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Unsolved challenges in hepatitis B and hepatitis C: From prevention to treatment

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Published in
Frontiers in Cellular and Infection Microbiology
Frontiers in Medicine
Frontiers in Public Health

ISSN 1664-8714
ISBN 978-2-8325-3634-6
DOI 10.3389/978-2-8325-3634-6

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Unsolved challenges in hepatitis B and hepatitis C: From prevention to treatment

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Citation

Yue, M., Lin, W., Guo, S., Dong, C., eds. (2023). *Unsolved challenges in hepatitis B and hepatitis C: From prevention to treatment*. Lausanne: Frontiers Media SA. doi: 10.3389/978-2-8325-3634-6

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frontiers | Frontiers in Cellular and Infection Microbiology

TYPE Editorial
PUBLISHED 25 October 2023
DOI 10.3389/fcimb.2023.1321432

OPEN ACCESS

EDITED AND REVIEWED BY
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RECEIVED 14 October 2023
ACCEPTED 17 October 2023
PUBLISHED 25 October 2023

CITATION
Yue M (2023) Editorial: Unsolved challenges in hepatitis B and hepatitis C: from prevention to treatment.
Front. Cell. Infect. Microbiol. 13:1321432.
doi: 10.3389/fcimb.2023.1321432

Editorial: Unsolved challenges in hepatitis B and hepatitis C: from prevention to treatment

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KEYWORDS

hepatitis B virus, hepatitis C virus, host, prevention, treatment

Editorial on the Research Topic
Unsolved challenges in hepatitis B and hepatitis C: from prevention to treatment

Currently, hundreds of millions of people worldwide are infected with hepatitis B virus (HBV) or hepatitis C virus (HCV). End-stage liver diseases caused by these viruses claim countless lives every day. Existing hepatitis B vaccine have effectively controlled the transmission of hepatitis B. However, there is currently no vaccine available for HCV. Over the past 10 years, in terms of hepatitis B treatment, the combination of nucleoside analogs (NAs) with pegylated interferon α-2b has significantly improved the clinical efficacy in the treatment of hepatitis B, and has even achieved clinical cure in some patients; as for hepatitis C, the approval of oral interferon-free direct-acting antivirals (DAAs) revolutionized the therapy of chronic hepatitis C infection and achieved the cure of hepatitis C in many patients. However, the rates of awareness, diagnosis and treatment for these two viral hepatitis are still suboptimal. In addition, what cannot be ignored is, the different genetic backgrounds of the host and the genetic diversity of viruses driven by genomic mutations may also lead to the clinical uncertainties in some patients. Therefore, there are still many unsolved challenges to achieve the WHO "eliminate hepatitis" goal, and we should not give up our attention and exploration.

The Research Topic: Unsolved challenges in hepatitis B and hepatitis C: From Prevention to Treatment of Journal: Frontiers in Cellular and Infection Microbiology focus on the new research findings made in these fields in recent years. This editorial will summarize and discuss these developments.

In the context of hepatitis B and C prevention, Meyer et al. conducted an assessment of prescription numbers for DAA medications within Germany during the COVID-19 pandemic. Their findings revealed a significant overall decrease in prescriptions, indicating a temporary gap in hepatitis C treatment. This highlights the need for hospitals and private clinics to adapt more quickly in future pandemics to ensure continued access to care. Furthermore, political strategies should place greater emphasis on maintaining essential healthcare services during periods of restricted access due to infectious disease outbreaks. Ndunguru et al. found that conducting promotional activities and mobilizing resources at primary healthcare facilities in Tanzania is crucial to promote hepatitis B vaccination.

In the field of epidemiological research, Qian et al. quantitatively analyzed the long-term spatiotemporal heterogeneity of hepatitis B and C incidence in China from 2010 to 2018, as well as the impact of socio-economic factors on their risk using Bayesian spatiotemporal hierarchical models. The results indicate that there is significant spatial and temporal heterogeneity in the risk of hepatitis B and C. These findings contribute to the effective allocation of resources and the design of intervention measures to combat viral hepatitis. Another research conducted by the Jia et al., utilizing Bayesian analysis, SNP genotyping, and sequencing methods, revealed that HCV 3b subtype is the predominant circulating strain in Yunnan Province, China and the prevalence of HCV 3a and 3b subtypes is rapidly increasing. He et al. found that the IFNL4 gene, along with the MxA and MxB genes, is associated with HCV infection in the Yunnan population or the liver function of HCV patients. These findings underscore the need for the implementation of more stringent public health measures for prevention and treatment to curb the spread of the virus.

In the context of auxiliary diagnosis and model prediction of hepatitis B and hepatitis C, Li S. et al. developed and validated a non-invasive model that includes parameters such as aspartate aminotransferase (AST), hepatitis B e-antigen (HBeAg), and platelet. This model aimed to identify pseudo-immune tolerance in chronic hepatitis B (CHB) patients by predicting significant liver fibrosis, thereby assisting in the formulation of more appropriate treatment strategies. Zhao et al. reported a correlation between serum hepatitis B surface antigen (HBsAg) and hepatitis B core-related antigen (HBcrAg) levels and inflammation grading in HBeAg-positive CHB patients before nucleos(t)ide analogues (NAs) therapy. Furthermore, the combination of HBsAg and AST showed excellent diagnostic capability for significant inflammation. These non-invasive biomarkers contribute to the diagnosis and grading of liver necroinflammation. Zhang Q. et al. established an effective individualized nomogram, including clinical and endoscopic features (such as the size of varices, red wale marks, ascites, spleen thickness, γ- glutamyltransferase, and hematocrit), to predict the risk of first variceal hemorrhage in HBV-related gastroesophageal varices patients. This can assist clinicians in formulating more appropriate prevention strategies. Wang et al. assessed the performance of pregenomic RNA (pgRNA) and HBcrAg kinetic in predicting HBeAg seroconversion and HBsAg reduction postpartum in HBeAg-positive pregnant women. They found that in chronic HBV carriers who were HBeAg-positive pregnant women receiving antiviral prophylaxis, a postpartum decrease in pgRNA and peak ALT levels helped identify patients with HBsAg reduction after treatment cessation.

In terms of hepatitis B treatment, Li J. et al. in a prospective multicenter study, found that factors associated with persistent HBV DNA positivity in patients with chronic hepatitis B treated with entecavir included high HBV DNA levels, low anti-HBc levels, and HBeAg serum positivity. However, persistent viremia patients had a lower rate of fibrosis progression and risk of developing HCC. Guo et al. found that in patients who achieved pegylated interferon-induced HBsAg loss who achieved functional cure with HBsAg loss, those who had both HBeAg negativity and higher anti-HBs levels at the end of PEG-IFN treatment had the low risk of HBsAg reversion after PEG-IFN discontinuation. Li M. et al. found that antiviral therapy could reverse decompensation of ascites in HBV-related first decompensated cirrhosis, and ALT and HBV DNA levels were associated with ascites recompensation. Cai et al. established a convenient *in vitro* cell model for screening compounds that target elongation factor Tu GTP-binding domain containing 2 (EFTUD2) as antiviral agents against hepatitis B. In this model, it was confirmed that small-molecule compounds, plerixafor and resatorvid, can inhibit HBV by upregulating the EFTUD2. This provides a new avenue for the development of novel anti-hepatitis B drugs that target host factors rather than viral enzymes.

HBV and HCV infections may also have some extrahepatic effects and can potentially co-infect with the human immunodeficiency virus (HIV). Tao et al. collected hepatitis test results and bone mineral density (BMD) from respondents in the NHANES database and compared BMD between respondents who were positive and negative for respondents related to hepatitis B and C. The results of multiple regression analysis revealed that positive tests for HBsAg and hepatitis C RNA were associated with a reduction in BMD. Positive serology for these hepatitis indicators may increase the risk of reduced BMD. Zaltron et al. reported a case of occult HBV infection (OBI) reactivation in a HIV/HCV co-infected patient who was lost to follow-up after DAAs treatment. Upon re-encounter with the patient, the individual exhibited high levels of plasma HIV-RNA, severe immunosuppression. After reintroducing antiretroviral treatment, an immune reconstitution inflammatory syndrome (IRIS) was diagnosed, along with high level of HBV-DNA load and transaminase. This case report highlights the dynamic balance between the virus and the host immune system, emphasizing the importance of strict monitoring of HBV serological and virological markers for patients with compromised immune systems receiving tenofovir or lamivudine-sparing regimens, even in the absence of a hepatitis flare. Zhang Q. et al. conducted a retrospective cohort study to investigate the clearance rate of HBsAg in Chinese HIV/HBV co-infected patients on long-term tenofovir disoproxil fumarate-containing antiretroviral therapy and found that advanced age, high CD4 cell count, and positive HBeAg at baseline could be regarded as potential predictors and biological markers for HBsAg clearance in patients with HIV/HBV coinfection.

This Research Topic also includes valuable reviews. CD73 is fundamentally an enzyme and a crucial component of the adenosine signaling pathway. It mediates the conversion of inflammatory ATP into the immunosuppressant adenosine, dynamically regulating processes such as hepatic steatosis, inflammation, and fibrosis in the liver. Shi et al. provided a comprehensive review of the close connection between CD73-mediated adenosine metabolism and the liver, as well as its role in the pathogenesis of various liver diseases. Functional cure for hepatitis B, characterized by the absence of detectable HBsAg in the patient's serum and HBV DNA, remains a rarity, achievable only in a fortunate few. The difficulty in achieving a cure for hepatitis B primarily stems from covalently closed circular DNA (cccDNA) of HBV, integrated HBV DNA, high viral loads, and compromised host immune responses. In the pursuit of developing more effective

treatments and achieving higher rates of functional cure, the industry continues relentless efforts in the development of innovative drugs. Pan et al. have summarized the functions and mechanisms of various synthetic molecules, natural products, and traditional herbal remedies and discussed therapeutic strategies for modulating the host immune response. DExD/H-box helicases play crucial roles in various biological processes, including hematopoiesis, cell proliferation, metabolism, signal transduction, immune responses, and inflammation. They can sense non-self viral nucleic acids and participate in regulating multiple antiviral immune signaling pathways, including Toll-like receptor and retinoic acid-inducible gene I-like receptor pathways. You et al. reviewed the current understanding of the effects of different DExD/H-box helicases on HBV replication regulation, the role of HBV in altering DExD/H-box helicases, and the potential of targeting DEAD/H-box helicase to eliminate HBV infection. Further understanding of the effects of DExD/H-box helicase on HBV infection may aid in the eliminate hepatitis B. 308 words.

In summary, this Research Topic encompasses the latest advancements in the prevention, diagnosis, and treatment of hepatitis B and hepatitis C. These developments provide a theoretical foundation and scientific basis for efforts aimed at preventing and ultimately eradicating these two viral liver diseases in the future.

Author contributions

MY: Funding acquisition, Writing – original draft, Writing – review & editing.

Funding

The author(s) declare financial support was received for the research, authorship, and/or publication of this article. The work is supported by the National Science Foundation (No. 82273691), Science Foundation for Distinguished Young Scholars of Jiangsu Province (No. BK20190106), and Open Research Fund Program of the State Key Laboratory of State Key Laboratory of Pathogen and Biosecurity (No. SKLPBS2137) to MY.

Conflict of interest

The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The author(s) declared that they were an editorial board member of Frontiers, at the time of submission. This had no impact on the peer review process and the final decision.

Publisher's note

frontiers | Frontiers in Cellular and Infection Microbiology

TYPE Review
PUBLISHED 25 November 2022
DOI 10.3389/fcimb.2022.1062553

OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to Virus and Host, a section of the journal Frontiers in Cellular and Infection Microbiology

RECEIVED 06 October 2022
ACCEPTED 11 November 2022
PUBLISHED 25 November 2022

CITATION
You H, Ma L, Wang X, Zhang F, Han Y, Yao J, Pan X, Zheng K, Kong F and Tang R (2022) The emerging role of DEAD/H-box helicases in hepatitis B virus infection.
Front. Cell. Infect. Microbiol. 12:1062553.
doi: 10.3389/fcimb.2022.1062553

The emerging role of DEAD/H-box helicases in hepatitis B virus infection

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DEAD/H-box helicases are an essential protein family with a conserved motif containing unique amino acid sequences (Asp-Glu-Ala-Asp/His). Current evidence indicates that DEAD/H-box helicases regulate RNA metabolism and innate immune responses. In recent years, DEAD/H-box helicases have been reported to participate in the development of a variety of diseases, including hepatitis B virus (HBV) infection, which is a significant risk factor for hepatic fibrosis, cirrhosis, and liver cancer. Furthermore, emerging evidence suggests that different DEAD/H-box helicases play vital roles in the regulation of viral replication, based on the interaction of DEAD/H-box helicases with HBV and the modulation of innate signaling pathways mediated by DEAD/H-box helicases. Besides these, HBV can alter the expression and activity of DEAD/H-box helicases to facilitate its biosynthesis. More importantly, current investigation suggests that targeting DEAD/H-box helicases with appropriate compounds is an attractive treatment strategy for the virus infection. In this review, we delineate recent advances in molecular mechanisms relevant to the interplay of DEAD/H-box helicase and HBV and the potential of targeting DEAD/H-box helicase to eliminate HBV infection.

KEYWORDS

DEAD/H-box helicases, hepatitis B virus, virus replication, molecular mechanisms, therapy

Introduction

To date, the prevalence of hepatitis B virus (HBV) infection remains high in the Western Pacific region and Africa. HBV is a well-known hepatotropic DNA virus capable of causing persistent infection, which further progresses to hepatic fibrosis, cirrhosis, and liver cancer. To date, the standard therapy for HBV infection has been limited to

interferon (IFN), an immunomodulatory agent, and nucleotide analogs, including tenofovir, entecavir, and tenofovir alafenamide, which function as HBV polymerase (HBp) inhibitors. However, these approaches rarely achieve complete viral clearance, and many undesirable adverse effects, including fatigue, headache, and dizziness, often limit the efficacy of the existing antiviral therapies (Tang et al., 2018; Yuen et al., 2018). The HBV genome contains the S, C, X, and P open reading frames (ORFs). S ORF codes HBsAg, preS1-Ag, and preS2-Ag. C ORF contributes to the expression of the structural protein HBcAg and secretory protein HBeAg. X and P ORFs facilitate the production of two regulatory proteins, HBX and HBp. After the virus binds to its receptor sodium taurocholate cotransporting polypeptide (NTCP) and enters liver cells, viral DNA can be transported to the host cell nucleus and converted into closed covalent circular DNA (cccDNA). Depending on a variety of host and viral factors, HBV cccDNA transforms into a minichromosome that acts as a template for viral transcription. Different viral RNAs are then translated into HBV proteins. Sequentially, viral pre-genomic RNA (pgRNA) is encapsulated and reverse-transcribed into HBV DNA. Subsequently, viral DNA-containing particles are enveloped and eventually secreted from the host cells (Fanning et al., 2019; Iannacone and Guidotti, 2022) (Figure 1). The interplay between HBV and cellular factors is important for viral replication (Ligat et al., 2021). Hence, targeting host factors that benefit viral infection is a promising strategy for eradicating the virus in HBV-infected individuals.

DExD/H-box helicase is an important RNA-binding protein superfamily of the large super family-2 (SF2) RNA helicases that contributes to the recognition and unwinding of RNA duplexes by specific amino acid motifs in an ATP-dependent manner. Based on the homology of their nucleotide sequences, DExD/H-box helicases are subdivided into DExD-box helicases (DDX) and DExH-box helicases (DHX). A conserved motif with a unique amino acid sequence, D-E-A-D (Asp-Glu-Ala-Asp), exists in DDX and D-E-A-H (Asp-Glu-Ala-His) in DHX (Ullah et al., 2022). Although DHX shares many sequences and structural similarities with DDX proteins, the molecular mechanisms related to RNA regulation, including RNA duplex unwinding, mediated by these two types of DExD/H-box helicases, are different (Gilman et al., 2017). To date, 37 DDX and 16 DHX have been discovered in humans (Andrisani et al., 2022), and accumulating data indicate that they are essential for cellular RNA metabolism, including RNA transcription, RNA splicing, RNA export, microRNA biogenesis, RNA translation,

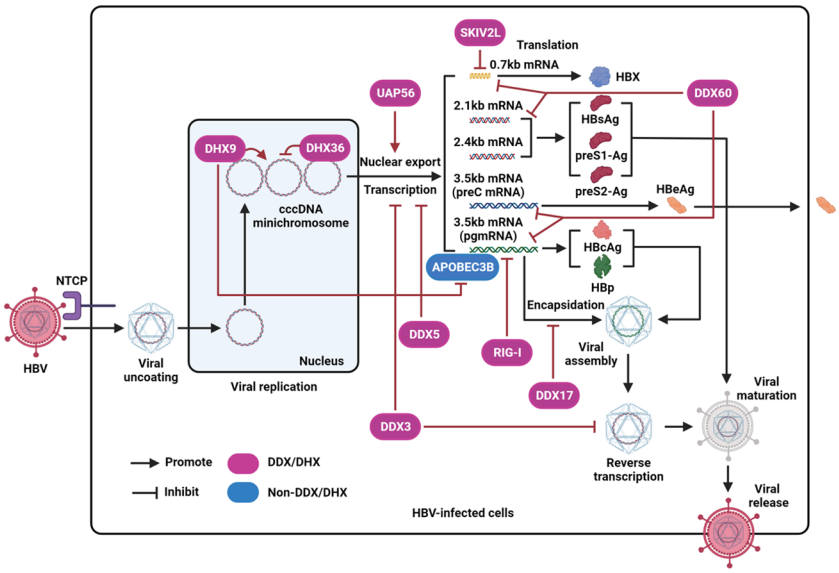

FIGURE 1
The effect of DEAD/H-box helicases on the regulation of HBV by disrupting different steps of the viral life cycle. After interacting with NTCP and entering host cells, the virus is uncoated and then transferred into the nucleus. Next, the HBV genome is converted into cccDNA, forms a mini-chromosome, and is further transcribed into various viral mRNA, including pregenomic RNA (pgRNA) (3.5kb), preC mRNA (3.5kb), two envelope mRNAs (2.1kb and 2.4kb), and X mRNA (0.7kb). The pgRNA is a translation template for viral polymerase proteins (HBp) and HBcAg. The preC mRNA encodes HBeAg antigen. The two envelope mRNAs encode HBsAg, preS1-Ag, and preS2-Ag. X mRNA codes HBX protein. Then, pgRNA is encapsulated into viral particles and further reverse-transcribed into DNA. Finally, intact viral particles are secreted from liver cells. RIG-I represses HBV pgRNA. DDX60 facilitates the degradation of HBV RNA. DDX3 inhibits the transcription of viral cccDNA and suppresses viral reverse transcription. SKIV2L is capable of degrading HBX mRNA. DDX5 inhibits HBV transcription. DHX9 promotes viral DNA replication, interacts with and inhibits the binding of APOBEC3B to viral pgRNA. DHX36 interacts with the G-quadruplex structure of HBV cccDNA. DDX17 interacts with HBV pgRNA to restrain its encapsidation. UAP56 could facilitate the nuclear export of HBV RNA.

and RNA decay (Ullah et al., 2022). In addition, some DExD/H-box helicases function as DNA sensors and participate in DNA regulation (Hu et al., 2020b; Antcliff et al., 2021). Especially, it has been demonstrated that DExD/H-box helicases play vital roles in multiple biological processes consisting of hematopoiesis, cell proliferation, metabolism, signal transduction, immune response, and inflammation, and are relevant to the development of several diseases, including autoimmune diseases and cancer (Cai et al., 2017; Andrisani et al., 2022; Samir and Kanneganti, 2022). In addition, a variety of DExD/H-box helicases can sense non-self-viral nucleic acids (Fullam and Schroder, 2013; Ullah et al., 2022) and participate in modulating diverse antiviral immune signaling pathways, including Toll-like receptor (TLR) and retinoic acid-inducible gene I (RIG-I)-like receptor (RLR) pathways (Su et al., 2021). Therefore, further advances in understanding the effect of DExD/H-box helicases on viral infection may contribute to the treatment of infectious diseases caused by these viruses.

Recently, many DExD/H-box helicases, including DDX3 (Ko et al., 2014), DDX5 (Sun et al., 2022), DHX9 (Chen et al., 2020), RIG-I (Sato et al., 2015), MDA5 (Lu and Liao, 2013), SKIV2L (DDX13) (Shiromoto et al., 2018), DDX17 (Mao et al., 2021), DHX36 (Meier-Stephenson et al., 2021), and UAP56 (DDX39B), have been identified to play vital roles in the development of chronic HBV infection (Hu et al., 2020a). Multiple molecular mechanisms, including the regulation of HBV replication cycle and the sensitization of the innate immune responses (Table 1), are identified to participate in the control of HBV replication and associated liver diseases mediated by these identified DEAD/H-box helicases. Here, we outline the current view of the effect of different DExD/H-box helicases on the modulation of HBV replication, the role of HBV in the alteration of DExD/H-box helicases, and the potential of DExD/H-box helicase-targeting strategies to eliminate HBV infection.

DDX3

DDX3 is a prominent member of the DEAD/H-box helicases involved in the regulation of RNA metabolism and has a pivotal role in antiviral innate immunity (Wang and Ryu, 2010; Ko et al., 2014). Current evidence suggests that DDX3 restricts HBV replication by targeting viral transcription and reverse transcription. For example, by relying on tetracycline-inducible HBV-producing cells, Ko et al. demonstrated that DDX3 inhibited the transcription of viral cccDNA. Although DDX3 interacts with viral transcriptase HBp, DDX3-mediated HBV transcription inhibition is independent of the interplay between HBp and DDX3 (Ko et al., 2014). A common transcription factor, which has not been well identified so far, may plausibly contribute to DDX3-mediated transcriptional suppression of the virus. Wang et al. showed that DDX3 does not affect pgRNA degradation. However, depending on the interaction of HBp

TABLE 1 The detailed information on the interaction between HBV and DEAD/H-box helicases.

Target molecules	The role of target molecules on HBV infection	The biological processes related to HBV mediated by target molecules	The role of HBV on target molecules	The viral protein related to target molecules	The small molecules against target molecules related to the repression of HBV	References
DDX3	Inhibition	HBV life cycle/ Innate immune response	Inhibition	HBp	5-HT; AS-19; Rg3	Wang et al., 2009; Yu et al., 2010; Choi et al., 2014; Ko et al., 2014; Kang et al., 2019
DDX5	Inhibition	HBV life cycle/ Innate immune response	Inhibition	HBX	Unknown	Zhang et al., 2016; Murphy et al., 2016; Sun et al., 2022
DHX9	Promotion	HBV life cycle	Promotion	HBX	Unknown	Murphy et al., 2016; Shen et al., 2020; Shen et al., 2020
RIG-I	Inhibition	HBV life cycle/ Innate immune response	Inhibition	HBX/HBp	poly-U/UC RNA, Poly(I:C)-HMW/ LyoVec, Inarigivir	Yu et al., 2010; Jiang and Tang, 2010; Sato et al., 2015Asadi-Asadabad et al., 2021; ; Lee et al., 2021; Fung et al., 2022
MDA5	Inhibition	Innate immune response	Inhibition	HBX	Poly(I:C)-HMW/LyoVec	Wang et al., 2010; Lu and Liao, 2013; Asadi-Asadabad et al., 2021
SKIV2L	Inhibition	HBV life cycle	Promotion	HBX	Unknown	Aly et al., 2016; Shiromoto et al., 2018
DDX17	Inhibition/ Promotion	HBV life cycle	Promotion	HBX	Unknown	Mao et al., 2021; Dong et al., 2022
DHX36	Inhibition	HBV life cycle	Unknown	Unknown	Unknown	Meier-Stephenson et al., 2021
DDX60	Inhibition	HBV life cycle	Unknown	Unknown	Unknown	Kouwaki et al., 2016
UAP56	Promotion	HBV life cycle	Unknown	HBX	Unknown	Hu et al., 2020a

with DDX3, DDX3 can be incorporated into nucleocapsids. Furthermore, encapsidated DDX3 had an inhibitory effect on viral reverse transcription. The suppression of HBV reverse transcription mediated by DDX3 may be associated with the disruption of the secondary structure of pgRNA, which is important for HBV biosynthesis in viral nucleocapsids (Wang et al., 2009). More importantly, the mutational analysis indicated that the ATPase activity of DDX3 is vital for the suppression of viral reverse transcription.

In addition, DDX3 can strengthen the activity of adapter molecules TANK-binding kinase 1 (TBK1) and IKKε, based on its interaction with IKKε or TBK1, which further phosphorylates IFN-regulatory factor (IRF) 3 to initiate IFN-β production (Schroder et al., 2008). However, a study by Wang et al. demonstrated that to facilitate HBV infection, HBp can restrain IFN-β production that is triggered by TLR3/TRIF and RIG-I/melanoma differentiation-associated gene 5 (MDA5)-associated RLR signaling pathways that are stimulated by Poly (I:C) in the medium. Poly(I:C) was administered by lipofectin transfection, or treated with Sendai virus, a stimulus of the RIG-I pathway (Wang and Ryu, 2010). Furthermore, pull-down coupled with mass spectrometry (MS) and associated functional experiments revealed that the suppression of TLR3 or RLR signaling pathways mediated by HBp depended on the interaction of HBp with DDX3 to suppress the activity of TBK1/IKKε by blocking the binding of DDX3 to IKKε to suppress the sensitization of IRF3 and restrain the expression of IFN-β (Figure 2) (Yu et al., 2010).

Furthermore, there is evidence that multiple compounds targeting DDX3 have satisfactory effects on the inhibition of HBV infection in cell models. For example, Kang et al. showed the interaction of the serotonin (5-HT) component with its receptor located in hepatocellular cells. This component can increase the DDX3 promoter activity to restrict HBV replication. As previously mentioned, DDX3 can inhibit HBV replication and sensitizes the innate immune response *via* TBK1/IKKε/IRF3-mediated IFN-β induction (Wang and Ryu, 2010). A wide variety of 5-HT receptors (from 5-HT1 to 5-HT7 receptors) have been identified, and the agonist of 5-HT7 receptor AS-19 [(2S)-(þ)-5-(1,3,5-trimethylpyrazol-4-yl)-2-(dimethylamino)tetralin] is also known to activate DDX3 and suppress HBV replication by increasing IFN-β expression (Kang et al., 2019). In addition to AS-19, Choi et al. found that ginsenoside Rg3, an active ingredient in ginseng, exerts anti-HBV activity by elevating DDX3 levels. Mechanistically, the upregulation of DDX3 expression mediated by Rg3 is related to the activation of the DDX3 promoter. Furthermore, p53 phosphorylation mediated by Rg3 contributes to the inhibition of Akt phosphorylation which facilitates DDX3 expression and further activates the TBK1/IKKε/IRF3 pathway to inhibit viral replication (Choi et al., 2014).

DDX5

DDX5 is one of the best-characterized DEAD-box helicases and participates in multiple RNA metabolic processes, including RNA transcription, translation, and decay (Xing et al., 2019). Current evidence indicates that it also plays a vital role in controlling viral replication (Cheng et al., 2018). Zhang et al. showed that DDX5 inhibits viral replication by suppressing the transcription of HBV cccDNA. Detailed investigations have indicated that although DDX5 is an RNA-binding protein, it does not bind HBV RNA. Nevertheless, inhibiting DDX5 can reduce polycomb repressive complex 2 (PRC2) occupancy along with decreased repressive H3K27me3 (Zhang et al., 2016), at the HBV minichromosome formed by viral cccDNA, may leading to increased transcription of HBV pgRNA. Especially, DDX5 could interact with chromatin regulating protein suppressor of zeste 12 (SUZ12), one core subunit of PRC2, and based on the helicase activity, DDX5 enhances the stabilization of SUZ12 by inhibiting ubiquitination-mediated degradation. A recent study demonstrated that SUZ12 has a significant antiviral effect against HBV infection (Wang et al., 2011). Therefore, SUZ12 is speculated to play a crucial role in the inhibition of viral transcription mediated by DDX5.

Additionally, DDX5 enhances the activation of interferon (IFN) signaling to suppress HBV replication. Activation of the JAK/STAT pathway plays a vital role in the antiviral response induced by IFN (Kong et al., 2021b; You et al., 2022). Sun et al. showed that DDX5 could bind to STAT1 mRNA and modulate its translation by resolving a secondary RNA structure, namely the G-quadruplex, which is in the STAT1 mRNA 5'-untranslated region (UTR) to accelerate its expression and activation to facilitate the sensitization of IFN-α signaling (Sun et al., 2022) (Figure 2). Conversely, the knockdown of DDX5 *via* small interfering RNA (siRNA) decreased IFN-α-stimulated anti-HBV effects by inhibiting the expression of STAT1.

It has been demonstrated that during HBV replication, DDX5 is downregulated, and reduced DDX5 in HBV-associated hepatocellular carcinoma (HCC) is related to poor prognosis (Mani et al., 2020). In particular, Mani et al. demonstrated that increased miR106b~25 and miR17~92 clusters, including miR-18a, miR-17, miR-19a, miR-20a, miR-19b1, and miR-106b, which bind to the 3'-UTR of DDX5, contribute to the repression of DDX5 induced by HBV (Mani et al., 2020) (Figure 2). Furthermore, the decrease in DDX5 induced by HBV activated the Wnt pathway, along with elevated mRNA expression of DVL1, SFRP4, FZD7, SFRP5, and MMP7. In addition, the interaction of DDX5 with lncRNA HOX transcript antisense RNA (HOTAIR) and SUZ12 contributes to the modulation of hepatocarcinogenesis (Zhang et al., 2016). In addition to HBV-associated HCC, the expression of DDX5

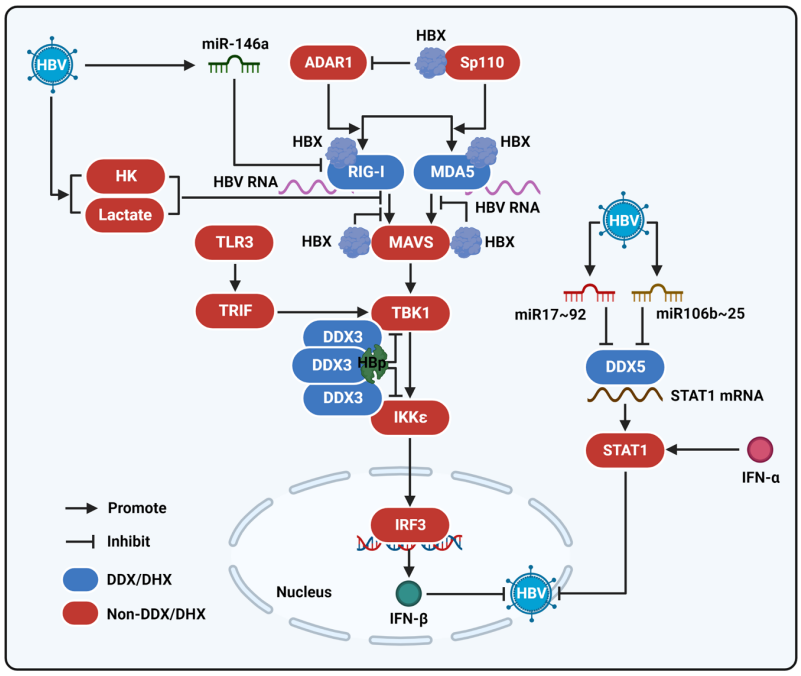

FIGURE 2
The interaction of HBV with DEAD/H-box helicases to regulate the innate immune response. MDA5 and RIG-I interact with HBV RNA and function as viral RNA sensors to sensitize the RLR signaling pathway. After being stimulated by HBV RNA, RIG-I and MDA5 activate MAVS and then sensitize TBK1. Next, TBK1 stimulates the activation of IKKε and IRF3 to produce IFN-β and then repress HBV infection. HBX can interact with MDA5, RIG-I, and MAVS to inhibit their interactions and thereby reduce the activation of the RLR signaling pathway. ADAR1 and Sp110 mediated by HBX also contribute to the modulation of the RIG-I/MDA5-mediated RLR signaling pathway. HBp could interact with DDX3 to suppress the binding of DDX3 to TBK1 or IKKε and inhibit the RIG-I/MDA5-mediated RLR signaling pathway, as well as the TLR3 signaling pathway. In addition, HBV can induce miR-164a expression to inhibit RIG-I-mediated innate immune response. HBV upregulates miR106b~25 and miR17~92 clusters to repress DDX5, which binds to STAT1 mRNA and regulates STAT1 translation when the cells are stimulated by IFN-α to suppress viral infection. HBV promotes the activity of hexokinase (HK) and the production of lactate to inhibit the interaction between RIG-I and MAVS.

declined in the liver tumor tissues of HBX/c-myc bitransgenic mice. In addition, Murphy et al. suggested that DDX5 is a substrate of HBX-DDB1-CUL4-ROC1 (CRL4 HBX) E3 ligase (Murphy et al., 2016). Therefore, it is possible that HBX contributes to DDX5 inhibition during HBV infection, and further investigation is needed to confirm this assumption.

DHX9

DHX9 also participates in various cellular pathways associated with RNA metabolism and contributes to the regulation of viral infections (Lee and Pelletier, 2016; Guo and Xing, 2021). To date, the expression levels of DHX9 were shown to be increased in HBV-replicating cells and transgenic mice. Shen et al. demonstrated that DHX9 was responsible for viral DNA replication, and the role of DHX9 in HBV biosynthesis relied on its helicase activity and nuclear localization. Furthermore, Nup98, an essential component of the nuclear pore, participates in DHX9-mediated HBV replication (Shen et al., 2020). In addition, HBV can produce circular viral RNAs. DHX9 can bind to HBV circular RNA to modulate the production of viral circular RNA (Sekiba et al., 2018). However, it is still unknown how DHX9 modulates the production of circular viral RNAs during the replication of HBV. APOBEC3B is known to inhibit HBV replication, and its antiviral effect relies on its deaminase activity. To date, the cellular factors that contributed to the anti-HBV effect mediated by APOBEC3B have not been fully defined. Based on co-immunoprecipitation, MS, and associated functional experiments, Chen et al. discovered that the interaction of DHX9 with APOBEC3B has a suppressive effect on the anti-HBV effect of APOBEC3B (Chen et al., 2020). Mechanistically, DHX9 did not affect APOBEC3B deamination activity but inhibited the binding of APOBEC3B to viral pgRNA (Figure 1).

The current study indicates that DHX9 upregulation mediated by the virus mainly relies on HBX. Based on tandem affinity purification (TAP)/MS analysis, DHX9 was found to serve as a substrate of the CRL4 HBX E3 ligase complex (Murphy et al., 2016). However, the effect of this E3 ligase complex on DHX9 remains unclear. In addition, Shen et al. suggested that HBX could increase DHX9 protein expression by

enhancing its stability. Furthermore, the elevation of DHX9 protein regulated by HBX in hepatocytes is relevant to the inhibition of E3 ligase mouse double minute 2 (MDM2)-associated degradation of DHX9 (Shen et al., 2020).

RIG-I

RIG-I, also called DDX58, is a well-known immune molecule with the ability to trigger RLR signaling, which is composed of three DExD/H-box RNA helicases: RIG-I, LGP2 (also called DHX58), and MDA5 in the cytoplasm to regulate MAVS activation (Kong et al., 2021b). The structures of RIG-I and MDA5 are similar. They have a helicase domain for RNA sensing, carboxy-terminal repressor domain (CTD) for activity modulation, and caspase activation and recruitment domains (CARDs) for signal transduction. RIG-I mainly binds to short dsRNA of no more than 300 bp, and viral RNA-bearing 5'-diphosphate can activate the RIG-I-mediated IFN response. To date, the molecular nature of MDA5 ligands has not been well identified, but it has been demonstrated that MDA5 is mainly activated by long dsRNAs of over 2 kb pairs. In particular, the presence of specific AU-rich sequences in mRNA, as well as the lack of 2'-O-methylation in mRNA, can be identified by MDA5 (Chan and Jin, 2022). Furthermore, because of their differing preferences for RNA binding, these two molecules can recognize different sections of the same viral genome independently and synergistically (Brisse and Ly, 2019). In HBV infection, whether RIG-1 and MDA5 can recognize HBV RNA in an independent or synergistic manner remains unclear. LGP2 contains only the CTD and helicase domains. Because of the lack of CARDs, LGP2 is considered incompetent and inhibits RIG-I- or MDA5-mediated signaling. After MAVS is activated, it further induces the sensitization of TBK1, which initiates IKK-dependent phosphorylation of NF-κB and leads to IRF3 activation to facilitate the production of inflammatory factors and IFN (You et al., 2022). Current evidence indicates that RIG-I is a viral RNA sensor with the ability to recognize the 5'-ϵ region of HBV pgRNA and induce IFN expression to inhibit infection (Figure 2) (Sato et al., 2015). RIG-I counteracts the interplay between HBp and viral pgRNA to restrain viral replication (Figure 1). Furthermore, Wu et al. found that RIG-I could increase the IFN-α-mediated immune response by elevating the levels of different antiviral proteins, including PKR, ADAR1, OAS, and Mx (Wu et al., 2018). Knockdown of RIG-I by a specific siRNA also inhibits the phosphorylation of STAT1, a signaling molecule in the IFN-associated immune pathway.

However, to facilitate viral infection, HBV utilizes N6-methyladenosine to repress the RIG-I-mediated recognition of viral RNA (Kim et al., 2020). The virus also blocks the expression of RIG-1 by inducing miR-146a (Hou et al., 2016). Furthermore, HBp and HBX were found to retrain virus-mediated RIG-I-associated signaling. For example, HBp can control RIG-I-induced IFN production by disrupting the interaction of TBK1 with DDX3 (Yu et al., 2010). HBX was found to bind to RIG-I (Figure 2) (Jiang and Tang, 2010), and residues Glu119 and Asn118 of the viral protein were mainly responsible for the suppression of RIG-I-related RLR signaling (Wang et al., 2020). HBX also has the ability to interact with MAVS and counteract the interplay between MAVS and RIG-I to block sensitization of the IFN-β promoter (Wang et al., 2010). Additionally, HBX regulates ADAR1 expression to block RIG-I transcription (Wang et al., 2021). Speckled at 110 kDa (Sp110), a transcription factor, can also control the expression of RIG-I (Sengupta et al., 2017; You et al., 2022). The interaction between HBX and Sp110 may downregulate RIG-I. Furthermore, targeting HBX using 5'-triphosphate siRNA can enhance the activation of RIG-I to stimulate the IFN-induced innate response in HBV-infected hepatocytes and pAAV-HBV-transfected mice (Han et al., 2011; Han et al., 2019). In addition, to inhibit RLR signaling, HBV promotes the activity of hexokinase (HK) and the production of lactate, which suppresses the interaction of RIG-I with MAVS and blocks IFN production (Zhou et al., 2021).

Lee et al. evaluated the effect of the RIG-I agonist 5'-triphosphate-poly-U/UC pathogen-associated-molecular-pattern (PAMP) RNA on restraining HBV cccDNA (Lee et al., 2021). The results demonstrated that treating HBV-infected cells with poly-U/UC RNA induces RIG-I signaling activation and causes the upregulation of various antiviral genes, including APOBEC3A, SAMHD1, and APOBEC3G, to repress cccDNA formation and accelerate the decay of HBV cccDNA. In addition, poly (I:C)-HMW/LyoVec, a RIG-1, and MDA5 agonist, was also found to inhibit HBV infection (Asadi-Asadabad et al., 2021), with a decline in HBsAg, HBeAg, and viral cccDNA. Furthermore, the effect of poly (I:C)-HMW/LyoVec was observed to be relevant to the upregulation of APOBEC3.

MDA5

MDA5 is also known as helicase-DEAD-box protein 116. As mentioned above (You et al., 2022), subsequent to MDA5 sensitization by cytoplasmic dsRNA, the molecule activates the MAVS-associated RLR signaling pathway to cause the production of IFN. A study by Lu et al. indicated that the expression levels of MDA5 were upregulated in HBV plasmid-transfected hepatoma cells and HBV plasmid-injected mouse livers (Lu and Liao, 2013). In particular, the authors revealed that MDA5 associates with HBV-specific nucleic acids, indicating that the molecule can sense HBV to induce the sensitization of RLR signaling, and triggers IFN-β-related innate immune responses with increased expression of MxA and OAS1. To date, the detailed mechanisms that are responsible for the recognition of HBV RNA by MDA5 are not well understood. However, Ebrahim et al. observed that the mRNA

levels of MDA5 are significantly attenuated in patients with chronic HBV infection (Ebrahim et al., 2015). Potential explanations for these inconsistencies include differences in the cell lines or clinical specimens used. Among the molecules encoded by the virus, HBX has been observed to suppress MDA5 activation *via* its interaction with MDA5 (Wang et al., 2010) (Figure 2). HBX also disrupts the interaction between MDA5 and MAVS to restrain the sensitization of the RLR signaling pathway (Wang et al., 2010). In addition, ADAR1 and Sp110 are linked to a reduction in MDA5 induced by HBX (Sengupta et al., 2017; Wang et al., 2021; You et al., 2022).

SKIV2L

SKIV2L is required for exosome-mediated RNA surveillance (Lee-Kirsch, 2022). Shiromoto et al. showed that the inflammatory factor IL-1β could upregulate the levels of transcription factor ATF3, which further binds to the cyclic AMP-responsive element sequence in the SKIV2L promoter to benefit its expression. Functionally, SKIV2L binds to HBX mRNA and promotes its degradation to restrict viral infection (Aly et al., 2016; Shiromoto et al., 2018). Mechanistically, with the help of SKIV2L, HBX mRNA can bind to the RNA exosome, and relying on HBS1L-dependent RNA quality control mechanisms, SKIV2L facilitates the decay of HBX mRNA in the RNA exosome (Figure 1). Furthermore, the suppression of HBV replication mediated by SKIV2L is IFN-independent. Shiromoto et al. found that HBX significantly increased SKIV2L expression. However, the underlying mechanism remains unknown.

DDX17

Mao et al. discovered that DDX17 can repress HBV replication in a helicase-dependent manner, by the RNA-binding activity of DDX17, which interacts with the stem-loop structure ϵ of viral pgRNA and then restrains its encapsidation (Mao et al., 2021) (Figure 1). However, Dong et al. found that HBX can enhance DDX17 expression (Dong et al., 2022). Upregulation of DDX17 further enhanced viral replication and transcription by increasing ZWINT expression. To date, the effects of DDX17 on HBV replication detected by separate groups have been inconsistent, as mentioned above, and are unknown.

DHX36

Current evidence shows that, as a member of the DEAD/H-box helicase family, DHX36 can enzymatically unwind G-quadruplex DNA and RNA, which are secondary nucleic acid structures with various roles in different cellular processes (Antcliff et al., 2021). Meier-Stephenson et al. revealed that DHX36 interacts with the G-quadruplex structure in the pre-core promoter region of HBV cccDNA, which is responsible for the generation of viral pgRNA (Meier-Stephenson et al., 2021). The binding of DHX36 to the G-quadruplex of the viral genome may contribute to the regulation of viral replication (Figure 1).

DDX60

DDX60 is an IFN-inducible cytoplasmic DEAD/H-box helicase. Kouwaki et al. found that DDX60 induced by IFN-γ enhanced the degradation of HBV RNA to inhibit HBV infection. Interestingly, the degradation of cytoplasmic viral RNA mediated by DDX60 is faster than that of nuclear viral RNA (Kouwaki et al., 2016) (Figure 1). Conversely, the downregulation of DDX60 by siRNA delayed the degradation of cytosolic HBV RNA, but not viral RNA in the nucleus.

UAP56

UAP56 is a cellular mRNA export factor (Morris et al., 2020). UAP56 benefits HBV replication by binding to HBX to facilitate the nuclear export of viral RNA (Figure 1). Furthermore, UAP56 facilitates viral RNA alternative splicing but not transcription. Moreover, the Q-motif in UAP56 is associated with helicase activity and contributes to the interaction between the protein and HBX (Hu et al., 2020a). Downregulation of UAP56 impairs cytosolic accumulation of viral RNA transcripts and reduces the levels of HBV pgRNA splicing variants.

Conclusion and future perspectives

Here, we show that, depending on various molecular mechanisms, different DEAD/H-box helicases participate in the regulation of HBV replication. On the one hand, some DEAD/H-box helicases, including DDX3, DDX17, DDX5, as well as UAP56, directly participate in the modulation of viral transcription, viral RNA nuclear export, pgRNA encapsidation, and viral reverse transcription in the viral life cycle (Figure 1). In contrast, different DEAD/H-box helicases, such as DDX3, MDA5, RIG-I, and DDX5, function as sensors of HBV RNA or modulate the function of adapter molecules or the translation of distinct genes in the RLR, TLR3, and IFN-related antiviral signaling pathways to trigger the innate immune response (Figure 2). In addition, inhibition of HBV replication mediated by DDX41 and DHX35 has been reported (Aly et al., 2016). Nevertheless, the underlying mechanisms are still unclear. It should be noted that, although DEAD/H-box helicases are involved in HBV infection, the effect of the identified DEAD/

H-box helicases on viral replication varies. Among the identified DEAD/H-box helicases, only DHX9 and UAP56 have been identified to benefit HBV replication, while other DEAD/H-box helicases exhibit an anti-HBV effect. As stated before, 53 DEAD/H-box helicases have been identified to date (Andrisani et al., 2022). However, only a few DEAD/H-box helicases, presented in this review, have been found to modulate HBV replication. In the future, more research is worthy to elucidate the interplay between DEAD/H-box helicases and HBV.

The current standard therapy for HBV is based on IFN and nucleus(t)ide analogs (Tang et al., 2018; Fanning et al., 2019). IFN treatment elicits the antiviral immune response. Nucleus(t)ide analogs restrict viral biosynthesis by disrupting HBp activity. However, these treatments cannot eliminate the virus, and the main treatment goal is to prevent disease progression and improve survival and quality of life. Moreover, current approaches often result in adverse side effects, therapeutic resistance, and recurrence of the disease (Feng et al., 2018). Therefore, new molecular targets are urgently required to improve the therapeutic effects. Because of the potential significance of DEAD/H-box helicases in the modulation of viral replication by targeting different steps in the HBV life cycle or participating in antiviral immune signaling pathways, targeting these molecules is an attractive strategy to attenuate HBV infection.

