

B cell-mediated humoral immune responses are essential for HBV control and clearance. Universal vaccination against HBV has remarkably decreased HBV infection rate, since anti-HBsAg antibodies (i.e. anti-HBs) induced by immunization could prevent HBV infection.[13] It is considered that those individuals with an anti-HBs concentration of ≥10 mIU/mL were immune against HBV infection, while those with an anti-HBs concentration of <10 mIU/mL might re-

Keywords: Chronic hepatitis B (CHB); B cell; T follicular helper (Tfh) cells; Antibody; Therapeutics.

Abbreviations: HBV, hepatitis B virus; CHB, Chronic hepatitis B; Tfh, T follicular helper; HBsAg, hepatitis B surface antigen; APCs, professional antigen-presenting cells; IA, immune active; IT, immune tolerance; TLR, Toll-like receptor; PD-1, programmed cell death receptor-1; atMBCs, atypical memory B cells; Bregs, regulatory B cells; Tregs, regulatory T-cells; IL-10, interleukin -10; GCs, germinal centers; CXCR, chemokine receptor.

[#]Contributed equally to this work.

[*]**Correspondence to:** Yuxin Chen, Department of Laboratory Medicine, Nanjing Drum Tower Hospital, Nanjing University Medical School, Nanjing, Jiangsu 210008, China. ORCID: https://orcid.org/0000-0001-5955-687X. Tel: +86-25-8968-3827, Fax: +86-25-8330-7115, E-mail: yuxin_chen2015@163.com; Wu Chao, Department of Infectious Diseases, Nanjing Drum Tower Hospital, Nanjing University Medical School, Nanjing, Jiangsu 210008, China. ORCID: https://orcid.org/0000-0002-1657-010X. Tel: +86-25-8310-5890, Fax: +86-25-8330-7115, E-mail: dr.wu@nju.edu.cn