More importantly, our review indicates that the use of different small molecules to target DDX3 and RIG-I can effectively repress HBV replication (Choi et al., 2014; Kang et al., 2019; Lee et al., 2021). In particular, the RIG-I agonist, inarigivir, has undergone clinical trials for the treatment of HBV infection in a small study. It has been demonstrated that inarigivir can cause HBsAg reduction of more than 1 log10 IU/ml in 55% of patients. Nevertheless, 17% of patients had significant ALT flares. One patient with necrotizing pancreatitis was dead, and drug-induced liver steatosis and injury were also observed. Owing to these serious adverse reactions, clinical trials of inarigivir have been terminated (Fung et al., 2022). In addition to inarigivir, the antiviral effects of other RIG-1 agonists, including a synthetic 5'-triphosphate dsRNA RIG-I ligand (3pRNA), stem-loop RNA 14 (SLR14), and a sequence-optimized RIG-I agonist (named M8), have been explored by different groups (Chiang et al., 2015; Mao et al., 2022; Marx et al., 2022). In the future, the role of these RIG-1 agonists in the treatment of HBV infections should be examined. However, compounds targeting other DEAD/H-box helicases, including DDX5, DHX36, and UAP56, have not yet been discovered. In recent years, many significant breakthroughs have been achieved in the development of DEAD/H-box helicase inhibitors to treat cancer, and the efficacy of some DEAD/H-box helicase inhibitors has been examined in preclinical studies (Cai et al., 2017). To better assess whether targeting DEAD/H-box helicase is an effective therapeutic strategy against HBV, more attention is needed to develop compounds targeting DEAD/H-box helicases and explore their effect on the treatment of HBV infection.

During HBV infection, viral proteins including HBX and HBp have evolved multiple strategies to regulate the expression of different DEAD/H-box helicases to facilitate infection. Especially, HBX is a multifunctional viral molecule that is essential for HBV replication and associated diseases (Kong et al., 2019; Kong et al., 2021a; Kong et al., 2021c; You et al., 2022). This review examines various evidence that indicates HBX regulates different DEAD/H-box helicases, including DHX9, RIG-I, and MDA5. However, the underlying mechanisms related to HBX-induced modulation of DEAD/H-box helicases remain poorly understood, and further investigation is required. In addition, recent studies have mainly focused on the effects of DEAD/H-box helicases on HBV replication. In addition, DDX3 (Chang et al., 2006), DDX5 (Mani et al., 2020), DHX15 (Xie et al., 2019), and DDX17 (Dong et al., 2022), are associated with HBV-associated HCC. The role of DEAD/H-box helicases in the progression of different liver diseases, including hepatic fibrosis, cirrhosis, and HCC, caused by HBV infection, is poorly understood. Hence, it is vital to assess the effects of different DEAD/H-box helicases, and the relevant molecular mechanisms involved in the modulation of diverse diseases induced by the viruses in the future.

Author contributions

HY and LM contributed the work equally. HY, LM, XW, FZ, YH, JY, and XP designed the artwork and wrote the manuscript. KZ, FK, and RT supervised the manuscript. All authors contributed to the article and approved the submitted version.

Funding

The study was supported by the Natural Science Foundation of Jiangsu Province (BK20211347), the Natural Science Foundation of the Jiangsu Higher Education Institutions (21KJA310004), Xuzhou Technology Bureau Foundation (KC21065), and a project funded by the Priority Academic Program Development of Jiangsu Higher Education Institutions (PAPD). The Figures used in the review was created by BioRender (https://biorender.com/).

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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frontiers | Frontiers in Cellular and Infection Microbiology

TYPE Original Research
PUBLISHED 12 December 2022
DOI 10.3389/fcimb.2022.1055774

OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to Virus and Host, a section of the journal Frontiers in Cellular and Infection Microbiology

RECEIVED 28 September 2022
ACCEPTED 28 October 2022
PUBLISHED 12 December 2022

CITATION
Wang C-R, Liu X-q, Li H, Zhang Q, Zhong G-C, Tang Q, Chang Y, Wang J-S, Duan Y-q and Hu P (2022) PgRNA kinetics predict HBsAg reduction in pregnant chronic hepatitis B carriers after treatment cessation. *Front. Cell. Infect. Microbiol.* 12:1055774. doi: 10.3389/fcimb.2022.1055774

PgRNA kinetics predict HBsAg reduction in pregnant chronic hepatitis B carriers after treatment cessation

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Background: Pregenomic RNA (pgRNA) and hepatitis B core-related antigen (HBcrAg) play significant roles in predicting discontinuing treatment outcomes. However, their role in pregnancy has rarely been reported. We aimed to evaluate the performance of pgRNA and HBcrAg kinetics in predicting HBeAg seroconversion and HBsAg reduction postpartum in HBeAg-positive pregnant women.

Methods: Pregnant HBeAg-positive patients receiving antiviral prophylaxis and ceasing treatment postpartum were included. PgRNA and HBcrAg levels were measured before treatment, at 32 weeks of gestation, and at treatment withdrawal postpartum. Other virological and biochemical parameters were regularly examined until 96 weeks postpartum.

Results: Of 76 pregnant chronic hepatitis B (CHB) carriers with a median treatment duration of 18.1 weeks, HBeAg seroconversion and HBsAg reduction >0.3 $\log_{10}$ IU/mL at 96 weeks postpartum occurred in 8 (10.5%) and 13 (17.1%) patients, respectively. HBsAg correlated most strongly with pgRNA, while HBeAg correlated most strongly with HBcrAg. Multivariable regression analysis revealed that postpartum pgRNA decline and peak ALT levels were independent predictors of HBsAg reduction. The area under the curve of the regression model was 0.79 and reached as high as 0.76 through bootstrapping validation. The calibration plot showed that the nomogram had a performance similar to that of the ideal model. A decision tree was established to facilitate application of the nomogram. In addition, HBcrAg kinetics, as an independent predictor, performed poorly in predicting HBeAg seroconversion.

Conclusions: Postpartum pgRNA decline together with peak ALT levels may identify patients with a higher probability of HBsAg reduction after treatment cessation postpartum among pregnant CHB carriers receiving antiviral prophylaxis.

KEYWORDS

novel biomarkers, prediction model, NAs prophylaxis, mother-to-child transmission (MTCT), pregnance

Introduction

Chronic hepatitis B (CHB) is a serious public health problem, resulting in approximately 800,000 deaths every year (Liu et al., 2019). China has the largest burden of hepatitis B virus (HBV) infection worldwide (The Polaris Observatory Collaborators, 2018). In China, approximately 6% of women giving birth live with HBV, among whom the hepatitis B e antigen (HBeAg)-positive rate is up to 30% (Jing et al., 2020). Mother-to-child transmission is the primary route of HBV transmission in China. Thus, antiviral prophylaxis in pregnant CHB patients is necessary in preventing HBV transmission (Zhou et al., 2020).

During pregnancy, the maternal immune system tolerates fetal antigens by suppressing cell-mediated immunity while retaining normal humoral immunity (Gaunt and Ramin, 2001). After delivery, these adaptations disappear and the immune system reconstructs, which could influence liver disease activity (ter Borg et al., 2008). The reported prevalence of alanine aminotransferase (ALT) flares after cessation postpartum varies from 5% to 62% (ter Borg et al., 2008; Xu et al., 2009; Han et al., 2011; Liu et al., 2013; Pan et al., 2016; Liu et al., 2017; Chang et al., 2018), potentially related to the flare definition, patient characteristics, or occurrence of virological rebound. It has been hypothesized that the rapid reactivation of the immune system against HBV antigens is responsible for postpartum ALT flares (ter Borg et al., 2008). Choi et al. (2021) concluded that ALT flares during PEG-IFN-α treatment were associated with subsequent HBsAg and HBV RNA decline, and predicted subsequent HBsAg loss. Serum pregenomic RNA (pgRNA) and hepatitis B core-related antigen (HBcrAg) are potential surrogate markers for covalently closed circular DNA (cccDNA) transcriptional activity (Wang et al., 2016). Currently, only three studies have analyzed pgRNA and HBcrAg in HBV-infected pregnant women (Zhang et al., 2018; Patel et al., 2019; Wang et al., 2022). However, the roles of pgRNA and HBcrAg levels in predicting long-term outcomes following treatment cessation postpartum have not been investigated. Hence, we aimed to investigate the performance of pgRNA and HBcrAg kinetics in predicting HBeAg seroconversion and HBsAg reduction in HBeAg-positive pregnant CHB carriers.

Materials and methods

This study strictly conformed to the Ethical Guidelines of the 1975 Declaration of Helsinki and was approved by the Ethics Committee of the Second Affiliated Hospital of Chongqing Medical University. Written informed consent was obtained from all the subjects. This study was registered with the Chinese Clinical Trial Registry (ChiCTR2100054116).

Study population

The study population comprised pregnant women with HBV infection who visited the Second Affiliated Hospital of Chongqing Medical University outpatient clinic from January 2019 to September 2021. HBeAg-positive pregnant CHB carriers who received antiviral prophylaxis at 24–28 weeks of gestation and ceased treatment at 4–8 weeks postpartum were also included. Patients were excluded if they received treatment before pregnancy or were coinfected with other hepatotropic virus infections, HIV infection, or were complicated with cirrhosis and pregnancy-related diseases.

Study setting

According to the 2019 Chinese Guidelines for Prevention and Treatment of CHB (Wang and Duan, 2021), antiviral prophylaxis was performed at 24–28 weeks of gestation among pregnant CHB patients with HBV DNA levels >200,000 IU/mL and ceased within 12 weeks postpartum. Patients with drug withdrawal were regularly followed-up at 1- or 3-month intervals for an additional 2 years to analyze 96 weeks postpartum outcomes, which included HBeAg seroconversion (defined as the loss of serum HBeAg and the development of anti-HBe antibodies during follow-up (EASL, 2017)) and HBsAg reduction (defined as HBsAg decrease >0.3 $\log_{10}$ IU/mL from baseline to the end of follow-up) (Zhang et al., 2020; Janssen et al., 2020).

Clinical and laboratory data collection

Serum samples were collected at 24–28 weeks of gestation (referred to as baseline), 32–36 weeks of gestation (referred to as near delivery), and treatment withdrawal postpartum (referred to as postpartum) for serum pgRNA and HBcrAg measurements. Serum pgRNA was measured using an ABI7500 quantitative real-time polymerase chain reaction (PCR) system (ABI Laboratories, USA) , with a detection range of 2×10 (The Polaris Observatory Collaborators, 2018) to 1×10 (Pan et al., 2016) copies/mL. Serum HBcrAg was measured using a chemiluminescent immunoassay, Lumipulse G1200 automated analyzer (Fujirebio, Tokyo, Japan), with a sensitivity of 2 log U/mL. Demographic and clinical data, including age, parity status, infant sex, antiviral therapy regimen, complete blood count, liver function test, and classic HBV markers, were collected during pregnancy and postpartum.

Statistical analysis

Five major statistical analyses were performed. First, a descriptive analysis was performed. Fisher's exact test and Mann–Whitney U test were conducted for categorical and continuous variables between groups, respectively. Spearman's correlation test was used to evaluate the correlations between continuous biomarkers. Second, multivariable logistic regression was used to identify independent factors associated with postpartum outcomes. Of note, baseline variables that were considered clinically relevant or showed a statistically significant association with the outcome of interest in the univariable logistic regression model were entered into the multivariable logistic regression model. Third, a nomogram was constructed based on the screened independent risk factors to predict the probability of an outcome of interest. Fourth, we further calculated the discrimination and calibration of this nomogram indicated by the area under the curve (AUC) and calibration curve, respectively, which were validated by 1000 bootstrap resamplings. Finally, by calculating the appropriate nodes and complex parameters, a decision tree was established to provide a simple decision-making process for clinicians. All data analyses were conducted using the SPSS software (version 26.0) and R software (version 3.6.1). The statistical significance level was set at $P<0.05$, using a two-tailed test.

Results

Characteristics of included patients

Of 152 HBV-infected pregnant women, 76 were included, with a median treatment duration of 18.1 weeks (range 14.6–20.7 weeks) (Figure S1). The demographic and clinical characteristics of the included patients are shown in Table 1. HBeAg seroconversion and HBsAg reduction at 96 weeks postpartum were observed in 8 (10.5%) and 13 (17.1%) patients, respectively. Compared with those in the non-seroconversion group, patients in the seroconversion group had increased ALT levels [56.5 (IQR 37.0–65.2) vs. 20.0 (IQR 15.8–28.2) U/L, $P<0.01$], decreased HBeAg [2.4 (IQR 1.7–3.1) vs. 3.2 (IQR 3.0–3.3) $\log_{10}$PEIU/mL, $P=0.02$], and HBsAg [4.3 (IQR 4.0–4.4) vs. 4.5 (IQR 4.3–4.7) $\log_{10}$IU/mL, $P=0.05$]. No significant differences were noted in HBV DNA, pgRNA, or HBcrAg levels between the two groups (all $P>0.05$). Compared with those in the non-reduction group, patients in the HBsAg reduction group had increased ALT levels [28.0 (IQR 19.0–38.0) vs. 20.0 (IQR 15.5–31.0) U/L, $P=0.01$] and increased peak ALT values postpartum (hereafter referred to as postpartum ALT_{max}) [67.0 (IQR 46.0–102.0) vs. 40.0 (IQR 27.0–83.6) U/L, $P=0.04$]. We further plotted changing patterns of HBsAg and ALT levels for each patient with HBsAg reduction (N=13) during follow-up (Figure S2) and found that peak ALT levels postpartum >40 U/L were observed in 10 (76.9%) patients. No significant differences were noted in other host or viral biomarkers between the two groups.

The kinetics of HBV biomarkers are summarized in Figure 1. HBV biomarkers in the five groups changed dynamically during pregnancy and postpartum in different patterns. The kinetics of HBcrAg and HBV DNA levels showed similar changing patterns between patients with and without HBeAg seroconversion. PgRNA decreased significantly postpartum in the seroconversion group, but not in the non-seroconversion group; HBsAg and HBeAg decreased significantly near delivery in the seroconversion group but not in the non-seroconversion group. The kinetics of HBcrAg and HBV DNA levels showed similar changing patterns between patients with or without HBsAg reduction; kinetics of HBsAg and HBeAg levels showed similar changing patterns between the two groups; pgRNA decreased significantly postpartum in the HBsAg reduction group but not in the non-reduction group.

No significant differences were found between tenofovir disoproxil fumarate and telbivudine groups in terms of HBeAg seroconversion rate [9.1% (3/33) vs. 11.6% (5/43), $P=0.72$] and HBsAg reduction [12.1% (4/33) vs. 20.9% (9/43), $P=0.31$] (Table S1). In addition, neither maternal (age, parity status, infant sex, alanine transaminase) nor viral biomarkers (pgRNA, HBcrAg, HBV DNA, HBsAg, and HBeAg) showed any significant difference between the tenofovir disoproxil fumarate and telbivudine groups (all $P>0.05$).

Serum HBsAg correlated most strongly with pgRNA levels during pregnancy and postpartum

We analyzed the overall correlation between serum pgRNA and HBcrAg levels and other HBV markers (Figure S3).

10.3389/fcimb.2022.1055774

TABLE 1 Demographics and clinical characteristics of 76 pregnant CHB carriers with HBeAg positive at baseline.

	All patients N=76	Without HBeAg seroconversion N=68	HBeAg seroconversion N=8	*P* value	Without HBsAg reduction N=63	HBsAg reduction N=13	*P* value
Age, years	28.0 (26.0-31.0)	28.0 (26.0-31.0)	26.5 (25.5-27.2)	0.06	28.0 (26.0-31.0)	26.0 (25.0-30.0)	0.60
Parity status				0.47			0.16
The first pregnancy	71 (93.4%)	64 (94.1%)	7 (87.5%)		60 (95.2%)	11 (84.6%)	
The second pregnancy	5 (6.6%)	4 (5.9%)	1 (12.5%)		3 (4.8%)	2 (15.4%)	
Male Infant, n (%)	34 (44.7%)	31 (45.6%)	3 (37.5%)	0.66	28 (44.4%)	6 (46.2%)	0.91
Antiviral prophylaxis TDF vs. LDT	33 (43.4%) vs. 43 (56.6%)	30 (44.1%) vs.38 (55.9%)	3 (37.5%) vs.5 (62.5%)	0.72	29 (46.0%) vs. 34 (54.0%)	4 (30.8%) vs. 9 (69.2%)	0.31
Treatment duration, weeks	18.1 (14.6-20.7)	18.3(14.7-20.9)	15.4 (12.4-17.4)	0.31	17.4 (14.2-21.1)	18.7 (16.4-20.0)	0.62
ALT, U/L	21.5 (16.0-32.0)	20.0 (15.8-28.2)	56.5 (37.0-65.2)	<0.01	20.0 (15.5-31.0)	28.0 (19.0-38.0)	0.01
Postpartum ALT $_{max}$	46.5 (30.2-87.0)	46.0 (28.0-85.5)	55.0 (39.0-129.8)	0.08	40.0 (27.0-83.6)	67.0 (46.0-102.0)	0.04
HBV DNA, log_{10}IU/mL	6.9 (6.6-7.4)	7.0 (6.6-7.4)	6.5 (5.7-6.9)	0.10	6.8 (6.5-7.3)	7.3 (6.8-7.9)	0.06
HBsAg, log_{10}IU/mL	4.5 (4.3-4.6)	4.5 (4.3-4.7)	4.3 (4.0-4.4)	0.05	4.5 (4.2-4.6)	4.7 (4.4-5.0)	0.06
HBeAg, log_{10}PEIU/mL	3.2 (2.9-3.3)	3.2 (3.0-3.3)	2.4 (1.7-3.1)	0.02	3.2 (2.9-3.3)	3.2 (3.0-3.3)	0.89
HBcrAg, log_{10}U/mL	8.6 (8.4-8.7)	8.6 (8.4-8.7)	8.6 (8.2-8.6)	0.83	8.6 (8.4-8.7)	8.7 (8.6-8.8)	0.43
PgRNA, log_{10}copies/mL	7.8 (7.6-8.1)	7.8 (7.7-8.1)	7.6 (7.1-8.4)	0.96	7.8 (7.6-8.0)	8.1 (7.9-8.6)	0.21

Continuous variables were expressed as median [interquartile range (IQR)], and categorical variables were expressed as counts (percentage). Baseline: at 26 ± 2 weeks of gestation; TDF, tenofovir disoproxil fumarate; LDT, telbivudine; HBV, hepatitis B virus; DNA, deoxyribonucleic acid; ALT, alanine aminotransferase; postpartum ALT_{max}, peak ALT level postpartum; HBsAg, hepatitis B surface antigen; HBeAg, hepatitis B e antigen; pgRNA, pregenomic RNA; HBcrAg, hepatitis B core-related antigen. HBsAg reduction, HBsAg decrease >0.3log_{10}IU/mL from baseline to last date of follow-up.

Overall, HBsAg levels were found to be most strongly related to pgRNA levels (r=0.59, P<0.01) and showed a slightly weaker association with HBcrAg levels, whereas serum HBeAg was correlated most strongly with HBcrAg (r=0.60, *P*<0.01), and showed a weaker association with pgRNA (r=0.41, *P*<0.01). Significant correlations between the above biomarkers were also observed during pregnancy and postpartum period.

Decreased pgRNA and HBcrAg levels in HBeAg seroconversion and HBsAg reduction groups

Next, we calculated the fold changes of HBV markers in the HBeAg seroconversion and HBsAg reduction groups (Figure 2). Serum PgRNA and HBcrAg levels decreased more rapidly in patients with HBeAg seroconversion than in those without; a similar decreased pattern was observed between the HBsAg reduction and non-reduction groups. No significant differences were noted in the fold changes of HBV DNA levels between the HBeAg seroconversion and non-seroconversion groups (*P*>0.05), or between the HBsAg reduction and non-reduction groups (*P*>0.05). We then compared the fold changes in HBV markers near delivery and postpartum in the five groups (Figure 3). Similar trends were noted in the comparisons of fold changes near delivery among the five groups. We observed significant differences in the fold changes near delivery between HBV DNA and pgRNA, HBcrAg, HBsAg, and HBeAg in the five groups (all *P*<0.05). During postpartum, we found significant differences in fold changes between HBV DNA and pgRNA, between HBcrAg and HBsAg, and between HBsAg and HBeAg in the non-seroconversion and non-HBsAg reduction groups (all *P*<0.01), whereas these differences were not found in the HBeAg seroconversion and HBsAg reduction groups (all *P*>0.05).

PgRNA kinetics independently predicted HBsAg reduction postpartum

Multivariable logistic regression analyses revealed that the pgRNA decline from baseline to postpartum (ΔpgRNA) and

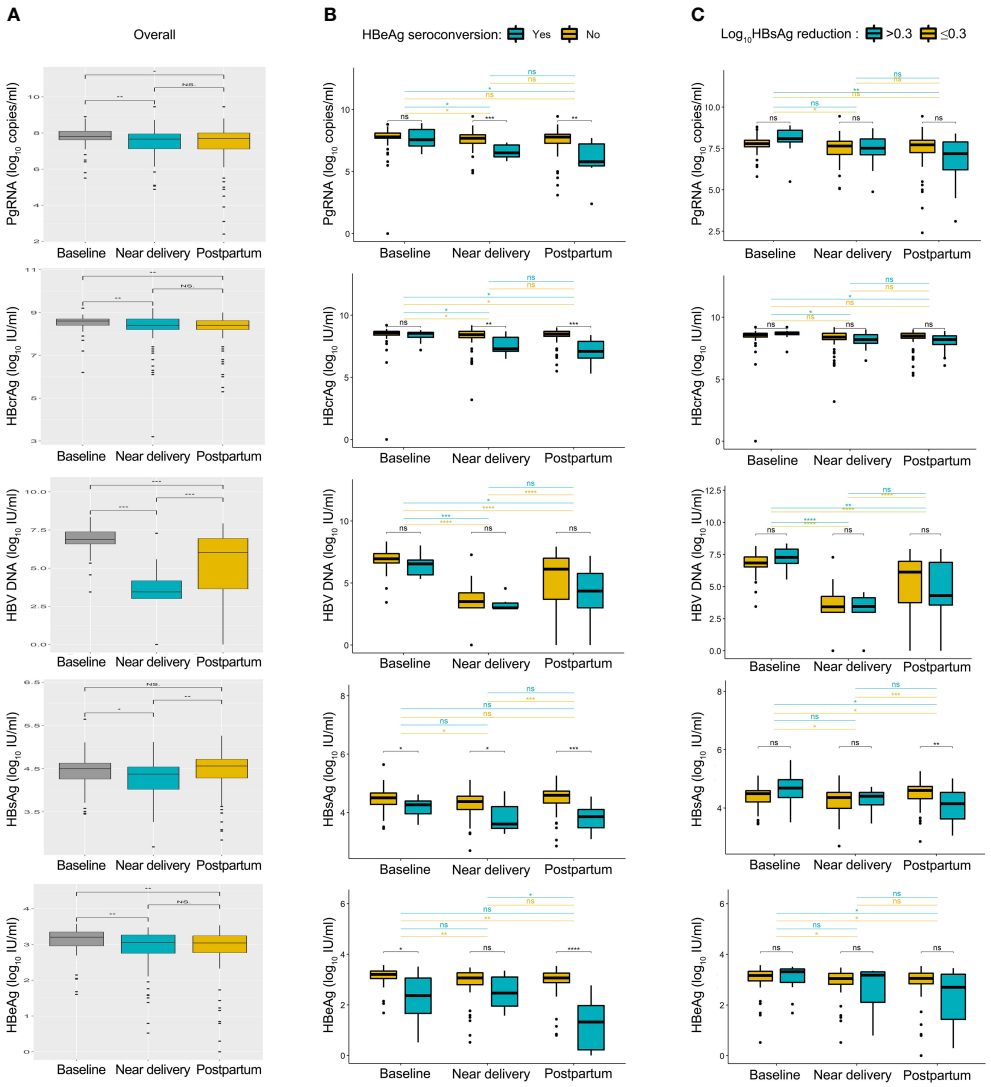

FIGURE 1
Summary of HBV biomarkers from baseline to postpartum. Kinetics of pgRNA, HBcrAg, HBV DNA, HBsAg and HBeAg levels was shown in all patients **(A)**, patients with or without HBeAg seroconversion **(B)**, patients with or without HBsAg reduction **(C)**. Baseline, 24-28 weeks of gestation; near delivery, 32-36 weeks of gestation; postpartum, at 2-6 weeks after delivery. NS = non-significant ($p > 0.05$), *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$.

postpartum ALT_{max} were independent predictors of HBsAg reduction (Table 2). The AUC value was calculated to estimate the advantages of ΔpgRNA, postpartum ALT_{max}, and their combined performance (Figure 4A). The AUC of the combined biomarkers was 0.79 (95% confidence interval (CI), 0.67–0.92] and reached as high as 0.76 *via* bootstrapping validation (Table S2). In addition, we found that ALT levels at baseline and HBcrAg decline from baseline to postpartum (ΔHBcrAg) were independent predictors of HBeAg seroconversion. The AUC value was calculated to estimate the advantage of ΔHBcrAg, ALT at baseline, and their combined performance (Table S3). We further calculated the calibration of the combined model consisting of baseline ALT and ΔHBcrAg. The prediction model of ΔHBcrAg plus ALT at baseline showed poor calibration performance, although it had a high AUC value (0.99) (Figure S4).

ΔpgRNA plus postpartum ALT_{max} improved the estimation of HBsAg reduction postpartum

Based on the results of the multivariate logistic regression analyses, we constructed a nomogram by combining predictive

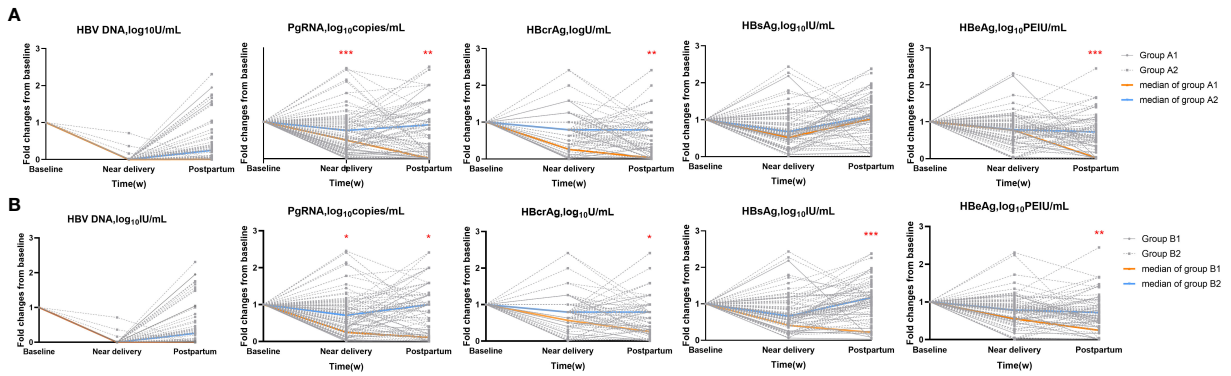

FIGURE 2
Fold changes of HBV markers in subgroups. **(A)** HBeAg seroconversion group (Group A1) and non-seroconversion group (Group A2). **(B)** HBsAg reduction (Group B1) and non-reduction group (Group B2). *=p < 0.05, **= p < 0.01, ***= p < 0.001.

factors, including postpartum ALT_{max} and ΔpgRNA, to facilitate the prediction of HBsAg reduction postpartum after treatment cessation in pregnant CHB carriers (Figure 4B). Calibration curves and decision trees were constructed to estimate the clinical utility of the nomogram. The calibration curve showed that the performance of our nomogram was similar to that of an ideal model (Figure 4C). The decision tree showed that the combined biomarkers had a superior performance in predicting the probability of postpartum HBsAg reduction (Figure 4D). When ΔpgRNA >0.50 log_{10} copies/mL plus postpartum ALT_{max} >40 U/L, the possibility of HBsAg reduction >0.3 log_{10} IU/mL reached as high as 47%.

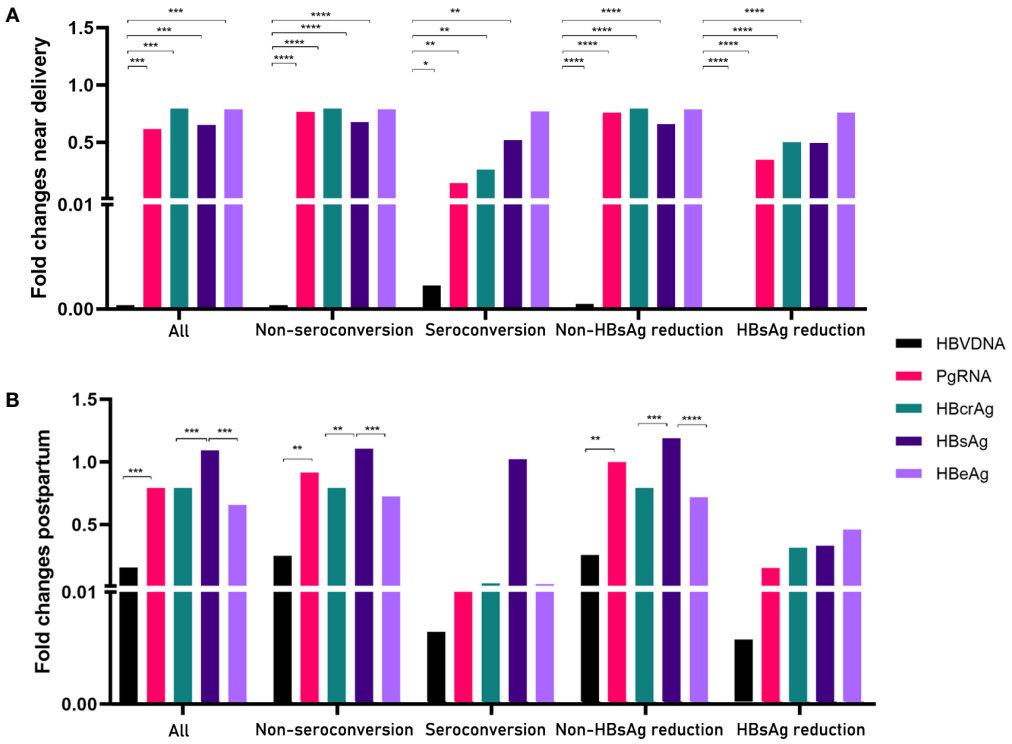

FIGURE 3
Comparisons of fold changes near delivery **(A)** and postpartum **(B)** in five groups. *= p < 0.05, **= p < 0.01, ***= p < 0.001, ****= p < 0.0001.

TABLE 2 Logistic regression analysis for factors of HBeAg seroconversion and HBsAg reduction postpartum in HBeAg positive carriers (N=76).

Variables*	HBeAg seroconversion				HBsAg decrease>0.3logIU/mL			
	Univariate analysis		Multivariate analysis		Univariate analysis		Multivariate analysis	
	OR (95% CI)	*P* Value	OR (95% CI)	*P* Value	OR (95% CI)	*P* Value	OR (95% CI)	*P* Value
Age, year	0.81 (0.65-1.02)	0.07			0.96 (0.82-1.12)	0.59		
Infant gender	0.72 (0.16-3.24)	0.66			1.07 (0.32-3.55)	0.91		
Type of therapy	1.32 (0.29-5.95)	0.22			1.92 (0.54-6.89)	0.32		
Pregnancy status	2.29 (0.22-23.44)	0.49			3.64 (0.54-24.34)	0.18		
HBVDNA, log_{10}IU/mL	0.54 (0.25-1.17)	0.12			2.54 (0.96-6.67)	0.06		
ALT, U/L	1.14 (1.06-1.23)	0.00	1.18(1.05-1.32)	0.01	1.04 (1.00-1.07)	0.03		
HBsAg, log_{10}IU/mL	0.18 (0.03-1.07)	0.06			5.46 (0.92-32.54)	0.06		
HBeAg, log_{10}PEIU/mL	0.19 (0.05-0.71)	0.01			0.87 (0.23-3.26)	0.84		
PgRNA, log_{10}copies/mL	0.72 (0.24-2.16)	0.57			2.76 (0.76-9.97)	0.12		
HBcrAg, log_{10}U/mL	0.45 (0.13-1.64)	0.23			2.35 (0.36-15.29)	0.37		
ΔHBVDNA, log_{10}IU/mL	1.18 (0.82-1.69)	0.35			1.37 (1.02-1.86)	0.04		
ΔPgRNA.log_{10}copies/mL	2.51 (1.36-4.63)	0.00			1.90 (1.14-3.17)	0.01	2.00(1.17-3.45)	0.01
ΔHBcrAg.log_{10}U/mL	4.99 (1.88-13.24)	0.00	6.5(1.41-29.87)	0.02	2.17 (0.99-4.72)	0.05		
ΔHBsAg, log_{10}IU/mL	5.62 (1.27-24.92)	0.02			74.04 (7.68-713.57)	0.00		
ΔHBeAg, log_{10}PEIU/mL	7.76 (2.54-23.78)	0.00			2.10 (0.95-4.60)	0.06		
Postpartum ALT $_{max}$	1.02 (0.99-1.04)	0.09			1.02 (1.00-1.03)	0.04	1.02(1.00-1.04)	0.04

*The variables enrolled in the logistic regression analysis were age, HBVDNA, ALT, HBsAg, HBeAg, pgRNA, HBcrAg level, ΔHBVDNA, ΔpgRNA, ΔHBcrAg, ΔHBsAg, ΔHBeAg (continuous variable), infant gender (male vs. female), type of therapy (TDF vs. LDT), pregnancy status (first vs. second pregnancy). Δ means variable decline from baseline to postpartum; Postpartum ALT $_{max}$ means peak ALT level postpartum.

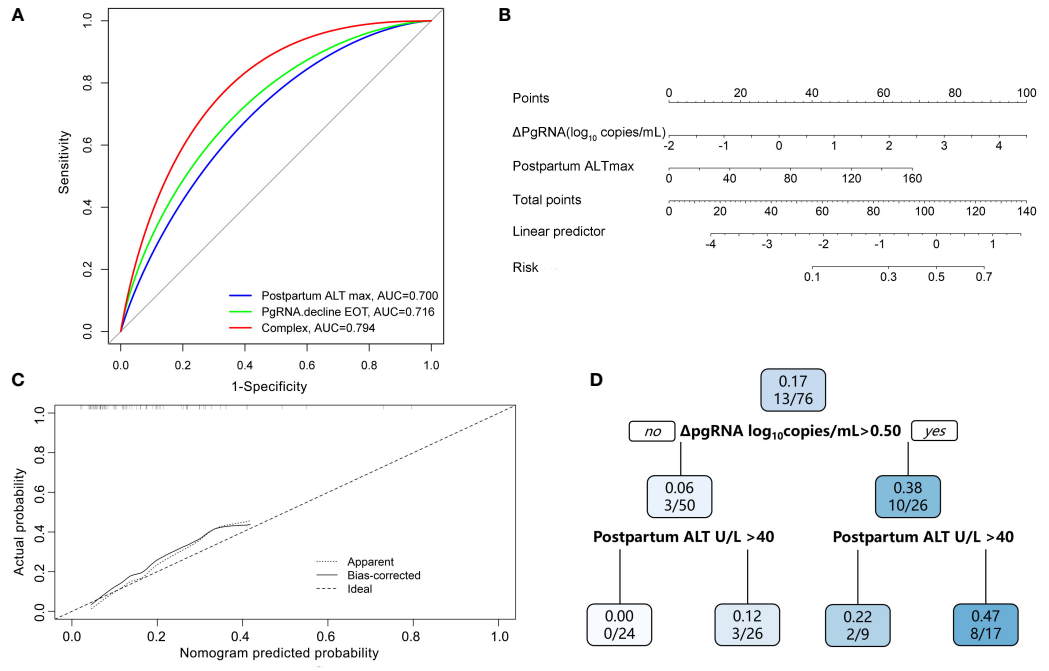

FIGURE 4
Predictive model of HBsAg reduction. **(A)** AUC performance of ΔpgRNA at the end of treatment (EOT), Postpartum ALT $_{max}$ and their combinations. **(B)** Calibration curve for the combined prediction model to predict probability of HBsAg reduction. **(C)** Nomogram to predict probability of HBsAg reduction in pregnant CHB carriers with HBeAg positive. **(D)** A decision tree for the HBsAg reduction outcome predictive model. PgRNA decline EOT means decline from baseline to postpartum; Postpartum ALT $_{max}$ means peak ALT level postpartum.

Discussion

Currently, only three studies have analyzed pgRNA or HBV RNA levels in HBV-infected pregnant patients (Zhang et al., 2018; Patel et al., 2019; Wang et al., 2022). One prospective study in 46 pregnant CHB patients described HBV total RNA and pgRNA levels during pregnancy and postpartum and their possible associations with established HBV viral markers. Another retrospective study showed that genotype had a certain influence on HBV RNA kinetics following NA therapy. The degree of decline in HBV RNA in patients with genotype B was significantly higher than that in patients with genotype C. In addition, an observational cohort study analyzed the relationship between HBV DNA, HBV RNA, and HBsAg and the predictive value for mother-to-child transmission. The role of HBcrAg levels in pregnant CHB carriers has rarely been reported. It is well known that the aim of nucleos(t)ide analog (NA) therapy is to maintain viral suppression during treatment (EASL, 2017). HBsAg loss is regarded as an effective treatment endpoint, termed "functional cure"; however, it is rarely achieved with current antiviral regimes (Cornberg and Höner Zu Siederdissen, 2014). In our study, after analyzing 76 pregnant CHB carriers with a median treatment duration of 18.1 weeks, we found that HBsAg reduction >0.3 $\log_{10}$ IU/mL at 96 weeks postpartum occurred in 13 (17.1%) patients and concluded that pgRNA was not only positively correlated with classic viral biomarkers but also acted as an independent predictor of HBsAg reduction. The combination of pgRNA decline postpartum and postpartum ALT_{max} performed satisfactorily in predicting HBsAg reduction after treatment cessation postpartum.

By estimating the overall correlation between HBV markers, we found the strongest correlation between serum pgRNA and HBcrAg (r=0.630, *P*<0.001) and a slightly weaker correlation between serum pgRNA and HBsAg or HBV DNA (r=0.590 and r=0.480, respectively, all *P*<0.001). Consistent with a previous study, Wang et al. (2021) reported that in HBeAg-positive patients, serum HBV RNA was weakly correlated with HBV DNA (r=0.449, P<0.001) but moderately correlated with HBsAg (r=0.557, P<0.001) and HBcrAg (r=0.601, P<0.001). In another Hong Kong study, Mak et al. (2021) concluded serum HBV RNA correlates best with HBcrAg in HBeAg-positive NA-treated CHB patients (r=0.795, P<0.001), and to a lesser extent with serum HBsAg (r=0.719, P <0.001) or HBV DNA (r=593, P<0.001). Both pgRNA and HBcrAg originated only from cccDNA, which may explain the strong correlation between them. We also found that the correlation between serum HBsAg and pgRNA (r=0.59, P<0.01) was the highest, but weakened between HBsAg and HBcrAg (r=0.53, P<0.001) and HBV DNA (r=0.40, P<0.001). The correlation between serum HBeAg and HBcrAg (r=0.60, P<0.01) was the highest, but weakened between HBeAg and pgRNA (r=0.41, P<0.001) or HBV DNA (r=0.39, P<0.001). Similar to a previous Hong Kong study (Mak et al., 2021), they showed good correlation between HBsAg and pgRNA (r=0.719, *P* <0.001) and weaker correlation between HBsAg and HBcrAg (r=0.632, *P*<0.001) and HBV DNA (r=0.547, *P*<0.001). It is well known that HBcrAg combines the antigenic reactivity resulting from denatured HBeAg, HBcAg, and core-related protein (Mak et al., 2018), which may explain the strong correlation between serum HBeAg and HBcrAg.

Our subsequent analysis also supports this result. Multivariable regression analyses revealed that ΔpgRNA postpartum levels were independently and positively correlated with HBsAg reduction. Several studies have reported that HBV RNA decline is associated with higher rates of off-treatment sustained response among patients treated with NAs or peginterferon alfa (PEG-IFN) (Huang et al., 2015; van Bömmel et al., 2018; Luo et al., 2020; van Campenhout et al., 2020). Janssen et al. (van Campenhout et al., 2020) investigated whether HBV RNA can predict serological response to peginterferon treatment and revealed that at week 12, a trend was observed toward lower HBV RNA levels in patients with HBsAg loss (3.8 vs. 4.9 log copies/mL, *P*=0.11) and more HBV RNA decline from baseline (−3.2 vs. −2.0 log copies/mL, *P*=0.06). Another study (Huang et al., 2015) reported that on-treatment low-serum HBV RNA levels at treatment week 12 (adjusted hazard ratio=0.908, 95% CI 0.829, 0.993, *P*=0.035) independently predicted the initial virological response in NA-treated patients with CHB. A previous American study (van Bömmel et al., 2018) demonstrated that at week 12, an HBV RNA cutoff of 5.5−log10 copies/mL identified a higher proportion of non-responders to PegIFN alfa-2a treatment (30%) than an HBV DNA cutoff of 8.9−log10 IU/mL (22%) or HBeAg cutoff of 2.7−log10 IU/mL (29%). Together with our results, these results indicate that HBV pgRNA may not only correlate well with classic viral biomarkers but also act as an independent predictor of HBsAg reduction.

Another independent predictor, postpartum ALT elevation, is associated with subsequent HBsAg decline. ALT flares are postulated to be primarily immune-mediated and may be beneficial for successful clearance of infection. The postpartum period appears to have an effect on HBeAg seroconversion, with several small studies showing higher than expected seroconversion rates in the early postpartum period (12% of 40 pregnancies (Lin et al., 2006); 17% of 30 women (Lin et al., 1989); in the Melbourne cohort study (Giles et al., 2015), 7% of 30). In addition, on-treatment flares have been associated with declines in serum HBV DNA and HBsAg (Flink et al., 2005; Sonneveld et al., 2013). Wong et al. (2018) reported that ALT flares are independently associated with HBsAg loss. Another study (Jeng et al., 2016) showed that early HBsAg reduction increased in an ALT (<5, 5–10, 10–20, and ≥20×ULN, *P*=0.001) level-dependent manner. In our study, peak ALT level postpartum was higher in subjects who achieved HBsAg reduction and independently associated with HBsAg response,

possibly associated with a stronger antiviral effect. The characteristics of T-cell immunity were distinct between mothers with postpartum ALT flare and those without (Huang et al., 2021). T cells in mothers with ALT flares produced more pro-inflammatory cytokines (IFN-γ, IL-21, TNF-α, and IL-2) or less anti-inflammatory cytokines (IL-10) than those in mothers without ALT flares. Next, we constructed a nomogram consisting of peak ALT levels and ΔpgRNA postpartum with satisfactory AUC values and calibration performance. As the decision tree shown, the incidence of HBsAg reduction was significantly higher in patients with postpartum ΔpgRNA >0.50 log_{10} copies/mL plus peak ALT level >40 U/L than in those with postpartum ΔpgRNA <0.50 log10 copies/mL or peak ALT level <40 U/L.

Notably, multivariate regression analysis revealed that ALT and ΔHBcrAg were independent factors for HBeAg seroconversion. However, the univariate model that included baseline ALT >30 U/L had an AUC value of 0.95. Hence, the contribution of ΔHBcrAg was marginal. The prediction model of ΔHBcrAg plus ALT at baseline showed an unsatisfactory calibration performance. Thus, we did not develop a nomogram or decision tree for the HBeAg seroconversion model. More detailed kinetic studies with larger sample sizes are needed to characterize pgRNA and HBcrAg kinetics under NA treatment among pregnant HBV carriers, which will help us to understand HBV-host interactions and the NA mode of action.

To the best of our knowledge, this is the first study to evaluate the performance of serum pgRNA and HBcrAg levels in predicting off-treatment HBeAg seroconversion and HBsAg decline in well-characterized cohorts of pregnant CHB carriers with close monitoring and comprehensive off-treatment data collection in China. Nonetheless, our study has several limitations. First, it was a retrospective study with a limited number of patients. Therefore, our results should be interpreted with caution. Prospective analyses are required to validate our findings in a large and diverse population. Second, because of the limited number of patients with HBsAg loss (1/76) in our postpartum 96-week cohort, we could not further assess the prediction of pgRNA and HBcrAg levels for HBsAg loss following treatment. Third, the study population included pregnant CHB carriers in the immune tolerance stage receiving antiviral prophylaxis. Therefore, the conclusions of this study cannot be generalized to all patients with HBV infection. More studies that include patients with different infection periods or patients with low viral loads are needed to verify the roles of pgRNA and HBcrAg in predicting HBeAg seroconversion and HBsAg reduction.

In conclusion, postpartum pgRNA decline together with peak ALT level, as independent predictors, were positively correlated with the rates of HBsAg reduction postpartum among pregnant HBeAg-positive CHB carriers, indicating that pgRNA could be a potential serological marker for monitoring HBV progression after treatment cessation in this special population.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author. .

Ethics statement

The study was reviewed and approved by the ethics committee of Second Affiliated Hospital of Chongqing Medical University. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

Author contributions

Study concept and design: C-RW, PH; Acquisition of data: C-RW, QZ, X-QL, Y-QD, QT; Analysis and interpretation of data: C-RW, HL, YC, J-SW; Drafting of the manuscript: C-RW; Critical revision of the manuscript for important intellectual content: PH, G-CZ; Statistical analysis: C-RW, G-CZ. Administrative and material support: PH. All authors contributed to the article and approved the submitted version.

Funding

This work was supported in part by grants from the National Science and Technology Major Project of China (2017ZX10202203008, 2017ZX10202203007), the National Natural Science Foundation of China (81772171, 82203391), the Chongqing Talents Project (cstc2021ycjh-bgzxm0150).

Acknowledgments

We thank all the patients who participated in this research.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2022.1055774/full#supplementary-material

SUPPLEMENTARY TABLE 1
Characteristics of biomarkers of 76 analyzed pregnant CHB carriers with HBeAg positive from the TDF and LDT treated group.

SUPPLEMENTARY TABLE 2
Prediction of HBsAg reduction by HBV biomarker cutoffs.

SUPPLEMENTARY TABLE 3
Prediction of HBeAg seroconversion by HBV biomarker cutoffs.

SUPPLEMENTARY FIGURE 1
The flow chart of a process for identifying eligible patients. CHB, Chronic hepatitis B; MTCT, Mother to child transmission; HBsAg reduction, HBsAg decrease >0.3log$_{10}$IU/mL from baseline to last date of follow-up.

SUPPLEMENTARY FIGURE 2
The changing pattern of HBsAg and ALT levels for each patient with HBsAg reduction (N=13) during follow-up.

SUPPLEMENTARY FIGURE 3
Correlation of pgRNA, HBcrAg levels and other HBV markers in pregnant CHB carriers with HBeAg positive. A) Correlation matrix of parameters. The size of circle is proportional to Spearman's correlation coefficient. B) Scatterplots of serum pgRNA or HBcrAg levels (y-axis) vs. other HBV parameters. R= Spearman 's correlation coefficient; *P* = *P* value of correlation t-test. Baseline, 24-28 weeks of gestation; near delivery, 32-36 weeks of gestation; postpartum, 2-6 weeks after delivery.