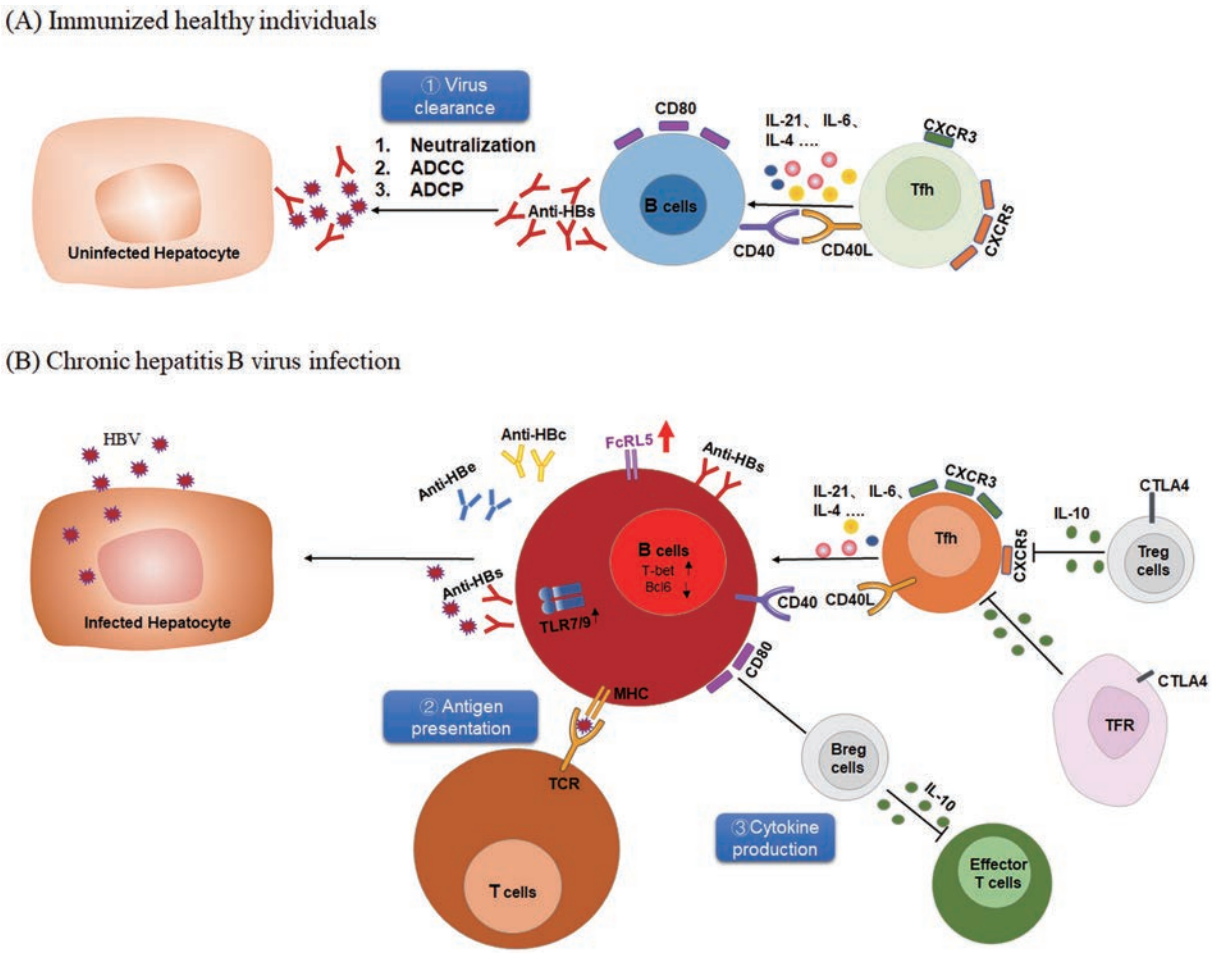

Fig. 1. B cell-mediated humoral immunity in immunized healthy individuals and CHB patients. (A) HBsAb production by HBsAg-specific B cells in immunized healthy individuals plays a pivotal role in the clearance of HBV. A major antiviral role for HBsAb is viral clearance, mediated by neutralization, antibody-dependent cellular cytotoxicity and antibody-dependent cellular phagocytosis. Tfh cells could assist B cell function by expressing cytokines such as IL-21, IL-6 and IL-4 and direct interactions through CD40L/CD40. (B) In CHB patients, B cells were phenotypically dysfunctional with increased expression of T-bet, TLR7/9 and FcRL5. During CHB infection, despite HBcAg-specific B cells being class-switched memory B cells and secrete anti-HBc, HBsAg-specific B cells fail to mature efficiently into antibody secreting cells, leading to the scarcity of serological anti-HBs. Beyond the traditional role of antibody production, HBV-specific B cells might efficiently serve as a primary source of APC for T cells and induce CTLs responses. Moreover, B cells can produce cytokines such as IL-10 to inhibit the function of effector T cells and enhance Treg cell function. TFR and Treg cells can impair the Tfh function by secreting IL-10 and expressing CTLA4. The dysregulated B cells, Tfh cells, TFR cells and Treg cells might contribute to the defective function of B cell mediated humoral immunity during CHB infection. HBV, hepatitis B virus; CHB, chronic hepatitis B; Tfh, T follicular helper; HBsAg, hepatitis B surface antigen; APCs, antigen-presenting cells; TLR, toll-like receptor; IL-10, interleukin-10.

quire an additional booster vaccine dose.[14–16]

The specific antibodies against different HBV protein components are one of the major approaches for B cells to be involved in anti-HBV infection, such as antibody to hepatitis B core antigen (anti-HBc), antibody to hepatitis B e antigen (anti-HBe) and anti-HBs. Anti-HBc and anti-HBe serve as diagnostic biomarkers for HBV infection, while anti-HBs antibody is the only antibody that can specifically recognize and bind to HBsAg,[17,18] thus serving an important role in HBsAg clearance.[19] First, anti-HBs can not only block HBV entry by binding to free HBV viral particles as protective neutralizing antibodies to reduce viral load *in vivo*[20–22] but it also can mediate antigen-dependent cellular cytotoxicity and antigen-dependent cellular phagocytosis to clear infected cells.[23] HBV reactivation and hepatitis are well recognized complications that occur in patients who have undergone cytotoxic chemotherapy or immunosuppressive therapy.[24] For example, high incidence of HBV reactivation was observed in lymphoma patients who were HBs-negative/anti-HBc-positive with or without anti-HBs and receiving rituximab-containing chemotherapy.[25] Negative anti-HBs at baseline is an independent risk factor for HBV reactivation in patients with resolved CHB, compared with higher titer of anti-HBs ≥100 mIU/mL.[26] Moreover, adoptive transfer of HBV-specific immunity with the liver from an immune living liver donor leads to successful transfer of HBV-specific humoral and cellular immunity, which might be responsible for the delay of reinfection and a reduction of viral load.[27] Therefore, anti-HBs is essential to alleviate disease advancement and prevent reinfection during CHB.

Several neutralizing monoclonal antibodies (referred to as mAbs) specific to HBsAg have been reported. For example, human mAbs including 2H5-A14[28] and Bc1.187[29] that block the engagement of HBsAg to sodium taurocholate co-transporting polypeptide potently neutralize HBV *in vitro*. In addition, they could decrease viremia *in vivo* in an HBV mouse model. E6F6 that recognizes an evolutionarily conserved epitope (GPCK(R)TCT) not only prevented initial HBV infection and reduced the viral dissemination in human-liver-chimeric mice but also facilitated the restora-

tion of anti-HBV T cell response in hydrodynamic injection-based HBV carrier mice.[30] Furthermore, *in vivo* delivery of a DNA-encoded monoclonal antibody plasmid can efficiently neutralize HBV virus *in vitro*.[31] These antibodies can serve as a promising immunotherapeutic regimen or immunoprophylaxis for HBV infection.

Beyond the traditional role of antibody production, B cells also may play a vital role as professional antigen-presenting cells (APCs) during CHB infection.[25,32] Compared to the classical non-B cell APCs, HBcAg-specific B cells might efficiently serve as a primary source of APCs for native HBcAg-specific T cells.[33–36] In addition, B cells can induce an HBcAg-specific cytotoxic T lymphocytes (CTLs) response and further prevent immune tolerance by the cross-presentation of HBcAg on major histocompatibility complex-I (i.e. MHC-I) to specific $CD8^+$ T cells. At the same time, HBsAg is a special exogenous antigen, which can be involved to MHC-I molecules expressed on B cells.[37,38]

Immune dysfunction of B cells during CHB infection

HBV infection has exerted a significant impact on the global B cell compartment and HBV-specific antibody secretion.[39,40] Global peripheral B cells were activated with reduced functional capacity, while anti-HBs-secreting B cells were rarely detected.[19,41] Additionally, although total immunoglobulin G (IgG) in the serum among CHB patients is remarkably greater than in that of healthy controls, the absence of HBV-specific antibodies was observed.[39,42,43] B cell hyperactivation, differentiation disorder, activation of inhibitory signal and regulatory B cells may contribute to immune dysfunctions observed in CHB patients.[44,45]

A hallmark of chronic hepatitis infections, such as hepatitis C virus is the presence of immune exhausted virus-specific $CD8^+$T cells, characterized by their inability to secrete antiviral cytokines and an upregulation of inhibitory receptors such as programmed cell death receptor-1 (referred to as PD-1).[46] B cell hyperactivation is characterized by enhanced expression of activation markers with displayed impaired function, especially in patients at immune active (IA) and immune tolerance (IT) stage.[45,47] Overall, the mechanism of the hyperactivation of B cells remains to be clarified. Xu *et al*[19] reported that the B cell hyperactivation could be induced by increased interferon (IFN)-α and sCD40 ligands in IA patients. The increased activation of CD71 and CD69 expressed on B cell accounts for the B cell hyperactivation.[48,49] A high level of Toll-like receptor (TLR) 9 expression likely contributes to the functional hyperactivation of B cells in CHB patients.[50] A recent study revealed that B cells from CHB patients had a markedly reduced capacity to generate CD39/CD73-dependent extracellular adenosine and exhibited increased activation markers after adenosine-production blockade, suggesting CD39/CD73/adenosine pathway might contribute to B cell hyperactivation.[51]

The frequency of HBsAg-specific B cells was comparable in both CHB patients and immunized healthy individuals, while anti-HBs in CHB patients were detected at low level or were even undetectable.[52] In CHB patients, there was a unique population of B cell subsets with high levels of inhibitory receptors, including PD-1, which resemble $CD21^-CD27^-$ atypical memory B cells (referred to as atMBCs). These atMBCs had elevated level of defective signals, which might be responsible for defective capacity of survival, cytokine production and differentiation into antibody-secreting cells. Such atMBCs were found to be expanded in CHB patients and to have accumulated quickly in the HBsAg-specific compartment, which might reduce anti-HBs secretion[47,53] and enhance B cell hyperactivation in CHB patients.[41,54,55] In addition, the transcription factor T-bet was also upregulated in $CD21^-$ B cells during murine and human HBV infections,[56] which may be correlated with the inadequate production of HBsAg-specific B cells among CHB patients.[57–59] Moreover, chemokine receptor 3 (CXCR3), Fc receptor-like 4 (FCRL4) and FCRL5 are upregulated in B cells and associated with B cell immune dysfunction during HBV infection.

A regulatory subset of B cells (regulatory B cells, Bregs) is elevated in CHB patients,[44] which has been reported to inhibit liver inflammation and immune disorders in mouse models.[60,61] Previous studies showed that the frequency of Bregs had a significant correlation with alanine aminotransferase (ALT) and glutamic oxaloacetic transaminase (AST).[62] Furthermore, CHB patients in the IA phase exhibit increased Bregs due to inflammatory responses.[63] However, the underlying mechanism of the Bregs' elevation during CHB infection remains unclear. Bregs could suppress $CD8^+$ T cell responses, which might serve a pathogenic role by secreting interleukin-10 (IL-10), enhancing the function of regulatory T-cells (Tregs),[63] and suppressing T cell from secreting proinflammatory cytokines in various autoimmune diseases.[63,64] During CHB infection, Bregs have a crucial role in suppressing antiviral immune response by producing IL-10.[65] Notably, in HBeAg-negative CHB patients, serum IL-10 level was correlated with high virus load and advanced liver inflammation,[66,67] while blockade of IL-10 could improve vaccine efficacy and disease resolution in CHB patients.[68–70]

A recent study elegantly characterized the phenotype and functional impairment of HBsAg-specific B cells and HBcAg-specific B cells.[71] Of note, B cell response against HBsAg and HBcAg is different during CHB infection. HBcAg-specific B cells are present at higher frequency than HBsAg-specific B cells. Further, HBcAg-specific B cells are class-switched memory B cells and secrete antibodies, while HBsAg-specific B cells failed to mature efficiently into antibody secreting cells. The transcriptomic analysis showed that HBV-specific B cells had an mRNA expression pattern that differs from global memory B cells and express cross-presentation and innate immune genes, suggesting additional roles of HBV-specific B cells beyond the production of antibodies.

Multifunctional roles of Tfh cell subsets in CHB infection

T follicular helper (Tfh) cells are a unique subset of $CD4^+$ T cells, which can directly help B cells secrete antibodies in germinal centers (referred to here as GCs).[72–74] By colocalizing with B cells and expressing costimulatory signals as well as various cytokines, Tfh cells directly interact with B cells, facilitate B cell differentiation into long-lived plasma cells and memory B cells with high affinity, and facilitate the formation of GCs.[75–77]

Peripheral $CD4^+CXCR5^+$ T cells are considered as circulating memory $CD4^+$ Tfh cells. Peripheral circulating memory Tfh cells had similar phenotypic and functional properties as Tfh cells in the GC, known as GC Tfh cells, such as enhanced expression of CXCR5, stimulation of B cell maturation, terminal differentiation of B cells into antibody-producing plasma cells, and isotype switching. By the dominant transcriptional factors and cytokines, the circulating human memory Tfh cells have been divided into three subsets: Tfh1 ($CXCR3^+CCR6^-$); Tfh2 ($CXCR3^-CCR6^-$); and Tfh17 ($CXCR3^-CCR6^+$).[78] It is considered that blood memory Tfh2 and Tfh17 cells can induce naïve B cells to produce IgGs. Interestingly, Tfh2 cells can preferably induce the secretion of IgG and IgE, and Tfh17 cells can effectively promote IgG and especially IgA secretion.[79] while Tfh1 cells enhance protective antibody responses, making the memory B cells differentiate into effector B cells.[79,80]

It has been well established that Tfh cells have an essential role in various infectious diseases, such as *Plasmodium vivax* infection,[81] acute malaria,[82] CHB,[72] human immunodeficiency virus,[83] and tuberculosis.[84] Indeed, Tfh cells also play a vital role during CHB progression. The frequency of circulating Tfh cells ($CXCR5^+CD4^+$ T cells, cTfh cells) was correlated with the serum levels of ALT and AST,[85] suggesting that cTfh cells may be involved in HBV-specific immune responses. Further evidence showed that CHB patients have a significant increase of Tfh cells compared to healthy controls.[12] The frequency of $CD4^+CXCR5^+$ T cells in IA patients was higher than that of IT patients and healthy individuals,[86,87] suggesting high frequency of $CD4^+CXCR5^+$ Tfh cells could be a biomarker to assess the immune status of CHB patients. cTfh cells secrete IL-21 to facilitate HBeAg seroconversion.[88] On the other hand, HBsAg is a T cell-dependent antigen, and seroconversion of HBsAg also requires the assistance of Tfh cells. A unique group of $CXCR5^+CD8^+$ T cells with minimal levels of inhibitory receptors exerted its potent cytotoxicity to control viral replication by migrating into B cells follicles during CHB.[51,89,90] A subset of $CD25^+FOXP3^+$ Treg-like cells in cTfh cells that was enriched in patients, known as follicular regulatory T (referred to as TFR) cells, could suppress helper function of Tfh cells.[91] In a mouse model with persistent HBV infection, the function of HBsAg-specific cTfh cells was blocked by Treg cells, whereas the depletion of Treg cells could restore the cTfh function.[92] Moreover, a group of type 1 regulatory T (i.e. Tr1)-like cells migrate from the liver to the draining lymph node and can inhibit peripheral anti-HBV immunity by negatively regulating GC B cells and Tfh cells.[93]