SUPPLEMENTARY FIGURE 4
Calibration curve for the combined prediction model to predict probability of HBeAg seroconversion.

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frontiers | Frontiers in Cellular and Infection Microbiology

TYPE Original Research
PUBLISHED 12 January 2023
DOI 10.3389/fcimb.2022.1053608

OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to Virus and Host, a section of the journal Frontiers in Cellular and Infection Microbiology

RECEIVED 26 September 2022
ACCEPTED 19 December 2022
PUBLISHED 12 January 2023

CITATION
Li M, Zong Z, Xiong X, Fan J, Zhong H, Liu N, Ye W and Jing J (2023) Ascites re-compensation in HBV-related first decompensated cirrhosis after anti-viral therapy. *Front. Cell. Infect. Microbiol.* 12:1053608. doi: 10.3389/fcimb.2022.1053608

Ascites re-compensation in HBV-related first decompensated cirrhosis after anti-viral therapy

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Effective antiviral therapy can significantly improve the long-term prognosis of HBV-related decompensated patients, and re-compensation may be achieved in part of the patients. To explore the re-compensation of ascites after HBV suppression and the risk factors, the clinical outcomes of 196 consecutive patients with HBV-related first decompensated cirrhosis of ascites treated with nucleos(t)ide analogue (NUC) were analyzed retrospectively. Among these patients, the median serum HBV DNA level was 5.0 (IQR, 3.0-6.0) $\log_{10}$ IU/mL before treatment. Most patients were given NUC with high barrier to resistance including ETV (152), TDF (1) and TAF (1). Initial combination of LAM plus ADV and LdT plus ADV was used in 41 patients and 1 patients, respectively. After NUC treatment, the percentage of patients with ascites regression was 77.6%, 81.4%, 70.5%, 93.8%, 80.8% at 12, 24, 36, 48, 60 months, respectively (P<0.001). The distribution of ascites severity showed that the patients' ascites improved, with the proportion of no ascites and mild ascites gradually increased. The proportion of re-compensation of ascites defined as negative HBV DNA, improved liver function and ascites regression (off diuretics) was 59.7%, 70.0%, 52.3%, 59.4%, 46.2% at 12, 24, 36, 48, 60 months (P<0.001). The rate of ascites regression was higher in viral response (VR) cohort when compared with that in non-VR cohort. Univariate and multivariable analysis showed that level of serum ALT (OR:0.988, 95%CI, p=0.029) and load of serum HBV DNA (OR:0.78895%CI, p=0.044) at baseline were risk factors of re-compensation of ascites. This study demonstrated that antiviral therapy could reverse decompensation of ascites in HBV-related first decompensated cirrhosis and the level of ALT and HBV DNA were risk factors of ascites re-compensation.

KEYWORDS

decompensated cirrhosis, antiviral therapy, re-compensation of ascites, hepatitis B virus, liver cirrhosis

1 Introduction

Hepatitis B virus (HBV) infection is a major cause of acute and chronic liver disease globally, which usually progresses to liver fibrosis, liver cirrhosis and hepatocellular carcinoma (HCC). Currently, despite the fact that preventive vaccines have been used for decades as well as the use of effective and well-tolerated viral suppressive medications since 1998, an updated estimate indicated that the total global HBV infection prevalence increased to 3.9%, corresponding to 292 million people globally, in 2016 (Nguyen et al., 2020). It is estimated that the prevalence rate of HBsAg positive in the general population in China is 5% ~ 6%, thus the chronic HBV infection is about 70 million cases, including 20 to 30 million cases of chronic Hepatitis B (CHB) (Liu et al., 2019).

The natural history of liver cirrhosis is characterized by an asymptomatic compensated phase followed by a decompensated phase (European Association for the Study of the Liver, 2018). Decompensated cirrhosis is a common cause of admissions, and these patients often have complex medical needs and are at high risk of in-hospital death. The typical clinical manifestations of decompensated cirrhosis include ascites, jaundice, hepatorenal syndrome, hepatic encephalopathy and variceal haemorrhage (Fontana, 2003). Among them, ascites is the most common manifestation of decompensation in cirrhosis, as 5% to 10% of patients with compensated cirrhosis per year develop to this complication (Ginés et al., 1987). Renal sodium retention due to the activation of sodium retention systems such as the renin-angiotensin-aldosterone system (RAAS) and sympathetic nervous system is the main cause of ascites. The resulting positive fluid equilibrium eventually causes the volume of the extracellular fluid to expand. The reduction of effective blood volume due to dilatation of visceral arteries is the main determinant of these changes (European Association for the Study of the Liver, 2018; Bernardi et al., 2015). The occurrence of ascites can affect social and economic life, which often leads to hospitalization, requires long-term treatment, and is the direct cause of further complications such as spontaneous bacterial peritonitis (SBP), restrictive ventilatory dysfunction, and abdominal hernia. The presence of ascites can predict a poor prognosis, with five-year survival rates decreasing from about 80% in compensated patients to about 30% in patients with decompensated cirrhosis of ascites (D'Amico et al., 2006).

Reversal of the decompensated state (defined as ascites re-compensation) in HBV-related decompensated cirrhosis of ascites treated with antiviral therapy is of significant prognostic significance. The concept of re-compensation implies that there is at least partial regression of the structural and functional changes of cirrhosis after removal of the aetiology of cirrhosis. At present, several studies showed that antiviral therapy had a significant effect on patients with decompensated HBV cirrhosis. Among them, a South Korean 10-year follow-up cohort study showed that MELD and CTP scores in patients with decompensated hepatitis B cirrhosis were decreased after effective antiviral therapy, suggesting that some patients might be re-compensated (Jang et al., 2018). The criteria of re-compensation and scoring system to predict re-compensation in patients with HBV-related decompensated cirrhosis were also explored in some studies (He et al., 2022; Kim et al., 2022; Wang et al., 2022). However, the definition of re-compensation had not been unified.

Ascites could present alone in 36% of patients and in combination with other complications in 37%. Therefore, it marked the transition to decompensation in 73% of cirrhotic patients. Moreover, it was also associated with worse clinical outcomes with higher mortality (D'Amico et al., 2022). As the clinical manifestations of patients with decompensated hepatitis B cirrhosis were complicated, it was still unclear whether different types of complications should be separately investigated or together. A multicenter retrospective case-control study included 553 patients with re-compensation and 3400 patients with acute decompensation with different causes. The regression analysis was conducted separately according to the different complications including gastrointestinal bleeding, bacterial infection, hepatic encephalopathy and ascites (Xu et al., 2021). In our study, we focused on ascites only as ascites was the most common reason of decompensation in cirrhosis patients. The re-compensation of ascites after HBV suppression and the factors associated with re-compensation of ascites in patients with HBV-related first decompensated cirrhosis of ascites were investigated.

2 Patients and methods

2.1 Study population

From September 2015 to November 2020, 265 patients with first decompensated HBV cirrhosis of ascites who were treated with antivirals were retrospectively screened in the Second Hospital of Nanjing. Clinical data of patients were collected using electronic medical record systems.

The inclusion criteria were as follows: (1) the reason for hospitalization was hepatitis B decompensated cirrhosis; (2) clinical symptoms of hospitalized patients included ascites; (3) HBV DNA>500 IU/mL; (4) patients were given antiviral drugs for the first time; (5) patients were followed up for at least 1 year. The exclusion criteria were as follows: (1) co-infection with other viral hepatitis; (2) death within 6 months; (3) less than one year of the follow-up time for the patients.

The study was approved by the ethical committee of the Second Hospital of Nanjing (2020-LY-kt043) and the requirement for informed consent was waived.

2.2 Diagnostic criteria, grading and definition

The presence of cirrhosis was determined based on liver histology findings, gross findings during surgery, or radiological findings of an irregular liver margin with ascites, varices, or thrombocytopenia (<10^5 cells/mm^3) (Kim et al., 2018). Decompensated cirrhosis was characterized by the development of overt clinical signs, including ascites, jaundice, hepatorenal syndrome, hepatic encephalopathy and variceal haemorrhage (Fontana, 2003).

Clinically, according to the amount of ascites, it could be divided into grade 1 (small amount), grade 2 (medium amount) and grade 3 (large amount). Grade 1 or a small amount of ascites: ascites could only be found through ultrasound examination; patients generally did not show abdominal distension; negative mobility dullness; under ultrasound, the ascites was located in each space with a depth < 3 cm. Grade 2 or moderate ascites: patients often had moderate abdominal distention and symmetrical abdominal heave; physical examination showed negative/positive mobility dullness; under ultrasound, the ascites flooded the intestine, but did not cross the middle abdomen, with a depth of 3 ~ 10 cm. Grade 3 or large ascites: abdominal distention was obvious; positive mobility dullness on physical examination; abdominal distention and even umbilical hernia might occur; under ultrasound, ascites occupied the entire abdominal cavity, and the middle abdomen was filled with ascites, with a depth of > 10 cm.

The primary endpoint was re-compensation of ascites. Clinically, some patients with decompensated HBV cirrhosis of ascites received effective antiviral therapy. Then, the patients' condition was stable (>3 months) with improved liver function, serum HBV DNA negative (<500 IU/mL) and ascites regression after discontinuing diuretics, which were considered as re-compensation of ascites. The improved liver function was defined as improved serum total bilirubin (TBIL), alanine aminotransferase (ALT), aspartate aminotransferase (AST), albumin (ALB) combined with the values of TBIL, ALT, AST less than 2 ULN and ALB more than 28g/L. In this way, we attempted to achieve complete recovery in patients with liver dysfunction and cirrhotic ascites.

2.3 Treatment

All patients were treated with standard medications after diagnosis, such as antiviral therapy, symptomatic relief and supportive treatment. Patients were given the following antivirals for life: entecavir (ETV), lamivudine (LAM) plus adefovir dipivoxil (ADV), tebivudine (LdT) plus ADV, tenofovir fumarate (TDF) or tenofovir alafenamide fumarate (TAF).

2.4 Data collection

We collected demographic, interview, clinical and routine laboratory data on the first contact visit to the hospital for patients with first decompensation of HBV cirrhosis of ascites in an electronic medical record system. Demographic characteristics and interview data included age, gender, history of drug use, and other diseases (such as hypertension and diabetes). The laboratory data included white blood cells (WBC), red blood cells (RBC), hemoglobin, platelet count, TBIL, indirect bilirubin, levels of serum ALB, ALT, AST, creatinine, alkaline phosphatase (ALP), sodium, γ -glutamyltransferase (γ-GT), prothrombin activity, prothrombin time(PT), international normalized ratio (INR), fibrinogen (FIB), serum HBV DNA (detected by real-time fluorescent quantitative PCR with the lower limit of detection of 500 IU/mL). Imaging studies included ultrasound, computed tomography (CT), and magnetic resonance imaging (MRI). The laboratory and imaging data for the study were derived from evaluations performed within a month on the date closest to the baseline and follow-up.

2.5 Propensity score matching

We used logistic regression model to fit three relevant variables ascites grade, HBV DNA level, Child-Pugh grade for propensity score. Nearest neighbor matching (1:1 propensity matching) was used to establish a propensity matched cohort of treatment with either the ETV monotherapy group or the LAM+ADV combination therapy group.

2.6 Statistical analysis

Measurement data were expressed as mean ± standard deviation or median (quartile range [IQR]), and count data were expressed as number of cases or constituent ratio. When comparing the differences between the two groups, the *t*-test was used for continuous variables and the Fisher exact test or chi-square test was used for categorical variables. Based on clinical experience, a number of indicators were included in the univariate logistic regression to identify the factors associated with re-compensation of ascites, and then the significantly associated factors in univariate analysis (P<0.1) were included in multivariate analysis. The area under the ROC curve (AUC)

with 95% confidence intervals (CI) was used to assess the predictive accuracy of the occurrence of re-compensation after at least 1 year of NUC treatment.

All statistical analyses were conducted using SPSS 22.0 and GraphPad Prism8.0.2. Among this study, two-tailed P<0.05 was considered statistically significant.

3 Results

3.1 Demographic characteristics

A total of 265 patients were enrolled in the study. Overall, 69 patients were excluded according to the exclusion criteria. Figure 1 presents the flow diagram of patient selection. 196 patients were included for the final analysis. The 196 patients were followed for 1-5 years with median follow-up time 13.58 months and the flow chart of the total cohort was shown in Figure 1. The baseline characteristics of the total cohort of 196 patients were shown in Table 1. Among these patients, males were predominated (69.9%) and the mean age was 52.0 years. The first decompensated event was ascites (100%). At the beginning of NUC treatment, the median serum HBV DNA level was 5.0 (IQR, 3.0-6.0) $\log_{10}$ IU/mL. The percentage of Child-pugh class A, B, C was 2.0%, 47.4%, 50.5%, respectively (Table 1). Most patients were given NUC with high barrier to resistance including ETV (152/196), TDF (1/196) and TAF (1/196). Initial combination of LAM plus ADV and LdT plus ADV was used in 41 patients and 1 patient, respectively (Table S1).

3.2 Clinical outcomes

During the follow-up period, the proportion of patients with ascites regression was 77.6%, 81.4%, 70.5%, 93.8%, 80.8% at 12, 24, 36, 48, 60 months, respectively (P<0.001) (Figure 2). The distribution of ascites severity showed that the patients' ascites improved, with the proportion of no ascites and mild ascites gradually increased and the proportion of moderate to severe ascites decreased (P<0.001) (Figure 2). The proportion of patients with moderate to severe ascites was 20.9%, 3.1%, 4.3%, 2.3%, 3.1%, 3.8% at baseline, 12, 24, 36, 48, 60 months, respectively. When the re-compensation of ascites was defined as HBV DNA negative (<500 IU/mL), no diuretics, liver function improvement and ascites regression, the proportion of patients with re-compensation of ascites was 59.7%, 70.0%, 52.3%, 59.4%, 46.2% at 12, 24, 36, 48, 60 months (P<0.001) (Figure 2). Then, the baseline characteristics were compared between the re-compensation of ascites group and no re-compensation of ascites group. There were no obvious differences for the percentage of ETV therapy, HBV DNA level, ALT and AST at baseline (Table S2).

To assess the effect of antiviral treatment on biochemical response in patients with decompensated HBV cirrhosis of ascites, total bilirubin, ALT, and AST were measured and compared at baseline and follow-up period (Supplementary Figure S1). The routinely tested parameters of total bilirubin (TB), ALT, AST improved after initiation of antiviral therapy (Figure S1). The rate of ALT normalization in ETV monotherapy group was higher compared with that in the LAM+ADV combination therapy group after 12-month treatment (Table S1). However, after Propensity Score Matching, the ALT normalization rates were similar betweeen these two groups (Figure S2A).

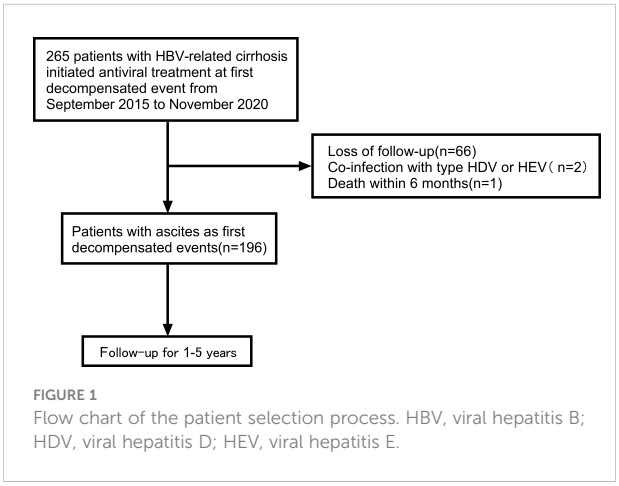

FIGURE 1
Flow chart of the patient selection process. HBV, viral hepatitis B; HDV, viral hepatitis D; HEV, viral hepatitis E.

3.3 Virological response

After antiviral treatment, the proportion of patients with high level of HBV DNA (>2×10^3 IU/mL) was decreased dramatically. The proportion of patients with HBV DNA <500 IU/mL increased to 89.8%, 88.6%, 90.9%, 96.9%, 92.3% at 12, 24, 36, 48, 60 months respectively (P<0.001) (Figure 3). The virological response (VR) rates of ETV monotherapy therapy and LAM+ADV combination therapy were 93.42% and 78.05% after 12-month antiviral treatments, respectively (P<0.05) (Table S1). After Propensity Score Matching, the virological response rates of these two types of antiviral therapy were 95.12% and 80.05%, respectively (P<0.05) (Figure S2B).

The incidence of ascites regression was 77.6%, 81.4% and 70.5% at 1-year, 2-year and 3-year, respectively. Among patients who achieved VR, the incidence of ascites regression was 80.2%, 83.9%, and 77.5% at 1-year, 2-year and 3-year respectively. Among the 20 patients who failed to achieve VR, the incidence of ascites regression was only 47.3%, 62.5%, and 25% at 1-year, 2-year, and 3-year, respectively. The rate of

TABLE 1 Baseline characteristics of the patients in the total cohort.

Varibles	Total (n=196)
Age, y	52.0 ± 10.3
Male, n	137 (69.9)
Diabetes mellitus, n	20 (10.2)
Hypertension, n	16 (8.2)
WBC, $\times10^9$/L	3.6 (2.7-5.1)
RBC, $\times10^{12}$/L	3.6 ± 0.6
Hemoglobin, g/L	115 (102.3-127.8)
Platelet, $\times10^9$/L	58.5 (41.5-90.8)
Total bilirubin, μmol/L	37.8 (23.5-67.5)
Indirect bilirubin, μmol/L	19.1 (13.0-30.0)
Albumin, g/L	30.9 (27.0-34.7)
ALT, IU/L	43.7 (31.4-123.7)
AST, IU/L	64.2 (42.8-131.2)
Creatinine, μmol/L	67 (57-78)
ALP, IU/L	108.5 (85.9-140.4)
Sodium, mmol/L	140 (138.1-141.8)
γ-GT, U/L	63.8 (33.1-114.5)
INR	1.4 (1.3-1.7)
Prothrombin activity, %	50.4 (42.1-58.5)
Prothrombin time (s)	15.2 (16.5-19.0)
FIB, g/L	1.4 (1.1-1.7)
HBV DNA, $\log_{10}$ IU/mL	5.0 (3.0-6.0)
Child-Pugh class	
A	4 (2.0)
B	93 (47.4)
C	99 (50.5)
MELD score	11.0 (8.0-15.0)

RBC, red blood cell count; WBC, white blood cell count; ALT, alanine aminotransferase; AST, aspartate aminotransferase; ALP, alkaline phosphatase; γ-GT, γ-glutamyl transferase; INR, international normalized ratio; FIB, fibrinogen; MELD, model for end-stage liver disease.

ascites regression in VR cohort was higher than that in non-VR cohort, which suggested that VR might be one of the determinants of disappearance of ascites in patients with first decompensated HBV cirrhosis of ascites after antiviral treatment (Figure 3). There was no obvious difference between the rate of ascites regression in ETV monotherapy group and that in LAM +ADV combination therapy at 1-year (Figure S2C).

3.4 Factors associated with re-compensation of ascites

In the entire cohort, univariate analysis based on the competitive risk model revealed a number of pre-treatment variables associated with re-compensation of ascites including hemoglobin, bilirubin, ALT, AST, ALP, PT (INR), HBV DNA, MELD score. These significant variables were incorporated into the multivariable analysis. The results showed that ALT (OR:0.988, 95%CI, p=0.029) and HBV DNA (OR:0.788, 95% CI, p=0.044) were risk factors of re-compensation of ascites (Table 2).

Then, The ROC curve analysis revealed that when setting the cut-off value of ALT<46.1 IU/L and the cut-off value of HBV DNA<5 $\log_{10}$ IU/mL, the area under curve (AUC) of ALT and HBV DNA were 0.6866 [95% confidence interval (95% CI):0.609-0.764, P<0.001] and 0.6167 [95% confidence interval (95%CI):0.534-0.699, P=0.012], respectively. (Figure 4) The sensitivity and specificity of ALT were 57.70% and 77.80%, with positive predictive value of 36.52%, negative predictive value of 25.93%, + LR of 0.74 and - LR of 0.57 and the sensitivity and specificity of HBV DNA were 33.35% and 81.95%, with positive predictive value of 57.02%, negative predictive value of 25.61%, + LR of 1.85 and - LR of 0.81.

4 Discussion

Antiviral therapy for chronic hepatitis B virus prevents progression to clinical complications associated with decompensation of cirrhosis and hepatocellular carcinoma (Marcellin et al., 2013). Several studies have generally focused on the risk of mortality and hepatocellular carcinoma, development of decompensation, and identification of related factors (Kim et al., 2022). However, considering the corrective effect of NUCs on liver function and fibrosis, it is also important to elucidate its potential effect on the reversal of decompensated cirrhosis complications. Re-compensation is a special phase of decompensated liver cirrhosis. After effective treatment over time, liver function enables the patient to perform daily activities without the complications associated with decompensated cirrhosis (Zhao et al., 2020). To date, there has been a lack of a comprehensive assessment to identify patients with a "re-compensated advantage". In this study, we observed the occurrence of ascites re-compensation after anti-viral therapy in patients with HBV-related first decompensated cirrhosis and analyzed factors associated with re-compensation of HBV-related decompensated cirrhosis of ascites. Most patients could achieve ascites re-compensation after effective NUC therapy. It was also demonstrated that ALT and HBV

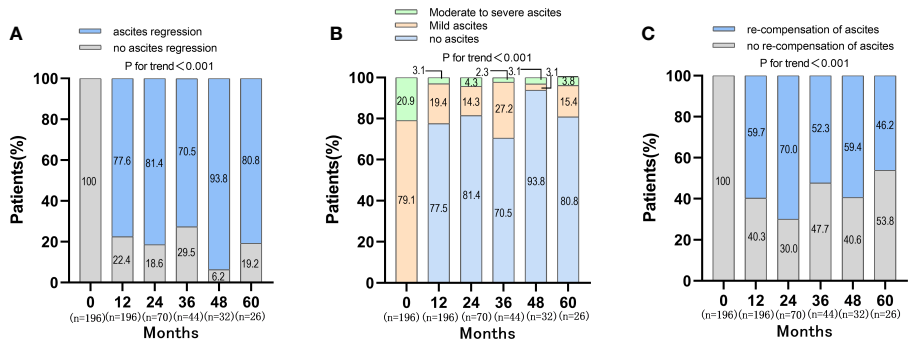

FIGURE 2
Changes of ascites and re-compensation of ascites in patients with first decompensated HBV cirrhosis of ascites after NUC treatment. **(A)** Changes of ascites in patients after NUC treatment at baseline, 12, 24, 36, 48 and 60 months; **(B)** Distribution of ascites severity in patients after NUC treatment at baseline, 12, 24, 36, 48 and 60 months; **(C)** Re-compensation of ascites in patients after NUC treatment at baseline, 12, 24, 36, 48 and 60 months.

DNA at baseline were predictors of re-compensation of the decompensated HBV-related cirrhosis of ascites.

The objective of treatment of decompensated cirrhosis was to improve liver function, reduce secondary decompensated events, prolong the survival time and even achieve the re-compensation. Clinically, the definition of re-compensation had not been unified. Simply, it should include persistent absence of complications (He et al., 2019). In Baveno VII concensus, the criteria of re-compensation should include removal/suppression/cure of the primary aetiology of cirrhosis; resolution of ascites (off diuretics), encephalopathy (off lactulose/rifaximin) and absence of recurrent variceal haemorrhage (for at least 12 months); stable improvement of liver function (albumin, INR, bilirubin) (De Franchis et al., 2022). However, these criteria did not define the cut-off values for stable improvement of liver function tests. Recent research suggested that MELD score <10 and/or liver function tests within Child-Pugh A would be used as a criterion for stable improvement of liver function tests (Wang et al., 2022). In this study, the values of TBIL, ALT, AST and ALB were used as the criteria for stable improvement of liver function, which could be easily obtained from the liver function test. There were 77.6%, 81.4%, 70.5%, 93.8%, 80.8% of patients achieving ascites regression at 12, 24, 36, 48, 60 months after anti-viral therapy in HBV-related first decompensated cirrhosis of ascites, respectively. However, when the ascites re-compensation was

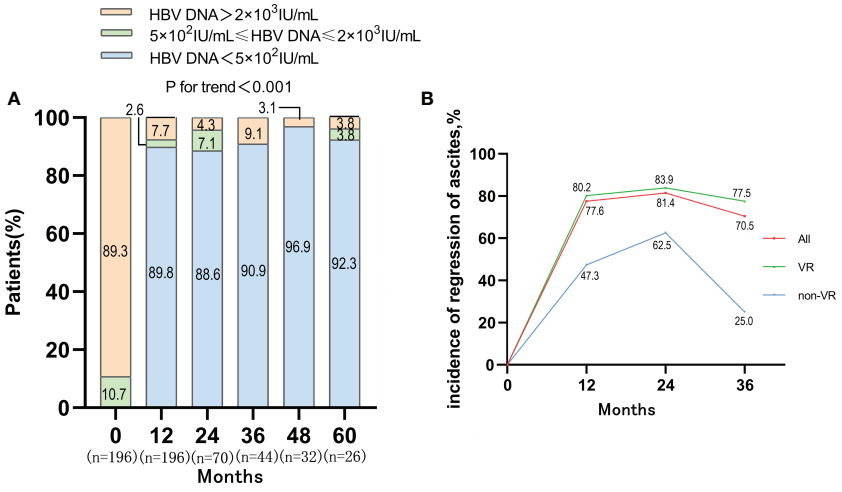

FIGURE 3
VR and the incidence of regression of ascites in VR and non-VR group in patients with first decompensated HBV cirrhosis of ascites after NUC treatment. **(A)** Dynamic change of HBV DNA level after antiviral treatment; **(B)** Regression of ascites occurred with incidences of 77.6%, 81.4% and 70.5% in the whole cohort at 1, 2 and 3 years, and the VR cohort achieved a higher re-compensated rate than the non VR cohort.

TABLE 2 Factors associated with re-compensation of ascites following NUC treatment in the total cohort.

Factors	Univariate analyses	P value	Multivariate analyses	P value
	OR (95% CI)		OR(95% CI)	
Male, n	1.032 (0.520-1.032)	0.929		
Diabetes mellitus, n	0.677 (0.255-1.799)	0.434		
Hypertension, n	0.454 (0.160-1.288)	0.138		
WBC, $\times10^9$/L	0.904 (0.786-1.040)	0.157		
RBC, $\times10^{12}$/L	0.825 (0.506-1.345)	0.44		
Hemoglobin, g/L	0.982 (0.968-0.997)	0.018	0.989 (0.972-1.005)	0.185
Platelet, $\times10^9$/L	0.996 (0.989-1.004)	0.345		
Total bilirubin, μmol/L	0.991 (0.983-0.998)	0.015	0.996 (0.986-1.005)	0.358
Albumin, g/L	1.004 (0.946-1.066)	0.893		
ALT, IU/L	0.993 (0.988-0.998)	0.006	0.988 (0.978-0.999)	0.029
AST, IU/L	0.994 (0.990-0.999)	0.012	1.008 (0.999-1.017)	0.089
Creatinine, μmol/L	0.995 (0.982-1.009)	0.521		
ALP, IU/L	0.993 (0.985-1.000)	0.066	0.996 (0.988-1.004)	0.322
Sodium, mmol/L	1.007 (0.969-1.047)	0.727		
Prothrombin activity, %	1.014 (0.991-1.037)	0.242		
Prothrombin time, s	0.900 (0.809-1.001)	0.052	0.869 (0.733-1.030)	0.104
FIB, g/L	1.108 (0.664-1.851)	0.694		
HBV DNA, $\log_{10}$ IU/mL	0.767 (0.624-0.943)	0.012	0.788 (0.625-0.994)	0.044
Child-Pugh class	0.941 (0.783-1.132)	0.521		
MELD score	0.925 (0.867-0.986)	0.017	1.024 (0.905-1.159)	0.71

RBC, red blood cell count; WBC, white blood cell count; ALT, alanine aminotransferase; AST, aspartate aminotransferase; ALP, alkaline phosphatase; FIB, fibrinogen; MELD, model for end-stage liver disease.

defined as disappeared ascites (off diuretics), HBV DNA negative (<500 IU/mL) and liver function improvement, there were only 59.7%, 70.0%, 52.3%, 59.4%, 46.2% patients at 12, 24, 36, 48, 60 months achieving the objective, respectively.

In the context of effective etiological control of cirrhosis, multiple studies have shown that patients in compensated cirrhosis stage may reverse or even return to the non-cirrhotic stage (Marcellin et al., 2013; Jang et al., 2018; Rong et al., 2022). Some patients with decompensated hepatitis B cirrhosis could achieve improvements in liver function and a reduction in portal-hypertension-related complications after effective antiviral therapies (Singal and Fontana, 2012). A retrospective study screened 311 patients with decompensated HBV cirrhosis and Child-Pugh scores of 7 or higher at baseline and re-compensation was defined as the restoration of cirrhosis status to a Child-Pugh score of 5, which was maintained for at least 2 months. Then, re-compensation occurred in 57.2% and 66.7% of the subjects in the derivation and validation cohorts after NUC therapies (Kim et al., 2022). In this study, the liver function and HBV DNA levels were significantly improved after NUC therapy for more than 1 year. Symptom of ascites was also relieved dramatically and the percentage of mild, moderate to severe ascites decreased from 79.1%, 20.9% to 19.4%, 3.1% separately after 1 year of NUC therapy. Further, our data showed that the regression of ascites was related to the viral response. The percentage of patients without ascites was higher in viral response group compared with non-viral response group after antiviral therapy (80.2% vs 47.3%, 83.9% vs 62.5%, 77.5% vs 25% at year 1, 2, 3, separately), which was similar to previous research (Hanafy et al., 2019). Our results strongly supported the possibility of ascites re-compensation after effective antiviral therapy for patients with HBV-related first-time decompensated cirrhosis of ascites.

In this study, most patients were given NUC with high barrier to resistance including ETV, TDF and TAF. Initial combination of LAM plus ADV was used in 20.9% patients as the combination treatment approach was also tried in some chinese hospitals 7 years ago. There were also some studies

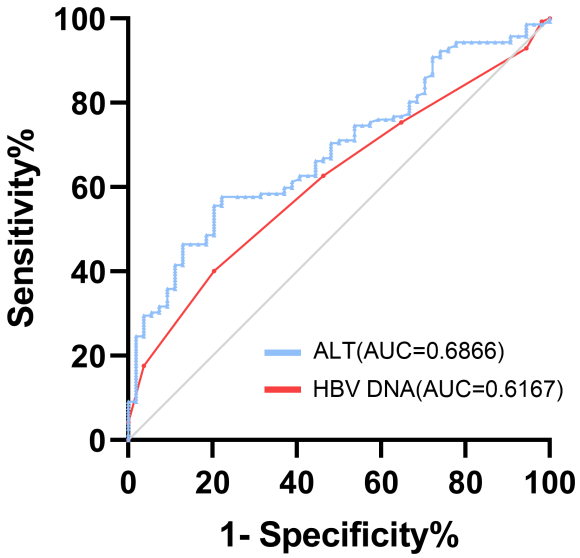

FIGURE 4
ROC curve analysis used to calculate the best cut-off point of ALT and HBV DNA level for predicting the occurrence of re-compensation of ascites in patients with HBV-related first decompensated cirrhosis of ascites. Best cut-off points of ALT and HBV DNA levels as 46.1 IU/L (sensitivity%,57.70% and specificity%, 77.80%) and 5 $\log_{10}$ IU/mL (sensitivity%,33.35% and specificity%, 81.95%). The area under curve (AUC) of ALT and HBV DNA were 0.6866 [95% confidence interval (95%CI):0.609-0.764, P<0.001] and 0.6167 [95% confidence interval (95% CI):0.534-0.699, P=0.012], respectively. AUC, area under the curve; ROC, receiver operating characteristic.

exploring the efficacy of LAM combined with ADV versus ETV monotherapy in patients with hepatitis B associated decompensated cirrhosis. The reduction of ALT levels, HBV DNA levels, the rate of ALT normalization, undetectable HBV DNA, HBV e antigen (HBeAg) loss, HBeAg seroconversion and mortality were similar between the two groups (Lian et al., 2013; Peng et al., 2014). Our data showed that both of the virological response rate and ALT normalization rate were higher after 12-month therapy in ETV monotherapy group compared with combination group. However, after propensity matching analysis there were no obvious differences for the ALT normalization rate between these two groups. ETV monotherapy might be a better choice for patients with HBV-related decompensation compared with LAM plus ADV. Most patients taking LAM plus ADV had switched to the first-line regimens as the price of ETV and TDF had been decreased dramatically in China.

The age and gender distribution is related to the severity of liver diseases. The average age was 52 years and the proportion of male patients was up to 69.9% as all of the included patients were in the stage of decompensation in this study, which suggested the disease progression was more likely in more advanced age and males might be more likely to have severe HBV-related diseases, consistent with prior findings. A study showed that the sex ratio (male/female) increased with severity of liver diseases: 1.2 in HBV carrier, 1.8 in chronic hepatitis, 3.3 in HBV-related cirrhosis and 3.9 in HBV-related HCC (Liu et al., 2022). HBV genotype plays a role in the progression of HBV-related liver cirrhosis and HCC as well as response to antiviral therapy (Lin and Kao, 2017). Genotype B or C are most prevalent in China. Some studies found that HBV genotype B is associated with a slower rate of progression to cirrhosis, and a lower rate of HCC development compared with genotype C. A significantly higher incidence of HCC had been reported in persons infected with genotypes C or F compared with the others (Ching et al., 2016; Terrault et al., 2018; McMahon et al., 2021). The genotype was not detected routinely in this study, thus its relationship with re-compensation of ascites could not be evaluated, which still need to be further explored in future research.

The significant differences in clinical outcomes based on the presence or absence of re-compensation highlighted the importance and necessity of predicting re-compensation in the treatment of patients with decompensated cirrhosis of ascites receiving antiviral therapy against infection (D'Amico, 2014; Vinaixa et al., 2017; Hanafy et al., 2019). In this study, we identified two pre-treatment variables, ALT and HBV DNA, that were associated with re-compensation of HBV-associated decompensated cirrhosis of ascites treated with NUC therapy. In our study, the rate of undetected HBV DNA patients increased after antiviral treatment. However, it decreased from month 48 (96.9%) to month 60 (92.3%), which might be related with the limited number of patients in month 60. VR was associated with the ascites regression, which had also been demonstrated by another research (Kim et al., 2022). The HBV DNA level at baseline was one of the variables in the multivariable analysis, which was in accord of the results about VR, as patients with higher level of HBV DNA might face more difficulty to achieve VR. Higher ALT levels before treatment in patients with hepatitis B, as markers of increased inflammatory activity for liver necrosis, was related to the worse clinical outcomes, which suggested that biochemical response might be also important for the ascites re-compensation after NUC therapy in HBV-associated decompensated cirrhosis of ascites. Besides, an upsurge of serum HBV DNA and hepatitis B surface antigen levels usually precedes the abrupt rise of ALT levels, which could explain the synergistic effect between level of HBV DNA and ALT (Chang and Liaw, 2014; Chien and Liaw, 2022). Further, it was found that the best cut-off value of ALT and HBV DNA levels was chosen at 46.1 IU/L and 5 $\log_{10}$ IU/mL by ROC curve analysis.

The advantage of this study was that the ascites re-compensation was strictly defined as disappeared ascites (off

diuretics), HBV DNA negative (<500 IU/mL) and liver function improvement according to the Baveno VII consensus and the occurrence was explored in patients with HBV-related first decompensation cirrhosis of ascites. However, there were several limitations to our study. First, this study was a retrospectively designed, thus inherent limitations of selection bias were inevitable. Patients who had less than 1 year follow up and who had death within 6 months were excluded from the criteria might overestimate the prognosis. Second, as our study was based on a limited number of patients from a single medical center, the factors associated with ascites re-compensation in HBV-related decompensated cirrhosis of ascites was unique, there was no other cohorts available for external validation. Besides, the sample size was too small in the 60-months group, which might affect the reliability of conclusion. Third, the low limit of HBV DNA was 500 IU/mL in this study and the proportion of patients with VR might change if the HBV DNA assay with 10 IU/mL detection limit was used.

In conclusion, this study showed that antiviral therapy could reverse ascites and the level of ALT and HBV DNA were risk factors of ascites re-compensation after antiviral therapy in patients with HBV-related decompensated cirrhosis of ascites. Overall, this study provided important information on the changes of ascites after HBV suppression in patients with decompensated HBV cirrhosis and shed light on the future treatment and care in HBV cirrhosis patients. These results may warrant further validation in prospective, multicenter, large-scale trials in future.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding authors.

Ethics statement

The studies involving human participants were reviewed and approved by the ethical committee of the Second Hospital of Nanjing. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements. Written informed consent was not obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

Author contributions

JJ and WY designed the study. ML, ZZ and XX implemented this research. HZ and NL collected medical records. ML, JF and WY drafted the manuscript. All the authors participated in the revision of the manuscript. All authors contributed to the article and approved the submitted version.

Funding

This study was supported by Medical Science and Technology Development Foundation, Nanjing Department of Health (grant number YKK20100 to WY); 333 Project of Jiangsu Province (WY) and Scientific research project of Jiangsu Provincial Health Commission (M2021074 to WY).

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2022.1053608/full#supplementary-material

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frontiers | Frontiers in Cellular and Infection Microbiology

TYPE Original Research
PUBLISHED 17 January 2023
DOI 10.3389/fcimb.2023.1062172

OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to Virus and Host, a section of the journal Frontiers in Cellular and Infection Microbiology

RECEIVED 05 October 2022
ACCEPTED 03 January 2023
PUBLISHED 17 January 2023

CITATION
Zhang Q, Niu S, Yang L, Zhu B, Shi K, Zhang X, Zhang Y, Bi Y, Mu Y and Wang X (2023) A novel prognostic model for predicting the risk of first variceal hemorrhage in patients with HBV-related cirrhosis.
Front. Cell. Infect. Microbiol. 13:1062172.
doi: 10.3389/fcimb.2023.1062172

A novel prognostic model for predicting the risk of first variceal hemorrhage in patients with HBV-related cirrhosis

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Background: Variceal hemorrhage (VH) is a life-threatening complication of cirrhosis. An accurate VH risk evaluation is critical to determine appropriate prevention strategies. We aimed to develop an individualized prediction model to predict the risk of first VH in hepatitis B virus (HBV)-related cirrhotic patients.

Methods: A nomogram was developed based on a retrospective analysis of 527 consecutive HBV-related cirrhotic patients with gastroesophageal varices (GEVs). The nomogram evaluation was performed using the area under the receiver operating characteristic curve (AUC), concordance index (C-index), calibration plot, and decision curve analysis (DCA). The results were verified using an external cohort (n = 187).

Results: We developed a nomogram based on clinical and endoscopic features, including the size of varices, red wale marks, ascites, spleen thickness, γ-glutamyltransferase, and hematocrit. The C-index of the nomogram in the derivation and validation cohort was 0.806 and 0.820, respectively, and the calibration plot fitted well. Compared with those of the North Italian Endoscopic Club (NIEC) and revised NIEC indexes, the AUC (derivation cohort: 0.822 vs. 0.653 vs. 0.713; validation cohort: 0.846 vs. 0.685 vs. 0.747) and DCA curves of this nomogram were better. Further, based on the risk scores, patients were classified into low-, medium-, and high-risk groups, and significant differences were noted in VH incidence among the three risk groups (P <0.001 for each cohort).

Conclusions: An effective individualized nomogram to predict the risk of first VH in HBV-related GEV patients was established, which can assist clinicians in developing more appropriate prevention strategies.

KEYWORDS

gastroesophageal varices, hepatitis B virus, liver cirrhosi, nomogram, variceal hemorrhage, prognostic model

1 Introduction

Variceal hemorrhage (VH) is a serious complication of cirrhotic portal hypertension and one of the leading causes of death worldwide (La Mura et al., 2020). The annual incidence of first bleeding events in patients with gastroesophageal varices (GEVs) is 5–15%, and more than 15% of the initial bleeding episodes are fatal (Shukla et al., 2016; Haq and Tripathi, 2017). Despite progress in diagnosis and therapy of VH, the mortality from the initial bleeding episode remains high (Reverter et al., 2014). Even if the bleeding is controlled, the patients still have very high-risk of recurrent bleeding, which is associated with mortality as high as that of the first bleed (O'Brien et al., 2013). Hepatitis B virus (HBV) infection is a serious global public health problem (Revill et al., 2020), and a large proportion of GEV cases is associated with HBV infection (Liu et al., 2016; Lv et al., 2019). Reducing the incidence of HBV-related VH is important to decrease the overall mortality rate associated with cirrhosis. For this purpose, routine bleeding risk assessment in patients with HBV-related GEVs is essential such that appropriate prophylactic measures are administered to avoid the first VH episode occurrence.

Hepatic venous pressure gradient (HVPG) is considered an excellent VH predictor (La Mura et al., 2020). Patients with HVPG ≥12 mmHg face high VH risk; in contrast, this bleeding risk significantly decreases when HVPG is <12 mmHg or when the HVPG is reduced by more than 20% from the baseline level (Eisenbrey et al., 2013; La Mura et al., 2020). However, the HVPG measurement is an invasive procedure, which is not suitable for routine clinical testing in most patients. Generally, the stratification strategy for GEVs is mainly based on endoscopic screening, which is currently recommended by the guidelines for cirrhotic patients (Garcia-Tsao et al., 2017). The size of varices >5 mm and appearance of red wale marks (RWM) are considered as high-risk features of VH (The North Italian Endoscopic Club for the Study and Treatment of Esophageal Varices, 1988). However, a study reported that only 30% of patients with actual bleeding presented with these endoscopic risk features (The North Italian Endoscopic Club for the Study and Treatment of Esophageal Varices, 1988). Therefore, endoscopic screening alone may not be enough to accurately identify patients with high-risk of VH occurrence.

The combination of the clinical indicators and endoscopic features is considered as the appropriate tool to provide a better assessment of VH risk (The North Italian Endoscopic Club for the Study and Treatment of Esophageal Varices, 1988). Currently, the most widely used indexes to stratify high-risk patients are the North Italian Endoscopic Club described NIEC index and revised (Rev)-NIEC index that was proposed by Carlo Merkel et al. (The North Italian Endoscopic Club for the Study and Treatment of Esophageal Varices, 1988; Merkel et al., 2000). Both indexes are a combination of the Child-Pugh classification and endoscopic parameters including the size of varices and RWM (The North Italian Endoscopic Club for the Study and Treatment of Esophageal Varices, 1988; Merkel et al., 2000). However, both the NIEC and Rev-NIEC indexes were developed primarily based on the data on populations with alcohol issues and/or hepatitis C virus infections. Whether they can accurately predict the VH risk of HBV-related GEV patients is unknown. In addition, these models were proposed more than two decades ago. Considerable advances have been made in liver disease treatment in the past 20 years, especially toward the antiviral treatment of HBV infection (Wang and Duan, 2021). As the medical environment changes, the predictive performance of these models may also change. Moreover, despite the greatly improved outcomes of antiviral therapy for HBV-associated cirrhosis, the incidence of HBV-related VH and the associated mortality remains high (Kim et al., 2011). Therefore, it is necessary to develop a new prediction model to predict the risk of first VH occurrence in HBV-related cirrhotic patients with GEVs, especially in the context of widespread antiviral therapy.

Nomograms are visualization tools of prognosis evaluation, which can accurately and quantitatively predict the prognosis for individual patients (Iasonos et al., 2008; Balachandran et al., 2015). Currently, nomograms to predict clinical outcomes have been widely used in many medical fields to help in decision-making (Liang et al., 2015; Guo et al., 2022). However, to date, no ideal nomogram has been established to predict the risk of HBV-related VH. In the current study, our aim was to develop a nomogram specifically dedicated to predict the risk of first VH for HBV-related cirrhotic patients with GEVs and to validate its predictive performance in an independent group of patients.

2 Materials and methods

2.1 Ethical concerns

Approval was obtained from the Ethical Review Committee of the Beijing Ditan Hospital (Beijing, China). This study followed the ethical principles of the Declaration of Helsinki. Because this was a retrospective observational study, the Ethics Committee waived the need for informed consent. All sensitive patient information was anonymized and deidentified prior to analysis.

2.2 Study population

In total, data on 1,824 consecutive HBV-related cirrhotic patients with GEVs hospitalized at the Beijing Ditan Hospital of Capital Medical University from February 2008 to February 2021 were retrospectively screened. All subjects included in the study met the following inclusion criteria: (1) age at diagnosis ≥20 years; (2) serum hepatitis B surface antigen positive and under antiviral treatment for at least 6 months; (3) diagnosed with cirrhosis (based on clinical manifestations, imaging and blood tests, or liver biopsy); and (4) presence of GEVs confirmed through an endoscopic examination without previous history of VH. Patients with any of the following

Abbreviations: VH, variceal hemorrhage; GEV, gastroesophageal varice; HBV, hepatitis B virus; HVPG, hepatic venous pressure gradient; RWM, red wale mark; NIEC index, North Italian Endoscopic Club index; Rev-NIEC index, revised NIEC index; GGT, γ-glutamyltransferase; HCT, hematocrit; MELD, model for end-stage liver disease; LASSO, least absolute shrinkage and selection operator; IQR, interquartile range; AUC, area under the receiver operating characteristic curve; C-index, concordance index; DCA, decision curve analysis; NPV, negative predictive value; PPV, positive predictive value; AHR, adjusted hazard ratio; CI, confidence interval; HR, hazard ratio.

exclusion criteria were excluded from the study: (1) combined with other liver diseases (such as alcoholic hepatitis, other viral hepatitis, and autoimmune hepatitis); (2) complicated with liver cancer or other space-occupying lesions; (3) having undergone splenectomy, endoscopic treatments, or transjugular intrahepatic portosystemic shunts before inclusion in the study; (4) complicated with other conditions that may cause bleeding, such as ulcers and coagulation disorders; and (5) follow-up of less than one year or missing data. Eventually, 527 patients formed the derivation cohort. In addition, according to the same inclusion and exclusion criteria as the derivation cohort, 187 patients were selected from the Shuguang Hospital affiliated to the Shanghai University of Traditional Chinese Medicine between October 2015 and March 2019 to form a separate validation cohort (Figure 1).