Novel CHB treatment strategies targeting B cells

The widely used clinical standard first-line antiviral therapeutics for chronic HBV infection include IFNs and nucleoside analogs (commonly known as NAs). IFNs have a strong antiviral effect and immune-mediated function, which promotes antiviral innate and adaptive immunity. Based on the genetic, structural and functional characteristics and their receptors on the cell surface, the IFN family is classified into three major types: type-I; type-II; and type-III. Type-I IFNs (IFN-α, IFN-β, IFN-ε, IFN-κ, and IFN-ω) has been approved for the treatment of CHB infection.[94] Pegylated-IFN-α eliminates the production of HBsAg and is well tolerated in HBeAg-negative CHB patients.[95–98] In addition to the previously reported efficiency of pegylated-IFN on T cells and natural killer cells,[99] B cells may also play an essential role in this process.[100–102] Pegylated-IFN-α treatment might exert the immunomodulatory effect by remodeling B cell compartments, which was correlated with a sustained increase in sCD30 levels and decrease of plasma HBsAg.[103,104]

TLR agonists and checkpoint inhibitors are an emerging treatment strategy for CHB patients. TLR7 is highly expressed on B cells and has been proven to inhibit antibody production. As an oral agonist of TLR7, GS9620 is currently in clinical assessment to treat CHB patients.[105] Preclinical study showed that GS9620 treatment significantly induced an intrahepatic transcriptional profile enriched with $CD8^+$ T cells and B cells, contributing to clearance of HBV in a chimpanzee model.[106] Also, TLR9 agonists such as CPG 7909 or 1018 ISS co-administrated with HBsAg induced robust antibody responses among CHB patients.[107] Therefore, combined immunotherapeutic agents might be necessary to restore B cell function and induce the desired B cell antibody response.

HBV therapeutic vaccines have also emerged as a promising treatment strategy to induce robust humoral responses by activating B cells. For example, the ferritin nanoparticle vaccine that delivers preS1 to specific myeloid cells, including $SIGNR1^+$ dendritic cells, that activate Tfh cells and lymphatic sinus-associated $SIGNR1^+$ macrophages that can activate B cells.[108] Furthermore, a recent study developed a B cell epitope-based vaccine, which was able to suppress serum HBsAg and HBV DNA by inducing SEQ13-specific antibody response.[109]

Conclusion

During the pathogenesis of CHB, defective HBV-specific B cells and antibodies were identified, in which global B cells were dysfunctional; whereas, HBV-specific antibodies were found to be insufficient and might be functionally limited. Tfh cells residing in peripheral blood, spleen and liver are pivotal to facilitate the seroconversion of HBeAg and HBsAg. Novel hepatitis B treatment strategies targeting B cells might facilitate the recovery of B cell function and develop the desired B cell responses, leading to functional cure of CHB.

Funding

This work was supported by National Natural Science Foundation of China (82002133 and 81600201) Nanjing Medical Science and Technique Development Foundation (QRX17141), Nanjing Department of Health (YKK19056 and YKK19078), and Yangzhou Key R&D Program (Social Development) (YZ2020101), Jiangsu Provincial Commission of Health and Family Planning (Q2017003).

Conflict of interest

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

Author contributions

Study conception and design (YC, CW, SY), drafted the first version of the manuscript (YL, RI), edited and revised the manuscript (SY, YC, YL, GW, GC, RI,DW, GC, RH,XT,JX,CC). All authors approved the final version of the article, including the authorship list.

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Journal of Clinical and Translational Hepatology **2021** vol. 9(4) | 598–602
DOI: 10.14218/JCTH.2020.00123

Case Report

Obstructive Jaundice Caused by Mucinous Cystic Tumor of Gallbladder: A Case Report and Literature Review

Sulai Liu[1,2,3#], Zhihua Zhang[1,2,3#], Chao Guo[1,2,3], Zhangtao Yu[1,2,3], Siyuan He[1,2,3], Junaid Khan[1,2,3], Bo Jiang[1,2,3], Yinghui Song[1,2,3*] and Chuang Peng[1,2,3*]

[1]Department of Hepatobiliary Surgery/Hunan Research Center of Biliary Disease, Hunan Provincial People's Hospital/The First Affiliated Hospital of Hunan Normal University, Changsha, Hunan, China; [2]Biliary Disease Research Laboratory of Hunan Provincial People's Hospital, Key Laboratory of Hunan Normal University, Changsha, Hunan, China; [3]Clinical Medical Technology Research Center of Hunan Provincial for Biliary Disease Prevention and Treatment, Changsha, Hunan, China

Received: 18 November 2020 | **Revised:** 3 January 2021 | **Accepted:** 19 March 2021 | **Published:** 6 May 2021

Abstract

Mucinous cystic tumor of the gallbladder is an extremely rare benign tumor, with potential for malignant degeneration. Mucinous cystic tumors of the cystic duct are divided into mucinous cystadenoma and mucinous cystadenocarcinoma. Currently, cystadenoma is generally considered to be a precancerous lesion of cystadenocarcinoma. At present, there are few cases reported worldwide, and there are no relevant guidelines for diagnosis and treatment of this disease. This article presents the collected clinical data of a patient with mucinous cystic tumor of the gallbladder who was admitted to the First Affiliated Hospital of Hunan Normal University, with the characteristics of the disease summarized in combination with a focused literature review.

Citation of this article: Liu S, Zhang Z, Guo C, Yu Z, He S, Khan J, *et al*. Obstructive jaundice caused by mucinous cystic tumor of gallbladder: A case report and literature review. J Clin Transl Hepatol 2021;9(4):598–602. doi: 10.14218/JCTH.2020.00123.

Introduction

The cystic duct mucinous cystic tumor is a rare cystic duct tumor with latent malignant lesions, and represents a special pathological type in cholangiocarcinoma.[1,2] Mucinous cystic tumors of the cystic duct are divided into mucinous cystadenoma and mucinous cystadenocarcinoma.[3] Currently, cystadenoma is generally considered to be a precancerous lesion of cystadenocarcinoma, so patients in whom this disease is suspected should be decisively operated, with their intraoperative fast frozen sections used to guide the operation.[2,3] The etiology of mucinous cystic gland tumors of the bile ducts is currently unclear. Some believe that this is a congenital disease, caused by fluid retention as a result of inflammatory hyperplasia or obstruction of some abnormal ducts that occurs during embryonic growth; others believe that mucinous cystic tumors of the cystic duct are related to preembryonic intestinal residual or ectopic ovarian tissue.[1–3]

The patient presented with painless jaundice that had lasted for a duration of 1 month. In this case, the large cystic duct tumor was found to have squeezed the common bile duct to cause obstructive jaundice and dilated intrahepatic and extrahepatic bile ducts.