2.3 Data collection

All study patients underwent detailed clinical, blood, endoscopic, and ultrasonographic evaluations within two days after admission, and relevant variables available were acquired from the electronic medical records. These variables included demographic characteristics (age and sex); complications (ascites, bacterial infection, and hepatic encephalopathy); routine laboratory parameters (aspartate aminotransferase, alanine aminotransferase, total bilirubin, γ-glutamyltransferase [GGT], alkaline phosphatase, albumin, white blood cell, red blood cell, platelet, neutrophil-lymphocyte ratio; hematocrit [HCT], potassium; sodium; blood urine nitrogen, creatinine; glucose, prothrombin time activity, prothrombin time, international normalized ratio, and HBV DNA levels), endoscopic parameters (the size of varices and RWM); and ultrasonography findings (portal vein diameter and spleen thickness). The Child-Pugh classification and model for end-stage liver disease (MELD) scores were calculated to evaluate the liver function status for each patient (Pugh et al., 1973; Kamath et al., 2001). All of the above variables were included in the least absolute shrinkage and selection operator (LASSO) Cox regression analysis to filter the candidate variables for the model. The NIEC and Rev-NIEC indexes were calculated according to previously published criteria (The North Italian Endoscopic Club for the Study and Treatment of Esophageal Varices, 1988; Merkel et al., 2000). All prognostic scores and definitions were applied at baseline.

2.4 Follow-up and outcome

The baseline date was defined as the date of admission to the hospital. All patients received regular outpatient or telephonic follow-ups and were hospitalized when needed. According to the guidelines, patients continued to receive antiviral treatment during the follow-up period, if indicated (Wang and Duan, 2021). All study subjects were followed up for one year (or until bleeding event occurred during the follow-up period). The end point of this study was the first gastric or esophageal VH occurrence confirmed by endoscopy. The VH diagnosis was based on the symptoms of hematemesis or/and melena with endoscopic variceal evidence or endoscopically evident active bleeding from gastric or esophageal varices without other possible source of hemorrhage (Garcia-Tsao et al., 2007).

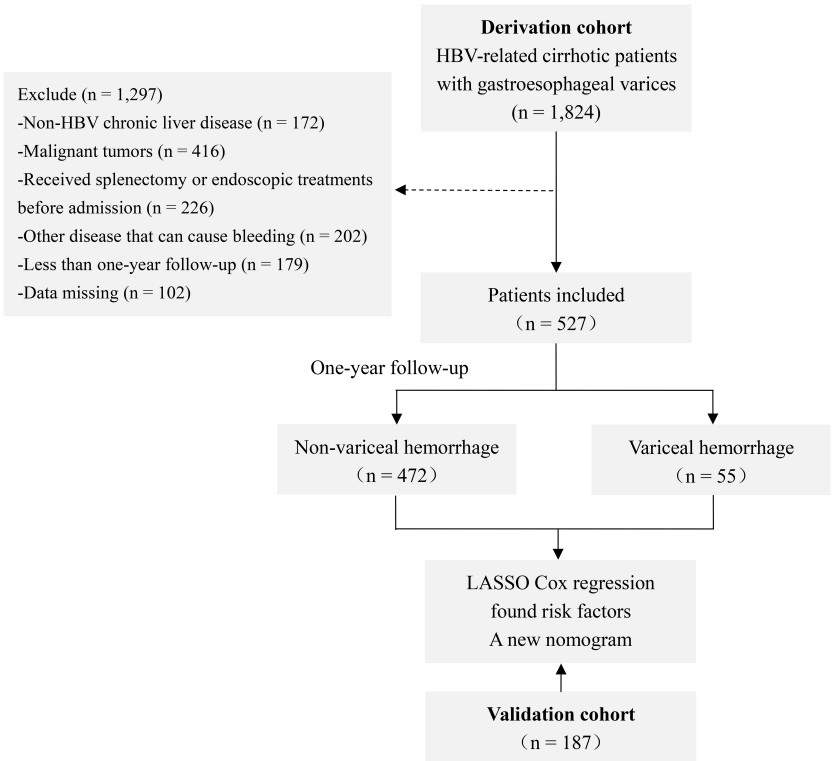

FIGURE 1
Study flow chart. Abbreviations: HBV, hepatitis B virus; LASSO, least absolute shrinkage and selection operator.

2.5 Statistical analysis

Patient baseline characteristics were compared between the derivation and validation cohorts using the Mann-Whitney U test or Student's *t*-test for continuous characteristics and the Pearson's $\chi 2$ test for categorical characteristics. Frequency (percentage) was reported to describe categorical characteristics, and median (interquartile ranges [IQR]) and mean ± standard deviation were reported to describe the continuous characteristics with skewed and normal distributions, respectively. Specific continuous variables were translated into categorized variables using the optimal cut-off value determined by the area under the receiver operating characteristic curve (AUC).

The LASSO regression analysis was performed to select the derivation cohort variables for inclusion in a multivariate Cox regression analysis to estimate the probability of VH (Tibshirani, 1997). The nomogram was elaborated according to the Cox regression coefficients of the identified prognostic factors. The discriminative capacity of the nomogram was assessed using AUC and concordance index (C-index) (Hanley and McNeil, 1982; Pencina and D'Agostino, 2015). The calibration curves were used to compare the consistency between actual observations and predicted probabilities (Van Calster et al., 2016; Alba et al., 2017). The decision curve analysis (DCA) curves were used to evaluate the clinical utility of the predictive model by quantifying the net benefit under different threshold probabilities (Vickers and Elkin, 2006). Patients were further classified into three risk groups using the 25th and 75th percentiles of the nomogram scores distribution as cut-off values (Papatheodoridis et al., 2020). Subsequently, we calculated the specificity, sensitivity, negative predictive value (NPV), and positive predictive value (PPV) for each cut-off. The cumulative VH incidence in the three risk groups was depicted using the Kaplan-Meier curves and compared using the modified log-rank test.

All statistical analyses were performed using the SPSS 22.0 statistical package (SPSS, Inc., Chicago, IL, USA) and R software (version 3.4.3). A two-tailed *P* value of <0.05 was considered to be statistically significant.

3 Results

3.1 Baseline characteristics of the study population

In total, 714 HBV-related GEV patients (527 *and 187* in the *derivation and validation cohorts, respectively*) were included in this study. A total of 55 (10.4%) and 20 (10.7%) patients in the derivation and validation cohorts, respectively, progressed to first VH occurrence at one-year follow-up. In the derivation cohort, the median age of patients was 55.0 years (IQR, 44.0–60.0 years) and 354 (67.2%) of them were males. The most common complication was ascites (57.3%), followed by bacterial infection (19.5%), and hepatic encephalopathy (3.8%). Endoscopy showed that the proportions of small, medium, and large varices were 44.4%, 27.5%, and 28.1%, respectively. RWM was observed in 34.9% of the patients. Most of the patients belonged to the Child-Pugh grade B (50.3%), followed by grade A (32.3%), and grade C (17.5%), while the median MELD score was 10.0 (IQR, 8.0–13.0). While comparing these baseline characteristics of the derivation cohort with that of the validation cohort, we found that patients in the derivation cohort had higher rates of bacterial infection and large varices (P <0.05) and had lower international normalized ratio level (P <0.05). Further details on clinical features of the patients in the two cohorts are summarized in Table 1.

3.2 Independent prognostic factors for VH

The LASSO regression output showed that the size of varices, RWM, ascites, spleen thickness, GGT, and HCT obtained from the derivation cohort were significant predictors of the first VH when the lambda was one standard error (Figures 2A, B). To elucidate the relationship between these potential predictors and the VH outcome, we further performed a multivariable Cox regression analysis using these six factors. However, the results revealed that the adjusted hazard ratios (AHR) of spleen thickness, GGT, and HCT were close to 1 (Supplementary Figure 1); thus, these three continuous variables were converted into categorical variables according to the optimal cut-off value. After adjustment, the above six categorical variables were reincorporated into the Cox regression analyses. Finally, we screened six independent categorical predictors in the derivation cohort: large varices (AHR, 2.566; 95% confidence interval [CI], 1.076–6.123; P = 0.034), RWM (AHR, 3.266; 95% CI, 1.646–6.481; P = 0.001), ascites (AHR, 2.287; 95% CI, 1.157–4.523; P = 0.017), spleen thickness >48 mm (AHR, 2.611; 95% CI, 1.395–4.886; P = 0.003), GGT >130 U/L (AHR, 5.475; 95% CI, 2.917–10.278; P <0.001), and HCT ≤32% (AHR, 2.260; 95% CI, 1.299–3.933; P = 0.004) (Figure 2C).

3.3 Construction and validation of the prognostic nomogram for the VH prediction

Based on the influential factors selected from the derivation cohort, a nomogram was developed to predict the risk of first VH occurrence within one year in HBV-related cirrhotic patients with GEVs (Figure 3). The detailed score for each variable and the estimated risk of first VH occurrence according to the nomogram score in the derivation cohort are presented in Supplementary Figure 2. The application of the nomogram is as follows: each prediction indicator was assigned a corresponding score based on its value on the nomogram. After calculating the total score, we draw a vertical line using the total score and the predicted risk corresponding to the total score is the individual probability of variceal bleeding. For example, one patient had larger varices (5.6 points) and RWM (6.9 points) diagnosed at endoscopy; ultrasonography examination revealed ascites (4.9 points) and spleen thickness was 35mm (0 points); blood tests showed GGT was 200U/L (10 piont) and HCT was 35% (0 points). His total points was 5.6 + 6.9+4.9+0+10+0 = 27.4, and the corresponding probability of bleeding within 1-year was 0.44 (44%) (Supplementary Figure 3). The C-index of the nomogram were 0.806 (95% CI, 0.753–0.859) and 0.820 (95% CI, 0.742–0.898) in the derivation and validation cohorts, respectively, which suggested a good discrimination potential of this nomogram. We also created a

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Table 1 *Baseline characteristics of the patients in the derivation and validation cohorts.*

Variables	Derivation cohort (n = 527)	Validation cohort (n = 187)	*P* value
Age (years)	55.0 (44.0-60.0)	52.0 (43.0-58.0)	0.202
Male sex	354 (67.2)	135 (72.2)	0.204
Ascites	302 (57.3)	106 (56.7)	0.883
Hepatic encephalopathy	20 (3.8)	7 (3.7)	0.975
Bacterial infection	103 (19.5)	20 (10.7)	0.006
The size of varices			0.027
Small	234 (44.4)	92 (49.2)	
Medium	145 (27.5)	61 (32.6)	
Large	148 (28.1)	34 (18.2)	
Red wale marks	184 (34.9)	63 (33.7)	0.762
Alanine aminotransferase (U/L)	36.8 (24.5-64.4)	35.4 (24.4-78.5)	0.702
Aspartate aminotransferase (U/L)	48.6 (33.2-87.5)	47.9 (32.8-91.8)	0.900
total bilirubin (μmol/L)	29.6 (18.6-47.4)	28.9 (17.4-57.4)	0.785
γ-Glutamyltransferase (U/L)	53.2 (28.6-110.7)	50.6 (26.1-128.5)	0.676
alkaline phosphatase (U/L)	99.3 (73.1-135.5)	98.2 (77.7-133.2)	0.835
Albumin (g/L)	31.4 (27.7-36.6)	31.1 (27.1-36.4)	0.593
White blood cell ($\times10^9$/L)	3.3 (2.5-4.7)	3.3 (2.5-4.5)	0.678
Red blood cell ($\times10^{12}$/L)	3.2 (3.7-4.1)	3.2 (3.6-4.2)	0.584
Platelet ($\times10^9$/L)	59.9 (43.6-85.0)	60.3 (44.7-84.0)	0.712
Neutrophil-lymphocyte ratio	1.9 (1.4-2.9)	1.9 (1.3-3.1)	0.988
Hematocrit (%)	35.0 (31.2-38.7)	34.8 (30.6-39.6)	0.965
Potassium (mmol/L)	3.7 (3.5-4.0)	3.7 (3.4-4.0)	0.595
Sodium (mmol/L)	140.1 (137.8-141.8)	140.0 (137.9-142.0)	0.841
Blood urine nitrogen (mmol/L)	5.0 (4.0-6.2)	5.0 (4.1-6.4)	0.502
Creatinine (μmol/L)	63.0 (55.0-74.7)	63.0 (55.0-73.0)	0.689
Glucose (mmol/L)	5.6 (5.0-7.0)	5.6 (4.9-7.5)	0.858
Prothrombin time (s)	14.6 (13.1-16.3)	15.1 (13.4-16.9)	0.074
Prothrombin time activity (%)	66.6 (55.0-78.0)	64.0 (52.8-75.1)	0.099
International normalized ratio	1.2 (1.1-1.4)	1.3 (1.1-1.5)	0.003
HBV DNA ($\log_{10}$IU/ml)	0.0 (0.0-3.6)	0.0 (0.0-3.9)	0.737
Spleen thickness (mm)	48.0 (42.0-56.0)	49.0 (42.0-55.0)	0.726
Portal vein diameter (mm)	12.0 (11.0-13.0)	12.0 (11.0-13.0)	0.405
Child-Pugh grade			0.299
A	170 (32.3)	54 (28.9)	
B	265 (50.3)	91 (48.7)	
C	92 (17.5)	42 (22.5)	
MELD score	10.0 (8.0-13.0)	10.2 (8.0-14.0)	0.424

Data are presented as n (%) or median (interquartile range).
MELD, model for end-stage liver disease.

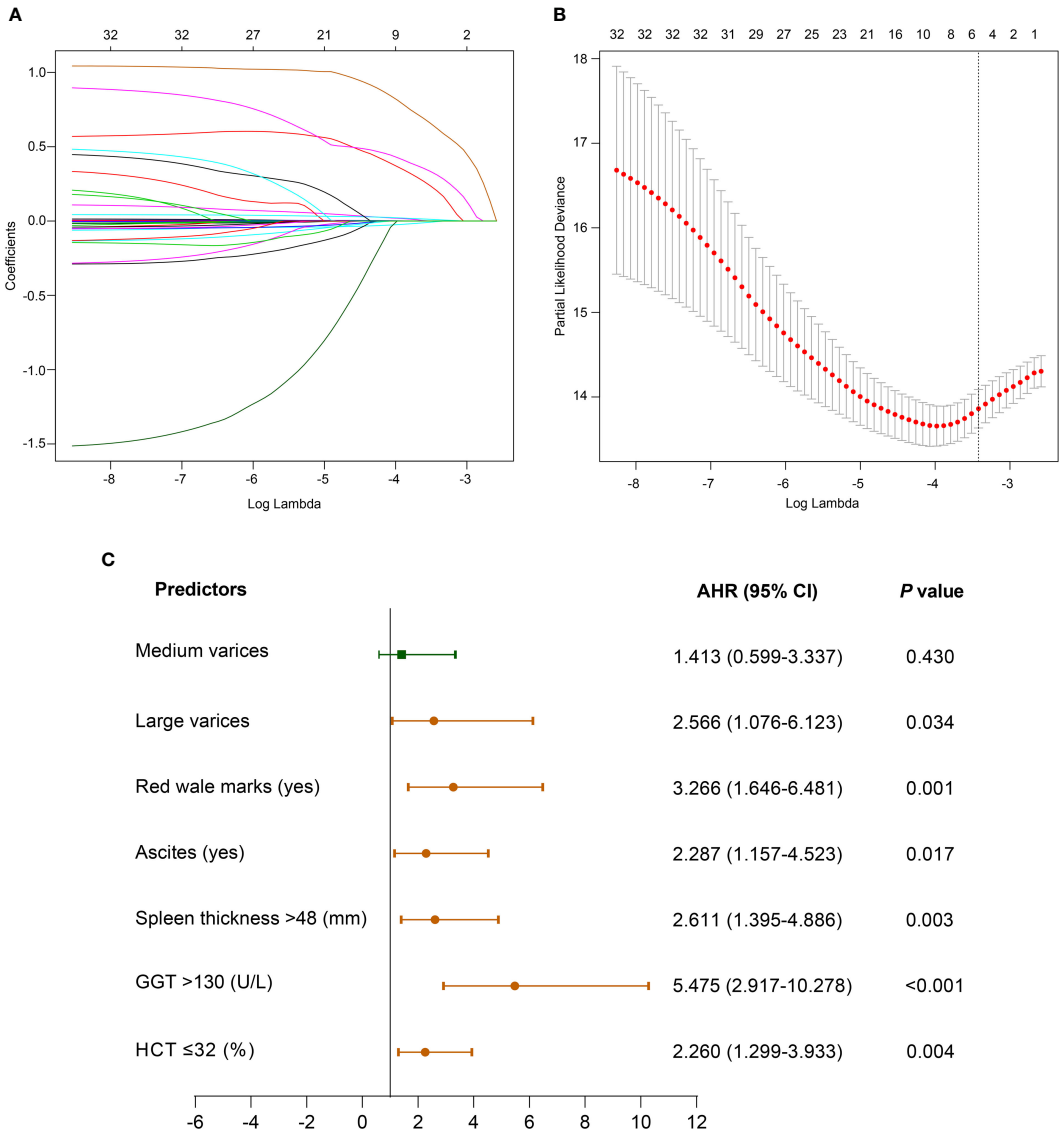

FIGURE 2
Clinical characteristics selection using the LASSO Cox regression model. **(A)** LASSO coefficient profiles of the 32 variables. **(B)** 10-fold cross-validation is applied to select the most suitable parameter using LASSO regression model. The vertical line on the right is plotted with six selected variables, which constructed a more concise model within one standard error. **(C)** Forest plot of multivariate Cox regression analysis of the selected variables in the derivation cohort. Abbreviations: AHR, adjusted hazard ratio; CI, confidence interval; GGT, γ-glutamyltransferase; HCT, hematocrit; LASSO, least absolute shrinkage and selection operator.

calibration plot, which indicated high consistency between the predicted outcomes and actual observations in the derivation (Figure 4A) and external validation cohorts (Figure 4B).

3.4 Comparison of the prognostic nomogram with NIEC and Rev-NIEC indexes

To compare the discriminative ability of the constructed nomogram with those of the traditional (NIEC and Rev-NIEC) indexes, we compared the AUC values of each model. The nomogram had an AUC value of 0.822 (95% CI, 0.787–0.854), which was significantly higher than that of the NIEC index (AUC, 0.653; 95% CI, 0.611–0.694) and Rev-NIEC index (AUC, 0.713; 95% CI, 0.673–0.752) in the derivation cohort (P <0.05 for each model) (Figure 4C). Moreover, the nomogram had the highest AUC value of 0.846 (95% CI, 0.786–0.894) compared with that of the NIEC index (0.685; 95% CI, 0.613–0.751) and Rev-NIEC index (0.747; 95% CI, 0.679–0.808) in the validation cohort (P <0.05 for both) (Figure 4D). We also used DCA to compare the net clinical benefit of these models. As shown in Figures 4E, F, compared with the NIEC and Rev-NIEC indexes, the new nomogram provided greater net benefits both in the derivation and validation cohorts.

3.5 Performance of the nomogram in stratifying risk of patients

As the nomogram points increased, the VH incidence also increased (Figure 5A). For convenience in the clinical evaluation,

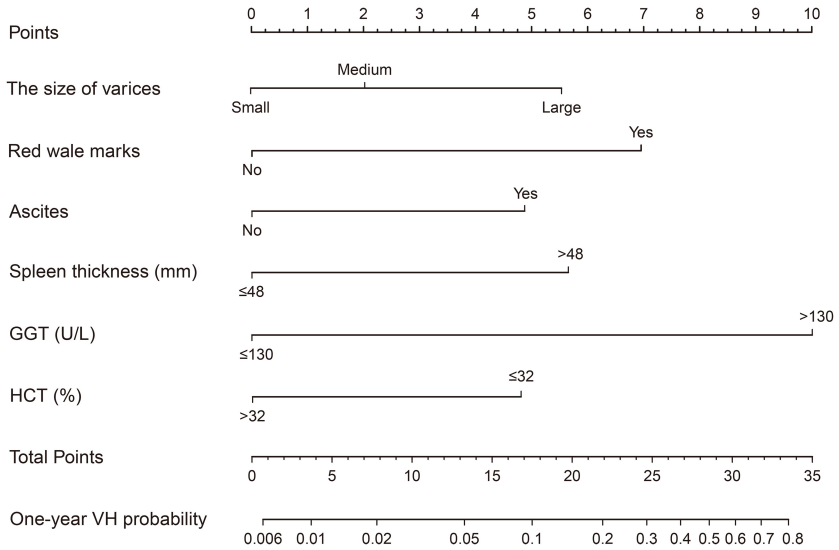

FIGURE 3
Nomogram for predicting the probability of VH within one-year in HBV-related cirrhotic patients with GEVs. The total points are calculated by adding the points of each covariate corresponding to the point on the upper pointing axis. A line is then drawn down to the probability axis at the bottom of the nomogram, which indicated the VH probability within one-year. Abbreviations: GGT, γ-glutamyltransferase; HCT, hematocrit; VH, variceal hemorrhage; HBV, hepatitis B virus; GEV, gastroesophageal varice.

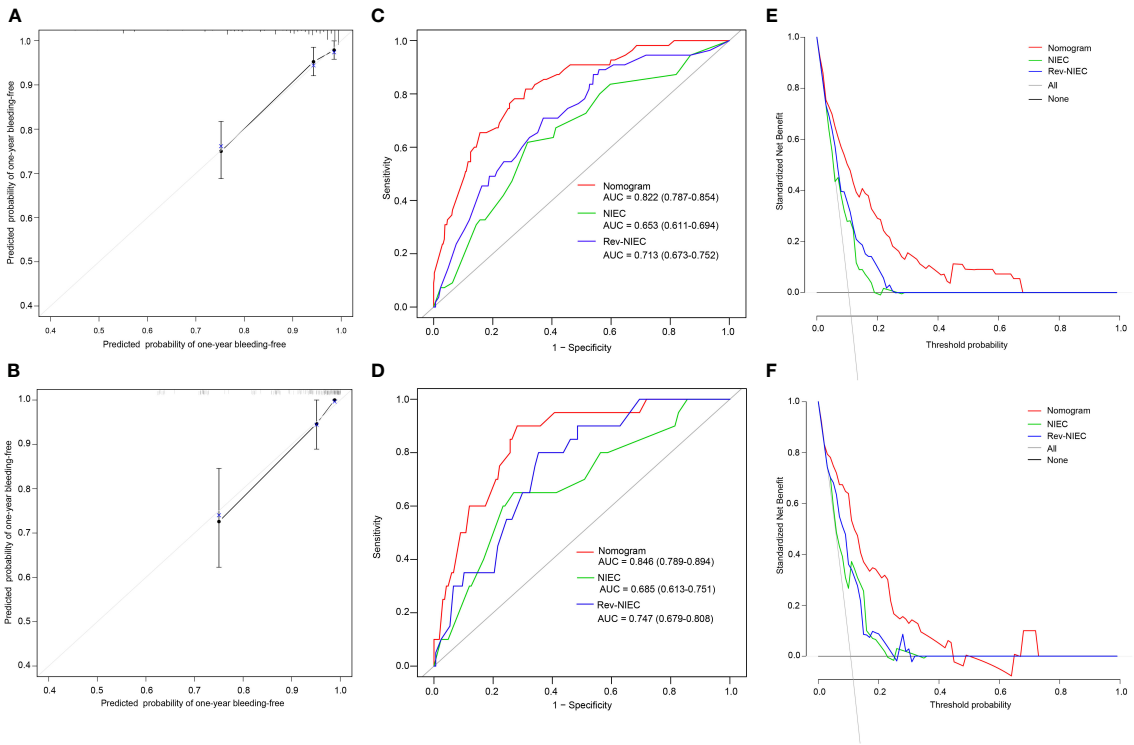

FIGURE 4
Calibration plots, ROC curves, and DCA curves for predicting VH in HBV-related cirrhotic patients with GEVs. The calibration plots of the nomogram for VH in the derivation **(A)** and validation cohorts **(B)**, in which the predicted probability of VH was compared with the actual bleeding probability. The ROC curves of the nomogram, NIEC index, and Rev-NIEC index in the derivation **(C)** and validation cohorts **(D)**. The DCA curves in the derivation **(E)** and validation cohorts **(F)** show that the net benefit of the nomogram is greater than the net benefit of the NIEC and Rev-NIEC indexes. Abbreviations: AUC, areas under the receiver operating characteristic curve; NIEC, North Italian Endoscopic index; Rev-NIEC, revised NIEC index; ROC, receiver operating characteristic; DCA, decision curve analysis; VH, variceal hemorrhage; HBV, hepatitis B virus; GEV, gastroesophageal varice.

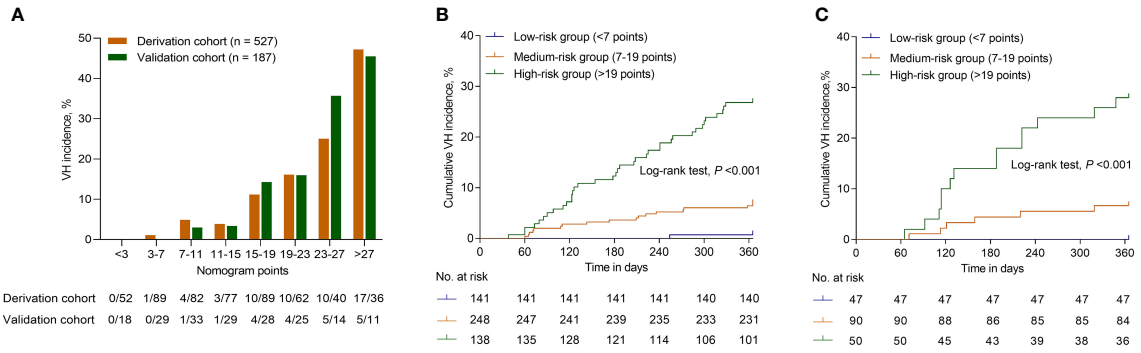

FIGURE 5
Risk for VH of patients with HBV-related GEVs with different scores. **(A)** The bars show the VH incidence for different score category in the derivation and validation cohorts. The cumulative VH incidence for patients categorized into different risk groups according to the nomogram in the derivation **(B)** and validation cohorts **(C)**. Abbreviations: VH, variceal hemorrhage; HBV, hepatitis B virus; GEV, gastroesophageal varice.

based on the 25th and 75th percentiles of the score distribution, *i.e.* <7, 7–19, and >19 points, 141 (26.8%), 248 (47.1%), and 138 (26.2%) patients were stratified into low-, medium-, and high-risk groups, respectively, in the derivation cohort. Moreover, in the derivation cohort, compared with patients in the low-risk group, those in the medium-risk and high-risk groups were at a significantly higher risk of VH, with a hazard ratio (HR) of 10.022 (95% CI, 1.334–75.306; P = 0.025) and 43.518 (95% CI, 5.970–317.217; P <0.001), respectively. In the derivation cohort, the cumulative VH incidence was 0.7%, 6.9%, and 26.8% in the low-, medium-, and high-risk groups, respectively (log-rank test, P <0.001; Figure 5B); and in the validation cohort, the cumulative incidence was 0.0%, 6.7%, and 28.0%, respectively (log-rank test, P <0.001; Figure 5C). The low cut-off value (>7) of the nomogram score achieved 98.2% sensitivity (95% CI, 90.3%–100.0%) and 99.3% NPV (95% CI, 96.3%–100.0%) in the derivation cohort and offered 100% sensitivity and NPV in the validation cohort to exclude patients who did not develop VH within one year (Table 2).

4 Discussion

In the current study, we developed and validated an individualized nomogram model specifically for HBV-related cirrhotic patients with GEVs to assess the risk of first VH occurrence within one-year. The robustness and predictive power of this nomogram was demonstrated to be superior compared with the traditional NIEC and Rev-NIEC indexes by internal and external validation tests. Importantly, this nomogram could be used as a risk stratification tool to identify patients at different risk levels of VH occurrence, which helps to promote the precise prevention of VH occurrence and personalized management of patients with GEVs.

VH prevention is the primary goal in the GEV patient management (Garcia-Tsao et al., 2017; de Franchis et al., 2022). Several decades ago, researchers noticed the importance of bleeding risk assessment in the development of prevention strategies and have established several models for this purpose (The North Italian Endoscopic Club for the Study and Treatment of Esophageal Varices, 1988; Merkel et al., 2000; Tacke et al., 2007). Among them, the NIEC index is the most widely used, and the risk stratification based on this index is still an important basis for primary prevention strategies (The North Italian Endoscopic Club for the Study and Treatment of Esophageal Varices, 1988; Garcia-Tsao et al., 2017; de Franchis et al., 2022). However, the predictive power of such models are expected to vary among different populations owing to differences in the etiologies of cirrhosis. Based on our research, the predictive performance of the traditional NIEC and Rev-NIEC indexes for

TABLE 2 Sensitivity, specificity, and predictive values of the nomogram classification determined by the 25th and 75th percentiles of the score distribution.

Cut-off value	Sensitivity (%) (95% CI)	Specificity (%) (95% CI)	PPV (%) (95% CI)	NPV (%) (95% CI)
Derivation cohort (n = 527)				
Nomogram <7	98.2 (90.3-100.0)	31.4 (27.2-35.8)	14.3 (10.9-18.2)	99.3 (96.3-100.0)
Nomogram <19	67.3 (53.3-79.3)	78.8 (74.8-82.4)	27.0 (19.8-35.3)	95.4 (92.8-97.2)
Validation cohort (n = 187)				
Nomogram <7	100.0 (83.2-100.0)	28.1 (21.5-35.6)	14.3 (8.9-21.2)	100.0 (92.5-100.0)
Nomogram <19	70.0 (45.7-88.1)	78.4 (71.4-84.4)	14.0 (8.2-21.8)	95.6 (90.7-98.4)

The patients were stratified into three groups according to the 25th percentile and the 75th percentile of the nomogram-predicted score: low-risk group (<25th percentile), medium-risk group (25th–75th percentile), and high-risk group (>75th percentile). The 25th percentile was 7 and the 75th percentile 19 points.
CI, confidence interval; PPV, positive predictive value; NPV, negative predictive value.

patients with HBV-related GEVs is lower than that for the patients with hepatitis C virus/alcohol abuse-related GEVs studied in the original report (The North Italian Endoscopic Club for the Study and Treatment of Esophageal Varices, 1988; Merkel et al., 2000). Furthermore, it is reported that the etiology is an independent VH predictor (Tacke et al., 2007), indicating different progression of VH between different etiologies. Therefore, different evaluation model based on different etiologies may be more effective for VH risk prediction. For patients with HBV infection, antiviral therapy is a very important strategy regardless of the disease stage, which can delay disease progression and improve long-term prognosis (Iloeje et al., 2006; Chen et al., 2006; European Association for the Study of the Liver, 2017; Wang and Duan, 2021). Previous studies have also demonstrated that antiviral therapy is effective in reducing the VH rate (Li et al., 2013; He et al., 2019). Nevertheless, the incidence and mortality of HBV-related VH remains high (Kim et al., 2011). Thus, apart from the antiviral therapy, it is essential to develop other targeted prevention strategies in accordance with bleeding risk of each individual, for reducing the HBV-associated first VH occurrence.

In the current study, we carefully analyzed data from 527 HBV-related cirrhotic patients with GEVs and explored the VH-related impact factors based on one-year follow-up outcomes. As expected from previous literature (The North Italian Endoscopic Club for the Study and Treatment of Esophageal Varices, 1988; Kleber et al., 1991; Merkel et al., 2000; Kim et al., 2019), the sizes of varices as well as the presence of RWM were strong predictors for VH. Endoscopy, as the gold standard technique for the diagnosis of varices, plays an important role in the bleeding risk assessment for patients with GEVs (de Franchis et al., 2022). Patients with large varices have approximately three times higher risk of VH than those with small varices, while the presence of RWM increased the risk of bleeding up to four times (Park et al., 2004; Aggeletopoulou et al., 2018). Further, the liver dysfunction severity, e.g., elevated GGT levels and ascites, was found to be an essential risk factor for VH. However, the liver function indicators were different from the previous studies (The North Italian Endoscopic Club for the Study and Treatment of Esophageal Varices, 1988; Merkel et al., 2000), in which the Child-Pugh classification as a liver function indicator was associated with bleeding. This may be because the Child-Pugh scoring system involves some subjective indexes (hepatic encephalopathy and ascites) and interrelated indexes (serum albumin and ascites) (Pugh et al., 1973), which virtually increase the instability of the prediction in different studies. It has also been previously reported that the Child-Pugh classification is not associated with the bleeding incidence (Kleber et al., 1991). Besides such well-recognized factors mentioned above, HCT and splenic thickness were also found as important factors affecting VH occurrence. HCT is one of the most important indicators of whole blood viscosity (Voerman and Groeneveld, 1989). A decreased blood viscosity is associated with higher bleeding risk and increased bleeding severity (Ohki et al., 1988). Previous reports also indicated that low hematocrit is a risk factor for variceal bleeding (Liu et al., 2006; Zhou et al., 2017). Splenomegaly, excluding diseases of the blood system, is an important manifestation of portal hypertension in cirrhosis (Li et al., 2017). The increased spleen thickness indicates the severity of liver morphological changes, indicating the portal hypertension and gastroesophageal variceal bleeding progression (Bolognesi et al., 2002; Berzigotti et al., 2008; Zhang et al., 2019).

We combined the above mentioned six independent predictors and established a novel and easy-to-use nomogram to evaluate the probability of the first VH occurrence. To our knowledge, this is the first report on developing a nomogram for predicting the risk of VH in HBV-related cirrhotic patients with GEVs. This established nomogram demonstrated better predictive performance for VH occurrence compared with the traditional NIEC and Rev-NIEC indexes, as supported by the AUC and DCA curves in the internal and external validation tests. Thus, this nomogram developed specifically to report that GEVs screening strategies for HBV-related cirrhosis offers unique advantages over the traditional risk predicting models.

For efficient prophylaxis, accurate risk stratification and prognosis assessment are important because patients in the high-risk group may benefit from earlier prevention strategies, while patients in the low-risk group could avoid undergoing unnecessary interventions (Tacke et al., 2007). In our study, we stratified patients into low- (<7 points), medium- (7–19 points), and high- (>19 points) risk groups according to their bleeding risk scores. In this hierarchical model, 26.8% of patients were categorized as low-risk group with an annual bleeding risk of 0.7%. Furthermore, the low cut-off point of 7 offered 98.2% sensitivity and 99.3% NPV for VH prediction in the derivation cohort, and 100% sensitivity and NPV in the validation cohort, which means that endoscopic screening and preventive measure should be reduced in patients with low-risk group features to avoid the risk of overdiagnosis and overtreatment. In contrast, the 26.8% annual VH incidence in the high-risk group signifies that patients in this group require closer surveillance and proactive preventive strategies, and it can be accepted that the higher the risk score, the more attention should be given. Therefore, the clinical significance of this study lies in the idea that clinicians can use this nomogram for assistance in understanding the risk for VH and choosing appropriate and individualized treatment strategies according to patient risk level, instead of adhering to a single treatment regimen, which may contribute to improved overall outcomes in all patients with GEVs and allocate medical resources efficiently.

This study has several limitations. First, this is a retrospective study and potential selection bias is inevitable. However, the nomogram was derived from a large sample size cohort of tertiary hospital and it was validated in an external cohort. Nonetheless, data from multicenter prospective studies with larger sample size are still needed to make the results generalizable. Second, the occurrence of bleeding events may be influenced by precipitating factors, such as severe coughing, and constipation. However, this challenge reflects those faced in daily clinical practice and also exists in all large-scale research studies. Although these precipitating factors may shorten the period of the VH event, this shortened period was considered meaningful for early warning of bleeding. Finally, the follow-up duration was one-year, and the model's predictive performance for the long-term prognosis remains unclear. Conversely, one-year is a reasonable time span for the development of VH risk prediction model. Despite these limitations, our research provides new guidance for the selection of prevention strategies for HBV-related VH and offers an idea to develop a predictive model for VH in patients with GEVs from other etiologies.

5 Conclusions

In summary, the nomogram established in our study can be useful for estimating the first VH probability within one-year and stratifying the bleeding risks in HBV-related cirrhotic patients with GEVs, which can assist clinicians in determining the appropriate prophylactic strategies. However, the clinical utility and true predictive value of this nomogram needs to be further verified in larger prospective studies.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The studies involving human participants were reviewed and approved by Ethical Review Committee of the Beijing Ditan Hospital (Beijing, China). Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements. Written informed consent was not obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

Author contributions

XW, YM, and QZ conceived and designed the project. QZ, SN, KS, XZ, YZ, and YB collected the data. QZ, BZ, and LY analyzed and interpreted the data. QZ, SN, and LY drafted the manuscript. All authors contributed to the article and approved the submitted version.

Funding

This work was supported by the National Natural Science Foundation of China [No. 81774234], and Beijing Municipal Science & Technology Commission [No. Z191100006619033].

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2023.1062172/full#supplementary-material

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frontiers | Frontiers in Cellular and Infection Microbiology

TYPE Original Research
PUBLISHED 19 January 2023
DOI 10.3389/fcimb.2023.1119805

OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to Virus and Host, a section of the journal Frontiers in Cellular and Infection Microbiology

RECEIVED 09 December 2022
ACCEPTED 03 January 2023
PUBLISHED 19 January 2023

CITATION
He M, Liu M, Geng J, Liu L, Huang P, Yue M, Xia X and Zhang A-M (2023) Polymorphisms of the *MxA* and *MxB* genes are associated with biochemical indices and viral subtypes in Yunnan HCV patients. *Front. Cell. Infect. Microbiol.* 13:1119805. doi: 10.3389/fcimb.2023.1119805

Polymorphisms of the *MxA* and *MxB* genes are associated with biochemical indices and viral subtypes in Yunnan HCV patients

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Introduction: Hepatitis C virus (HCV) infection was the primary reason causing critical hepatic Q7 diseases. Although direct-acting antiviral agents (DAA) were widely used in clinics, anti-drug mutation, the outcome of patients with different viral subtypes, and recurrence suggested that HCV pathogenic mechanism should be studied further. HCV infection, replication, and outcome were influenced by the IFNL4 and itsdownstream genes (MxA and MxB). However, whether genetic polymorphisms of these genes played necessary roles required verification in the Yunnan population.

Methods and Results: After analyzing the genotypes and allele frequencies of seven single nucleotide polymorphisms (SNP), we found the association between the genotype and allele frequencies of rs11322783 in the IFNL4 gene and HCV infection in Yunnan population. Furthermore, the genetic polymorphisms of the MxA and MxB genescould influence liver function of HCV patients. The indirect bilirubin (IBIL) and albumin (ALB) levels showed significant differences among HCV patients, who carried various genotypes. The IBIL levels were associated with genotypes of rs17000900 (P= 0.025) and rs2071430 (P= 0.037) in the MxA gene, and ALB levels were associated with genotypes of rs2838029 (P= 0.010) in the MxB gene. Similarly, the genotypes of SNPs also showed significant difference in patients infected with subtype 3a (P=0.035) and 2a (P=0.034). However, no association was identified between expression level and SNPs of the MxA and MxB genes. Furthermore, HCV subtype 3b was found to be the predominantly epidemic strain in Yunnan Province.

Conclusion: In conclusion, the association between biochemical indices/HCV subtypes and SNPs in the MxA and MxB genes was identified in Yunnan HCV population.

KEYWORDS

HCV infection, biochemical indices, subtype, *MxA*, *MxB*

Introduction

Hepatitis C virus (HCV) infection could lead to critical hepatitis diseases, such as cirrhosis and hepatocellular carcinoma (HCC). Although direct-acting antivirals (DAAs) drugs are used worldwide, approximately 1.75 million new HCV infections occurred in 2015 (W.H.O, 2017). The mortality trends of infectious hepatitis disease were increasing from 2000 to 2015, differing from other popular infectious diseases, such as human immunodeficiency virus (HIV), tuberculosis (TB), and malaria. Thus, the pathogenic mechanisms of HCV infection should be studied further.

Interferons (IFNs) are the common used drugs for HCV infection therapy, and they could active Jak/STAT signaling pathway and further induce some interferon-stimulated genes (ISGs) (Heim and Thimme, 2014). The expression and function of Interferon lambda 4 (*IFNL4*) gene is controlled by its genetically transcriptional regulation (Zhou et al., 2020). The *MyXovirus resistance* (*Mx*) genes belongs to the ISGs family. Firstly, the *MyXovirus resistance 1* (*MxA* or *Mx1*) gene was identified as one of the main protected factors for mice against influenza viruses (Lindenmann, 1964). Then, it is investigated that the *MxA* gene belonged to natural immune system and had wide-spectra anti-virus activity, which is induced by interferon (Haller et al., 2018). Although the antiviral activity of the *MxB* gene is limited compared to the *MxA* gene, it could interfere with HCV RNA replication by interacting with the NS5A protein (Yi et al., 2019). These reports suggested the *Mxs* genes were necessary natural factors for inhibiting HCV in patients.

To date, the genetic polymorphisms of the *MxA* gene have been reported to be associated with HCV infection or clearance of those with HCV infection (Garcia-Alvarez et al., 2017), but whether single nucleotide polymorphisms (SNPs) of the *MxB* gene could influence HCV infection, biochemical characteristics, or outcome of HCV patients are not clear. In this study, the SNPs of the *MxA* and *MxB* genes in HCV patients and controls from Yunnan Province were analyzed.

Materials and methods

Individuals and clinical data

We recruited 347 patients with chronic HCV infection and 448 normal controls from the First People's Hospital of Yunnan Province from 2019 to 2022. 3 mL of whole blood was collected from each individual. The patients were diagnosed as chronic HCV infected persons by the symptoms and liver function test. All HCV-infected patients were identified to be anti-HCV positive by HCV ELISA Kit (ORTHO, USA), and all patients were without any medical treatment when we collected the samples. All patients were identified without serious liver disease, such as fibrosis, cirrhosis and hepatocellular carcinoma). The basic information and liver function data of all samples [including alanine transaminase (ALT), aspartate transaminase (AST), total bilirubin (TBIL), direct bilirubin (DBIL), indirect bilirubin (IBIL), total protein (TP), albumin (ALB), globin (GLOB)] of HCV patients were collected for further analysis. Based on the clinical phenotype and biochemical indexes, all chronic HCV infected patients were identified without Hepatitis B virus (HBV) or HIV infection by using Quantitative CLIA Kit (Autobio, China) and Anti-HIV ELISA Kit (WANTAI, China). Written informed consents of the Declaration of Helsinki were obtained from all participants prior to the study. The institutional review board of Kunming University of Science and Technology approved this study.

HCV RNA loading quantification and genotyping

HCV RNA was abstracted from 100 μL serum of patients by using TIANamp Virus RNA Kit (TIANGEN, China), and viral RNA load was quantified by using the primers and probe in our previous study (Song et al., 2019). The detection limit was set at 1,000 copies/mL. HCV RNA was further transformed as log10-transformation. The NS5B gene of HCV was genotyped in each patient by using the method described in our previous study (Zhang et al., 2014).

SNP selection and genotyping

Genomic DNA was extracted from whole blood by using the Blood Genomic DNA Miniprep Kit (Axygen, USA). Seven tagSNPs were genotyped by using SnapShot method according to previous study (Zheng et al., 2022), including two SNPs (rs11322783 and rs117648444) in the *IFNL4* gene, two SNPs (rs2071430 and rs17000900) in the *MxA* gene and three SNPs (rs9982944, rs408825, and rs2838029) in the *MxB* gene,. Then, 10% of the genotyping results were further identified by using Sanger sequencing method.

RNA expression level detection

Total RNA was extracted from 200 μL whole blood of HCV patients using the RNAsimple Total RNA Kit (TIANGEN, China). Then, the level of RNA expression of the *MxA* and *MxB* genes was detected using fluorescent quantitative real-time PCR. The primers for quantifying target genes were GTGCATTGCAGAAGGTCAGA/CTGGTGATAGGCCATCAGGT (for the *MxA* gene) and CAGAGGCAGCGGAATCGTAA/TGAAGCTCTAGCTCGGTGTTC (for the *MxB* gene). The *GAPDH* gene was used as the reference gene, and the primers for quantification were GGCATCCTGGGCTACACTGAG/CATACCAGGAAATGAGCTTGAC. The ChamQ SYBR® qPCR Master Mix (Vazyme, China) and Quant Gene 9600 were used to determine the RNA expression levels of the *MxA* and *MxB* genes of HCV patients.

Data analysis

The genotype and allele frequencies between HCV patients and controls are analyzed by using Chi-square text. A student t test (two

tailed) was used to compare viral load between two HCV subtypes, biochemical indices among patients with different genotypes, and RNA level expression of *MxA* and *MxB* genes. The correlation analysis was used to determine the relationship between viral load of HCV and RNA level of the *MxA* or *MxB* gene (two-sided). A *P*-value less than 0.05 is considered statistically significant.

Results

The gender ratio (male: female) was similar between HCV patient group (1.4:1) and controls (1.6:1). There were 201 males and 146 females in the HCV patients, and 275 males and 173 females in the control group. The mean age was 44.70 ± 0.89 and 40.58 ± 0.53 years in the HCV patients and controls, respectively. The genotype and allele frequencies, excluding SNP rs11322783, showed no significant difference between HCV patients and controls. Genotype ΔG/T of rs11322783 showed significantly lower frequency in HCV patients (0%) than that in controls (5.58%); however, the frequency of genotype TT was statistically higher in HCV patients (99.42%) than that in controls (94.20%) (Table 1). Similarly, allele ΔG of rs11322783 was a protective factor for HCV infection in Yunnan, with a frequency of 0.58% (4/694) and 3.01% (27/896) in patients and controls, respectively. This result suggested genotype and allele of rs11322783 could influence HCV infection in the Yunnan population.

The biochemical data was analyzed among 270 HCV patients with different genotypes of each SNP (Table 2). The IBIL levels showed a significant difference among various genotypes of two SNPs in the *MxA* gene of HCV patients. In HCV patients with genotype AC of rs17000900 or genotype GT of rs2071430, the IBIL level were significantly higher than that in the other patients. The ALB levels were much lower in HCV patients carried genotype AA and AG of rs2838029 than other patients. These results suggested genetic polymorphisms of the *MxA* and *MxB* genes could influence the liver function of HCV patients.

In total, HCV subtypes were determined in 339 HCV patients (Figure 1). Subtype 3b was the main subtype in Yunnan HCV patients occurring in 42.18% of individuals (n= 143). The frequencies of HCV subtype 3a (n= 59, 17.40%), 1b (n= 51, 15.04%), 2a (n= 45, 13.27%), and 6 (n= 40, 11.80%) were similar in Yunnan population. Only one sample belonged to subtype 5b. Although HCV viral load was detected in only 137 patients, the viral load was compared among HCV patients with different subtypes (Figure 2). The results showed significantly higher viral load existed in the patients with subtype 6 than in patients with subtype 2a (P= 0.029) or 3b (P= 0.003). The viral load was statistically higher in the patients with subtype 1b than those with subtype 3b. These results suggested that the viral load of HCV patients might be affected by HCV subtypes.

The frequencies distribution of subtypes in HCV patients with different genotypes of each SNP in the *MxA* and *MxB* genes were analyzed (Table 3). The frequency of subtype 3a was lower in patients carried genotype GG of rs9982944 (11.69%, 18/154) than in those with genotype AA (25%, 7/28) or AG (22.08%, 34/154). The frequency of subtype 2a was significantly lower in HCV patients with genotype AG of rs408825 (6.84%, 8/117) than in those with genotype AA (16.98%, 36/212) or GG (11.11%, 1/9). These results suggested the possible

TABLE 1 Frequencies of SNPs in the *IFNL4*, *MxA*, and *MxB* gene in Yunnan HCV patients and controls.

SNP genotype/allele	HCV patients (N=347)	Controls (N=448)	*P*-value	OR (95% CI)
rs11322783 (*IFNL4*)				
GenotypeΔG/ΔG	2	1	0.584	2.591 (0.300-37.63)
ΔG/T	0	25	<0.0001	0.0001 (0-0.165)
TT	345	422	<0.0001	10.63 (2.695-45.68)
Allele ΔG	4	27	0.0004	0.187 (0.070-0.517)
T	690	869		5.360 (1.936-14.32)
rs117648444 (*IFNL4*)				
Genotype AA	0	0	–	–
AG	6	7	0.922	1.109 (0.368-3.025)
GG	341	441	0.922	0.902 (0.331-2.718)
Allele A	6	7	0.922	1.108 (0.371-3.018)
G	688	889		0.903 (0.331-2.699)
rs2071430 (*MxA*)				
Genotype GG	162	217	0.675	0.932 (0.706-1.229)
GT	150	188	0.776	1.053 (0.793-1.396)
TT	35	43	0.913	1.057 (0.656-1.669)
Allele G	474	622	0.672	0.949 (0.757-1.178)
T	220	274		1.054 (0.849-1.305)
rs17000900 (*MxA*)				
Genotype AA	6	13	0.401	0.589 (0.229-1.457)
AC	91	114	0.867	1.041 (0.753-1.431)

(Continued)

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TABLE 1 Continued

SNP genotype/allele	HCV patients (N=347)	Controls (N=448)	*P*-value	OR (95% CI)
CC	250	321	0.966	1.020 (0.745-1.387)
Allele A	103	140	0.719	0.941 (0.716-1.239)
C	591	756		1.063 (0.807-1.397)
rs9982944 (*MxB*)				
Genotype AA	29	40	0.876	0.930 (0.555-1.552)
AG	159	222	0.331	0.861 (0.652-1.135)
GG	159	186	0.253	1.191 (0.900-1.578)
Allele A	217	302	0.330	0.895 (0.724-1.103)
G	477	594		1.118 (0.907-1.382)
rs408825 (*MxB*)				
Genotype CC	11	18	0.659	0.782 (0.354-1.619)
CT	119	131	0.149	1.263 (0.940-1.696)
TT	217	299	0.247	0.832 (0.622-1.114)
Allele C	141	167	0.438	1.113 (0.870-1.431)
T	553	729		0.899 (0.699-1.149)
rs2838029 (*MxB*)				
Genotype AA	2	5	0.477	0.514 (0.102-2.390)
AG	51	58	0.543	1.159 (0.765-1.739)
GG	294	385	0.705	0.908 (0.618-1.348)
Allele A	55	68	0.878	1.048 (0.727-1.520)
G	639	828		0.954 (0.658-1.376)

(Continued)

TABLE 1 Continued

347 chronic HCV infected persons and 448 controls were used to analyze genotypes and allele frequencies of seven SNPs.

protective role of genotype GG of rs9982944 and genotype AG of rs408825 against HCV subtype 3b and 2a infection, respectively.

When comparing the RNA expression level of the *MxA* gene and *MxB* gene in the patients with various genotypes of each SNP of the corresponding gene (Table 4), no significant difference was identified. Similarly, no correlation was identified between the RNA expression level of the *MxA* gene (r= 0.083, *P*= 0.343) or the *MxB* gene (r= 0.036, *P*= 0.691) and the viral load of HCV patients.

Discussion

Although over 90% of HCV patients could be cured by using DAA, some were still treated by using IFN-α combined Ribavirin owing to the high price of DAA and anti-drug mutation of DAA. Thus, the mechanism of IFN against HCV was necessary to further study. IFN-α could activate the Jak-STAT signaling pathway and further induce MxA production, which could inhibit HCV replication (Stevenson et al., 2011). The MxB belongs to the same family as MxA and plays wide anti-virus roles. However, reports regarding how MxB inhibited HCV replication were few. Li et al. found that MxB competitively interacted with NS5A of HCV and reduced the combination between NS5A and CypA, which provided formation of HCV infection (Li et al., 2022). These results suggested the importance of MxA and MxB in inhibiting HCV infection and replication.

The genetic polymorphisms of the *MxA* gene were considered to influence HCV infection, viral clearance, and outcome in many studies (Hassany et al., 2017; Zang et al., 2018), but no association study was performed between SNPs of the *MxB* gene and Chinese HCV patients. In this study, although no association was identified between SNPs of the *MxA* and *MxB* genes with HCV infection in the Yunnan population, HCV subtypes and biochemical indices demonstrated significant differences among patients with various genotypes. Zang et al. reported that genotype TT of rs2071430 in the *MxA* gene preferred to induce patients into chronic HCV infection (Zang et al., 2018). In Egyptian and Japanese HCV patients, the promoter SNPs of the *MxA* gene were considered independently influenced factors in response to IFN therapy (Suzuki et al., 2004; Hassany et al., 2017).

In liver allograft patients with or without HCV infection, the expression level of the *MxA* gene was positively associated with AST levels (>40 U/L) in patients' liver biopsies (Borgogna et al., 2009). SNP

TABLE 2 Biochemical indexes analysis among HCV patients with various SNP genotypes.

Biochemical features (unit)	rs17000900				rs2071430			
	AA	AC	CC	*P*- value	GG	GT	TT	*P*- value
AST (U/L)	88.67 ± 21.70	10.7.4 ± 30.30	72.11 ± 7.05	0.271	76.11 ± 10.48	90.14 ± 19.53	76.13 ± 8.80	0.775
ALT (U/L)	115.2 ± 23.16	124.0 ± 28.82	91.13 ± 9.71	0.371	92.42 ± 14.29	105.5 ± 18.69	115.4 ± 14.95	0.742
TBIL (μmol/L)	15.60 ± 4.03	28.06 ± 5.61	19.97 ± 2.37	0.262	19.24 ± 1.99	27.11 ± 4.74	13.87 ± 1.43	0.120
DBIL (μmol/L)	7.27 ± 2.22	14.71 ± 4.18	10.57 ± 1.86	0.533	9.86 ± 1.57	14.82 ± 3.62	6.23 ± 0.69	0.228
IBIL (μmol/L)	8.33 ± 1.83	13.35 ± 1.81	9.47 ± 0.57	0.025	9.49 ± 0.56	12.29 ± 1.34	7.64 ± 0.78	0.037
TP (g/L)	74.33 ± 3.05	74.29 ± 0.91	73.98 ± 0.46	0.940	74.14 ± 0.55	73.93 ± 0.69	74.36 ± 1.23	0.944
ALB (g/L)	39.17 ± 2.44	40.85 ± 0.71	40.72 ± 0.40	0.781	40.63 ± 0.52	40.78 ± 0.52	40.86 ± 1.04	0.971
GOLB (g/L)	35.17 ± 2.60	33.47 ± 0.68	33.34 ± 0.41	0.737	33.63 ± 0.55	33.20 ± 0.50	33.43 ± 1.03	0.845
	rs9982944				rs408825			
Biochemical features (unit)	AA	AG	GG	*P*- value	CC	CT	TT	*P*- value
AST (U/L)	74.65 ± 10.88	85.78 ± 17.83	79.85 ± 11.04	0.929	77.33 ± 17.05	100.6 ± 23.89	72.00 ± 7.98	0.379
ALT (U/L)	97.77 ± 17.90	102.5 ± 17.06	99.30 ± 15.07	0.986	83.67 ± 17.47	115.1 ± 22.52	93.46 ± 11.17	0.595
TBIL (μmol/L)	15.65 ± 2.19	23.52 ± 4.21	21.83 ± 2.41	0.640	20.09 ± 3.07	27.72 ± 5.77	19.10 ± 1.76	0.202
DBIL (μmol/L)	7.11 ± 1.11	12.84 ± 3.26	11.21 ± 1.78	0.655	9.88 ± 2.39	16.50 ± 4.62	9.04 ± 1.11	0.129
IBIL (μmol/L)	8.54 ± 1.18	10.74 ± 1.09	10.66 ± 0.85	0.639	10.21 ± 1.03	11.22 ± 1.25	10.14 ± 0.78	0.731
TP (g/L)	74.13 ± 1.30	74.23 ± 0.60	73.89 ± 0.63	0.922	75.11 ± 2.48	73.56 ± 0.76	74.30 ± 0.49	0.621
ALB (g/L)	39.48 ± 1.23	40.96 ± 0.46	40.71 ± 0.56	0.509	40.22 ± 2.07	40.11 ± 0.60	41.09 ± 0.43	0.403
GOLB (g/L)	34.61 ± 1.16	33.24 ± 0.49	33.38 ± 0.55	0.562	34.78 ± 2.74	33.51 ± 0.658	33.30 ± 0.40	0.733
	rs2838029							
Biochemical features (unit)	AA & AG	GG	*P*- value					
AST (U/L)	106.6 ± 42.92	77.25 ± 7.90	0.961					
ALT (U/L)	112.5 ± 39.99	98.30 ± 9.73	0.794					
TBIL (μmol/L)	25.73 ± 5.53	21.40 ± 2.50	0.517					
DBIL (μmol/L)	14.90 ± 4.57	11.05 ± 1.89	0.581					
IBIL (μmol/L)	10.83 ± 1.26	10.45 ± 0.732	0.599					
TP (g/L)	73.31 ± 1.33	74.22 ± 0.42	0.411					
ALB (g/L)	38.88 ± 0.90	41.07 ± 0.37	0.010					
GOLB (g/L)	34.40 ± 1.11	33.23 ± 0.36	0.498					

Biochemical indices were analyzed in 270 HCV patients with different genotypes of each SNP. The indices were expressed by mean ± SE.

at nt-88 of the *MxA* gene was identified to be an independent determining factor for outcome of IFN therapy (Suzuki et al., 2004). Similarly, genotype TT of rs2071430 in the *MxA* gene influenced HCV infection chronicity of patients (Zang et al., 2018). Similarly, we found that genotype GT of rs2071430 was associated with high IBIL levels of patients. These suggested that genetic polymorphisms of the *MxA* gene might influence the pathogenic progress and outcome of patients. To the best of our knowledge, this was the first study to identify the association between genetic polymorphisms of the *MxB* gene and biochemical indices and subtypes of HCV patients.

The genotypes of rs11322783 in the *IFNL4* gene were widely reported as an important host genetic factor influencing viral clearance and outcome of HCV patients (Aka et al., 2014; O'Brien et al., 2015). The allele ΔG and T of rs11322783 were found to play protective and risk roles in HCV infection. However, we could not analyze the association between genotypes of rs11322783 and biochemical indices of patients owing to the few numbers of patients with genotype ΔG/ΔG. These studies suggested that SNPs of the *IFNL4* gene could influence HCV infection, disease progress, and treatment effect.

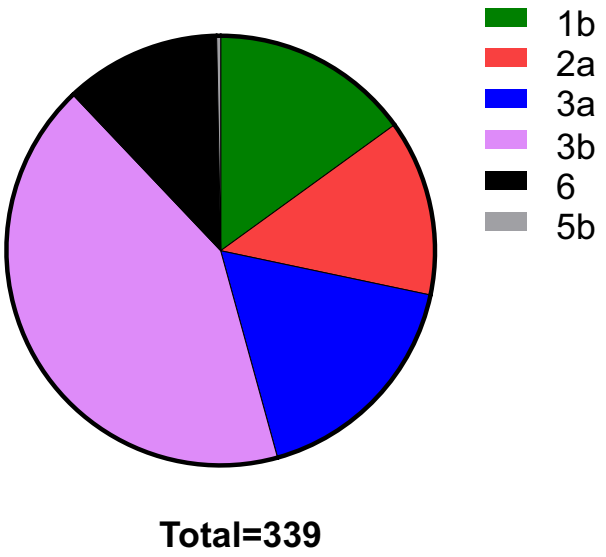

FIGURE 1
The distribution of HCV subtypes in this study. Totally 339 HCV patients were used to detect HCV subtypes. The subtype 3b was dominant in Yunnan Province.

TABLE 3 Continued

HCV subtypes	SNP Genotypes			*P*-value
6	5	23	12	0.098
	rs408825			
	GG	AG	AA	
1b	2	19	30	0.732
2a	1	8	36	0.034
3a	1	21	37	0.873
3b	5	56	82	<0.0001
6	0	13	27	0.489
	rs2838029			
	AA	AG	GG	
1b	0	11	40	0.256
2a	0	4	41	0.434
3a	0	10	49	0.688
3b	2	21	120	0.251
6	0	3	37	0.349

HCV subtypes were studied in 339 HCV patients with different genotypes of each SNP.

TABLE 3 HCV subtypes frequencies in patients carried with different SNP genotypes.

HCV subtypes	SNP Genotypes			*P*-value
	rs2071430			
	GG	GT	TT	
1b	29	20	2	0.150
2a	24	19	2	0.335
3a	25	28	6	0.695
3b	66	58	19	0.310
6	15	19	6	0.354
	rs17000900			
	AA	AC	CC	
1b	0	11	40	0.362
2a	0	9	36	0.318
3a	0	19	40	0.325
3b	5	37	101	0.121
6	1	14	25	0.396
	rs9982944			
	AA	AG	GG	
1b	3	20	28	0.334
2a	2	18	25	0.288
3a	7	34	18	0.035
3b	11	61	71	0.434

(Continued)

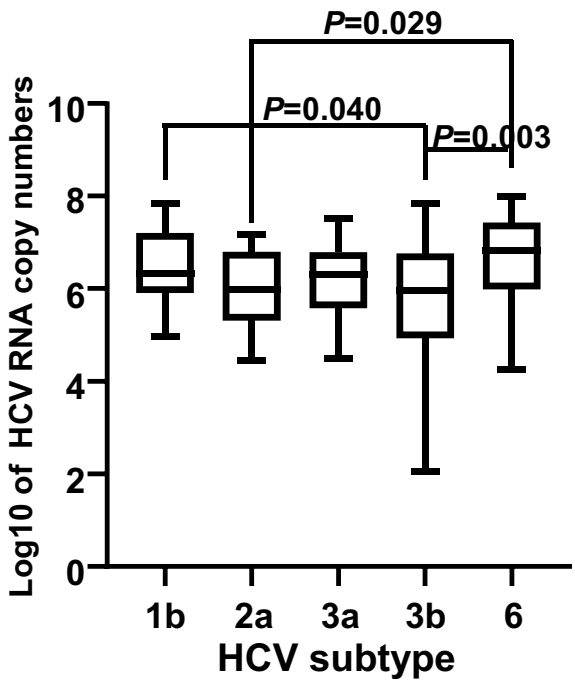

FIGURE 2
Comparison of viral load among 137 HCV patients with different subtypes. The viral loads were transformed into log10 of the copy numbers. A *P* value less than 0.05 was considered as significant difference.

TABLE 4 RNA expressing level of the *MxA* and *MxB* gene in HCV patients with various genotypes of these two genes.

Gene	RNA expressing level in various genotypes			*P*-value
rs17000900	AA	AC	CC	
MxA	3.461 ± 0.696	3.076 ± 0.321	2.573 ± 0.225	0.386
rs2071430	GG	GT	TT	
MxA	2.541 ± 0.296	2.930 ± 0.210	3.244 ± 0.455	0.332
rs9982944	AA	AG	GG	
MxB	7.401 ± 0.910	6.511 ± 0.374	6.209 ± 0.442	0.495
rs408825	CC	CT	TT	
MxB	7.022 ± 2.344	6.463 ± 0.387	6.460 ± 0.375	0.955
rs2838029	AA	AG	GG	
MxB	–	6.372 ± 0.508	6.495 ± 0.312	0.874

In summary, we firstly identified that genetic polymorphisms of the *MxA* and *MxB* genes were associated with biochemical indices and HCV subtypes of patients in Yunnan.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary Material.

Ethics statement

The studies involving human participants were reviewed and approved by The institutional review board of Kunming University of Science and Technology approved this study. The patients/participants provided their written informed consent to participate in this study.

Author contributions

A-MZ and XX designed this study. MH and ML performed the experiments. JG, PH and MY collected the samples. A-MZ and LL analyzed the data. A-MZ and MH prepared the manuscript. All authors contributed to the article and approved the submitted version.

Funding

This study is supported by the National Natural Science Foundation of China (32160148), Leading Reserve Talents of Academy and Science and Technology in Yunnan Province (2019HB002), and Yunnan Ten Thousand Talents Plan Young & Elite Talents Project.

Acknowledgments

We thank all the participants in this study. This study is supported by the National Natural Science Foundation of China (32160148), Leading Reserve Talents of Academy and Science and Technology in Yunnan Province (2019HB002), and Yunnan Ten Thousand Talents Plan Young & Elite Talents Project.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2023.1119805/full#supplementary-material

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frontiers | Frontiers in Cellular and Infection Microbiology

TYPE Original Research
PUBLISHED 08 February 2023
DOI 10.3389/fcimb.2023.1130485

OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to Virus and Host, a section of the journal Frontiers in Cellular and Infection Microbiology

RECEIVED 23 December 2022
ACCEPTED 23 January 2023
PUBLISHED 08 February 2023

CITATION
Zhang Q, Wang H, Jin Y, Zhou N, Sun L, Wu H, Chen H and Jiang T (2023) Incidence and predictors of HBV functional cure in patients with HIV/HBV coinfection: A retrospective cohort study. *Front. Cell. Infect. Microbiol.* 13:1130485. doi: 10.3389/fcimb.2023.1130485

Incidence and predictors of HBV functional cure in patients with HIV/HBV coinfection: A retrospective cohort study

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Background: This study was the first to examine the association of baseline clinical factors with the rate of HBsAg clearance in a large retrospective cohort of Chinese patients with HIV/HBV coinfection treated with combination antiretroviral therapy (ART).

Methods: Our retrospective cohort included 431 patients with HIV/HBV coinfection treated with TDF-containing ART. The median follow-up was 6.26 years. Logistic regression was used to investigate the association of baseline variables with HBsAg clearance, and Cox regression was used to investigate the association of baseline variables with time to HBsAg clearance.

Results: The clearance rate of HBsAg in our study was 0.072 (95% CI 0.049~0.101). In the multivariate logistic regression, advanced age (OR=1.1, P=0.007), high CD4 cell count (OR=2.06, P=0.05), and HBeAg positivity (OR=8.00, P=0.009) were significantly associated with the rate of HBsAg clearance. The AUC of the model integrating the above three predictors was 0.811. Similar results were found in the multivariate Cox regression (HR = 1.09, P = 0.038 for age, HR = 1.05, P = 0.012 for CD4 count and HR = 7.00, P = 0.007 for HBeAg).

Conclusions: Long-term TDF-containing ART can lead to HBsAg clearance of 7.2% in Chinese patients with HIV/HBV coinfection. Advanced age, high CD4 cell count, and positive HBeAg at baseline could be regarded as potential predictors and biological markers for HBsAg clearance in patients with HIV/HBV coinfection.

KEYWORDS

chronic hepatitis B, human immunodeficiency virus, HBsAg, antiretroviral therapy, HBeAg

Introduction

The prevalence of chronic hepatitis B virus (CHB) infection is 8.4% among patients with human immunodeficiency virus (HIV) worldwide (Leumi et al., 2020). Compared with chronic HBV mono-infection, HIV/HBV coinfection accelerates the progression of chronic HBV to liver cirrhosis, hepatocellular carcinoma (HCC), or end-stage liver disease (Cheng et al., 2021). The combination of tenofovir disoproxil fumarate (TDF) with lamivudine (3TC) or emtricitabine (FTC) is the most widely recommended combined antiretroviral therapy (ART) regimen in the treatment of patients with HIV/HBV coinfection (Panel on Guidelines for the Prevention and Treatment of Opportunistic Infections in Adults and Adolescents with HIV, 2022).

Even though more than 90% of patients with HIV/HBV coinfection could ultimately gain HBV DNA suppression after TDF-containing ART (Avihingsanon et al., 2010; Huang et al., 2019; Audsley et al., 2020), they do not achieve the same therapeutic effect of HBV clinical remission as observed in TDF-treated HBV mono-infected patients (Boyd et al., 2017; Kim et al., 2021; Sterling et al., 2021). Furthermore, some studies showed that approximately 10% of patients with HIV/HBV coinfection exhibited HBV viral rebound after achieving an undetectable plasma HBV DNA level (Matthews et al., 2013; Huang et al., 2019). This evidence indicates that virological response, such as HBV DNA suppression, might not be a reliable therapeutic goal in patients with HIV/HBV coinfection. Currently, the clearance of hepatitis B surface antigen (HBsAg), whether with the acquisition of anti-HBs or not, is commonly considered a functional cure and the ultimate therapeutic goal for CHB infection (Yuen et al., 2018).

Previously, we conducted a meta-analysis that showed that the rate of HBsAg clearance in patients with HIV/HBV coinfection treated with TDF-containing regimens was approximately 7.3% (Jiang et al., 2019), which is similar to the finding reported by Boyd et al. (Boyd et al., 2021). Recently, several studies in Caucasians and Thailand found that ethnicity, HBV viral load, CD4 cell count, HBeAg, and quantification of HBsAg were significantly associated with HBsAg clearance among patients with HIV/HBV coinfection treated with long-term TDF-containing ART (Zoutendijk et al., 2012; Matthews et al., 2013; Jiang et al., 2019; Audsley et al., 2020; Bremen et al., 2020). However, the rate of HBsAg clearance in the above study varies greatly, and the significant predictors they found are inconsistent or even contradictory to some extent, possibly because of the differences in the race, sample size, and follow-up time of each cohort.

It is important to understand the potential predictors and biological markers associated with the clearance of HBsAg among patients with HIV/HBV coinfection, which will deepen our understanding of the mechanism of HIV/HBV coinfection and may help physicians make more effective treatment decisions. However, a similar study has not been conducted in mainland China. The current study included the largest cohort of Chinese patients with HIV/HBV coinfection to date and investigated the association of baseline clinical variables with HBsAg clearance.

Methods

Characteristics of participants

Between 2005 and 2022, a total of 507 patients with HIV/HBV coinfection treated with ART at Beijing Youan Hospital were recruited in this study. The inclusion criteria were as follows: 1) enrollment of both patients with HIV and patients with chronic HBV; 2) age >18 years; 3) history of 3TC or 3TC/FTC co-formulated TDF-based antiretroviral therapy (ART); and 4) HBV surface antigen (HBsAg) positivity at baseline. Finally, 431 patients with HIV/HBV coinfection were included in this study (Figure 1).

This study was approved by the Ethics Committee of Beijing Youan Hospital. All patients signed an informed consent form before participating in this study.

Detection of HBV infection

HBV-specific antigens in patient plasma were detected in the clinical laboratory at Youan Hospital using the Elecsys HBsAg Immunoassay (Roche Diagnostics GmbH, Mannheim, Germany) and the immunoassay analyzer Cobase411 (Roche Diagnostics GmbH) according to the manufacturer's instructions. HBV serological markers were measured using the Beijing Wantaisheng China Pharmaceutical Co., Ltd.

Plasma HBV DNA monitoring

HBV DNA testing was conducted with real-time polymerase chain reaction (PCR) using the RealART HBV LC PCR kit (Artus GmbH, Hamburg, Germany; with a lower limit of detection [LLOD] of 12 IU/mL or 69.84 copies/mL), Abbott RealTime HBV DNA (Abbott Molecular, Des Plains, Illinois) or Hunan Xiang Sheng Biotechnology Co., Ltd., with an LLOD of 200 copies/mL.

Liver function tests

Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels were determined in the patient's plasma using ultraviolet-lactate dehydrogenase method test kits (Fortress Diagnostics Limited, United Kingdom).

Markers of HIV disease progression

Absolute blood $CD4^+$ T-cell counts were measured using a FACSCalibur flow cytometer. Viral load was measured by the Amplicor HIV monitor ultrasensitive method with a detection limit of 40 copies/mL of plasma.

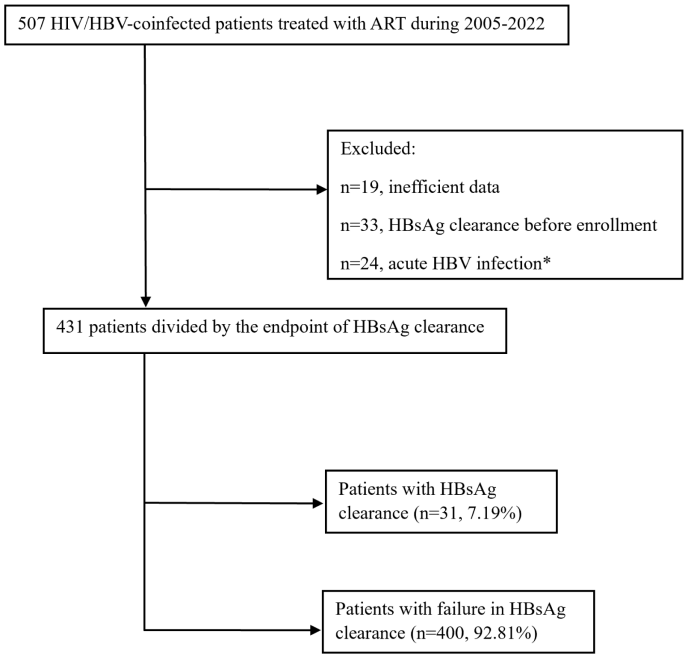

FIGURE 1
Flowchart of the study population inclusion. *Acute HBV infection was defined as the persistence of HBsAg within 6 months.

Efficacy measures

Chronic HBV infection was defined as the persistence of HBsAg for at least 6 months. The aspartate aminotransferase (AST)-to-platelet ratio index (APRI) and fibrosis index based on four factors (FIB-4) were used to evaluate liver fibrosis. The endpoint of this study was HBsAg loss, which was defined as having undetectable levels of HBsAg in serum.

Statistical analyses

Data are expressed as counts and percentages for categorical variables and as medians and interquartile ranges (IQRs) for continuous variables. The Mann–Whitney U test was used to assess the differences in the medians of continuous variables between the two groups when variables were nonnormally distributed. Differences in binary variables were tested using the chi-squared test. Fisher's exact test was used if 25% of cells had expected counts less than 5. Multivariate logistic regression analyses were performed using a stepwise selection process in which only factors with a $P < 0.05$ in the univariate logistic regression were included. A P value of 0.05 was used in the stepwise selection procedure for a factor to remain in the model. The discrimination ability of the prediction model derived from multiple logistic regression analysis was quantified by the area under the receiver-operating characteristic curve (AUC), and Delong's test was used to compare two AUCs of different models (DeLong et al., 1988). Subgroup analyses were conducted to investigate the association of risk factors with HBsAg loss in different age groups (< 35 years and ≥ 35 years) and different HBeAg groups (baseline HBeAg-positive group and baseline HBeAg-negative group). Kaplan–Meier survival curves were depicted to assess the survival probability of HBsAg loss for patients of different ages and HBeAg groups. Univariate and multivariate Cox proportional hazards models were used to investigate the association of baseline factors with time to HBsAg clearance. All analyses were performed using R Studio Version 4.2.1 (R Foundation for Statistical Computing, Vienna, Austria). A two-tailed $P < 0.05$ was considered statistically significant.

Results

Demographic and clinical characteristics of participants

A total of 431 patients were included in this study, and they were divided into the HBsAg clearance group (n=31, 7.19%) and HBsAg nonclearance group (n=400, 92.81%) according to the endpoint (Figure 1). The median age of patients with HBsAg clearance was 37, which was significantly higher than that of HBsAg nonclearance patients (37 vs. 32, P=0.016). The majority of participants were male (97.4%). Most of these patients were infected with HIV by MSM transmission (n=418, 85.8%). The median follow-up time for the HBsAg clearance patients was 3.02 years, while for HBsAg nonclearance patients, it was 6.34 (P<0.001). The first-line anti-HBV treatment for most of the patients was TDF-containing ART (n=390, 90.9%). There was a difference in CD4 cell counts between the HBsAg clearance group and the HBsAg nonclearance group (P=0.041). The proportions of HBeAg-positive patients at baseline were 65.2% and 34.1% in HBsAg clearance patients and HBsAg nonclearance patients, respectively (P=0.005). No differences were found for CD4/CD8, AST, ALT, ALP, APRI, and FIB-4 (Table 1).

10.3389/fcimb.2023.1130485

TABLE 1 Baseline clinical characteristics of 431 patients with HIV/HBV coinfection.

	All (n=431)	HBsAg non-clearance at endpoint (n=31)	HBsAg clearance at endpoint (n=400)	P
Age	33 (11)	37 (16)	32 (11)	0.016
Gender				0.728
Male	418 (97.4)	31 (100.0)	387 (97.2)	
Female	11 (2.6)	0	11 (2.8)	
BMI	21.45 (3.65)	21.63 (2.76)	21.40 (3.65)	0.256
Transmission				0.599
MSM	370 (85.8)	28 (90.3)	342 (85.5)	
Not MSM	61 (14.2)	3 (9.7)	58 (14.5)	
Years since admission	6.26 (3.20)	3.02 (2.70)	6.34 (3.00)	<0.001
Treatment regimen				0.754
TDF-containing ART	390 (90.9)	28 (90.3)	362 (91.0)	
Not TDF-containing ART	39 (9.1)	3 (9.7)	36 (9.0)	
HIV VL (10^4)				0.116
≤10^4	110 (40.7)	11 (61.1)	99 (39.3)	
>10^4	160 (59.3)	7 (38.9)	153 (60.7)	
HBV DNA (10^3)				0.222
≤10^3	101 (46.5)	5 (29.4)	96 (48.0)	
>10^3	116 (53.5)	12 (70.6)	104 (52.0)	
CD4 count				0.041
≤100	45 (27.3)	5 (35.7)	40 (26.5)	
(100,350]	75 (45.5)	2 (14.3)	73 (48.3)	
(350,500]	33 (20.0)	5 (35.7)	28 (28.5)	
>500	12 (7.3)	2 (14.3)	10 (6.6)	
CD4/CD8	0.24 (0.28)	0.2 (0.4)	0.24 (0.27)	0.126
≤0.5	141 (85.5)	10 (71.4)	131 (86.8)	
>0.5	24 (14.5)	4 (28.6)	20 (13.2)	
ALT	28.3 (23.9)	33.20 (22.50)	28.25 (24.17)	0.167
AST	27.8 (13.4)	26.40 (11.10)	27.90 (13.42)	0.890
ALP	73.0 (26.7)	71.20 (28.00)	73.2 (26.5)	0.993
Positive HBeAg	123 (36.2)	15 (65.2)	108 (34.1)	0.005
APRI	0.36 (0.32)	0.36 (0.29)	0.36 (0.32)	0.884
FIB-4	0.006 (0.007)	0.004 (0.007)	0.005 (0.006)	0.250

MSM, men who have sex with men.

Association of baseline variables with HBsAg clearance

We evaluated the association of baseline clinical variables with HBsAg clearance by using logistic regression analyses (Table 2). In the univariate logistic regression analyses, increased age (OR=1.05, p = 0.008) and HBeAg positivity (OR=3.63, p = 0.004) were significantly associated with a higher rate of HBsAg clearance. In the multivariate logistic regression analysis, age (OR = 1.08, P=0.018) and HBeAg (OR=6.12, P=0.016) remained significant after adjusting for each other. The AUC of the model consisting of age and HBeAg was 0.738 (Figure 2). As CD4 count was another significant variable in Table 1, we fit another model by including age, HBeAg, and CD4 count. We found that all three variables remained significant after adjusting for

TABLE 2 Association of baseline variables with HBsAg clearance in univariate and multivariate logistic regression analyses.

	Univariate logistic		Multivariate logistic 1		Multivariable logistic 2	
	OR (95% CI)	*P*	OR (95% CI)	P	OR (95% CI)	*P*
age	1.05 (1.01-1.09)	0.008	1.08 (1.01-1.15)	0.018	1.10 (1.03~1.18)	0.007
BMI	1.05 (0.9-1.21)	0.551				
Strategy	1.08 (0.31-3.72)	0.906				
HIV VL	0.41 (0.15-1.1)	0.076				
HBV DNA	2.22 (0.75-6.52)	0.149				
CD4 count	1.34 (0.73-2.45)	0.341			2.06 (1.00~4.39)	0.050
CD4/CD8	2.62 (0.75-9.16)	0.131				
HBeAg	3.63 (1.49-8.83)	0.004	6.12 (1.41-26.58)	0.016	8.00 (1.90~46.63)	0.009
APRI	0.96 (0.66-1.40)	0.821				

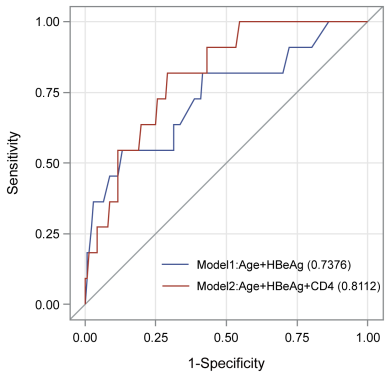

FIGURE 2
AUC of the two models in the multivariate logistic regression.

other variables (Table 2), and the AUC of the model increased from 0.738 to 0.811 (Figure 2). However, the P value for DeLong's test to compare two AUCs is 0.217, which may be attributed to the different sample sizes of the two models.

We further performed a subgroup analysis by dividing all patients into two groups according to age (age < 35 years and age ≥ 35 years) and HBeAg status (HBeAg positive and HBeAg negative). As shown in Supplementary Table 1, age was significant in the age < 35 years group, while HBeAg was significant in the age ≥ 35 years group. Age was significant in the HBeAg -positive group, while the CD4 count was significant in the HBeAg -negative group.

Association of baseline variables with time to HBsAg clearance

By using univariate and multivariate Cox regression, we evaluated the association of baseline variables with time to HBsAg clearance. In the univariate Cox regression, only age (HR=1.05, p = 0.012) and HBeAg status (HR=3.37, p = 0.005) were significantly associated with the time to HBsAg clearance (Table 3). Similar to the previous multivariate logistic regression, we fit two models with or without CD4, and we found that age (HR = 1.09, P=0.004), HBeAg (HR = 1.98, P = 0.038) and CD4 count (HR=7.00, P = 0.007) remained significant after adjusting for other variables.

We also depicted two Kaplan–Meier plots to evaluate the cumulative hazard of HBsAg clearance rate for different age groups (age < 35 years and age ≥ 35 years) and HBeAg status groups (HBeAg positive and HBeAg negative). We found that the cumulative hazard of HBsAg clearance rate in the age ≥ 35 group and HBeAg -positive group was significantly higher than that in the age < 35 group and HBeAg -negative group (P=0.032 for age and P=0.003 for HBeAg, Figure 3).

Plateau effect of HBsAg clearance during follow-up

The cumulative number of HBsAg-loss patients in different age groups and different HBeAg statuses are shown in Figure 4. The clearance rate of HBsAg increased sharply in the first few years of ART treatment and then reached a plateau by the 8th year of follow-up. A similar trend was observed in subgroups stratified by age and HBeAg status at baseline.

Discussion

For the first time, we investigated the association of baseline clinical variables with HBsAg clearance in 431 Chinese patients with HIV/HBV coinfection. We found that age, baseline HBeAg status, and CD4 count were significantly associated with the HBsAg clearance rate. In addition, age, HBeAg status, and CD4 count were also significantly associated with time to HBsAg in the survival analyses.

In previous studies, the rate of HBsAg clearance varied from 4.1% to 21% in patients with HIV/HBV coinfection (Zoutendijk et al., 2012; Hamers et al., 2013; Matthews et al., 2013; Boyd et al., 2015; Boyd et al., 2016; Huang et al., 2019; Audsley et al., 2020; Bremen et al., 2020; Jain et al., 2021). The HBsAg clearance rate in our study (0.072, 95% CI: 0.049~0.101) was very close to that in the American cohort, France cohort, and Netherlands cohort. Similar to our study, these

TABLE 3 Association of baseline variables with time to HBsAg clearance in univariate and multivariate Cox regression analyses.

	Univariate Cox		Multivariate Cox 1		Multivariable Cox 2	
	HR (95% CI)	*P*	HR (95% CI)	*P*	HR (95% CI)	*P*
age	1.05 (1.01-1.09)	0.012	1.06 (1.02-1.10)	0.002	1.09 (1.03-1.16)	0.004
BMI	1.05 (0.91-1.22)	0.492				
Strategy	0.52 (0.12-2.19)	0.370				
HIV VL	0.42 (0.16-1.07)	0.070				
HBV DNA	2.24 (0.79-6.36)	0.129				
CD4 count	1.31 (0.74-2.30)	0.349			1.98 (1.04-3.78)	0.038
CD4/CD8	2.36 (0.74-7.54)	0.146				
HBeAg	3.37 (1.43-7.95)	0.005	3.91 (1.65-9.27)	0.002	7.00 (1.68-29.27)	0.007
APRI	1.01 (0.51-1.99)	0.979				

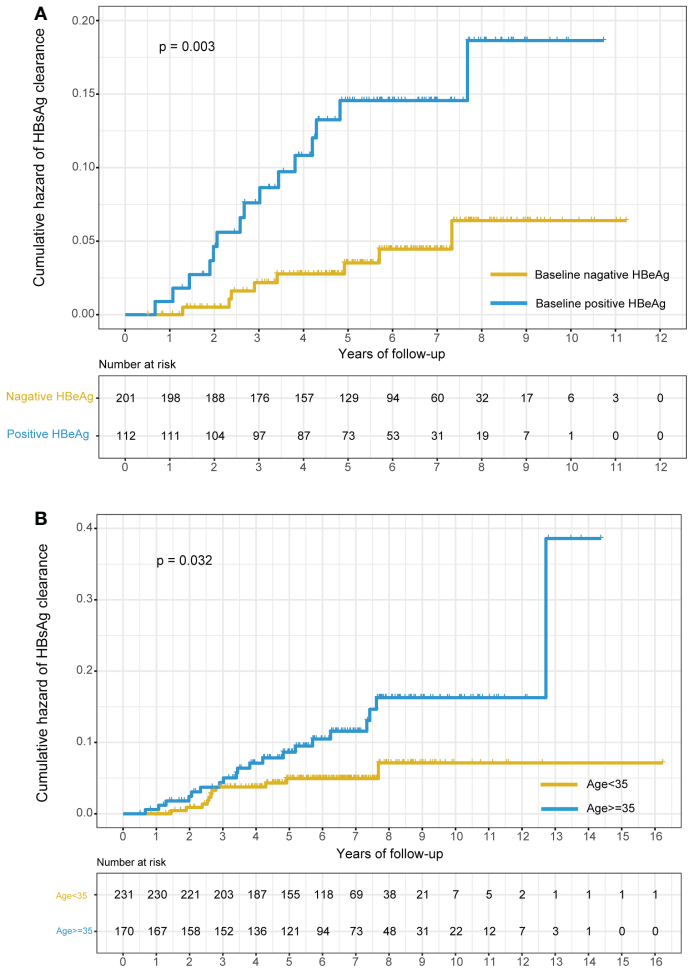

FIGURE 3
Kaplan–Meier plots of the cumulative hazard of HBsAg clearance stratified by the baseline status of HBeAg and age. **(A)** Stratified by the baseline status of HBeAg (positive HBeAg at baseline or negative HBeAg at baseline); **(B)** stratified by age (age<35 or age>=35).

three cohorts all have a long follow-up time of at least 5 years and a large sample size (Zoutendijk et al., 2012; Boyd et al., 2015; Jain et al., 2021). The HBsAg clearance rate in Taiwan's cohort was slightly lower than that in our study (Huang et al., 2019), which may be explained by the fact that some patients in the Taiwan cohort were treated with lamivudine (FTC) monotherapy, while TDF/FTC combination therapy has a better effect on the clearance of HBsAg than FTC monotherapy (Gantner et al., 2019; Hawkins et al., 2022).

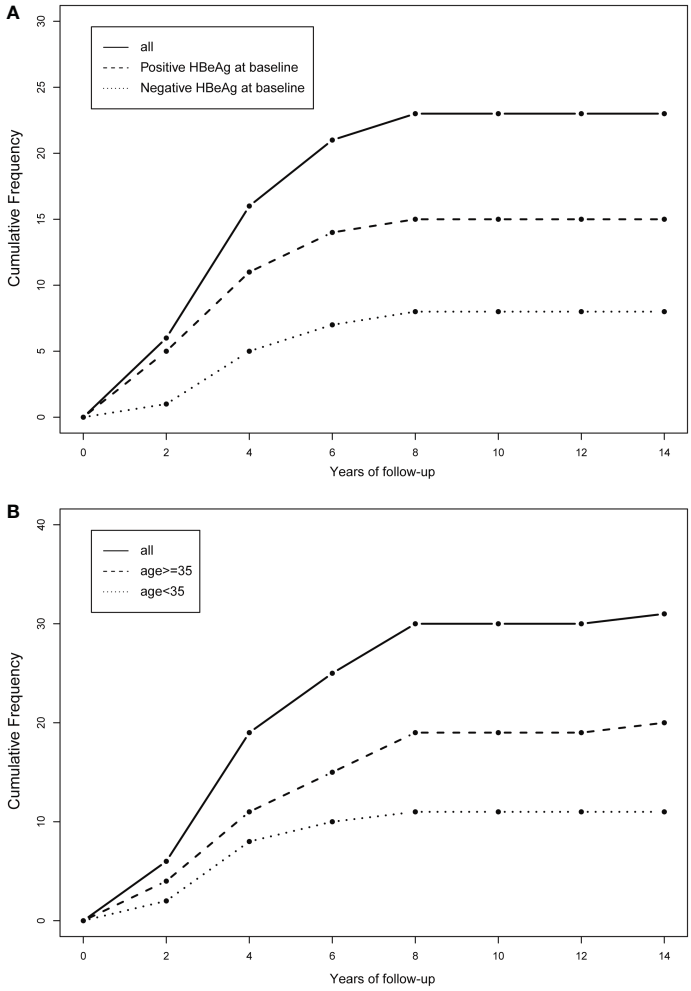

FIGURE 4
Cumulative frequency of HBsAg clearance stratified by the baseline status of HBeAg and age. **(A)** Stratified by the baseline status of HBeAg (positive HBeAg at baseline or negative HBeAg at baseline); **(B)** stratified by age (age<35 or age>=35).

The distribution of HBV genotypes varies in different areas. In China, the major prevalent genotypes are B and C, and different genotypes also determine the cure of HBV after treatment. Therefore, larger clinical studies are needed for further confirmation.

Our study found that the rate of HBsAg clearance increased sharply in the first few years of TDF-containing ART and then reached a plateau by 8 years of follow-up. The plateau of HBsAg clearance was also observed after 6 years of TDF-containing ART in an Australian study (Audsley et al., 2020). However, more studies have reported that the plateau occurred earlier, such as 24 weeks, one year, or three years after TDF-containing ART (Zoutendijk et al., 2012; Matthews et al., 2013; Boyd et al., 2015). This difference can be attributed to the fact that these studies analyzed the change in quantitative HBsAg rather than HBsAg clearance observed in our study. Another explanation was that some of these reported studies had shorter follow-up, and patients in their study usually had a history of antiretroviral drug use.

We found that advanced age was more likely to lead to HBsAg clearance after long-term TDF-containing ART (OR = 1.10, p = 0.007; HR = 1.09, p = 0.038). Most studies of patients with HIV/HBV coinfection have reported that age had no impact on the occurrence of HBsAg clearance (Zoutendijk et al., 2012; Boyd et al., 2015; Audsley et al., 2020; Bremen et al., 2020). We speculate that one of the reasons may be that our cohort has a median age of 33 years, which is evidently younger than cohorts with a median age of more than 40 years. Younger age always means greater potential in virus control. This finding was supported by a chronic HBV monoinfection study in which they found that advanced age was associated with a higher rate of HBsAg clearance (Tai et al., 2010).

There is no surprise that we found that a high CD4 cell count and HBeAg -positive status at baseline were conducive to HBsAg clearance after long-term TDF-containing ART. Netherlandish and Sub-Saharan African studies showed that long-term TDF-containing ART led to a significant decrease in HBsAg in HBeAg-positive patients with HIV/HBV coinfection with high CD4 cell counts (Zoutendijk et al., 2012; Boyd et al., 2016). The sub-Saharan African team also demonstrated that a high-level CD4 cell count at baseline is associated with faster HBsAg declines (Maylin et al., 2012). The HBeAg -positive status could be suggested as a surrogate

marker for HBV viral load usually accompanied by high HBV DNA in patients with HIV/HBV coinfection (Li et al., 2016), which ultimately results in immune activation and restoration. The HBeAg -positive stage generates less HBsAg than the HBeAg -negative stage because the chromosome integration level caused by HBV double -stranded linear DNA (dslDNA) becomes low (Bill and Summers, 2004; Wu et al., 2022). Mutations in the precore region that occur over time in chronic untreated HBV infection lead to viral mutants that do not produce HBeAg and therefore HBeAg-negative disease, which is associated with periods of high viral replication and necro-inflammatory activity in the liver.

Our study does have some limitations due to the nature of the retrospective study. Some medical records were partly incomplete, such as HBV DNA load, HBV genotype, and ART-adherence data. Despite the above limitation, this study is one of the largest and longest cohorts of HIV/HBV coinfection in China. We set strict inclusion criteria to ensure that all subjects included were chronic HBV patients.

In conclusion, our study found that long-term TDF-containing ART successfully leads to a 7.2% functional cure in 431 patients with HIV/HBV coinfection. Furthermore, we found that advanced age, high CD4 cell count, and positive HBeAg at baseline were significantly associated with a higher rate of HBsAg clearance in patients with HIV/HBV coinfection after long-term TDF-containing ART.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

Ethics statement

The studies involving human participants were reviewed and approved by the ethics committee of Beijing Youan Hospital. The patients/participants provided their written informed consent to participate in this study.

Author contributions

HC, and TJ contributed to conception and design of the study. QZ, NZ, HWa, LS, and HWu extracted data and assessed the methodological quality of included studies. QZ performed the statistical analysis. QZ and YJ wrote the first draft of the manuscript. All authors contributed to manuscript revision and approved the submitted version.