Case report

The patient was a 57 year old female, with complaint nausea and vomiting for more than 1 month. She had been treated at a local community hospital 1 month prior to presentation at our hospital. However, after having received intravenous fluids and antibiotics, her symptoms did not alleviate. Laboratory examination upon presentation to our hospital showed the following: total bilirubin of 143.9 μmol/L; direct bilirubin of 109.6 μmol/L; alanine aminotransferase of 87.7 U/L; aspartate aminotransferase of 78.25 U/L; alkaline phosphatase of 201 U/L; gamma-glutamyltransferase of 682.0 U/L; carbohydrate antigen 19–9 of 252.02 U/mL; cancer antigen 72–4 of 7.76 U/mL; and, negativity for the panel of antinuclear antibodies. Findings for cancer antigen 125, blood routine, and serum C-reactive protein were basically normal. Abdominal computed tomography (Fig. 1A, B) showed obstruction of the lower part of the common bile duct, dilatation of the upper bile duct, and chronic cholecystitis. Magnetic resonance cholangiopancreatography (Fig. 1C) showed thickening of the lower part of the common hepatic duct with dilatation of the bile ducts inside and outside the liver. For preoperative jaundice reduction and cholangiography, percutaneous transhepatic cholangial drainage (referred to as PTCD) was performed. The PTCD angiography (Fig. 1D) showed filling defect in the common bile duct and bile duct dilation.

In order to clarify the nature of the space occupied by the bile duct and relieve the patient's biliary obstruction, abdominal cavity exploration, biliary exploration, preparation of biliary and enteral drainage were performed. A fro-

Keywords: Mucinous cystic tumor; Jaundice; Gallbladder; Case report; Literature review.
Abbreviations: MCN, mucinous cystic neoplasm; PTCD, percutaneous transhepatic cholangial drainage.
[#]These authors contributed equally to this work.
[*]**Correspondence to:** Yinghui Song and Chuang Peng, Department of Hepatobiliary Surgery, Hunan Provincial People's Hospital/The First Affiliated Hospital of Hunan Normal University, Changsha, Hunan 410004, China. Tel: +86-731-83929520, Fax: +86-731-83929520, E-mail: sissysyh@163.com (YS) or pengchuangcn@163.com (CP)

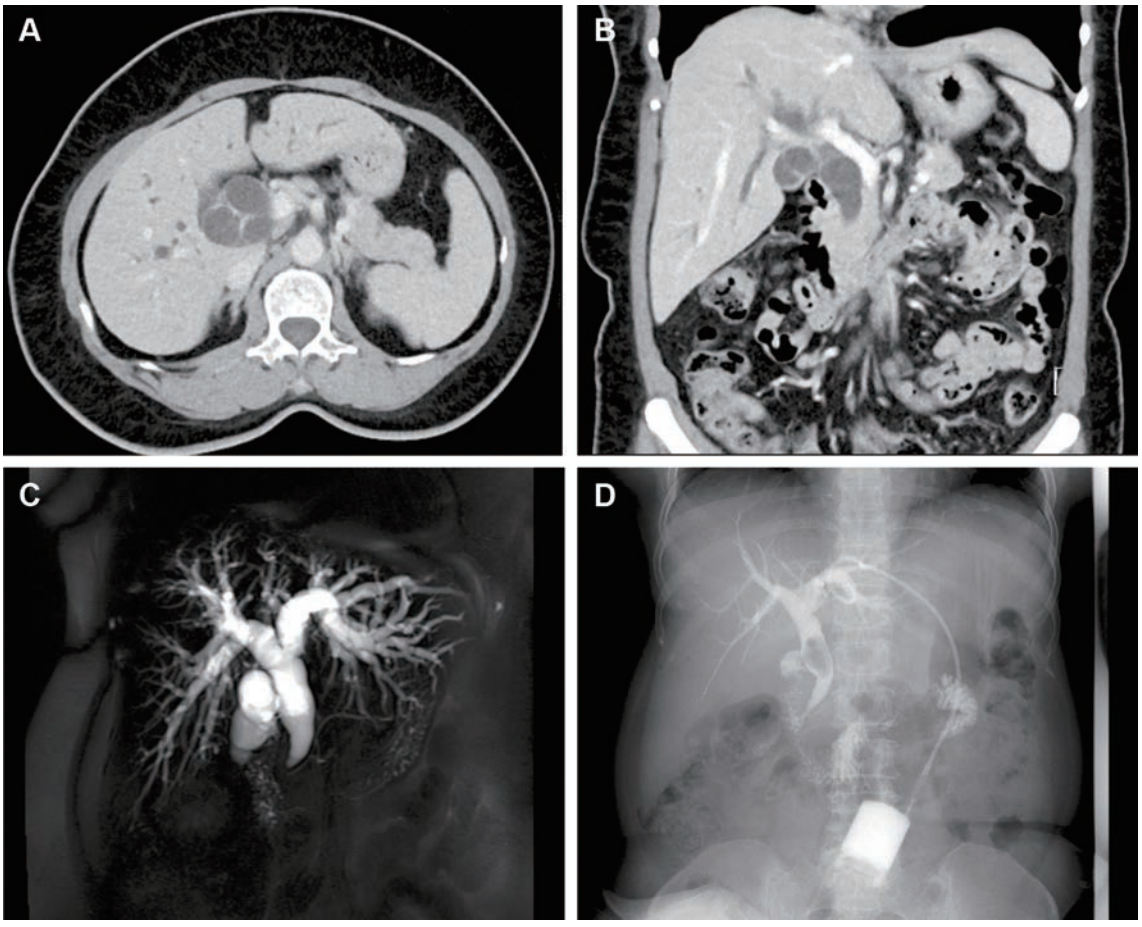

Fig. 1. Preoperative imaging revealed biliary obstruction. (A, B) Abdominal plain computed tomography scan plus enhanced (C) Magnetic resonance choliangiopancreatography and (D) PTCD angiography showed both intrahepatic bile duct dilation and common hepatic duct dilation, as well as a space-occupying lesion at the confluence of the cystic duct.

zen section was assessed intraoperatively, and the results were reported as: "*Consider gallbladder cyst adenoma*". We then performed an open cholecystectomy, biliary exploration, and bile duct repair with shaping and T-tube drainage.