Funding

This study was supported by the National Natural Science Foundation of China (NSFC, 82272312 to HC) and Beijing Key Laboratory for HIV/AIDS Research (BZ0089). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2023.1130485/full#supplementary-material

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TYPE Original Research
PUBLISHED 20 February 2023
DOI 10.3389/fcimb.2023.1118801

OPEN ACCESS

EDITED BY
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SPECIALTY SECTION
This article was submitted to Virus and Host, a section of the journal Frontiers in Cellular and Infection Microbiology

RECEIVED 08 December 2022
ACCEPTED 06 February 2023
PUBLISHED 20 February 2023

CITATION
Cai J, Li Y, Hu P, Xu R, Yuan H, Zhang W, Feng T, Liu R, Li W and Zhu C (2023) Plerixafor and resatorvid inhibit hepatitis B virus *in vitro* by upregulating elongation factor Tu GTP-binding domain containing 2.
Front. Cell. Infect. Microbiol. 13:1118801.
doi: 10.3389/fcimb.2023.1118801

Plerixafor and resatorvid inhibit hepatitis B virus *in vitro* by upregulating elongation factor Tu GTP-binding domain containing 2

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Background: An increase in the demand for a functional cure has accelerated research on new methods of therapy for chronic hepatitis B, which is mainly focused on restoring antiviral immunity for controlling viral infections. Previously, we had described elongation factor Tu GTP-binding domain containing 2 (EFTUD2) as an innate immune regulator and suggested that it might be an antiviral target.

Methods: In this study, we generated the Epro-LUC-HepG2 cell model for screening compounds that target EFTUD2. Plerixafor and resatorvid were screened from 261 immunity and inflammation-related compounds due to their ability to highly upregulate EFTUD2. The effects of plerixafor and resatorvid on hepatitis B virus (HBV) were examined in HepAD38 cells and HBV-infected HepG2-NTCP cells.

Results: The dual-luciferase reporter assays showed that the EFTUD2 promoter hEFTUD2pro-0.5 kb had the strongest activity. In Epro-LUC-HepG2 cells, plerixafor and resatorvid significantly upregulated the activity of the EFTUD2 promoter and the expression of the gene and protein. In HepAD38 cells and HBV-infected HepG2-NTCP cells, treatment with plerixafor and resatorvid strongly inhibited HBsAg, HBV DNA, HBV RNAs, and cccDNA in a dose-dependent manner. Furthermore, the anti-HBV effect was enhanced when entecavir was administered along with either of the previous two compounds, and the effect could be blocked by knocking down EFTUD2.

Conclusion: We established a convenient model for screening compounds that target EFTUD2 and further identified plerixafor and resatorvid as novel HBV inhibitors *in vitro*. Our findings provided information on the development of a new class of anti-HBV agents that act on host factors rather than viral enzymes.

KEYWORDS

hepatitis B virus, small-molecule agents, plerixafor, resatorvid, EFTUD2

Introduction

Chronic hepatitis B (CHB) is a major risk factor for liver cirrhosis and hepatocellular carcinoma (Xia and Liang, 2019). Interferon (IFN) can effectively perform virological clearance in chronic hepatitis B virus (HBV) infection, but its clearance rate is low and only a few patients benefit from IFN-based therapy (Geng et al., 2018). Additionally, although nucleoside analogs (NAs) can inhibit the replication of HBV DNA, they are inefficient in removing the hepatitis B surface antigen (HBsAg). Therefore, efficacious anti-HBV therapeutic methods need to be developed (Levrero et al., 2018).

The innate immunity of the host is the first line of defense against the invasion of viruses and determines the outcome of infection (Horner and Gale, 2013). In another study, we identified the elongation factor Tu GTP-binding domain-containing 2 (EFTUD2) as a novel host factor that can counter hepatitis C virus (HCV) infection (Zhu et al., 2015). EFTUD2 encodes a GTPase responsible for pre-mRNA splicing (Janssen et al., 2005) and can regulate the innate immune response by alternatively splicing the mRNA of myeloid differentiation factor 88 (MyD88) (De Arras et al., 2014), which is a key factor involved in type I IFN response and many viral infections (Saikh, 2021). HBsAg and HBeAg can suppress the binding of MyD88 and decrease IFN signaling (De Arras et al., 2014). Single-nucleotide polymorphism analysis showed that EFTUD2 rs3809756 polymorphism is significantly associated with susceptibility to HBV infection (Tian et al., 2022). However, the effect of EFTUD2 on HBV replication is not known. In this study, we found that HBV is inhibited by EFTUD2 in different cell models, and then, proposed an approach for screening new anti-HBV agents to provide more options for the immunotherapy of CHB patients with poor response to IFN therapy.

We screened 261 compounds associated with immunity and inflammation and found that plerixafor and resatorvid could significantly upregulate the expression of EFTUD2 and reduce HBV replication *in vitro*. Moreover, plerixafor and resatorvid inhibited the transcriptional activity of cccDNA to suppress HBV RNA synthesis and HBsAg secretion and showed anti-HBV activity. These findings provided insights into the pharmacodynamics of plerixafor and resatorvid. Additionally, their antiviral effects increased when combined with entecavir (ETV). Thus, plerixafor and resatorvid may be considered as valuable candidates for the treatment of HBV in the future.

Materials and methods

Chemical compounds

The 261 screened compounds associated with immunity and inflammation were purchased from MedChemExpress (MCE, HY-L007) and stored at −80°C at the concentration recommended by the manufacturer. They were diluted in a medium to the desired concentration before use.

Cell culture

The HepG2 cells were purchased from the Cell Bank of Type Culture Collection of the Chinese Academy of Sciences. The HepAD38 and HepG2-NTCP cells were gifts from the Institute of Blood Transfusion, Chinese Academy of Medical Sciences and Peking Union Medical College, Chengdu, Sichuan Province, China. All cells were cultured in Dulbecco's Modified Eagle medium (DMEM, Gibco) containing 10% fetal bovine serum (FBS, Gibco) and incubated in a humidified atmosphere containing 5% CO_2 at 37°C. To maintain the stably transfected HBV genome, the HepAD38 cells were grown with 1 μg/mL doxycycline (MCE) and 400 μg/mL G418 (Thermo Fisher Scientific).

Virus extraction and infection

The supernatants of the HepaAD38 cells were concentrated 100-fold by ultracentrifugation as HBV inoculums. The HBV stock titer (genome equivalents [GEq] per milliliter) was measured by performing qPCR.

The HepG2-NTCP cells were incubated with 1,000 GEq/cell of HBV in a medium containing 4% (w/v) polyethylene glycol 8000 (PEG 8000) for 16 h. After discarding the viral mixture, the cells were rinsed thrice with PBS, and cultured in a fresh medium containing different agents at various concentrations. The medium was replaced every 2 days.

Detection of HBsAg

Briefly, different concentrations of agents were added to the plates 24 h after seeding the cells. Then, the supernatants were collected at predetermined time points and the secretion of HBsAg was detected using enzyme-linked immunosorbent assay (ELISA) kits (KHB, Shanghai, China) following the manufacturer's instructions. The absorbance was measured at 450 nm. The supernatant from the HepAD38 cells was diluted 20 times and the supernatant from the other cells was used as primary samples. All OD values were between 0.5 and 3.5.

Plasmid and siRNA

The small interfering RNA (siRNA) targeting EFTUD2 and the plasmid pEFTUD2 were purchased from GenePharma (Shanghai, China). They were transfected into HepAD38 cells and HBV-infected HepG2-NTCP cells with the Lipo3000 reagent (Thermo Fisher Scientific, USA) following the manufacturer's instructions. The knockout efficiency was determined by performing RT-qPCR for detecting the EFTUD2 mRNA and a Western blotting assay was performed for detecting the EFTUD2 protein.

Cell cytotoxicity assay

The effect of the compounds on cell cytotoxicity was measured by performing the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay (Sigma-Aldrich). The cells (2×10^3/well) were seeded into 96-well plates in 100 μL of DMEM and cultured at 37°C for 24 h. Then, the culture medium was replaced with a fresh medium containing various concentrations of compounds for a certain number of days. After the cells were cultured for a specific period, 10 μL of MTT (5 mg/mL) was added to each well. After incubation for 4 h, the supernatant was removed and the cells were lysed in 100 μL of DMSO (Solarbio). Then, the cytotoxicity was determined by analyzing MTT absorbance at 490 nm.

Luciferase reporter assay

The HepG2 cells were seeded in 12-well-plates at a concentration of 2×10^5 cells/well for 24 h, and then, transfected with a promoter-reporter plasmid plus vectors containing the gene of interest by Lipofectamine™ 3000 (Invitrogen). The Renilla luciferase reporter plasmid was used as the internal control of transfection efficiency. After transfection for 48 h, the luciferase activity was measured using a GloMax microplate luminometer (Promega).

All vectors used in this study were purchased from GenePharma (Shanghai, China). The restriction enzymes, different modification enzymes, and T4-DNA ligase were purchased from MBI Fermentas (Ontario, Canada).

Hirt extraction of cccDNA and analysis

To selectively extract HBV cccDNA, infected HepG2-NTCP cells were lysed in 6-cm dishes with 1 mL of lysis buffer at 37°C for 60 min, and then, incubated with 0.25 mL of 2.5 M KCl overnight at 4°C. The lysis buffer contained 50 mM Tris–HCl (pH 7.4), 10 mM EDTA, 150 mM NaCl, and 1% SDS, without proteinase K. The lysate was clarified by centrifugation at 12,000 g for 30 min at 4°C. Viral DNA was extracted with phenol and phenol: chloroform, precipitated in an equal volume of isopropanol containing 20 μg glycogen (Roche), and dissolved in TE buffer. The prepared DNA sample was then treated with plasmid-safe adenosine triphosphate (ATP)-dependent deoxyribonuclease DNase (Epicentre Technologies) following the manufacturer's instructions.

The treated Hirt DNA was subjected to Taq-man probe RT-qPCR for detecting the HBV cccDNA levels; the specific primers and the probe used are listed in the Supplementary (Table. S1).

Real-time PCR

HBV DNA was extracted using the QIAamp DNA Mini kit (Qiagen, Germany) and total RNA was extracted using TRIzol reagent (Invitrogen) following the manufacturer's instructions. The DNA and RNA samples were quantified by Nanodrop 2000 (Thermo scientific). The cDNA was synthesized from about 1,000 ng of RNA using the PrimeScript RT kit (Takara).

The levels of HBV genomic DNA, HBV RNAs, and EFTUD2 mRNA were detected by real-time PCR analysis, using SYBR Green (Roche, Germany) in the Applied Biosystems QuantStudio 3 Real-Time PCR System. The expression of the target genes was normalized by glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The primers used are detailed in the Supplementary (Table. S1).

Western blot

The proteins were separated by performing SDS polyacrylamide gel electrophoresis. Then, they were transferred onto polyvinylidene difluoride (PVDF; Thermo Scientific) membranes, blocked with 5% gelatin in TBST, and incubated with the primary antibodies at 4°C overnight. After washing the membranes thrice, the secondary antibodies (1:8000) conjugated to horseradish peroxidase (HRP) were added, and the mixture was incubated for 1 h at room temperature. The images were recorded using the enhanced chemiluminescence (ECL) system (Invitrogen). The mouse anti-GAPDH and the rabbit anti-EFTUD2 were purchased from Abcam. The HRP-conjugated enhanced ECL goat anti-rabbit immunoglobulin G (IgG) and HRP-conjugated ECL goat anti-mouse IgG were purchased from Bioss (Beijing, China).

Southern blot

The DNA samples were separated on a 0.9% agarose gel and transferred onto a nylon membrane overnight (Roche, Germany). After UV cross-linking and prehybridization, hybridization was performed by rotating and incubating the membrane with digoxigenin-labeled HBV-specific DNA probes, using a random primed DNA labeling kit (Roche, Germany). The radioactive signals were detected using the GelDocXR System (Bio-Rad).

Statistical analysis

Statistical analyses were performed using GraphPad Prism (GraphPad version 7). All experiments were repeated at least thrice. The data were presented as the mean ± standard deviation (SD) and comparisons were made by performing unpaired Student's t-tests. All differences were considered to be statistically significant at $p < 0.05$.

Results

EFTUD2 has an anti-HBV effect *in vitro*

To elucidate the role of EFTUD2 in HBV infection, we first silenced EFTUD2 in HepAD38 and HepG2-NTCP cells. Real-time PCR and western blotting assays showed efficient knockdown of

EFTUD2 (>70%, $P < 0.001$) in both cell lines (Figure 1A). We found that knocking down EFTUD2 significantly enhanced HBV replication, based on the results of real-time PCR analysis (Figure 1B). In contrast, overexpression of EFTUD2 resulted in decreased HBV DNA levels at 24, 48 and 72 h postinfection (Figure 1C), indicating its role in restricting HBV infection at the viral postentry stage. Moreover, EFTUD2 expression decreased in HepG2-NTCP cells at 48 h postinfection ($P < 0.05$) (Figure 1D), which may be caused by immune escape after HBV entry.

Analysis of the activity of the EFTUD2 promoters and construction of the Epro-LUC-HepG2 cell line

According to the ENCODE data, we predicted a promoter region near the exon1 of EFTUD2, where DNA endonuclease hypersensitive site (DHS) and transcriptional activity enhancement marker H3K27Ac are enriched (Figure 2A). Four fragments of 0.5 kbp, 1 kbp, 1.5 kbp, and 2 kbp from –1 to –2 kbp upstream of the transcriptional initiation site were selected. The PCR products were digested with the Nhel and BglII enzymes and cloned into the psiCHECK-2 vector to generate the recombinant plasmid of the promoter-luciferase reporter gene. All constructs were checked for the correct size by agarose gel electrophoresis (Figure S1A), verified by DNA sequencing, and transfected into HepG2 cells for 48 h. The results of the luciferase analysis suggested that the hEFTUD2pro-0.5 kb promoter had the strongest activity (3.2-fold, $P < 0.05$) (Figure 2B).

Therefore, the hEFTUD2pro-0.5 kb promoter sequence was fused to the firefly luciferase reporter gene, digested with Nhel and BamHI enzymes, and then, inserted into the LV6 vector to construct the LV6-Epro0.5-LUC luciferase reporter plasmid (Figure S1B). After transfection with the plasmid *via* lentivirus and selection with puromycin, the HepG2 transfected cell line was processed by monoclonal screening and the Epro-LUC-HepG2 cell line (2#) was obtained (Figure 2C).

Identification of plerixafor and resatorvid as upregulators of EFTUD2

The established Epro-LUC-HepG2 cell line was used for screening the 261 compounds from the HY-L007 compound library provided by MCE. The cytotoxicity of these compounds was evaluated by performing the MTT assay (partly shown in Figure S2). During the screening process, 0.1% DMSO was used as the negative control in each experimental setup. We found that

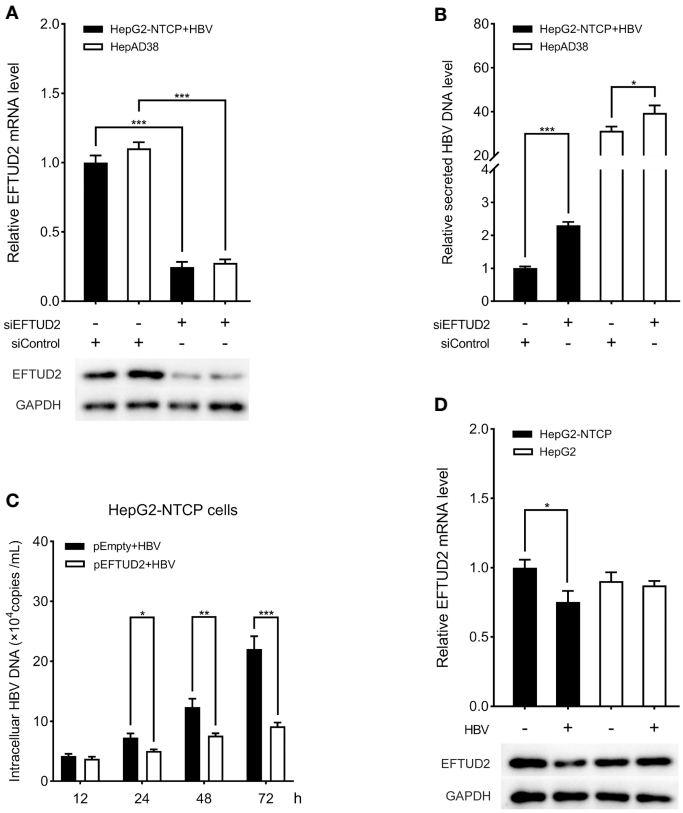

FIGURE 1
EFTUD2 had an anti-HBV effect *in vitro*. **(A)** The efficacy of the knockdown of EFTUD2 was determined by qPCR and Western blot analyses in HepAD38 cells and HepG2-NTCP cells. **(B)** The knockdown of EFTUD2 improved the HBV DNA levels. **(C)** EFTUD2 overexpression inhibited HBV replication at 12, 24, 48, 72 h postinfection. **(D)** Real-time PCR and Western blot analyses showed that EFTUD2 was downregulated in HepG2-NTCP and HepG2 cells treated with HBV particles at 48 h. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

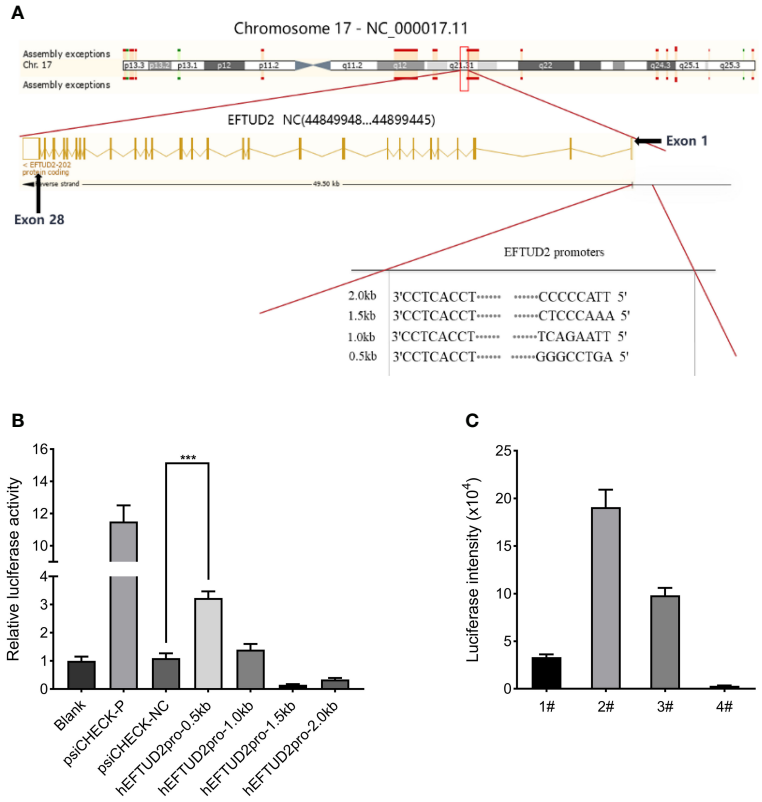

FIGURE 2
Construction of the Epro-LUC-HepG2 cell line. **(A)** Prediction of the EFTUD2 promoter sequences was performed according to the ENCODE data. **(B)** The luciferase analysis showed that the hEFTUD2pro-0.5 kb promoter had the strongest activity. **(C)** Stable transgenic Epro-LUC-HepG2 cell line (2#) was selected and the luciferase intensity was displayed. ***, $P < 0.001$.

33 compounds increased luciferase activity by more than 2 times, 11 of which were selected as the candidates for further experiments and increased luciferase activity by more than 3 times (Figure 3A). To verify that they upregulate the expression of EFTUD2, Epro-LUC-HepG2 cells were treated with these compounds for 3 days and the expression of the EFTUD2 mRNA and protein was quantified. Among the 11 candidates, plerixafor and resatorvid increased the EFTUD2 mRNA level by more than 10 times and increased the EFTUD2 protein level by more than two times. Thus, plerixafor and resatorvid were selected in this study due to their low cytotoxicity and ability to significantly upregulate the EFTUD2 promoter activity, along with its mRNA and protein levels (Figure 3B). The characteristics of the two agents and their effects on the viability of HepAD38 and HepG2-NTCP cells were shown in Figure S3.

Inhibitory effects of plerixafor and resatorvid on HBV replication

To further determine the anti-HBV activity of plerixafor and resatorvid, the HBV markers, including HBV RNAs, DNA, and HBsAg, were assessed in HepAD38 cells after treatment for 3, 6, and 9 days with the agent, respectively. The results of the ELISA showed that plerixafor significantly reduced the level of HBsAg in the supernatant. Specifically, after administering 0.1 nM plerixafor, the level of HBsAg decreased on the third (90%, $P < 0.05$), sixth (81%, $P < 0.05$), and ninth (68%, $P < 0.01$) day, compared to the level of HBsAg after DMSO treatment (Figure 4A). Similar results were found after administering 0.2 nM and 0.5 nM plerixafor (Figure 4A). Furthermore, treatment with 0.1 nM resatorvid reduced the level of HBsAg on the third (87%, $P < 0.05$), sixth (79%, $P < 0.01$), and ninth (73%, $P < 0.01$) day, compared to the level of HBsAg after DMSO treatment (Figure 5A). Similar results were found after treatment with 0.2 nM and 0.5 nM resatorvid (Figure 5A). Real-time PCR analysis showed that the secreted HBV DNA level and intracellular HBV DNA level also decreased significantly after treatment with plerixafor in a dose-dependent manner (Figures 4B, C, 5B, C). To further validate our findings, Southern blot analysis was performed. The bands were approximately 3.2 kbp (Figures 4F, 5F), which was similar to the full-length HBV genome; thus, indicating that intracellular HBV DNA was correctly detected. The HBV DNA in the cells decreased significantly after treatment for 10 days, indicating that plerixafor and resatorvid inhibited the replication of HBV DNA.

HBV cccDNA serves as the template for transcription of all four viral mRNAs (3.5, 2.4, 2.1, and 0.7 kb) (Ren et al., 2019). To determine whether the reduction of HBV DNA was due to a decrease in the mRNA levels, HBV RNAs were analyzed by real-time PCR analysis. The results showed that plerixafor and

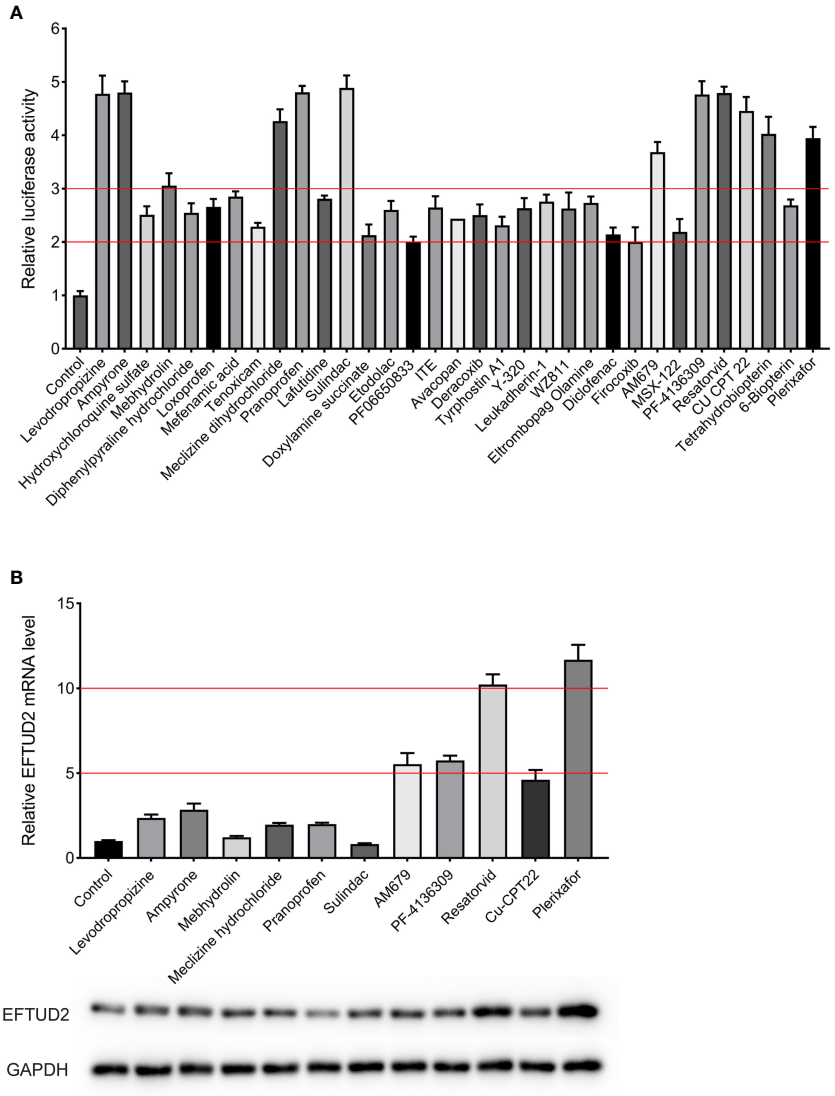

FIGURE 3
Screening compounds that upregulated the EFTUD2 promoter activity, genes, and proteins. **(A)** The luciferase analysis showed that 33 compounds increased the activity of the EFTUD2 promoter by more than two times, and 12 compounds increased the activity by more than three times. **(B)** Real-time PCR and Western blot analyses showed that plerixafor and resatorvid had the strongest ability to upregulate the activity of the EFTUD2 promoter among the 11 compounds (tetrahydrobiopterin was abandoned due to its obvious cytotoxicity).

resatorvid significantly decreased the levels of total HBV RNAs and 3.5-kb RNA in a dose-dependent manner, whereas, ETV had no such effects (Figures 4D, E, 5D, E). Together, these results suggested that plerixafor and resatorvid inhibited HBV replication in HepAD38 cells.

Inhibitory effect of plerixafor and resatorvid on HBV replication in the HBV infection model

Viral entry into the hepatocyte is mediated by the binding of the NTCP receptor to the pre-S1 domain of L-HBsAg (Ren et al., 2019). Based on the susceptibility of HepG2-NTCP cells to hepatitis B virus particles, a cell model of hepatitis B virus infection was established. To further investigate the effect of plerixafor and resatorvid in the HBV infection model, HepG2-NTCP cells were infected with a normalized amount of HBV particles (1,000 GEq/cell). Then, the HBV-infected HepG2-NTCP cells were treated with 0.5 nM plerixafor/resatorvid and 25 nM ETV as the positive control for 10 days. Plerixafor and resatorvid significantly reduced the levels of HBsAg ($P < 0.01$ and $P < 0.01$) (Figures 6A, 7A). Moreover, the results of real-time PCR analysis showed that the content of HBV DNA in the HepG2-NTCP cells treated with plerixafor and resatorvid decreased significantly ($P < 0.01$ and $P < 0.01$) (Figures 6B, 7B). A similar change in the intracellular HBV DNA content was also found ($P < 0.001$ and $P < 0.001$) (Figures 6C, 7C), which was comparable to the results of the Southern blot assay

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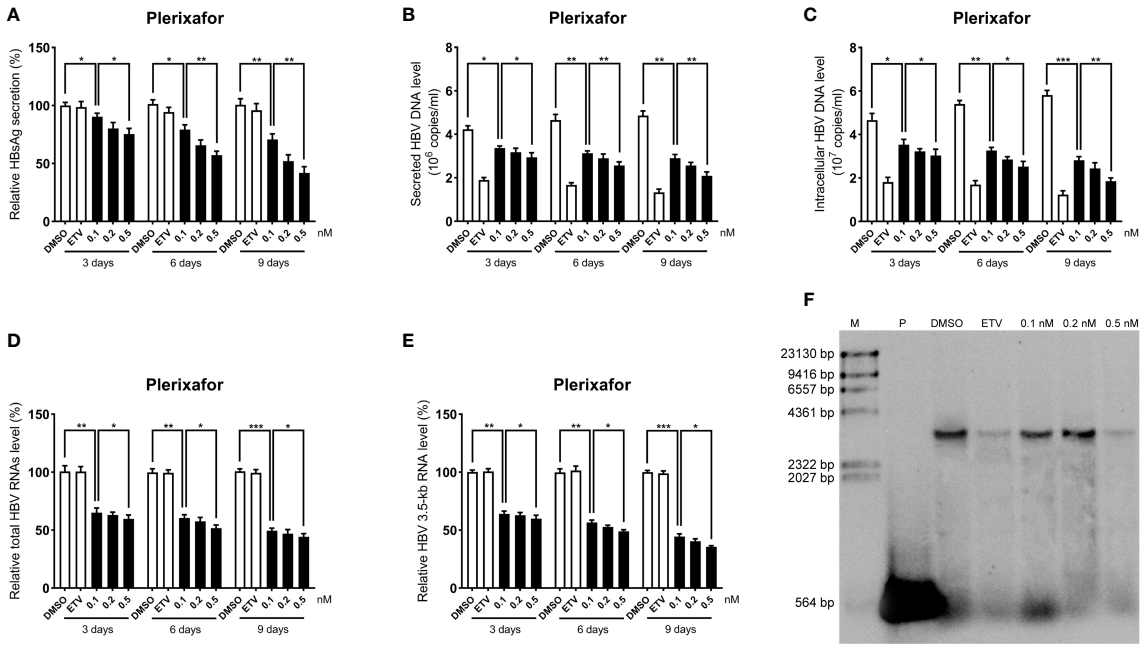

FIGURE 4
Plerixafor inhibited HBV replication in HepAD38 cells. HepAD38 cells were treated with 0.1% DMSO, 0.1, 0.2, or 0.5 nM plerixafor and 25 nM ETV for 3, 6 and 9 days. The levels of HBsAg, secreted and intracellular HBV DNA, and HBV RNAs were detected. **(A)** The results of the ELISA showed a decrease in the level of HBsAg in the supernatant. **(B-E)** Real-time PCR analysis showed that plerixafor inhibited HBV DNA both in the supernatant and cells, as well as, HBV total RNAs and 3.5-kb RNA. **(F)** A Southern blot analysis was performed to determine the level of HBV DNA after 9 days of treatment. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

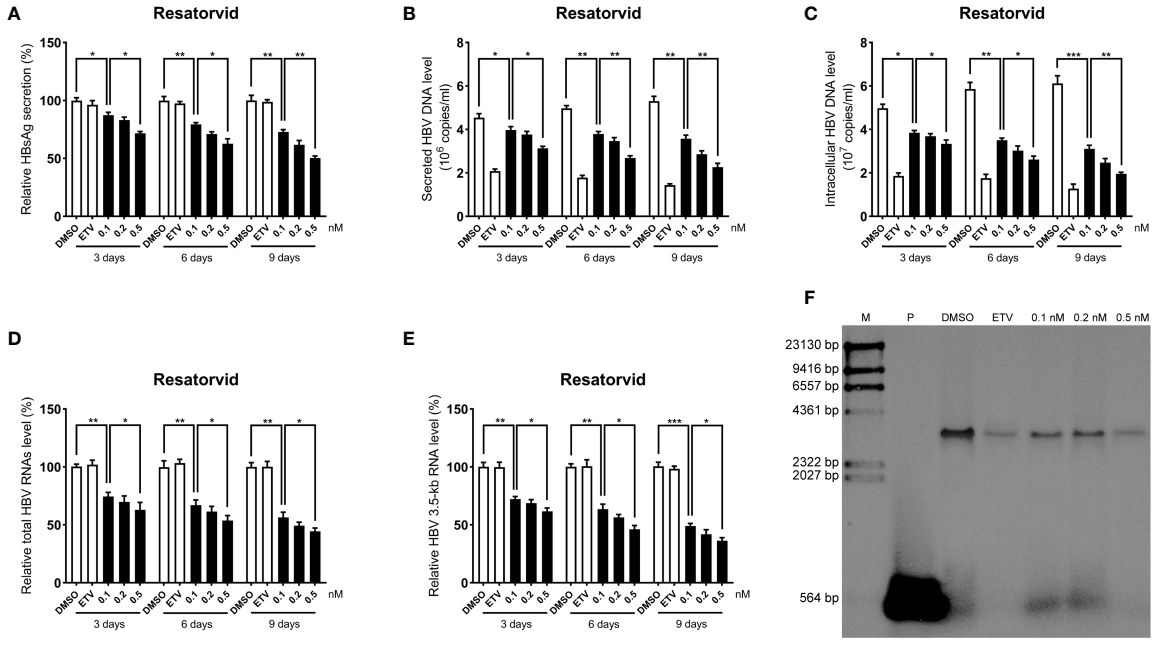

FIGURE 5
Resatorvid inhibited HBV replication in HepAD38 cells. HepAD38 cells were treated with 0.1% DMSO, 0.1, 0.2, or 0.5 nM resatorvid and 25 nM ETV for 3, 6 and 9 days. The levels of HBsAg, secreted and intracellular HBV DNA, and HBV RNAs were detected. **(A)** The results of the ELISA showed a decrease in the level of HBsAg in the supernatant. **(B-E)** Real-time PCR analysis showed that resatorvid inhibited HBV DNA both in the supernatant and cells, as well as, HBV total RNAs and 3.5-kb RNA. **(F)** A Southern blot analysis was performed to determine the level of HBV DNA after 9 days of treatment. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

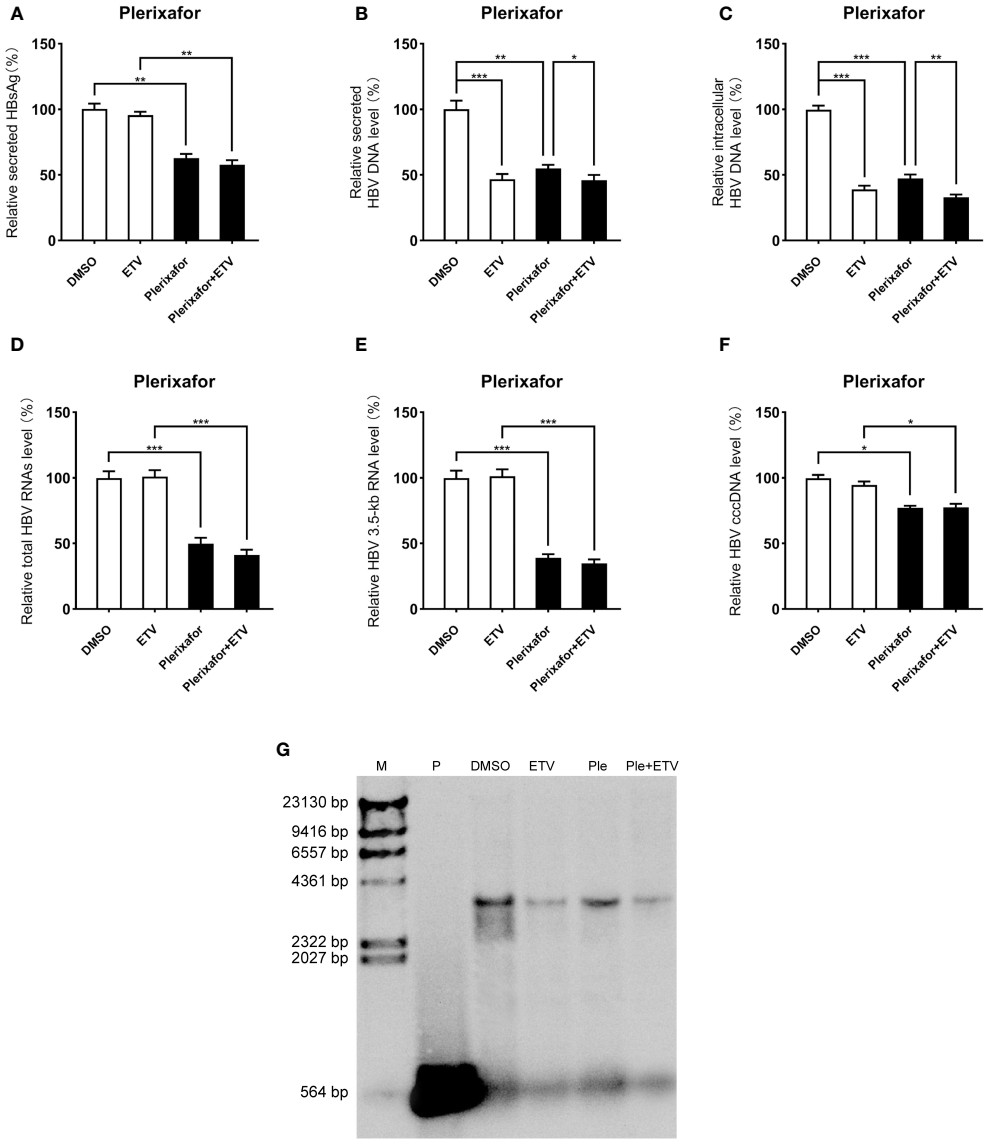

FIGURE 6
Plerixafor showed anti-HBV activity in the HBV infection model. HepG2-NTCP cells were infected with HBV particles (1,000 GEq/cell). Then, the HBV-infected cells were treated with 0.1% DMSO, 0.5 nM plerixafor or/and 25 nM ETV for 10 days. **(A)** The results of the ELISA showed that plerixafor decreased the HBsAg level in the supernatant. **(B–E)** Plerixafor significantly inhibited secreted and intracellular HBV DNA, total HBV RNAs, and the 3.5-kb RNA. **(F)** Plerixafor partly decreased the level of cccDNA. **(G)** A Southern blot analysis was performed to determine the reduction of HBV DNA. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

(Figures 6G, 7G), along with changes in the total HBV RNAs and 3.5-kb RNA ($P < 0.001$ and $P < 0.001$; $P < 0.001$ and $P < 0.001$) (Figures 6D, E, 7D, E). Additionally, the results of the qPCR analysis showed that plerixafor and resatorvid reduced the level of the transcription template cccDNA moderately in the HepG2-NTCP cells ($P < 0.05$ and $P < 0.05$) (Figures 6F, 7F). ETV alone only reduced the level of HBV DNA ($P < 0.001$) without affecting the levels of HBsAg and HBV RNAs, but the combination improved the antiviral activity. Collectively, these results suggested that plerixafor and resatorvid could inhibit HBV replication in the HBV infection model, probably by reducing HBV RNAs and cccDNA levels.

Plerixafor and resatorvid inhibit HBV replication by targeting EFTUD2

To determine the mechanism by which plerixafor and resatorvid act on HBV, we silenced EFTUD2 in HepAD38 and HBV-infected HepG2-NTCP cells and tested the antiviral activity of plerixafor and resatorvid. The results showed that knocking down EFTUD2 greatly impaired the effect of plerixafor and resatorvid, both in HepAD38 cells (Figures 8A, B) and in HepG2-NTCP cells (Figures 8C, D). Taken together, plerixafor and resatorvid act as positive regulators of EFTUD2 and inhibit HBV by upregulating EFTUD2.

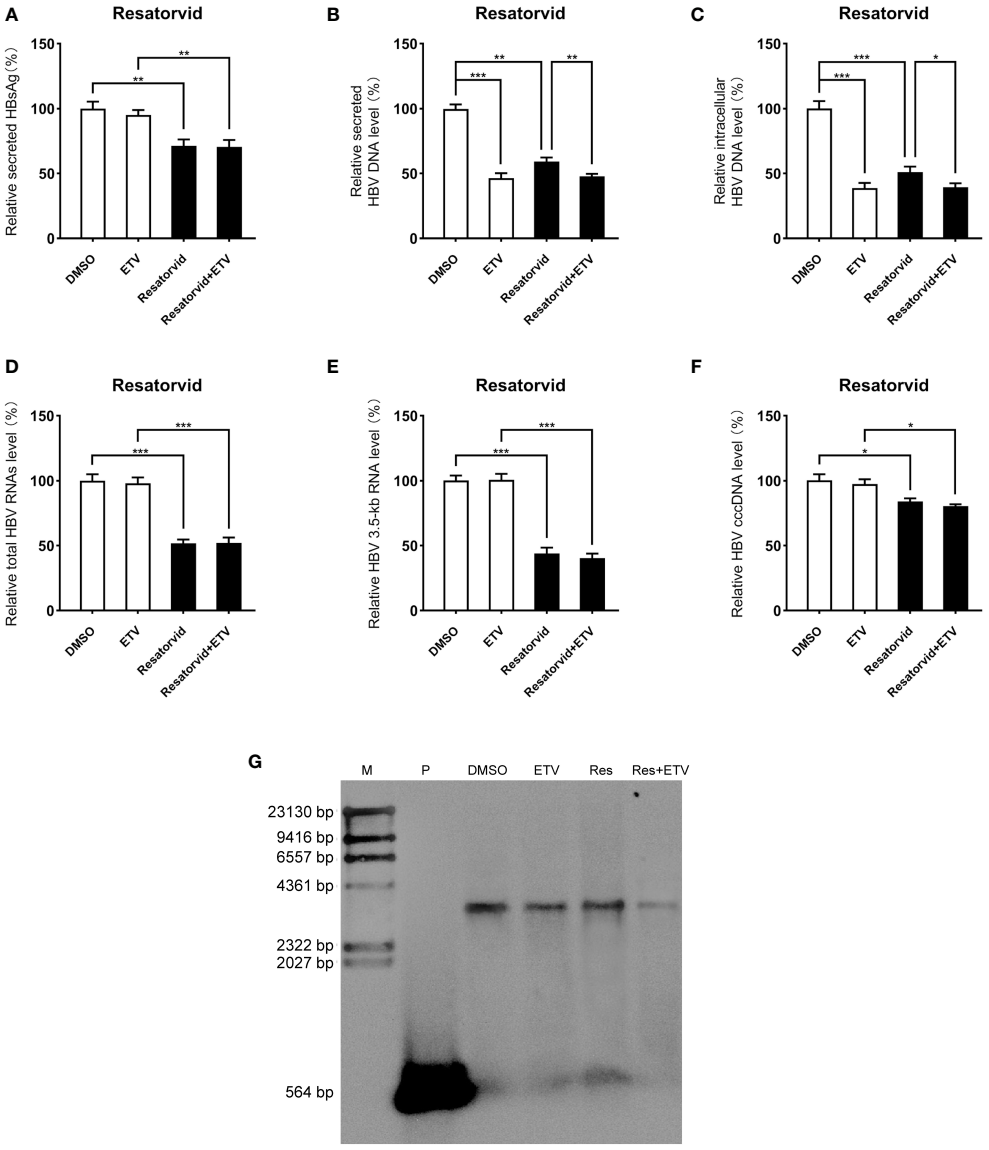

FIGURE 7
Resatorvid showed anti-HBV activity in the HBV infection model. The HBV-infected cells were treated with 0.1% DMSO, 0.5 nM resatorvid or/and 25 nM ETV for 10 days. **(A)** The results of the ELISA showed that resatorvid decreased the HBsAg level in the supernatant. **(B–E)** Resatorvid significantly inhibited secreted and intracellular HBV DNA, total HBV RNAs, and the 3.5-kb RNA. **(F)** Resatorvid partly decreased the level of cccDNA. **(G)** A Southern blot analysis was performed to determine the reduction of HBV DNA. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Discussion

Although advancements have been made in the regulation of HBV infection, it is still a threat to public health, especially in less-developed regions. Approximately 292 million people worldwide are living with HBV, and 86 million are affected in China, where only 11% of CHB patients have access to antiviral treatment (Jia et al., 2020). Known treatment strategies mostly involve the use of NAs and interferon-α (IFN-α), which reduce the viral load and improve long-term outcomes, but rarely achieve functional cures (Alonso et al., 2017). Therefore, finding better treatment strategies for HBV infection is extremely important. Although several small molecules have been reported recently, only a few are both safe and efficacious. For example, inarigivir is an agonist of the retinoic acid-inducible gene I (RIG-I) with high anti-HBV efficacy, but it was abandoned because it had adverse effects, and even led to death (Yuen et al., 2019). Vesatolimod (GS-9620) is a safe and well-tolerated TLR-7 agonist and can effectively suppress HBV DNA but does not affect HBsAg (Janssen et al., 2018). The TLR8 agonist selgantolimod (GS-9688) showed seroconversion in the woodchuck model of CHB (Daffis et al., 2021), but limited clinical activity was observed in patients, probably due to dose-limiting events such as gastrointestinal toxicity (Janssen et al., 2021). Thus, searching for new therapeutic targets and agents is necessary. We found a novel class of immunomodulators that targeted EFTUD2 and promoted HBV clearance in different cell models. Furthermore, they showed high anti-HBV efficacy, including the ability to reduce cccDNA.

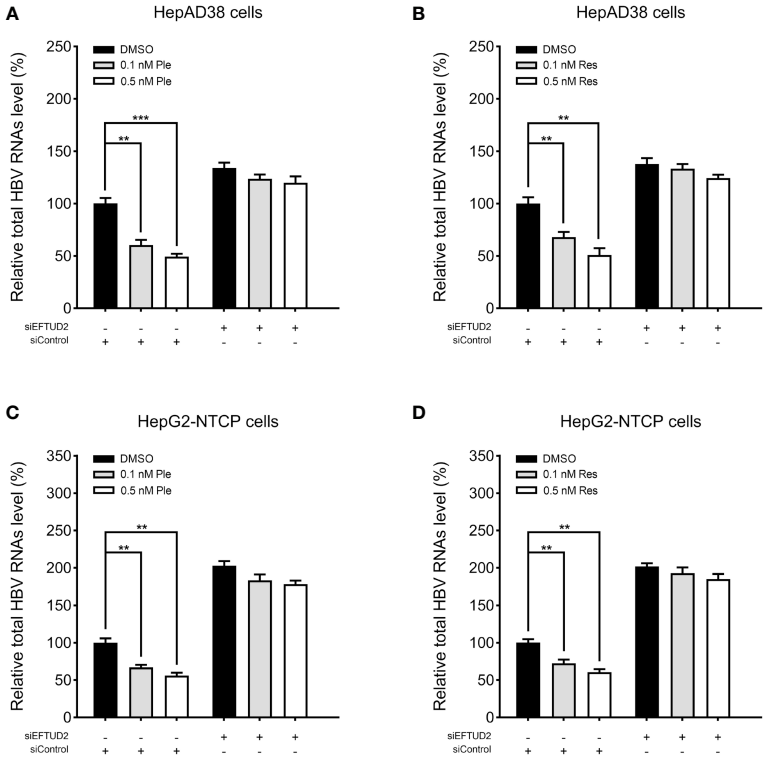

FIGURE 8
Plerixafor and resatorvid inhibited HBV by targeting EFTUD2. HepAD38 and HepG2-NTCP cells were transfected with siRNAs targeted EFTUD2 (siEFTUD2) or negative control (siControl) , and then, treated with DMSO, plerixafor or resatorvid at indicated concentrations for 7 days. The total HBV RNAs were extracted and quantified by qPCR. The results showed that knocking down EFTUD2 substantially impaired the anti-HBV effect of plerixafor and resatorvid, both in HepAD38 cells **(A, B)** and HepG2-NTCP cells **(C, D)**. **, $P < 0.01$; ***, $P < 0.001$.

EFTUD2 is a component of the U5 snRNP, which controls mRNA splicing along with the rest of the spliceosome (Malinová et al., 2017). As a general splicing factor, EFTUD2 participates in various physiological processes such as the organization of myofilaments (Meissner et al., 2009) and P granule development (Updike and Strome, 2009), as well as, pathophysiological processes of some genetic disorders (Löb et al., 2020; Wang et al., 2021; Yang et al., 2022). In this study, we found that silencing EFTUD2 increased the HBV load while overexpressing EFTUD2 reduced the HBV load in different cell models. Moreover, the regulation of EFTUD2 did not significantly affect overall cell viability, suggesting that EFTUD2 can protect against HBV. These results are similar to those regarding the inhibitory effect of EFTUD2 on HCV by modulation of the RIG-I pathway through mRNA splicing (Zhu et al., 2015). Several other viruses have similar characteristics and act on the U5 snRNP components; thus, inducing changes in host cell alternative splicing and affecting virus-host interactions. For example, 3D polymerase from enterovirus 71 directly binds to pre-mRNA processing factor 8 (PRPF8) and disrupts the pre-mRNA splicing processes, contributing to the invasion of the virus (Liu et al., 2014). The NS5 protein of the dengue virus hijacks the splicing machinery by targeting CD2 Cytoplasmic Tail Binding Protein 2 (CD2BP2) and DEAD-Box Helicase 23 (DDX23), creating a favorable environment for the replication of the virus (De Maio et al., 2016). Mammalian orthoreovirus infection leads to a decrease in the expression of EFTUD2 and alterations in cellular splicing, which benefits its oncolytic potential (Boudreault et al., 2022). Several transcriptomic studies have shown that the compatibility between a virus and its host is related to alternative splicing mediated by spliceosomes that are not limited to the U5 snRNP (Batra et al., 2016; Ashraf et al., 2019; Boudreault et al., 2019). We have addressed the role of the U5 core component EFTUD2 in HBV replication in another study (Hu et al., unpublished). In this study, we found that the EFTUD2 gene and protein levels decreased during HBV infection, probably because HBV can downregulate EFTUD2 to reduce its restriction.

A single nucleotide polymorphism analysis showed that the EFTUD2 mutation rs3809756A>C is associated with a decrease in the promoter activity and an increase in the susceptibility to HBV infection (Tian et al., 2022), which supported our findings. In this study, we identified the most active hEFTUD2pro-0.5 kb promoter among the different lengths of the EFTUD2 promoter predicted by bioinformatics, suggesting that it might be a key element in regulating the activity of EFTUD2. Then, the HepG2 cells were stably transfected with a luciferase reporter construct controlled by the hEFTUD2pro-0.5 kb promoter, and the Epro-LUC-HepG2 cell line was constructed to check the promoter activity. By screening 261 compounds from a small-molecule library related to immunity and inflammation, we identified 11 compounds that increased the promoter activity in the Epro-LUC-HepG2 cells by 3–5 folds

without showing any signs of obvious cytotoxicity. In these cells, plerixafor and resatorvid showed the most prominent effect on upregulating the expression of EFTUD2.

Plerixafor (AMD3100) is a well-known inhibitor of CXC chemokine receptor 4 (CXCR4) and has specific effects on T4-lymphotropic HIV strains (Hendrix et al., 2000). It was first developed as an anti-HIV drug and has now been repositioned and clinically applied to peripheral blood stem cell mobilization in non-Hodgkin's lymphoma and multiple myeloma (DiPersio et al., 2009). Plerixafor and its derivatives can also be administered along with other chemicals to increase the effectiveness of treatment in many solid cancers, such as ovarian (Righi et al., 2011), breast (Peng et al., 2015), and pancreatic cancers (Galsky et al., 2014). Resatorvid (TAK-242) is a newly developed, highly selective Toll-like receptor 4 (TLR4) antagonist that is effective for treating pulmonary inflammation (Wang et al., 2016), rheumatoid arthritis (Samarpita et al., 2020), and acute kidney damage (Mohammad et al., 2018). Resatorvid also has hepatoprotective effects against different forms of hepatic dysfunction, such as liver ischemia/reperfusion injury (Shao et al., 2016), along with acute and acute-on-chronic liver failure caused by endotoxemia (Oya et al., 2014; Engelmann et al., 2020). However, the effect of plerixafor or resatorvid on HBV replication in hepatocytes has not been reported. Here, we reported that plerixafor and resatorvid have potent anti-HBV activity *in vitro*.

Our results showed that plerixafor and resatorvid significantly reduced HBsAg secretion even at a low concentration of 0.1 nM with no signs of obvious cytotoxicity in the working concentration range. Furthermore, we found that plerixafor and resatorvid can substantially decrease both intracellular and extracellular HBV DNA levels in a dose-dependent and time-dependent manner. To determine the mechanism, we evaluated the levels of total HBV RNAs and 3.5-kb RNA and found that they decreased similarly. We further investigated the effect of plerixafor and resatorvid on cccDNA, and found a decrease in the cccDNA levels, albeit to a minor degree. However, considering that there was a significant decrease in the ratios of HBV RNAs to cccDNA, the HBV transcription activity was probably suppressed, which explains the decrease in the HBV DNA levels. We speculated the decreased HBV DNA might be due to the diminished upstream HBV RNAs, especially the 3.5-kb RNA.

We proposed that plerixafor and resatorvid act on EFTUD2 rather than other classical targets. Our results showed that silencing EFTUD2 substantially impaired the activity of plerixafor and resatorvid even at a relatively higher concentration of 0.5 nM, but not completely, which might probably be due to the incomplete knockout of EFTUD2. Plerixafor targets CXCR4, but this might not occur in hepatocellular carcinoma (HCC) cells. Many HCC cells express abundant CXCR4 receptors, but its principal ligand C-X-C Motif Chemokine Ligand 12 (CXCL12) is absent, indicating plerixafor inhibits HBV independent of its classical receptor CXCR4 (Li et al., 2014; Kawaguchi et al., 2019). Although some studies reported TLR4 was increased in persistent HBV infection, it was mainly detected in immune cells, not in hepatocytes (Li et al., 2020). In this study, the working concentration of resatorvid was so low that TLR4 was not significantly inhibited (data not shown). What's more, the synergistic administration of plerixafor and resatorvid for the treatment of HBV was tested, but there was no significant benefit (data not shown). This could be attributed to competition in upregulating EFTUD2.These findings indicated that EFTUD2 is indispensable for the antiviral effect of plerixafor and resatorvid, although the presence of other non-primary targets cannot be excluded.

ETV can act directly on DNA synthesis and strongly inhibit HBV DNA replication. However, it has a minor effect on host immune function or cccDNA micro-chromatin because the transcriptional template cccDNA is found in infected hepatocytes (Marcellin et al., 2008). In this study, we found that the HBV markers in cells and supernatants decreased significantly after treatment with plerixafor or resatorvid alone, but not with ETV alone, which was similar to the findings of previous studies (Marcellin et al., 2008; Buti et al., 2018). Moreover, ETV primarily reduces the HBV DNA levels, while plerixafor and resatorvid primarily decrease HBsAg and HBV RNA levels. Thus, we combined ETV with plerixafor or resatorvid to complement the effects of each other. We found a balanced situation that comprehensively exerted antiviral activity, suggesting that this combination therapy might be better than monotherapy in functional cure.

This study had some limitations besides those mentioned above. First, the effect on the stability of HBV RNAs or the CMV promoter activity in the HepAD38 cells was not further determined, although plerixafor and resatorvid were found to play an anti-HBV role in HepG2-NTCP cells. Also, the antiviral effects of plerixafor and resatorvid on other genotypes or strains, as well as, their effects *in vivo* need to be further studied.

Conclusion

In this study, we established an EPro-Luc-HepG2 cell line for the first time, which provided a reliable and convenient method for screening small-molecule compounds targeting EFTUD2. The screened small-molecule agents plerixafor and resatorvid significantly upregulated EFTUD2 and decreased HBsAg, HBV DNA, RNAs, and cccDNA levels *in vitro*. The effects of plerixafor and resatorvid complement those of ETV. Thus, these compounds are promising and might be considered while developing complementary or alternative therapeutic strategies for anti-HBV treatment.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

Author contributions

JC, YL and CZ conceived the manuscript. JC and YL acquired and analyzed the data, and contributed equally to this work. PH and

RX wrote the original draft. HY, WZ, TF, RL and WL revised the manuscript. All authors contributed to the article and approved the submitted version.

Funding

This work was supported by Science and Technology Plan of Hainan Province (Clinical Research Center) (LCYX202103 and LCYX202204), Hainan Province Science and Technology Special Fund (ZDYF2022SHFZ067), and the grants from Hainan Province Clinical Medical Center, the National Natural Science Foundation of China (No.81770591).

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2023.1118801/full#supplementary-material

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frontiers | Frontiers in Cellular and Infection Microbiology

TYPE Original Research
PUBLISHED 24 February 2023
DOI 10.3389/fcimb.2023.1120300

OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to Virus and Host, a section of the journal Frontiers in Cellular and Infection Microbiology

RECEIVED 09 December 2022
ACCEPTED 02 February 2023
PUBLISHED 24 February 2023

CITATION
Guo Y, Han J, Zhang Y, Jin C, Zhang Y, He J, Chen S, Guo Y, Lin Y, Li F, Yang F, Shen Z, Mao R, Zhu H and Zhang J (2023) End-of-treatment anti-HBs levels and HBeAg status identify durability of HBsAg loss after PEG-IFN discontinuation. *Front. Cell. Infect. Microbiol.* 13:1120300. doi: 10.3389/fcimb.2023.1120300

End-of-treatment anti-HBs levels and HBeAg status identify durability of HBsAg loss after PEG-IFN discontinuation

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Background: Hepatitis B surface antigen (HBsAg) loss, namely, the functional cure, can be achieved through the pegylated interferon (PEG-IFN)-based therapy. However, it is an unignorable fact that a small proportion of patients who achieved functional cure develop HBsAg reversion (HRV) and the related factors are not well described.

Methods: A total of 112 patients who achieved PEG-IFN-induced HBsAg loss were recruited. HBV biomarkers and biochemical parameters were examined dynamically. HBV RNA levels were assessed in the cross-sectional analysis. The primary endpoint was HRV, defined as the reappearance of HBsAg after PEG-IFN discontinuation.

Results: HRV occurred in 17 patients during the follow-up period. Univariable analysis indicated that hepatitis B e antigen (HBeAg) status, different levels of hepatitis B surface antibody (anti-HBs), and hepatitis B core antibody (anti-HBc) at the end of PEG-IFN treatment (EOT) were significantly associated with the incidence of HRV through using the log-rank test. Additionally, time-dependent receiver operating characteristic (ROC) analysis showed that the anti-HBs was superior to anti-HBc in predictive power for the incidence of HRV during the follow-up period. Multivariable Cox proportional hazard analysis found that anti-HBs ≥1.3 $\log_{10}$IU/L (hazard ratio (HR), 0.148; 95% confidence interval (CI), 0.044-0.502) and HBeAg negativity (HR, 0.183; 95% CI, 0.052-0.639) at EOT were independently associated with lower incidence of HRV. Cross-sectional analysis indicated that the HBV RNA levels were significantly correlated with the HBsAg levels in patients with HRV (r=0.86, p=0.003).

Conclusions: EOT HBeAg negativity and anti-HBs ≥1.3 log_{10}IU/L identify the low risk of HRV after PEG-IFN discontinuation.

KEYWORDS

chronic hepatitis B, HBsAg reversion, functional cure, PEG-IFN, HBeAg, anti-HBs

1 Introduction

Chronic hepatitis B virus (HBV) infection remains a major global health issue, affecting approximately 250 million people (WHO., 2017). Currently, hepatitis B surface antigen (HBsAg) loss with or without hepatitis B surface antibody (anti-HBs) is considered as the optimal endpoint for antiviral treatment and referred to as 'functional cure', but it is rarely achieved, putting patients at risk for severe liver diseases, including cirrhosis, hepatic decompensation, and hepatocellular carcinoma (HCC) (WHO, 2017).

Nucleos(t)ide analogues (NAs) are the first-line treatment and can provide sustained suppression of HBV replication (Sarin et al., 2016; Terrault et al., 2016; European Association for the Study of the Liver, 2017), but previous studies suggested that patients with pegylated interferon (PEG-IFN)-based therapy exhibited higher rates of achieving HBsAg loss compared to patients with NAs therapy (Marcellin et al., 2016; Yip et al., 2017; Wu et al., 2020; Tout et al., 2021). Unfortunately, a small proportion of patients who achieved HBsAg loss develop HBsAg reversion (HRV) during follow-up. The cumulative probability of HRV was 9.66% for 597 weeks in patients treated with interferon (IFN) (Wu et al., 2020). To date, however, fewer studies have focused on predicting HRV in patients with PEG-IFN-induced HBsAg loss.

Considering the likelihood of HRV and subsequent adverse outcomes, in this study, we focused on evaluating the factors affecting HRV after PEG-IFN discontinuation. The results of our study could be useful for clinicians to select the appropriate time to discontinue PEG-IFN treatment for patients who achieved HBsAg loss.

2 Materials and methods

2.1 Patients

A retrospective cohort study was performed in Huashan Hospital from Jan 2014 to Dec 2019. A total of 163 chronic hepatitis B (CHB) patients who had at least one undetectable HBsAg result were consecutively enrolled, and those 112 patients who achieved PEG-IFN-induced HBsAg loss were ultimately analyzed. Exclusion criteria were NAs-induced HBsAg loss; conventional IFN-induced HBsAg loss; unconfirmed HBsAg loss (Figure 1). The study was approved by the Ethics Committee of Huashan Hospital of Fudan University and carried out in accordance with the current version of the Helsinki Declaration.

2.2 Laboratory measurements

Liver biochemical parameters were determined by a biochemistry analyzer (7600 Series; Hitachi, Tokyo, Japan). Platelet was measured by Sysmex XN-2000 (Kobe, Japan). Serum HBsAg, anti-HBs, hepatitis B e antigen (HBeAg), hepatitis B e antibody (anti-HBe), and hepatitis B core antibody (anti-HBc) were detected using an enzyme-linked immunosorbent assay kit (ARCHITECT i2000 SR; Abbott Architect, USA). Serum HBsAg were retested (Roche Cobas e602; Roche, Switzerland) when it exceeded the upper linearity limit (250 IU/mL). HBV DNA was quantified by using a real-time PCR assay (DAAN Diagnostics, Guangzhou, China). Detection limits of HBsAg, anti-HBs, HBeAg, anti-HBe, anti-HBc, and HBV DNA were 0.05 IU/mL, 10 IU/L, 1 S/CO, 1 S/CO, 1 S/CO, 500 IU/mL, respectively. The upper limit of normal (ULN) of ALT has been defined as 40 U/L for women and 50 U/L for men. The ULN of bilirubin has been defined as 21 μmol/L for women and 26 μmol/L for men. The normal range of albumin was 40-55 g/L. In addition, hepatitis B core-related antigen (HBcrAg) was not detected due to the lack of serum samples.

2.3 HBV RNA concentration measurement

HBV RNA, as reverse transcribed pre-genomic HBV RNA, was extracted from 200 μL serum with the nucleic acid extraction or purification kit (magnetic beads method) (Sansure Biotech, Changsha, China). Next, the extraction was treated with DNase I (Thermo Fisher Scientific, Waltham, MA, USA). Finally, DNase-I-treated HBV RNA was quantitatively measured with the HBV RNA

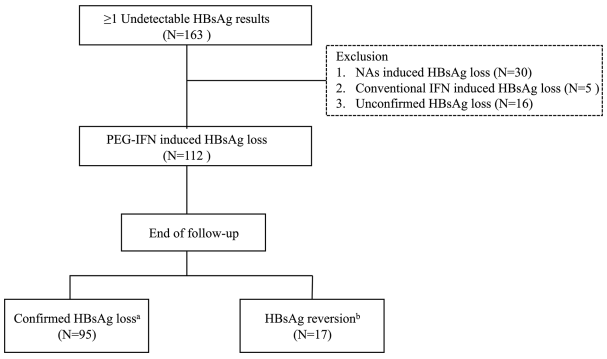

FIGURE 1
Flowchart of patients' selection process. HBsAg, hepatitis B surface antigen; NA, nucleos(t)ide analogue; IFN, interferon; PEG-IFN, pegylated interferon. [a]Two negative HBsAg results (<0.05 IU/mL) at least 6 months apart. [b]Reappearance of HBsAg after HBsAg loss.

quantitative kit (Sansure Biotech, no.010106025). The detection limit of the assay was 100 copies/mL. HBV RNA detection was conducted in 64 patients, not all patients, due to the lack of serum samples.

2.4 Definitions and endpoints

The primary outcome was the development of HRV. Follow-up duration was measured from the date of the end of PEG-IFN treatment (EOT) to the date of HRV or the last follow-up visit. Consolidation treatment duration was measured from the date of HBsAg loss to the date of EOT. Confirmed HBsAg loss (CHL) was defined as two negative HBsAg results (<0.05 IU/mL) at least 6 months apart; HRV was defined as the reappearance of HBsAg after HBsAg loss. PEG-IFN monotherapy was defined as PEG-IFN therapy in naïve chronic hepatitis B (CHB) patients. Add-on PEG-IFN was defined as combination therapy after at least 48 weeks of nucleot(s)ide therapy. Switch-to PEG-IFN was defined as PEG-IFN monotherapy in patients who received NAs for at least 48 weeks.

2.5 Statistical analysis

Continuous variables were expressed as mean (interquartile range [IQR]). Categorical variables were expressed as counting and percentage. Comparison between two-group of continuous variables was operated using the Student's t-test or Mann-Whitney U test. The Chi-squared test was used for categorical variables. Correlations between variables were tested with Pearson Correlation. The cumulative HRV rates were performed using the Kaplan-Meier method and comparisons were tested with the log-rank test. Predictive factors for HRV were evaluated *via* the univariable and multivariable Cox proportional hazard analyses. Variables with p <0.10 in the univariable Cox proportional hazard analyses were used in multivariable analyses. Time-dependent receiver operator characteristic (ROC) curve analysis was conducted to investigate the predictive performance of anti-HBs and anti-HBc using the "timeROC" package in R (version 4.1.1, http://www.r-project.org). The optimal cut-off values of anti-HBs and anti-HBc were determined by ROC analysis. The cut-off value of the consolidation treatment duration was based on the previous study (Li et al., 2019). A P-value <0.05 was considered statistically significant. Analyses were performed using the SPSS version 20.0 (SPSS, Chicago, USA).

3 Results

3.1 Patient characteristics and HRV development

Clinical characteristics of 112 CHB patients who achieved PEG-IFN-induced HBsAg loss were presented in Table 1. The median age of total patients was 37.0 years (IQR, 31.0-43.0 years) and the majority of the patients were male (91/112, 81.3%). The median duration of PEG-IFN treatment and consolidation treatment was 48.0 weeks

TABLE 1 Summary of 17 patients with HBsAg reversion after PEG-IFN therapy discontinuation.

Patient	Gender	Age (y)	Therapy	HBeAg status[a]	Anti-HBe status[a]	Anti-HBs (IU/L)[a]	Anti-HBc (s/co)[a]	Follow-up time (month)[c]	HBsAg (IU/mL)[b]	Anti-HBs (IU/L)[b]	HBV DNA (IU/mL)[b]	ALT (U/L)[b]
1	Male	37	PEG-IFN	Negative	Negative	<10	11.4	29	0.69	<10	<500	14
2	Male	51	PEG-IFN	Negative	Negative	142.3	11.3	48	250	<10	2.02E+07	225
3	Female	33	TDF +PEG-IFN	Negative	Positive	17.9	8.8	12	1.6	<10	<500	9
4	Male	46	TDF +PEG-IFN	Positive	Negative	16.2	5.4	4	0.33	<10	<500	53
5	Female	30	ETV +PEG-IFN	Positive	Negative	30.7	8.8	15	7.92	<10	<500	16
6	Male	46	TDF +PEG-IFN	Positive	Negative	186.7	9.3	24	0.25	<10	<500	29
7	Female	39	PEG-IFN	Negative	Positive	47.4	8.9	2	0.32	<10	<500	13
8	Male	39	TDF +PEG-IFN	Negative	Positive	14.1	9.3	10	0.08	<10	<500	16
9	Male	48	ETV +PEG-IFN	Negative	Negative	<10	7.5	4	0.08	<10	<500	23

(Continued)

TABLE 1 Continued

Patient	Gender	Age (y)	Therapy	HBeAg status[a]	Anti-HBe status[a]	Anti-HBs (IU/L)[a]	Anti-HBc (s/co)[a]	Follow-up time (month)[c]	HBsAg (IU/mL)[b]	Anti-HBs (IU/L)[b]	HBV DNA (IU/mL)[b]	ALT (U/L)[b]
10	Male	42	ADV +PEG-IFN	Negative	Positive	<10	8.1	31	0.26	<10	<500	29
11	Female	37	PEG-IFN	Positive	Negative	143.8	9.4	12	1.8	513.2	<500	10
12	Male	43	PEG-IFN	Negative	Positive	<10	8.1	3	0.14	<10	<500	31
13	Female	31	PEG-IFN	Negative	Positive	<10	8.9	3	0.75	<10	<500	19
14	Male	45	ADV +PEG-IFN	Negative	Positive	<10	7.1	1	2.34	<10	<500	226
15	Male	38	PEG-IFN	Negative	Positive	<10	9.5	1	0.22	<10	<500	27
16	Female	29	PEG-IFN	Negative	Positive	60.2	8.2	14	0.13	<10	<500	10
17	Male	40	PEG-IFN	Negative	Positive	<10	——	3	0.07	<10	<500	——

HBeAg, hepatitis B e antigen; HBsAg, hepatitis B surface antigen; Anti-HBs, hepatitis B surface antibody; Anti-HBc, hepatitis B core antibody; Anti-HBe, hepatitis B e antibody; ALT, alanine transaminase; PEG-IFN, pegylated interferon; ETV, entecavir; TDF, tenofovir; ADV, adefovir.
[a]At the end of PEG-IFN treatment.
[b]At the time of HBsAg reversion.
[c]Months from the date of the end of PEG-IFN treatment to the date of HBsAg reversion.

(48.0-60.0 weeks) and 24.0 weeks (12.0-30.0 weeks), respectively. The median follow-up duration was 12 months (5.3-32.5 months).

17 patients developed HRV during the follow-up period (Table 1). The HRV peak was within 15 months (13 patients with HRV were observed). The overall cumulative incidence of HRV by Kaplan-Meier analysis was shown in Figure 2A. The 6-, 12-, 18-, 24-, 30-, 36-month cumulative incidence rates of HRV were 7.6%, 12.1%, 15.4%, 17.7%, 20.3%, 22.9% respectively.

3.2 Comparison of clinical characteristics between patients with CHL and HRV

Of the total patients, 95 (84.8%) remained HBsAg negativity during the follow-up period whereas 17 (15.2%) experienced HRV (Table 2). The positivity of anti-HBs at EOT was more frequent in the CHL group than in the HRV group (87.4% *vs.* 64.7%, p=0.047). During the follow-up period, the disappearance of anti-HBs more commonly occurred in the HRV group (p <0.001).

3.3 Cumulative incidence of HRV based on HBV markers and ALT level at EOT

The cumulative incidence curves of HRV for each HBV marker and ALT level at EOT were presented in Figures 2B–F. Based on the ROC analysis, the optimal cut-off values of anti-HBs and anti-HBc at EOT were 1.3 $\log_{10}$IU/L and 9.6 S/CO, respectively (Supplementary Figure 1). The incidence of HRV differed significantly between the following subgroups: Anti-HBc ≥9.6 S/CO *vs.* <9.6 S/CO (p=0.003); HBeAg positivity *vs.* negativity (p=0.039); Anti-HBs ≥1.3 $\log_{10}$IU/L *vs.* <1.3 $\log_{10}$IU/L (p<0.001). Conversely, the incidence of HRV did not differ between anti-HBe positivity *vs.* negativity (p=0.382); ALT ≥1 upper limit of normal (ULN) *vs.* <1 ULN (p=0.611).

3.4 Time-dependent ROC analysis for incidence of HRV

The respective ROC curves of the anti-HBs level and anti-HBc at EOT for the incidence of HRV at 6, 12, 18, 24, 30, and 36 months after the start of follow-up were shown in Figures 3A, B respectively. The AUCs for anti-HBs at the above time points were 0.885, 0.882, 0.809, 0.795, 0.757, 0.772, respectively. The AUCs for anti-HBc at the above time points were 0.774, 0.698, 0.669, 0.714, 0.636, 0.791, respectively.

The plots of AUCs of anti-HBs and anti-HBc for the incidence of HRV from 6 to 36 months after the start of follow-up were shown in Figure 3C. The predictive power of anti-HBs was superior to that of anti-HBc from 6 to 36 months in general.

3.5 Predictive factors of HRV

Based on the multivariable analysis, anti-HBs ≥1.3 $\log_{10}$IU/L at EOT (hazard ratio (HR), 0.148; 95% confidence interval (CI), 0.044-0.502; p=0.002) and HBeAg negativity at EOT (HR, 0.183; 95%CI, 0.052-0.639, p=0.008) were independently associated with lower incidence of HR (Table 3).

We then analyzed the association between the cumulative incidence of HRV and the combination of HBeAg status and anti-HBs level at EOT (Figure 4). Among the patients with HBeAg negativity at EOT, the 36-month cumulative incidence rates of HRV in patients with anti-HBs ≥1.3 $\log_{10}$IU/L and <1.3 $\log_{10}$IU/L were 2.8% and 59.1% (P <0.001), respectively. Among the patients with anti-HBs ≥1.3 $\log_{10}$IU/L at EOT, the 36-month cumulative incidence rates of HRV in patients with HBeAg negativity and positivity were 2.8% and 52.4% (P=0.001), respectively. Moreover, the percentage of patients with HRV or CHL based on the different anti-HBs levels at EOT was shown in Figure 5.

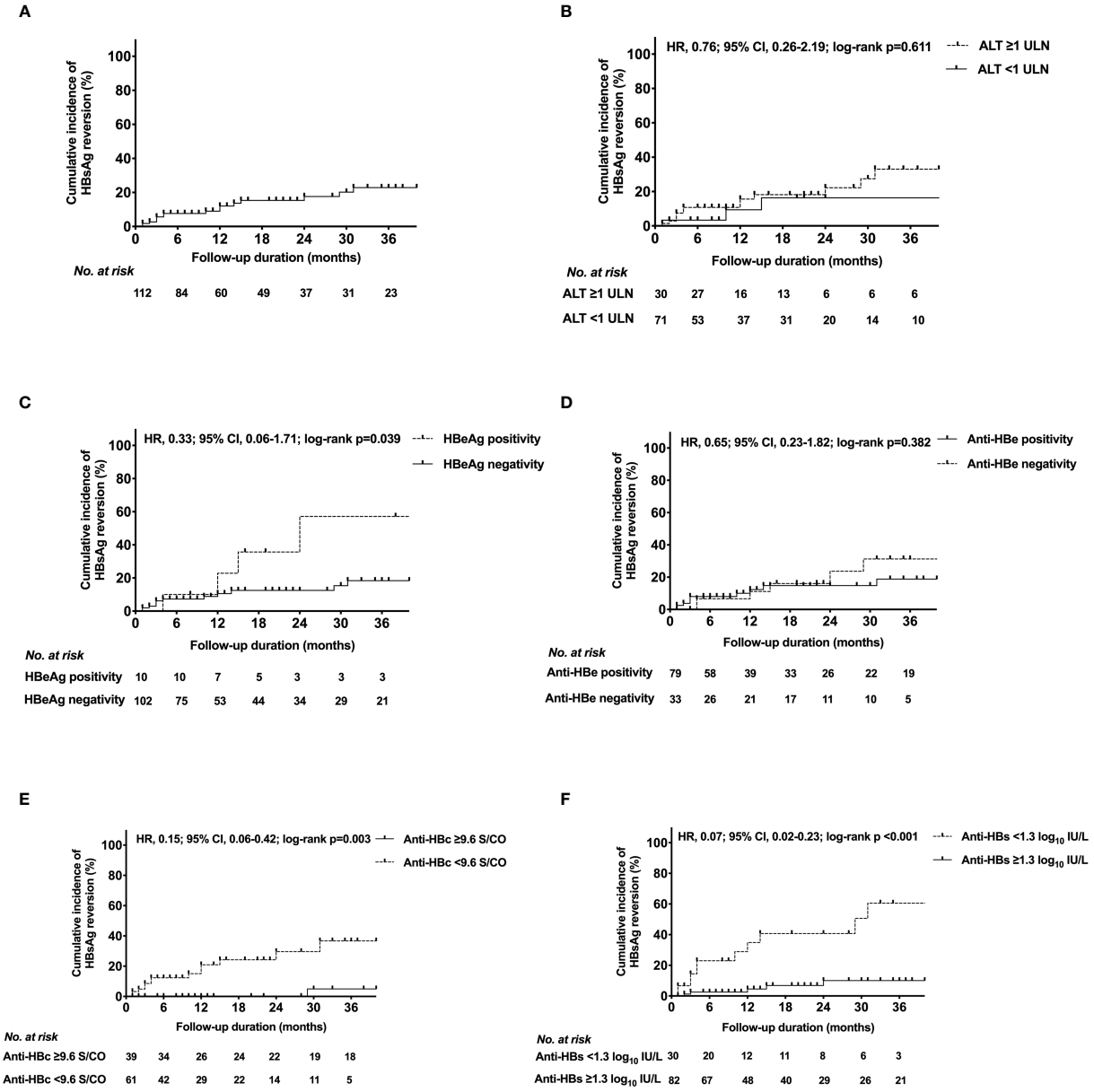

FIGURE 2
Kaplan-Meier estimates of cumulative incidence of HBsAg reversion in **(A)** the entire population, patients with different **(B)** ALT levels, **(C)** HBeAg status, **(D)** anti-HBe status, **(E)** anti-HBc level, **(F)** anti-HBs level at EOT. HBeAg, hepatitis B e antigen; HBsAg, hepatitis B surface antigen; Anti-HBs, hepatitis B surface antibody; Anti-HBc, hepatitis B core antibody; Anti-HBe, hepatitis B e antibody; ALT, alanine transaminase; ULN, upper limit of normal; HRV, hazard ratio; CI, confidence interval; EOT, end of PEG-IFN treatment.

3.6 Cross-sectional analysis of serum HBV RNA level

To understand the serum HBV RNA level in patients with CHL and HRV, a cross-sectional analysis of serum HBV RNA levels was performed in samples of 64 patients (Supplementary Table 1). There were 55 patients with CHL and 9 patients with HRV. No significant difference between the CHL and HRV groups (p=0.1502) (Figure 6A). Among the patients with CHL, the HBV RNA levels in patients at 24-60 months after the start of follow-up was significantly higher than that in patients at 0-6 months (p=0.0135), 6-13 months (p=0.0019), and 14-24 months (p=0.0052) (Figure 6B). Different therapeutic regimens including "PEG-IFN monotherapy", "Add-on", and "Switch-to" showed no difference in HBV RNA level (Figure 6C). HBV RNA levels were strongly correlated with HBsAg levels in patients with HRV (r=0.86, p=0.003) (Figure 6D). Anti-HBs titers of 64 patients were not correlated with HBV RNA levels (r=-0.24, p=0.0536) (Figure 6E).

4 Discussion

This retrospective cohort study mainly identified predictive factors associated with HRV in CHB patients who achieved PEG-IFN-induced HBsAg loss. Univariable analysis using the log-rank test showed that the status or level of HBeAg, anti-HBs, and anti-HBc

TABLE 2 Clinical characteristics of 112 CHB patients with HBsAg loss during and post PEG-IFN treatment.

	Total N=112	Confirmed HBsAg loss (CHL)[a] N=95	HBsAg reversion (HRV)[b] N=17	P value
Age (years)	37.0 (31.0-43.0)	36.0 (30.0-43.0)	39.0 (35.0-45.5)	0.355
Male, n (%)	91 (81.3%)	79 (83.2%)	12 (70.6%)	0.376
ALT (U/L)	36.0 (26.5-58.0)	36.5 (27.0-58.5)	33.0 (23.0-52.0)	0.516
HBeAg negativity, n (%)	98 (87.5%)	86 (90.5%)	13 (76.5%)	0.750
Therapeutic regimen, n (%)				0.636
PEG-IFN monotherapy	35 (31.2%)	29 (30.5%)	6 (35.3%)	
Add-on PEG-IFN[c]	46 (41.1%)	38 (40.0%)	8 (47.1%)	
Switch-to PEG-IFN[d]	31 (27.7%)	28 (29.5%)	3 (17.6%)	
Therapeutic medication, n (%)				0.393
PEG-IFN only	66 (58.9%)	57 (60%)	9 (52.9%)	
ETV+PEG-IFN	5 (4.5%)	3 (3.2%)	2 (11.8%)	
TDF+PEG-IFN	28 (25%)	24 (25.3%)	4 (23.5%)	
TAF+PEG-IFN	1 (0.9%)	1 (1.1%)	0 (0%)	
LAM+PEG-IFN	5 (4.5%)	5 (5.3%)	0 (0%)	
ADV+PEG-IFN	7 (6.3%)	5 (5.3%)	2 (11.8%)	
Platelet ($\times10^9$/L)	129.5 (103.8-171)	129.5 (104.0-163.8)	120.5 (90.0-181.5)	0.748
Albumin (g/L)	45.8 (45.0-48.7)	47.0 (45.0-49.0)	46.0 (44.0-47.8)	0.314
Bilirubin (μmol/L)	10.7 (8.0-15.4)	11.0 (8.1-16.1)	8.9 (8.0-13.0)	0.319
Positive anti-HBs at the time of HBsAg loss, n (%)	49 (43.8%)	42 (44.2%)	7 (41.2%)	0.816
Positive anti-HBs at EOT, n (%)	94 (83.9%)	83 (87.4%)	11 (64.7%)	0.047
Disappearance of anti-HBs during the follow-up period, n (%)	17 (15.2%)	8 (10.1%)	9 (81.8%)	<0.001
Positive anti-HBe, n (%)	78 (69.6%)	69 (73.4%)	9 (52.9%)	0.089
Anti-HBc (S/CO)	9.3 (8.1-10.1)	9.4 (8.2-10.1)	8.9 (8.1-9.4)	0.157
Undetectable HBV DNA	112 (100%)	95 (100%)	17 (100%)	>0.05
Consolidation treatment duration (weeks)[e]	24.0 (12.0-30.0)	24.0 (12.0-36.0)	12.0 (4.0-24.0)	0.177
PEG-IFN duration (weeks)	48.0 (48.0-72.0)	48.0 (48.0-72.0)	48.0 (48.0-80.0)	0.517
Follow-up duration (months)[f]	12.0 (5.3-32.5)	14.0 (6.0-35.0)	10.0 (3.0-19.5)	0.061

ALT, alanine transaminase; HBsAg, hepatitis B surface antigen; HBeAg, hepatitis B e antigen; Anti-HBs, hepatitis B surface antibody; Anti-HBc, hepatitis B core antibody; Anti-HBe, hepatitis B e antibody; PEG-IFN, pegylated interferon; ETV, entecavir; TDF, tenofovir; TAF, tenofovir alafenamide; LAM, lamivudine; ADV, adefovir; EOT, end of PEG-IFN treatment.
[a]Two negative HBsAg results (<0.05 IU/mL) at least 6 months apart.
[b]Reappearance of HBsAg after HBsAg loss.
[c]Combination therapy after at least 48 weeks of nucleot(s)ide therapy.
[d]PEG-IFN monotherapy in patients who received NAs for at least 48 weeks.
[e]Weeks from the date of HBsAg loss to the date of the end of PEG-IFN treatment.
[f]Months from the date of the end of PEG-IFN treatment to the date of HR or the last follow-up visit.

were significantly associated with the incidence of HRV. The predictive power of anti-HBs was superior to that of anti-HBc during the follow-up period (from 6 to 36 months) in general. Furthermore, multivariable Cox proportional hazard analysis showed that anti-HBs ≥1.3 $\log_{10}$IU/L, and HBeAg negativity at EOT were independently associated with lower incidence of HRV.

The role of the anti-HBs in the durability of HBsAg loss remains controversial (Paul et al., 2017; Yip et al., 2017; Li et al., 2019; Alawad et al., 2020; Wu et al., 2020; Song et al., 2021; Huang et al., 2022). For patients with PEG-IFN-induced HBsAg loss, Wu et al. reported in their retrospective cohort study that the average HRV time for anti-HBs ≥100 IU/L was longer than that for anti-HBs <100 IU/L (107 weeks *vs*. 41 weeks, P <0.001) (Wu et al., 2020). Li et al. reported that consolidation treatment ≥12 weeks and high anti-HBs levels were strong predictors of HRV in HBeAg-negative patients (Li et al., 2019). Huang et al. reported that HBcrAg <4 $\log_{10}$U/mL and anti-HBs >2 $\log_{10}$IU/L could predict the sustained functional cure (Huang et al., 2022). However, for patients with spontaneous HBsAg loss and NAs-

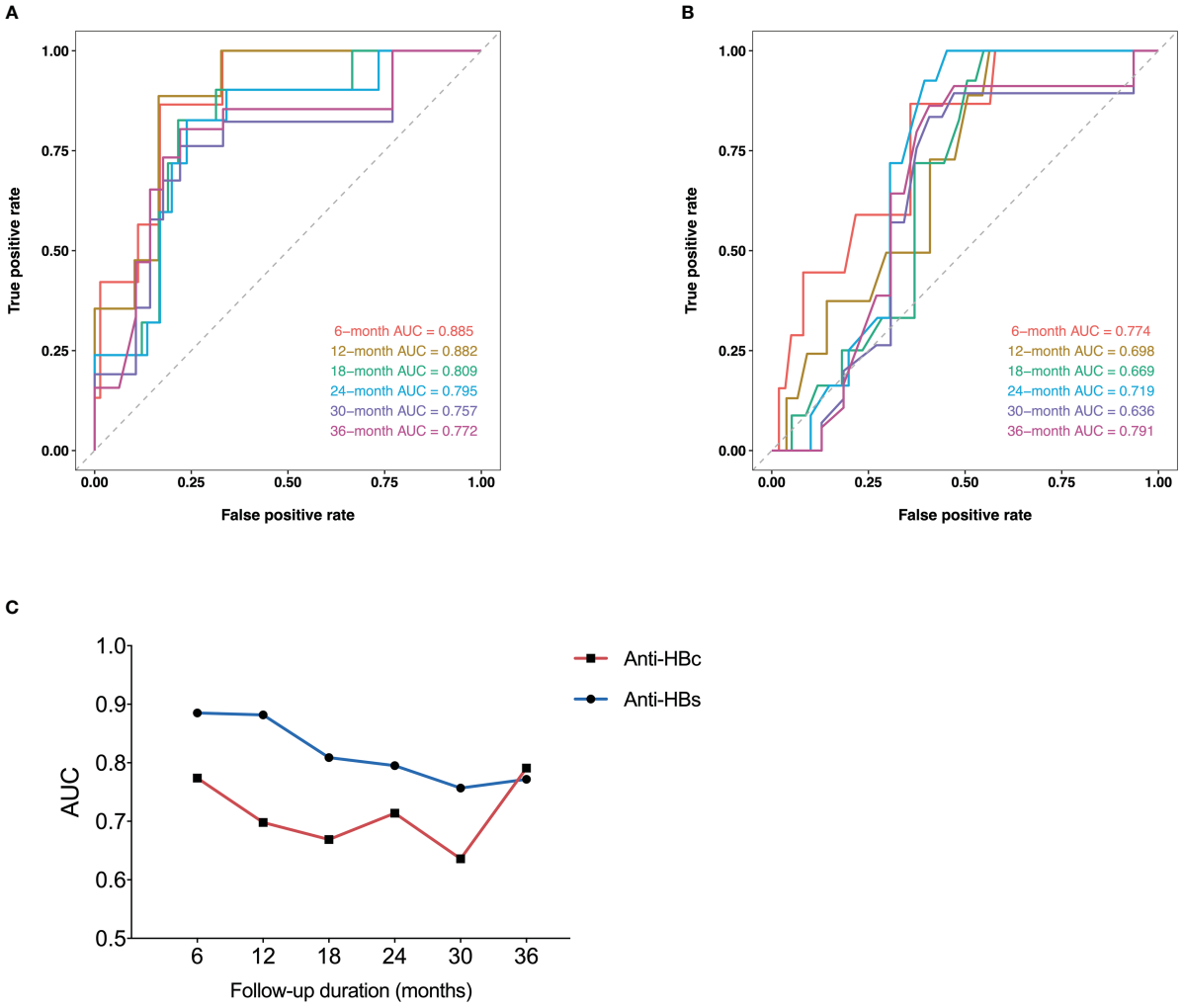

FIGURE 3
Time-dependent ROC curves of anti-HBs **(A)** and anti-HBc **(B)** at EOT for HRV incidence at 6, 12, 18, 24, 30, and 36 months after the start of follow-up. Plots of AUCs of anti-HBs and anti-HBc at the same time point as above **(C)**. ROC, receiver operating characteristic curve; Anti-HBs, hepatitis B surface antibody; Anti-HBc, hepatitis B core antibody; HRV, HBsAg reversion; AUC, area under the receiver operating characteristic curve; EOT, end of PEG-IFN treatment.

TABLE 3 Predictive factors of HBsAg reversion.

	Univariate analysis			Multivariable analysis[a]		
Variables	HR	95% CI	p value	HR	95% CI	p value
Age ≥36.5 years	1.761	0.551 to 5.626	0.340			
Male	0.460	0.161 to 1.314	0.147			
HBeAg negativity at EOT	0.323	0.104 to 1.007	0.051	0.183	0.052 to 0.639	0.008
ALT ≥1 ULN at EOT	0.562	0.182 to 1.711	0.316			
Consolidation treatment ≥12 weeks[b]	0.529	0.195 to 1.439	0.212			
Anti-HBs ≥1.3 $\log_{10}$IU/L at EOT	0.214	0.078 to 0.583	0.003	0.148	0.044 to 0.502	0.002
Anti-HBe positivity at EOT	0.652	0.248 to 1.718	0.387			
Anti-HBc ≥9.6 S/CO at EOT	0.134	0.029 to 0.622	0.010	0.328	0.064 to 1.682	0.181
NA-experienced	0.958	0.353 to 2.602	0.933			

ALT, alanine transaminase; HBeAg, hepatitis B e antigen; Anti-HBs, hepatitis B surface antibody; Anti-HBc, hepatitis B core antibody; Anti-HBe, hepatitis B e antibody; EOT, end of PEG-IFN treatment; NA, nucleos(t)ide analogue; HR, hazard ratio; CI, confidence interval; ULN, upper limit of normal.
[a]Multivariable analysis including variables with p<0.10 at univariate analysis.
[b]Weeks from the date of HBsAg loss to the date of the end of PEG-IFN treatment.

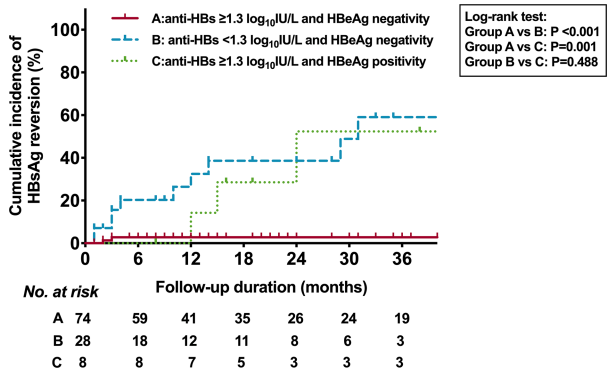

FIGURE 4
Kaplan-Meier estimates of cumulative incidence of HBsAg reversion based on HBeAg status and anti-HBs of 1.3 log10IU/L at EOT. HBsAg, hepatitis B surface antigen; HBeAg, hepatitis B e antigen; Anti-HBs, hepatitis B surface antibody; EOT, end of PEG-IFN treatment.

induced HBsAg loss, Yip et al. reported that anti-HBs negativity at EOT was not associated with HRV (Yip et al., 2017). In the present study, we found that not only was anti-HBs disappearance more common in the HRV group but also the anti-HBs levels at EOT were associated with HRV in univariable and multivariable analyses. The cut-off anti-HBs level at EOT for predicting HRV was 1.3 $\log_{10}$IU/L with a sensitivity of 0.65 and a specificity of 0.80 in our cohort. In addition, the optimal anti-HBs titer for predicting sustained functional cure remained unclear. We also used the cut-off anti-HBs level (2 $\log_{10}$IU/L) based on previous studies (Wu et al., 2020; Huang et al., 2022). The incidence of HRV in patients with anti-HBs ≥2 $\log_{10}$IU/L was significantly lower than that in patients with anti-HBs <2 $\log_{10}$IU/L (p=0.002) (Supplementary Figure 2). The multivariable analysis also showed that anti-HBs ≥2 $\log_{10}$IU/L and HBeAg negativity at EOT were significantly associated with HRV (Supplementary Table 2). Furthermore, to our knowledge, no previous studies have used time-dependent ROC analysis to evaluate anti-HBs or anti-HBc in terms of their association with the incidence of HRV after PEG-IFN discontinuation. We used this analysis to show that the anti-HBs level was mainly superior to the anti-HBc level regarding the predictive power for HRV over 36 months. Collectively, our results highlighted the clinical significance of anti-HBs levels at EOT in predicting HRV. However, the underlying mechanisms remain unclear. In contrast to the constant production of anti-HBc, the anti-HBs production displays functional defects in CHB patients. In a study on the HBV humoral immunity, researchers found that HBcAg-specific B cells exhibited higher frequency and more mature phenotype than HBsAg-specific B cells (Le Bert et al., 2020). The appearance of anti-HBs may indicate sustained and profound anti-HBV immunity elicited by PEG-IFN treatment, NAs treatment, or other factors, which need to be examined in great detail.