During the operation, a 4.0×2.0 cm mass of the cystic duct protruded into the bile duct lumen (Fig. 2A–D). The mass grew on the wall of the cystic duct with a pedicle, and a few stones were seen in the gallbladder.

The predischarge inspection showed that the total bilirubin was 25.8 μmol/L and the direct bilirubin was 18 μmol/L. Computed tomography of the abdomen showed that the dilatation of the bile ducts, inside and outside the liver, was significantly less than before. Postoperative pathology showed that there was a multicystic mass in the cystic duct, 4×2×2 cm in size, multicystic at the cut surface, and containing light-yellow, clear liquid in the cyst. The pathological diagnosis was mucinous cystic tumor with mild atypical hyperplasia with chronic cholecystitis immunohistochemistry of cytokeratin 7 (+), cytokeratin 19 (epithelial +), estrogen receptor (+), progesterone receptor (+), P53 (−), and Ki67 (scattered +) (Fig. 3).

Informed consent

Prior written informed consent was provided from the patient and this study was approved by the Ethics Review Board of Hunan Provincial People's Hospital/The First Affiliated Hospital of Hunan Normal University.

Discussion

Mucinous cystic neoplasms (MCNs) were first reported in pancreatic tissue and, subsequently, there has been much research devoted to investigating pancreatic MCNs. However, there are still many controversies about pancreatic MCN disease and even less is known about gallbladder MCN. According to the authors' search of the PubMed database, the earliest case of gallbladder MCN was reported by Bishop in *The Lancet* in 1901,[1] and there have been 16 literature reports on gallbladder MCN (Table 1).

Similar to pancreatic MCN, gallbladder MCN can manifest unilocular or multilocular cystic changes, containing septa. In the World Health Organization Classification of Digestive System Tumors (2010 Edition), biliary MCN is listed separately, as a special tumor of the gallbladder, and is classified into "*mucocystic tumors with low-grade or medium-grade epithelium according to the status of intraepithelial neoplasia. Internal neoplasia (8470/0) (8470/2)", "Invasive mucocystic carcinoma (8470/3)*". The existing literature data divides MCN into at least two types.[1] One is non-invasive and has ovarian-like stroma under the epithelium, which is characterized by a high cell density. It appears as a dense

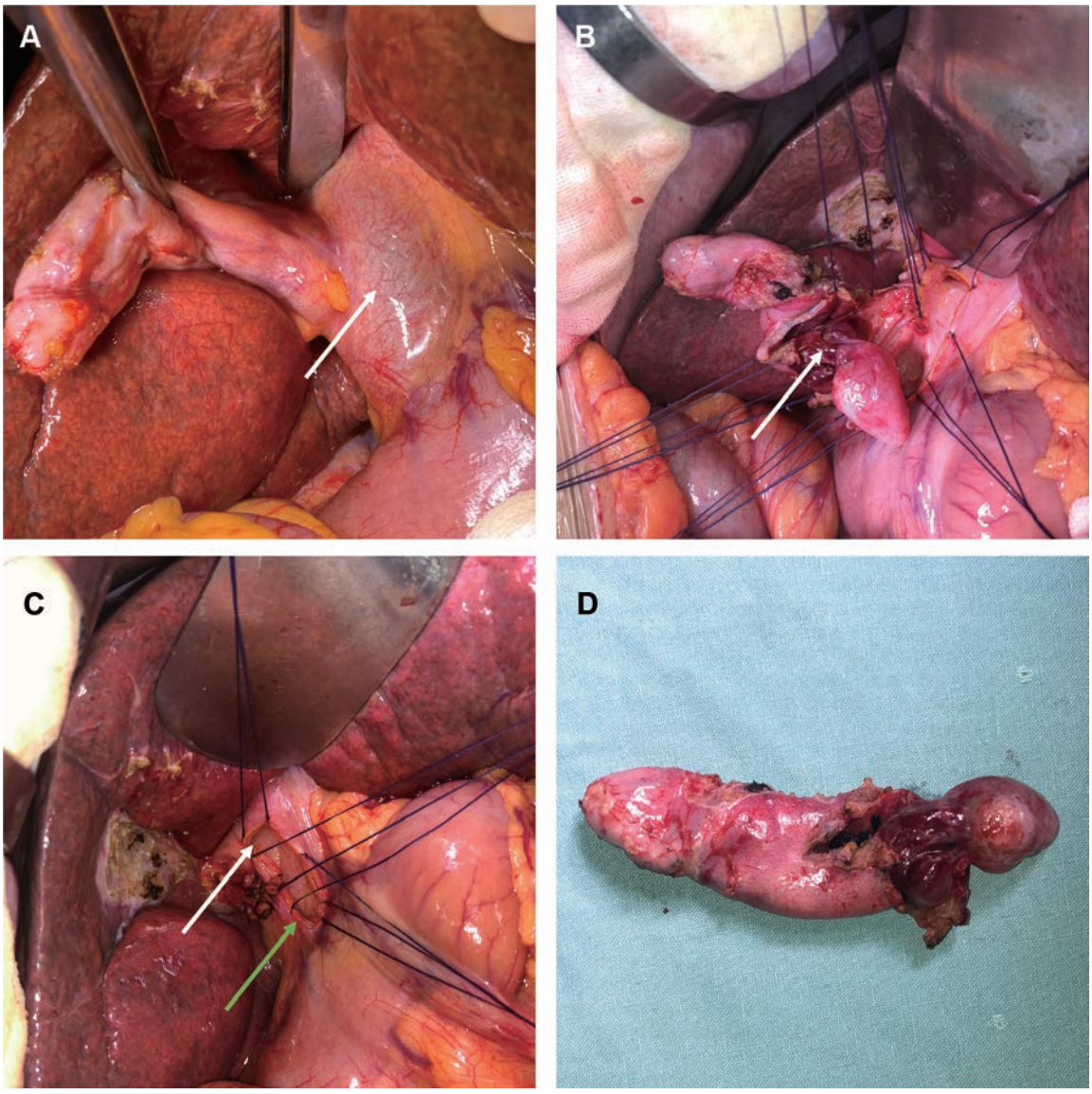

Fig. 2. A cystic duct-origin mass was found during the operation to block the common bile duct. (A) 4.0×2.0 cm mass of the cystic duct was seen protruding into the bile duct cavity (white arrow). (B) The mass was found on the wall of the cystic duct (white arrow). (C) The upper common hepatic duct (white arrow) and the lower common bile duct (green arrow) did not show stenosis nor any masses. (D) A cystic duct-origin mass was observed.

collection of spindle-shaped cells lacking cytoplasm and is immune to estrogen and progesterone receptors. This subtype affects middle-aged women. The other type is more aggressive, has no ovarian-like stroma, and affects men between 75 and 88 years-old. There are others who classify MCN using three subtypes, based on epithelial atypia and infiltration; the subtypes are mucinous cyst-adenoma, non-invasive mucinous cystadenocarcinoma, and invasive mucinous cystadenocarcinoma.[2]

Both gallbladder MCN and pancreatic MCN are common in women. The difference is that pancreatic MCN often occurs in the body and tail of the pancreas, which do not often cause obstructive jaundice.[3] In the case of gallbladder MCN, as the tumor increases, some patients will show painful or painless jaundice.[4] The overall prognosis of the disease is good, but there is a certain malignant potential. According to a Japanese study encompassing 156 cases of pancreatic MCN resection, the 10-year survival rate after resection was 95% for adenoma and 63% for cancer, among which microinvasive carcinoma also reached more than 90%.[4] Another study showed that the 5-year survival rate of untreated pancreatic MCN with invasive carcinoma was about 30% and the prognosis was poor.[5] Such statistics are still lacking for gallbladder MCN. In pancreatic MCN, the maximum tumor diameter is an independent risk factor affecting malignant transformation, and the level of carbohydrate antigen 19–9 has greater diagnostic significance for male patients.[3] In gallbladder MCN, as the tumor size increases, the likelihood of jaundice and malignancy increases together. In our case, the cystic duct tumor was large and it compressed the common bile duct, which then caused obstructive jaundice and intrahepatic bile duct dilation. Additionally, since gallstones were present, the case could have been misdiagnosed as common bile duct stones or Mirizzi syndrome.

Therefore, preoperative examination is particularly important. For this disease, ultrasound is more sensitive to the in-

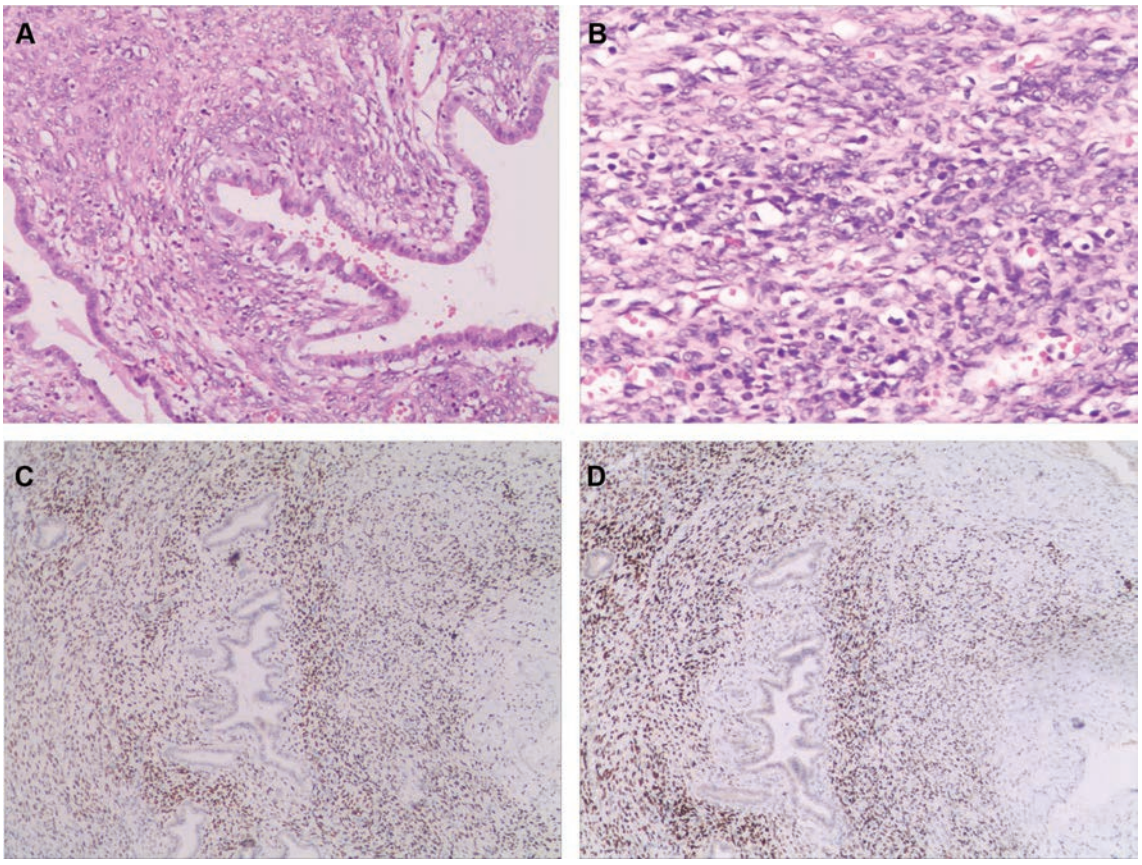

Fig. 3. Postoperative pathology showed that there was a multicystic mass in the cystic duct. (A) A 100× cyst, lined with a single layer of mucin-producing epithelial cells and showing low-grade dysplasia was observed. (B) Most segments of the 400× cyst wall contained ovarian-like stroma. (C, D) 40× ovarian-like stroma immunohistochemical analysis showed positivity for estrogen receptor (ER) and progesterone receptor (PR).

ternal features of the tumor (i.e. separation and fragments) and should be the first choice. Computed tomography can determine the location of the tumor and whether there is infiltration of surrounding tissues, which can help guide the scope of surgical resection. Magnetic resonance cholangiopancreatography can help determine the bile duct compression and involvement, determine the cause of jaundice in patients, and determine whether biliary reconstruction surgery is appropriate.[6] Assessment of a quick-frozen section during the operation will help guard against the possibility of malignancy.

For asymptomatic patients, such as those who have tumors found on physical examination or imaging, one might use the pancreatic MCN endoscopic ultrasound-fine needle aspiration data on fluid collection to evaluate glucose (sensitivity of 92%, specificity of 87%, accuracy of 90%) and carcinoembryonic antigen (sensitivity of 58%, specificity of 96%, accuracy of 69%), for evaluation before an invasive operation, since there is always risk of tumor dissemination and surgical complications.[3] It is important to comprehensively consider the patient's sex, age, family history, and surgical conditions. Interestingly, almost all gallbladder MCN patients are female[1,7–21] (Table 1). In treatment, surgical resection is recommended for patients with clinical symptoms, such as abdominal pain, bloating, jaundice, or asymptomatic patients with gallbladder stones.[22] It is important to send fast frozen sections during the operation to guide the operation method. After the surgical resection, it is recommended to check the confluence of the cystic duct, the wall of the gallbladder, and the common bile duct for other malignant tumors.

In summary, there is currently a lack of consistent evidence for the malignant potential of gallbladder MCN, and there is also a lack of guidelines or consensus in diagnosis and treatment. However, the consensus reached after we compiled the literature is that due to the potential malignancy of gallbladder MCN, early diagnosis of such diseases should be paid attention to in clinical work, surgical treatment should be actively performed, and changes should be made according to the rapid intraoperative pathological examination results. Operating or expanding the scope of surgery will likely improve the prognosis and reduce recurrence and malignant transformation.

Funding

This work was financially supported by following funds: Huxiang Youth Talent Support Program (Grant No. 2020RC3066); Postdoctoral Innovation Talents Project (Grant No. 2020RC2064); Hunan Provincial Natural Science Foundation of China (Grant No. 2019JJ50320/2020JJ5610); The Project of Improving the Diagnosis and Treatment Capacity of Hepatobiliary, Pancreas and Intestine Disease in Hunan Province (Xiangwei [2019] Grant No. 118).

Conflict of interest

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

Table 1. Gallbladder MCN reported cases

Case	Year	Age	Sex	Jaundice	Abdominal pain	Tumor size in cm	Carbohydrate antigen 19-9	Reference
1	1901	42	Female	Y	Null	Size of a child's head	Null	7
2	1930	Null	Null	Null	Null	Null	Null	8
3	1933	24	Female	Y	Null	15	Null	9
4	1977	52	Female	N	Null	Null	Null	10
5	1989	65	Female	Y	Y	14	Null	11
6	1994	Null	Null	Null	Null	Null	Null	12
7	2003	47	Female	Y	Y	4.6×4.2×4.4	Null	13
8	2003	88	Male	Y	Y	3.5×3×3	Normal	14
9	2005	38	Female	N	Y	1.2×0.8×0.8	Null	15
10	2006	75	Female	N	Y	17	High	16
11	2008	32	Female	N	Y	12	Null	17
12	2009	50	Female	N	Y	11×7.5×11.2	Null	18
13	2010	33	Female	N	Y	0.67 × 0.28	Null	19
14	2014	75	Female	Y	Y	Null	Null	1
15	2017	29	Female	N	Y	3	Null	20
16	2018	70	Female	N	N	6.7×6.8 ×7.2	High	2
17	2019	70	Female	N	Y	3×2×1	Null	21
18	2020	57	Female	Y	N	4.0 x 2.0	High	Current study

N, no; Null, not mentioned; Y, yes.

Author contributions

Patient management (CG), drafting of the manuscript (SL, ZZ, JK), statistical analysis (YS, SH), data collection (SL, ZZ, YS, ZY, CP, BJ), and revision of the manuscript for important intellectual content (YS,CP)

Data sharing statement

All data are available upon request.

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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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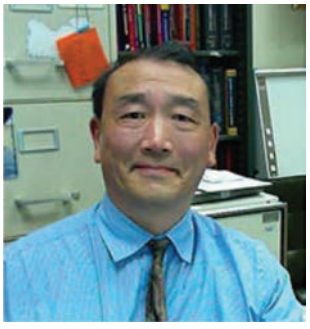

Prof. Wu obtained his MD and PhD degree at Albert Einstein College of Medicine in 1976. He was a resident at Harlem Hospital Center from 1976 to 1979. He worked as a postdoctoral fellow at Albert Einstein College of Medicine from 1979 to 1982. From 1983, he worked as Assistant Professor and then Professor at University of Connecticut School of Medicine. He is now the Director of Hepatology Section, Division of Gastroenterology-Hepatology. Dr. Wu's awards include the following: Research Prize awarded by the American Liver Foundation in 1982; Industry Research Scholar Award from the American Gastroenterological Association for 1985 to 1988; Gastroenterology Research Group Young Scientist Award from the American Gastroenterological Association in 1990; Herman Lopata Chair in Hepatitis Research from 1992 to date; Scientific Award from the Chinese American Medical Society in 1992; He was elected to membership in exclusive societies: American Society for Clinical Investigation in 1989; Association of American Physicians in 1995; and Top Doctor in the U.S. awarded by U.S. News and World Report in 2011. He has published about 180 peer-reviewed academic articles, 11 books, and is series editor for Clinical Gastroenterology book series by Springer-Nature, and is Senior Associate editor of J. Digestive Diseases.

Prof. Harry Hua-Xiang Xia (Editor-in-Chief)

Prof. Xia obtained his PhD in 1994 and worked as a postdoctoral fellow at Trinity College, Dublin University, Ireland. He spent 5 years as a senior Research Officer at Nepean Hospital, University of Sydney, Australia, and 6 years as an Assistant Professor at Queen Mary Hospital, University of Hong Kong to continue his research on Helicobacter pylori and associated diseases. He has achieved an academic reputation worldwide in the field. He was elected as a fellow of the American College of Gastroenterology in 2008. He joined Novartis Pharmaceuticals Corporation, USA, in 2006 for clinical development of new investigational drugs in different therapeutic areas. He is currently an Adjunct Professor of Beijing Friendship Hospital, Capital Medical University, Beijing; Municipal Hospital, Qingdao University, Qingdao; and First Affiliated Hospital, Guangdong Pharmaceutical University, Guangdong, China. He has published about 180 peer-reviewed academic articles. He has published two books, namely, "Helicobacter pylori infection: Basic Principles and Clinical Practice" (1997), and A Comprehensive Guide to English Medical Manuscript Writing and Publication (2017).

Updated on August, 2021

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