In addition to anti-HBs levels, patients with HBeAg positivity at EOT showed a significantly higher incidence of HRV. Moreover, HBeAg status at EOT was also identified as an independent predictor of HRV by multivariable analysis. However, a recent prospective study suggested that HBeAg at EOT was not indispensable for maintaining PEG-IFN-induced HBsAg loss (Huang et al., 2022). The inconsistency may partly be ascribed to the following differences in our patient cohort: (a) The cut-off value of HBeAg was based on negativity (<1 S/CO) and positivity (>1 S/CO) rather than the Youden's index; (b) Our patient cohort was relatively large; and (c) HBeAg was still positive in a small proportion of patients at EOT. Intriguingly, the same study reported that hepatitis B core-related antigen (HBcrAg) <4 $\log_{10}$U/mL was one of the significant protectors from HRV during the off-treatment follow-up. Furthermore, the HBcrAg level was positively correlated with the HBeAg level at EOT (Huang et al., 2022). Further studies with a larger population are warranted to clarify the role of HBeAg and HBcrAg in the durability of HBsAg loss.

Consolidation treatment duration has been reported to be associated with HRV following therapy discontinuation (Yip et al., 2017; Li et al., 2019). Conversely, the duration of consolidation treatment displayed no significant difference between the CHL and the HRV group in this study. We further analyzed the incidence of HRV using 12 weeks as the cut-off value, the incidence of HRV did not differ between patients with consolidation treatment <12 weeks *vs.* ≥12 weeks (Supplementary Figure 3).

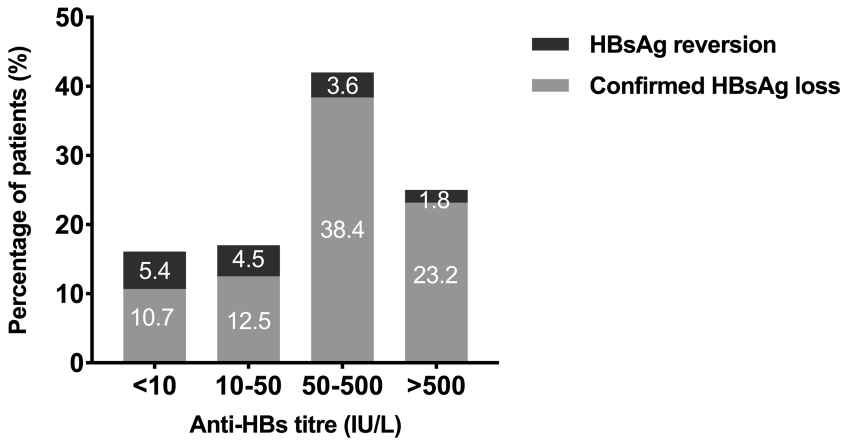

FIGURE 5
Percentage of patients with HRV according to different anti-HBs levels at EOT. HRV, HBsAg reversion; Anti-HBs, hepatitis B surface antibody; EOT, end of PEG-IFN treatment.

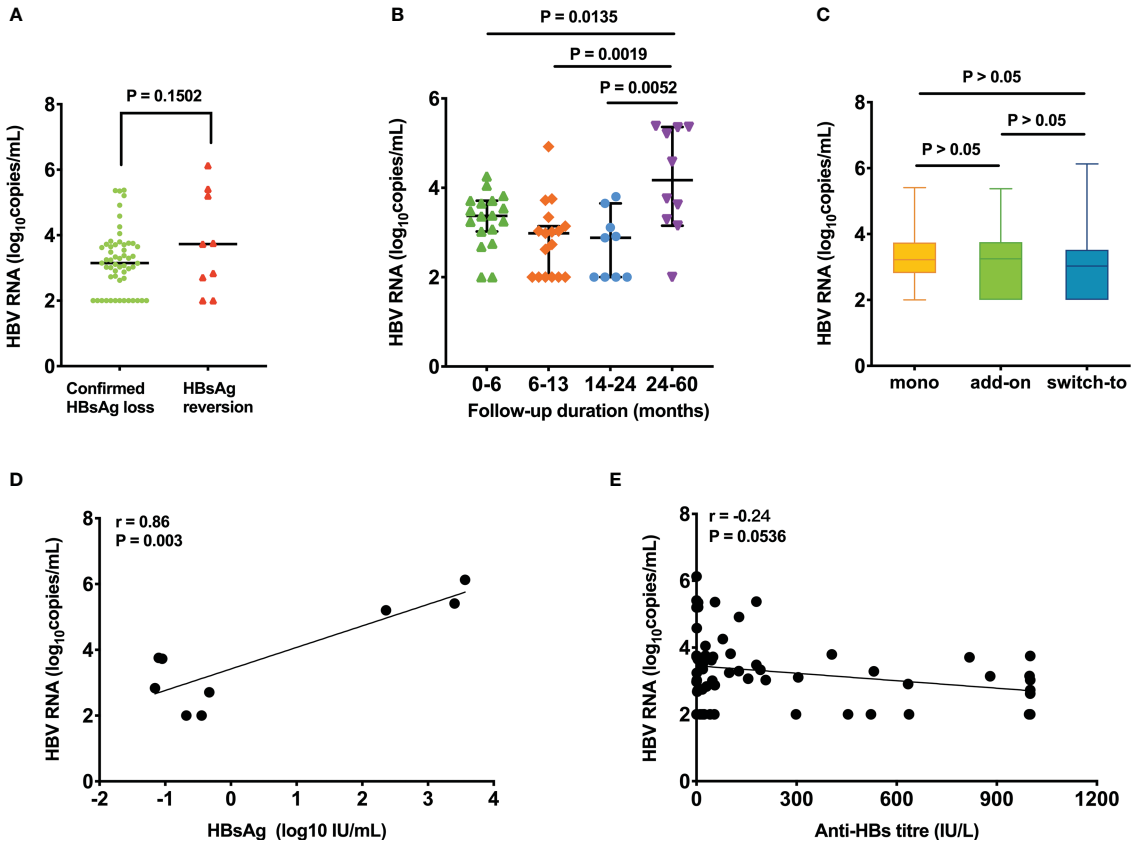

FIGURE 6
Cross-sectional analysis of serum HBV RNA level. Comparison of HBV RNA level in patients between **(A)** the CHL and HRV group, **(B)** different follow-up periods, and **(C)** different therapeutic regimens. Correlation between serum HBV RNA and **(D)** HBsAg level in patients with HRV, **(E)** anti-HBs level in the entire population. CHL, confirmed HBsAg loss; HRV, HBsAg reversion; HBsAg, hepatitis B surface antigen; Anti-HBs, hepatitis B surface antibody. Mono, pegylated interferon (PEG-IFN) monotherapy in patients who did not receive nucleot(s)ide therapy ever; Add-on, combination therapy after at least 48 weeks of nucleot(s)ide therapy; Switch-to, PEG-IFN monotherapy in patients who received NAs for at least 48 weeks.

Serum HBV RNA is regarded as an alternative biomarker for cccDNA activity (Wang et al., 2016; Gao et al., 2017; Huang et al., 2018). In this regard, several studies have suggested that HBV RNA can be used to predict virologic and clinical relapse after discontinuation of antiviral therapy (Wang et al., 2016; Carey et al., 2020; Fan et al., 2020). Nonetheless, HBV RNA alone may not be insufficient to assess sustained response, researchers found that many patients experiencing HBV RNA decline during PEG-IFN treatment did not achieve HBsAg and/or HBcrAg decline (Brakenhoff et al., 2021). In the present study, there were significant associations between higher HBV RNA levels and HBsAg levels in patients with HRV, in line with previous studies (Lin et al., 2020; Ghany et al., 2021). Thus, low HBsAg levels in the HRV group may account for no significant difference between the CHL and HRV groups. We also found that there was a trend that anti-HBs level in patients with CHL was negatively correlated with HBV RNA. For patients with CHL, HBV RNA levels did not differ across different therapeutic regimens, consistent with the observation that there was no statistical difference in the therapeutic regimens between the CHL and HRV groups. Similarly, Pan et al. also reported that add-on interferon treatment and interferon monotherapy exhibited equivalent efficacy in sustained functional cure (Pan et al., 2021). Additionally, HBV RNA at 24-60 months had higher HBV RNA levels than in other periods. The exact reasons for the discrepancy are unclear. Longitudinal studies on HBV RNA levels after PEG-IFN discontinuation are warranted to validate these above results.

This study has several limitations. First, this was a retrospective study and not all patients were regularly monitored. Thus, we cannot exclude the possibility of transient HRV between adjacent visits. Second, genotype data cannot be obtained in this study. Whether the genotype plays a role in the durability of HBsAg loss has not been determined. Third, as another novel biomarker, HBcrAg may be associated with HRV and could not be analyzed because serum samples were not available (Huang et al., 2022). Fourth, serum HBV RNA was investigated in a cross-sectional analysis. We could not analyze the longitudinal changes of this novel biomarker, especially after the PEG-IFN discontinuation in patients with CHL.

In conclusion, HBeAg status (negativity) and anti-HBs level (≥1.3 $\log_{10}$IU/L) at EOT were associated with durability of PEG-IFN-induced HBsAg loss. These findings should be further investigated on a larger sample size.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The studies involving human participants were reviewed and approved by Huashan Hospital of Fudan University(KY2015-212). The patients/participants provided their written informed consent to participate in this study.

Author contributions

All authors contributed equally to the concept and preparation of the manuscript. YFG and JZ completed the final preparation and editing of the manuscript. YFG analyzed the data and created the figures and tables. All authors contributed to the article and approved the submitted version.

Funding

This work was supported by the National Natural Science Foundation of China under Grant No. 81871640, 82172255, Shanghai Municipal Science and Technology Major Project (ZD2021CY001) and Shanghai Key Clinical Specialty Construction Program (ZK2019B24).

Acknowledgments

We gratefully acknowledge the assistance of Haiyan Lv collecting the data.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2023.1120300/full#supplementary-material

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frontiers | Frontiers in Cellular and Infection Microbiology

TYPE Original Research
PUBLISHED 27 February 2023
DOI 10.3389/fcimb.2023.1115087

OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to Virus and Host, a section of the journal Frontiers in Cellular and Infection Microbiology

RECEIVED 03 December 2022
ACCEPTED 13 February 2023
PUBLISHED 27 February 2023

CITATION
Qian J, Yue M, Huang P, Ai L, Zhu C, Wang C, Luo Y, Yue N, Wu Y, Zhang Y, Wang C and Tan W (2023) Spatiotemporal heterogeneity and impact factors of hepatitis B and C in China from 2010 to 2018: Bayesian space–time hierarchy model.
Front. Cell. Infect. Microbiol. 13:1115087.
doi: 10.3389/fcimb.2023.1115087

Spatiotemporal heterogeneity and impact factors of hepatitis B and C in China from 2010 to 2018: Bayesian space–time hierarchy model

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Introduction: Viral hepatitis is a global public health problem, and China still faces great challenges to achieve the WHO goal of eliminating hepatitis.

Methods: This study focused on hepatitis B and C, aiming to explore the long-term spatiotemporal heterogeneity of hepatitis B and C incidence in China from 2010 to 2018 and quantify the impact of socioeconomic factors on their risk through Bayesian spatiotemporal hierarchical model.

Results: The results showed that the risk of hepatitis B and C had significant spatial and temporal heterogeneity. The risk of hepatitis B showed a slow downward trend, and the high-risk provinces were mainly distributed in the southeast and northwest regions, while the risk of hepatitis C had a clear growth trend, and the high-risk provinces were mainly distributed in the northern region. In addition, for hepatitis B, illiteracy and hepatitis C prevalence were the main contributing factors, while GDP per capita, illiteracy rate and hepatitis B prevalence were the main contributing factors to hepatitis C.

Disussion: This study analyzed the spatial and temporal heterogeneity of hepatitis B and C and their contributing factors, which can serve as a basis for monitoring efforts. Meanwhile, the data provided by this study will contribute to the effective allocation of resources to eliminate viral hepatitis and the design of interventions at the provincial level.

KEYWORDS

hepatitis B, hepatitis C, Bayesian model, spatiotemporal heterogeneity, GDPta, illiteracy rate

1 Introduction

In 2015, the global hepatitis virus caused 10 million new infections and 1.3 million deaths, of which 96% were caused by chronic infection caused by hepatitis B virus (hepatitis B) and hepatitis C virus (hepatitis C). Hepatitis B virus and hepatitis C virus have parallel transmission routes, so a certain proportion of patients can have dual virus infection (Raimondo and Saitta, 2008; Brass and Moradpour, 2009; Riaz et al., 2011). Patients with co infection have a 2-3 fold increased risk of advanced liver disease (Liu et al., 2014). In 2016, the World Health Organization called on the world to fight against viral hepatitis and eliminate hepatitis by 2030 through expanded prevention, detection and treatment (Organization W.H., 2016). Eliminating hepatitis is defined as reducing the incidence rate by 90% and mortality by 65% on the basis of 2015. Studies(2017; WHO, 2016; Ward and Hinman, 2019) have shown that eliminating viral hepatitis was feasible because of the characteristics of HBV and HCV, the availability of HBV vaccines and other interventions to prevent transmission, reliable diagnosis, and drugs to treat HBV and cure HCV before the onset of serious disease and premature death. The burden of hepatitis B and C in China is enormous, with an estimated 70 million hepatitis B surface antigen (HBsAg) carriers (prevalence 5%-6%) in China (Liu et al., 2019), while the prevalence of hepatitis C is estimated at 1.3% (Gower et al., 2014). Although China has invested heavily in basic research, vaccine and drug development, and mandated hepatitis C screening (usually before blood transfusion) and hepatitis B immunization schedules (Wang et al., 2014), much remains to be done to meet the requirements for hepatitis elimination.

At present, the domestic research on viral hepatitis mainly focuses on its etiology, clinical features, epidemiology, and prevention and control policies, while few studies have been conducted on its temporal and spatial transmission. Nevertheless, relevant research results show that the spread of infectious diseases such as viral hepatitis and HIV is related to spatial factors (Busgeeth and Rivett, 2004; Rosenberg et al., 2018; Clipman et al., 2021). Ren et al. (Ren et al., 2022) have analyzed the distribution of HIV in Luzhou using Bayesian spatiotemporal model. Tian et al. (Tian et al., 2018) have used spatiotemporal analysis to study the impact of urbanization on hantavirus. Meanwhile, socioeconomics, income, education, occupation, and blood transfusion are all closely related to hepatitis B and C (Akbar et al., 1997; Salemi et al., 2017; Ahn et al., 2018). Additionally, accurate data is an important prerequisite for sound public health and health care policies and guidelines, allowing the health burden to drive resource allocation decisions and disseminating accurate information to health professionals, patients and the public. Therefore, this study used Bayesian spatiotemporal hierarchical model to analyze the influence of socioeconomic factors on the spatiotemporal distribution of hepatitis B and C in China from 2010 to 2018, and revealed provincial cold and hot spots in the time dimension. The posterior distribution was used to map the disease risk of hepatitis B and C, which provided new insights for the precise prevention and control of hepatitis B and C.

2 Methods

2.1 Ethics statement

This study has been approved by the ethics committee of Nanjing Bioengineering (Gene) Technology Center for Medicines (No:2021BY07). Patient consent was not required because no patients' individual information was included in this study and population data were collected from the public database of China.

2.2 Data Sources

Annual data of hepatitis B and C cases for the period from 2010 to 2018 were obtained from the Chinese Center for Disease Control and Prevention (https://www.phsciencedata.cn/Share/). The case definition is based on the unified diagnostic criteria formulated by the Chinese Ministry of Health (MOH). The following demographic information used in the Bayesian space-time hierarchy model were acquired from the Chinese economic Statistical Year book (http://www.stats.gov.cn/): (1) population by region at the end of the year; (2) the proportion of illiterate population in the population aged 15 and above (%); (3) per capita gross domestic product (GDP) (the GDP divided by the population of the region); (4) road mileage by region (kilometer); (5) the urbanization rate (which is divided by the urban resident population); (6) the number of hygienic personnel per 1000 people; (7) beds in medical and health institutions per 1000 people; (8) and population density (the number of permanent residents divided by the total area of the province). The data and code used in this article are uploaded to the sharing platform Github: https://github.com/ykjjqian/BSTHM1/tree/master.

2.3 Bayesian space–time hierarchy model

In this study, we used the BSTHM (Richardson et al., 2004; Li et al., 2014a) with Poisson distribution to capture spatial and temporal heterogeneity of hepatitis B and C and quantity the association between the potential driving factors and the incidence of hepatitis B and C. In the model, we let y_{it} , n_{it} and u_{it} represent the hepatitis B or C cases in province $i(i=1,\ldots,31)$ and year $t(t=1,\ldots,9)$, the risk population, and the spatiotemporal risk of hepatitis B or C. β_1 to β_8 denote the regression coefficients of the potential driving factors.

$$y_{it} \sim Poisson(n_{it}u_{it})$$

$$\log(u_{it}) = \alpha + s_i + b_0 t^* + v_t + \sum_{k=1}^{8} \beta_k x_{ik} + b_{1i} t^* + \epsilon_{it}$$

where α is the overall logarithm of hepatitis B or C risk in China over the nine years and $t^*=t-4.5$ (centering at the mid-observation period). The spatial term s_i describes the spatial distribution of disease risk throughout the study period. The $\exp(s_i)$ is the spatial

disease risk, which is influenced by some related factors in the study period, such as economic conditions, and medical resources. The temporal term $(b_0t^\star+v_t)$ describes the overall temporal trend common to all provinces, and the overall temporal trend is specified as a linear trend $(b_0t^\star)$ with $v_t \sim N(0,\sigma_v^2)$, which allows for nonlinearity of the overall trend pattern. The term $b_{1i}t^\star$ allows each province to have its own trend and capture the departure extent from b_0 for each region. A positive estimate represents a relatively rapid increase (or even decrease) of disease risk in that particular province over time. The last term $\epsilon_{it} \sim N(0,\sigma_\epsilon^2)$ (Gelman, 2006) is the Gaussian random noise variable and captures additional variability not yet explained by other model components. For such overdiscrete count data, this additional source of variability is mainly that the observed variability exceeds the variability that can be explained by the Poisson model (Johnson and Bowers, 2004). The prior distribution of the global spatial random effect term s_i is BYM model (Besag et al., 1991). The BYM model is a convolution of spatially structured random effects and spatial unstructured random effects, the latter following a Gaussian distribution. Meanwhile, the conditional autoregressive (CAR) prior with a space adjacency matrix $W_{31\times31}$ was used to impose spatial structure. If the country i and j shared a common border, then W_{ij}=1 , otherwise, W_{ij}=0 . $b_{1i}t^\star$ has the same BYM prior as s_i . The CAR prior to the spatial random effect showed that neighboring provinces tended to have a similar overall risk of disease. Finally, a strict positive half-Gaussian prior $N_{+\infty}(0,10)$ is assigned to all random effects standard deviations. x_{ik} is a covariate incorporated on the basis of the previous model that helps explain space/time patterns (Li et al., 2014a). K=8 represents the number of covariates, including illiteracy percentage aged 15 years and above, GDP per capita, regional road mileage, regional urbanization rate, number of hygienic personnel, beds in medical and health institutions, population density, and incidence rate of hepatitis B or hepatitis C. Assign the non-informational prior to the regression coefficient β . In Bayesian simulations, any interval that contains 95% of the posterior mass is a frequency confidence interval (CI), often called a credible interval (CRI), and sometimes called a Bayesian confidence interval. Generally, the 2.5th and 97.5th percentiles of the posterior sample are selected as the 95% CRI.

The provinces were classified into nine categories (3 risk categories × 3 trend categories) according to a two-stage classification rule (Richardson et al., 2004). In the first stage, a province was defined as a hotspot for posterior probability $P(exp(s_i)>1|data)\in[0.8,1]$ and as a coldspot for $P(exp(s_i)>1|data)\in[0,0.2]$. If $P(exp(s_i)>1|data)\in(0.2,0.8)$, the province is defined as neither hotspots nor coldspots. Hot and cold spots represent the province's consistently above/below the average disease risk in China, which changes over time. In the second stage, according to the the local slopes b_{1i} , the provinces corresponding to each risk category in the first stage were classified into three trend patterns: level 1, the variation trend of the disease is faster than the overall trend, if $P(b_{1i}>0|h_i,data)\in[0.8,1]$; level 2, the variation trend of the disease is slower than the overall trend, if $P(b_{1i}>0|h_i,data)\in[0,0.2]$; level 3, the variation trend in the disease has no difference with the mean level, if $P(b_{1i}>0|h_i,data)\in(0.2,0.8)$. This is used to highlight provinces that have not yet become hot/cold spots but have a tendency to become hot spots. Richardson et al. (Richardson et al., 2004) have demonstrated that the probability cut-off used above to identify areas of very high/very low disease risk strikes a good balance between sensitivity (i.e., the ability to detect hot spots/cold spots when overall risk is indeed above/below the mean) and false-positive rates (i.e., the ratio of declared hot spots/cold spots where actual risk does not differ from the mean).

The whole BSTHM was performed in OpenBUGS (Richardson et al., 2004). The posterior distribution of model parameters was obtained by Markov chain Monte Carlo (MCMC) simulation. We ran two Markov chain Monte Carlo (MCMC) chains for 45,000 iterations and discarded the first 15,000 iterations as aging. The diagnosis of convergence of Bayesian estimates was assessed by the Brooks-Gelman-Rubin (BGR) ratio (Brooks and Gelman, 1998). The closer the ratio is to 1.0, the better the model converges (Li et al., 2014b). Of the total 236 parameters of the Bayesian space-time model, only 1.69% had a BGR ratio greater than 1.05.

3 Results

3.1 Demographic characteristics

From 2010 to 2018 in China, a total of 9018099 cases of hepatitis B and 1782618 cases of hepatitis C were reported in the study regions, with the average annual incidence of 75.93 and 15.52 per 100,000 people respectively. Of the total hepatitis B cases, 5693525 cases were males and 3324574 cases were females, with a sex ratio of 1.71. 1001808 cases of hepatitis C were males and 780810 cases were females, with the sex ratio of 1.28. All age groups were susceptible, and 90.25% (8138559/9018099) and 85.58% (1525579/1782618) of hepatitis B and C cases occurred in aged 20-60, respectively (Figure 1). Farmers were the majority group of hepatitis B and C, accounting for 53.79% (4312756/8018114) and 48.33% (755510/1563243), respectively (Tables 1, 2). Geographically, Qinghai had the highest average incidence (195.50 cases per 100000 population) of hepatitis B from 2010 to 2018, 17.89 times higher than that in Beijing (10.93 cases per 100000 population), which had the lowest average incidence rate of hepatitis B. Xinjiang had the highest average incidence (47.75 cases per 100000 population) of hepatitis C, 40.47 times higher than that in Tibet(1.18 cases per 100000 population), which had the lowest average incidence rate of hepatitis C. Figure 2 showed the incidence change in hepatitis B and hepatitis C in China from 2010 to 2018, respectively.

3.2 Temporal trend

On the time perspective, the overall temporal variation of hepatitis B showed a slow downward trend, while the relative risks (RRs) of hepatitis C showed an overall upward trend from 2010 to 2018 (Figure 3). The highest disease risk of hepatitis B (RRs: 1.22, 95%CRI: 1.05-1.43) occurred in 2010 and the lowest disease risk (RRs: 0.87, 95%CRI: 0.80-0.94) occurred in 2016. In 2010, the hepatitis C temporal RRs (0.64, 95%CRI: 0.56-0.73) was the lowest,

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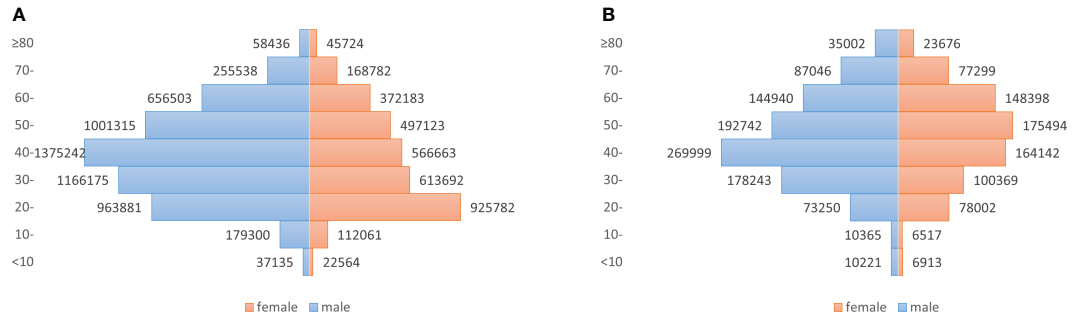

FIGURE 1
Number of newly diagnosed hepatitis B and hepatitis C cases for male and female patients in different age groups, in China, 2010–2018. **(A)** Hepatitis B, with a sex ratio of 1.71; **(B)** Hepatitis C, with a sex ratio of 1.28.

while in 2018, the temporal RRs of hepatitis C (1.30, 95%CRI:1.14-1.46) was the highest.

3.3 Spatial heterogeneity

Geographically, the spatial relative risks (RRs) of hepatitis B and C calculated using the BHSTM were different substantially, indicating significant heterogeneity in both hepatitis B and C incidence risk in the study region. Figure 4 showed the spatial RRs of hepatitis B and C at the province level from 2010 to 2018. The provinces with a higher spatial risk of hepatitis B were mainly distributed in the southeast and northwest regions, while the risk of hepatitis C was relatively higher in northern China.

Figure 5 showed the spatial patterns of hot and cold spots of hepatitis B and C in 2010-2018. For hepatitis B, 3/31 (9.68%) and 4/31 (12.90%) provinces were identified as cold spots and hot spots, respectively. The remaining 24/31(77.42%) provinces were defined

TABLE 1 Demographic characteristics of hepatitis B cases in mainland China, 2010–2018.

Hepatitis B group	2010	2011	2012	2013	2014	2015	2016	2017	2018	Total
Gender										
Male	675781 (0.64)	685544 (0.63)	679325 (0.62)	602913 (0.63)	587062 (0.63)	592930 (0.63)	597432 (0.63)	636961 (0.64)	635577 (0.64)	5693525 (0.63)
Female	384801 (0.36)	407791 (0.37)	407761 (0.38)	360061 (0.37)	348640 (0.37)	341285 (0.37)	344836 (0.37)	364991 (0.36)	364408 (0.36)	3324574 (0.37)
Sex ratio	1.76	1.68	1.67	1.67	1.68	1.74	1.73	1.75	1.74	1.71
Age										
0~	9931 (0.01)	8561 (0.01)	7695 (0.01)	6348 (0.01)	5901 (0.01)	5671 (0.01)	5060 (0.01)	5316 (0.01)	5216 (0.01)	59699 (0.01)
10~	62007 (0.06)	49891 (0.05)	41335 (0.04)	31809 (0.03)	26374 (0.03)	23253 (0.02)	20323 (0.02)	19380 (0.02)	16989 (0.02)	291361 (0.03)
20~	258060 (0.24)	270762 (0.25)	258664 (0.24)	216558 (0.22)	201159 (0.21)	186002 (0.20)	176813 (0.19)	170311 (0.17)	151334 (0.15)	1889663 (0.21)
30~	224181 (0.21)	223587 (0.20)	215381 (0.20)	187848 (0.20)	178316 (0.19)	175911 (0.17)	180524 (0.19)	195952 (0.20)	198167 (0.20)	1779867 (0.20)
40~	215394 (0.20)	231177 (0.21)	233778 (0.22)	209722 (0.22)	204620 (0.22)	205756 (0.22)	205636 (0.22)	219598 (0.22)	216224 (0.22)	1941905 (0.22)
50~	151925 (0.14)	156906 (0.14)	162973 (0.15)	154100 (0.16)	156574 (0.17)	163683 (0.18)	170883 (0.18)	186043 (0.19)	195351 (0.20)	1498438 (0.17)
60~	89984 (0.08)	99456 (0.09)	109268 (0.10)	103616 (0.11)	107904 (0.12)	115762 (0.12)	122621 (0.13)	136504 (0.14)	143571 (0.14)	1028686 (0.11)
70~	40364 (0.04)	43392 (0.04)	47251 (0.04)	42773 (0.04)	43762 (0.05)	46194 (0.05)	48182 (0.05)	54422 (0.05)	57980 (0.06)	424320 (0.05)

(Continued)

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TABLE 1 Continued

Hepatitis B group	2010	2011	2012	2013	2014	2015	2016	2017	2018	Total
80~	8736 (0.01)	9603 (0.01)	10741 (0.01)	10200 (0.01)	11092 (0.01)	11983 (0.01)	12226 (0.01)	14426 (0.01)	15153 (0.02)	104160 (0.01)
Occupation										
Farmers	513975 (0.48)	573538 (0.52)	581847 (0.54)	529647 (0.55)	513294 (0.55)	517927 (0.55)	524154 (0.56)	558374 (0.56)	–	4312756 (0.54)
Housework and unemployment	98654 (0.09)	117979 (0.11)	122827 (0.11)	125158 (0.13)	128704 (0.14)	132906 (0.14)	136909 (0.15)	149163 (0.15)	–	1012300 (0.13)
Worker	111147 (0.10)	76051 (0.07)	65956 (0.06)	57631 (0.06)	55909 (0.06)	53187 (0.06)	51501 (0.05)	53000 (0.05)	–	524382 (0.07)
Retiree	45571 (0.04)	47430 (0.04)	49247 (0.05)	45044 (0.05)	43429 (0.05)	44479 (0.05)	47089 (0.05)	48406 (0.05)	–	370695 (0.12)
Business service personnel	26762 (0.03)	29260 (0.03)	30691 (0.03)	32525 (0.03)	33597 (0.04)	34013 (0.04)	37054 (0.04)	43015 (0.04)	–	266917 (0.03)
Cadres and staff	44032 (0.04)	36427 (0.03)	32845 (0.03)	27877 (0.03)	25026 (0.03)	23639 (0.03)	24727 (0.03)	24829 (0.02)	–	239402 (0.03)
Others	31203 (0.21)	34668 (0.19)	37589 (0.19)	28459 (0.15)	27997 (0.15)	27552 (0.14)	26064 (0.13)	25774 (0.12)	–	239306 (0.16)

TABLE 2 Demographic characteristics of hepatitis C cases in mainland China, 2010–2018.

Hepatitis C group	2010	2011	2012	2013	2014	2015	2016	2017	2018	Total
Gender										
Male	88041 (0.58)	99179 (0.57)	113315 (0.56)	111954 (0.55)	112162 (0.55)	115616 (0.56)	116092 (0.56)	120731 (0.56)	124718 (0.57)	1001808 (0.56)
Female	64998 (0.42)	74693 (0.43)	88307 (0.44)	91201 (0.45)	90641 (0.45)	92281 (0.44)	90740 (0.44)	93292 (0.44)	94657 (0.43)	780810 (0.44)
Sex ratio	1.35	1.33	1.28	1.23	1.24	1.25	1.28	1.29	1.32	1.28
Age										
0~	2006 (0.01)	2170 (0.01)	2647 (0.01)	2266 (0.01)	2001 (0.01)	1860 (0.01)	1666 (0.01)	1408 (0.01)	1110 (0.01)	17134 (0.01)
10~	2749 (0.02)	2814 (0.02)	2961 (0.01)	2361 (0.01)	1660 (0.01)	1261 (0.01)	1103 (0.01)	1069 (0.00)	904 (0.00)	16882 (0.01)
20~	15988 (0.10)	17726 (0.10)	18932 (0.09)	19234 (0.09)	18297 (0.09)	17234 (0.08)	16084 (0.08)	14872 (0.07)	12885 (0.06)	151252 (0.08)
30~	31144 (0.20)	33725 (0.19)	35830 (0.18)	33944 (0.17)	31913 (0.16)	30065 (0.14)	28646 (0.14)	27608 (0.13)	25737 (0.12)	278612 (0.17)
40~	34093 (0.22)	42194 (0.24)	50187 (0.25)	50854 (0.25)	51080 (0.25)	51685 (0.25)	50800 (0.25)	51489 (0.24)	51759 (0.24)	434141 (0.24)
50~	26368 (0.17)	30402 (0.17)	37674 (0.19)	39791 (0.20)	41811 (0.21)	45056 (0.22)	45766 (0.22)	48382 (0.23)	52986 (0.24)	368236 (0.21)
60~	20626 (0.13)	23766 (0.14)	29032 (0.14)	30668 (0.15)	32586 (0.16)	35740 (0.17)	36920 (0.18)	40419 (0.19)	43581 (0.20)	293338 (0.16)
70~	14981 (0.10)	15777 (0.09)	18153 (0.09)	17746 (0.09)	17341 (0.09)	18329 (0.09)	18854 (0.09)	20948 (0.10)	22216 (0.10)	164345 (0.09)
80~	5084 (0.03)	5298 (0.03)	6206 (0.03)	6291 (0.03)	6114 (0.03)	6667 (0.03)	6993 (0.03)	7828 (0.04)	8197 (0.04)	58678 (0.03)

(Continued)

TABLE 2 Continued

Hepatitis C group	2010	2011	2012	2013	2014	2015	2016	2017	2018	Total
Occupation										
Farmers	58856 (0.38)	74173 (0.43)	93015 (0.46)	100110 (0.49)	101184 (0.50)	106432 (0.51)	107351 (0.52)	114389 (0.53)	–	755510 (0.48)
Housework and unemployment	21183 (0.14)	26463 (0.15)	30241 (0.15)	34444 (0.17)	34549 (0.17)	35243 (0.17)	35584 (0.17)	36821 (0.17)	–	254528 (0.16)
Worker	14563 (0.10)	10999 (0.06)	11310 (0.06)	10942 (0.05)	10487 (0.05)	9991 (0.05)	9065 (0.04)	8769 (0.04)	–	86126 (0.06)
Retiree	18127 (0.11)	18635 (0.10)	19939 (0.09)	19255 (0.09)	18725 (0.09)	19315 (0.09)	19424 (0.09)	18758 (0.09)	–	152178 (0.10)
Business service personnel	2830 (0.02)	3425 (0.02)	4066 (0.02)	4803 (0.02)	5126 (0.03)	4851 (0.02)	5238 (0.03)	5647 (0.03)	–	35986 (0.02)
Cadres and staff	6277 (0.04)	5509 (0.03)	5462 (0.03)	5142 (0.03)	4735 (0.02)	4513 (0.02)	4106 (0.02)	3865 (0.02)	–	39609 (0.03)
Others	220441 (0.20)	212650 (0.20)	203673 (0.19)	145092 (0.14)	135743 (0.14)	128064 (0.13)	120834 (0.13)	125165 (0.12)		1291662 (0.15)

as neither cold spots nor hot spots. The provinces in hotspots with a high spatial RRs value were located mainly in the southeast (Jiangxi, Fujian, Guangdong, and Hainan). Thus, the hepatitis B risk was relatively high in these provinces. The provinces in cold spots with a low spatial RRs value were located mainly in southwest (Heilongjiang, Tianjin, and Beijing), indicating a low level of hepatitis B. For hepatitis C, all provinces were defined as neither cold spots nor hot spots (Figure 5).

For hepatitis B, among the four hot spots, 75% (Hainan, Guangdong and Jiangxi) of all hotspots showed a faster temporal decreasing trend than the overall decreasing trend. Consequently, these regions might become lower risk regions or even non-hotspots in the future. Meanwhile, 25%(Fujian) of the hotspots showed the same trends as the overall trend, which indicated that these regions would still be hot areas over time (Figure 5). Therefore, the public health sector should focus on these provinces. Among the three cold spots, the provinces showed the same trends as the overall trend, indicating that these regions would likely remain cold areas, with a low risk (Figure 5).

Among the remaining twenty-four provinces of neither hot spots nor cold spots, approximately 45.83% (Inner Mongolia, Liaoning, Jilin, Ningxia, Gansu, Qinghai, Sichuan, Shaanxi, Henan, Zhejiang, and Yunnan) showed a slower decreasing trend than the overall decreasing trend, indicating that these regions might become higher risk regions or change into hot spots over time. Meanwhile, 29.17% (Tibet, Guangxi, Anhui, Jiangsu, Shandong, Shanghai, and Hunan) showed a faster decreasing trend than the overall trend, indicating that these regions might become lower risk areas or even cold spots over time. The remaining six provinces were consistent with the trend, with the current risk level over time (Figure 5).

For hepatitis C, among all provinces, 41.94% (Tibet, Guizhou, Hunan, Chongqing, Shandong, Shaanxi, Ningxia, Hubei, Anhui, Jiangsu, Shanghai, Hainan, and Tianjin) of neither hot spots nor

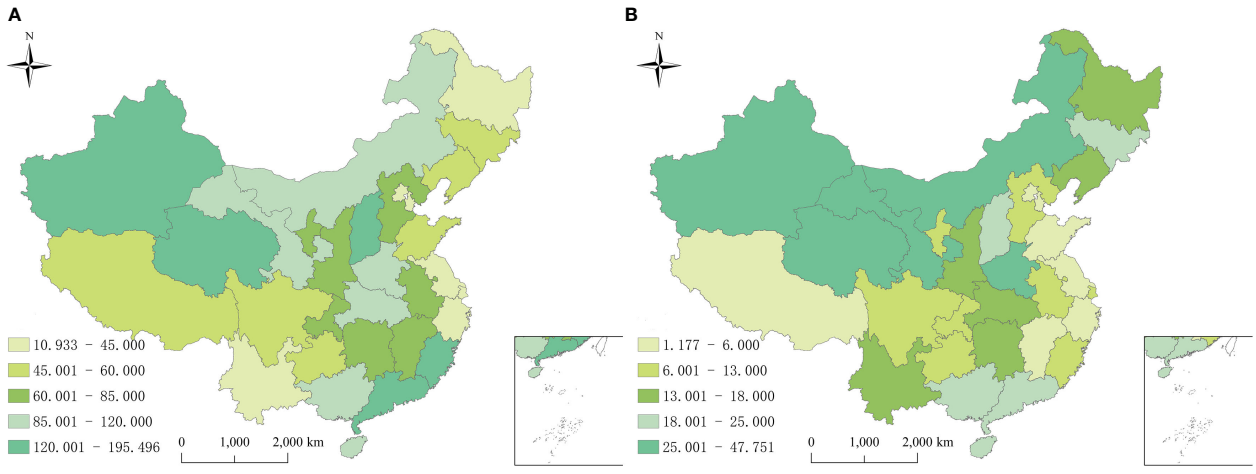

FIGURE 2
Annual average incidence rates of reported hepatitis B and C by province in China from 2010 to 2018. **(A)** Hepatitis B; **(B)** Hepatitis C.

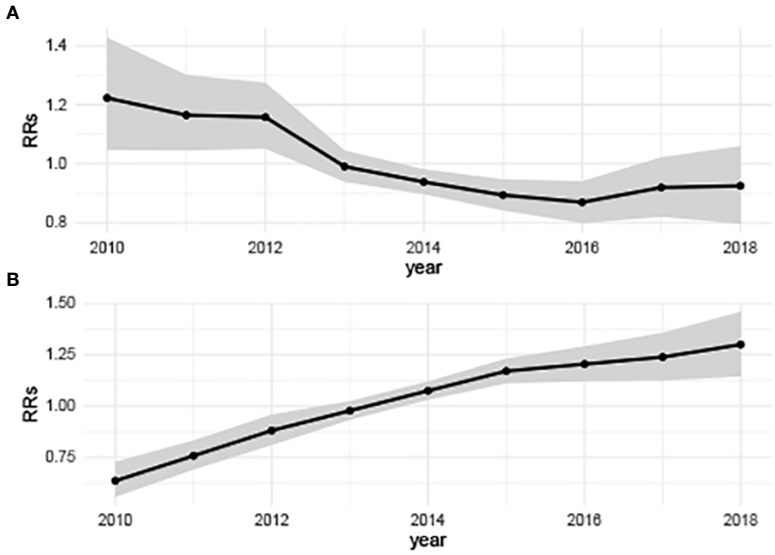

FIGURE 3
Annually temporal relative risks of hepatitis B and hepatitis C in China from 2010 to 2018. **(A)** Hepatitis B, showing a slow downward trend; **(B)** Hepatitis C, showing a significant upward trend. ($b_0t^*+v_t$) describes the overall time trend common to all counties with $v_t \sim N(0, \sigma_v^2)$.

cold spots exhibited a faster increasing trend than the overall increasing trend, indicating that these regions might become higher risk regions or even become hot spots in the future. 33.33% (Xinjiang, Heilongjiang, Jilin, Liaoning, Beijing, Shanxi, Henan, and Guangxi) of neither hot spots nor cold spots showed a slower upwards than the overall increasing trend. Consequently, the risk in these provinces would likely be lower than the overall risk, and they might become cold spots over time. The remaining provinces were consistent with the overall trend. Thus, the current risk level in these provinces will remain constant in the future (Figure 5).

3.4 Risk factor detection

We used a Bayesian space-time hierarchy model to analyze the impact of the factors, such as urbanization rate, per capita GDP, illiterate rate, road mileage, hygienic personnel, beds, and density, on hepatitis B and C. The results found that the increase of the incidence rate of hepatitis C, and illiteracy rate increased the RRs of having hepatitis B (Table 3). For hepatitis C, the increase of illiteracy rate, and per capita GDP were protective factors, while the increase of incidence rate of hepatitis B increased the RRs of having hepatitis C (Table 4).

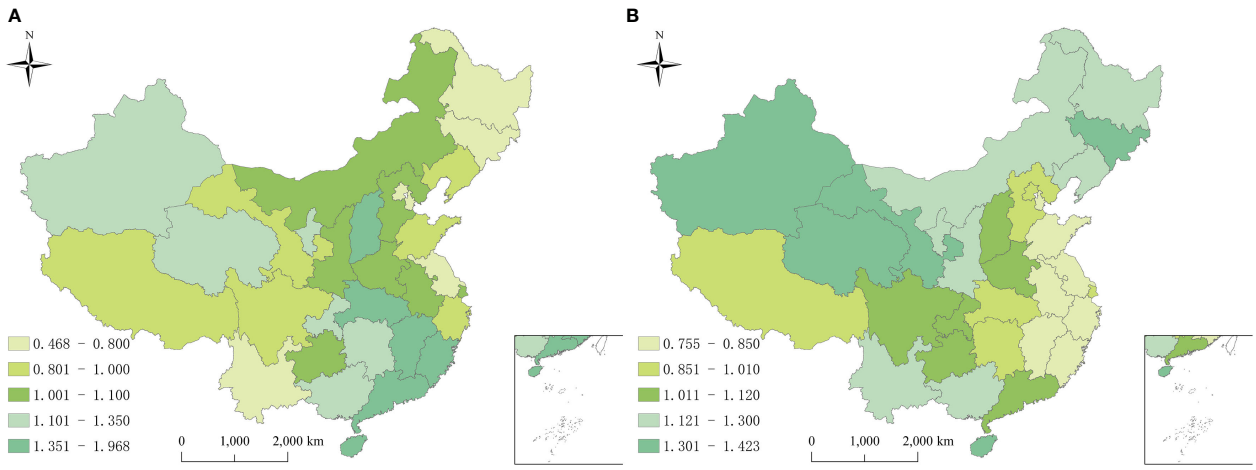

FIGURE 4
Spatial relative risks of hepatitis B and hepatitis C in China from 2010 to 2018. **(A)** Hepatitis B, high risk areas were mainly distributed in the southeast and northwest regions; **(B)** Hepatitis B, high risk areas were mainly distributed in the north. The $exp(s_i)$ is the spatial risk of this disease, which is influenced by some related factors in the study period, such as economic conditions, local prevention and control policies, and medical resources.

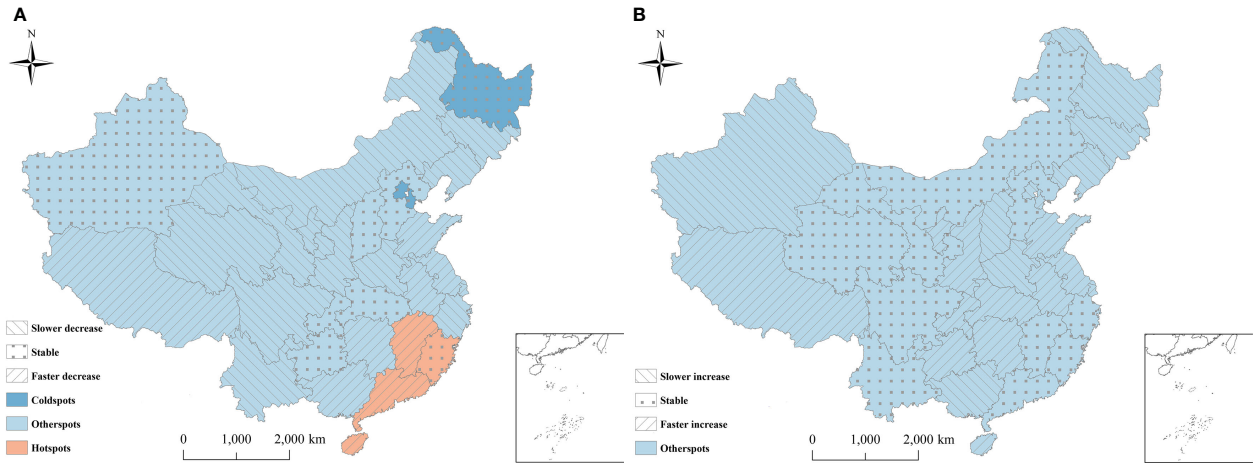

FIGURE 5
Distribution of hot and cold spots of hepatitis B and hepatitis C in China. **(A)** Hepatitis B; **(B)** Hepatitis C. The provinces were classified into nine categories (3 risk categories × 3 trend categories). 3 risk categories: hotspots: $P(exp(s_i)>1|data)\in[0.8, 1]$, representing high-risk area; coldspots: $P(exp(s_i)>1|data)\in[0,0.2]$, representing low-risk area; neither hotspots nor coldspots: $P(exp(s_i)>1|data)\in(0.2,0.8)$. 3 trend categories: level 1, the variation trend of the disease is faster than the overall trend, if $P(b_{1i}>0|h_i,data)\in[0.8,1]$; level 2, the variation trend of the disease is slower than the overall trend, if $P(b_{1i}>0|h_i,data)\in[0,0.2]$; level 3, the variation trend in the disease has no difference with the mean level, if $P(b_{1i}>0|h_i,data)\in(0.2,0.8)$.

An increase of 1 yuan in per capita GDP was related to a decrease of 0.0008% (-0.0015, -0.0003) in the risk of hepatitis C (RRs: 1.0000; 95%CRI: 1.0000-1.0000). Every 1% increase in illiteracy rate was related to a 2.5430% (0.9444, 4.2530) increase in hepatitis B risk, with a corresponding RRs of 1.0264(95%CRI: 1.0090-1.0430). By contrast, a 1% increase in illiteracy rate was related to a 3.1830% (-4.7090, -1.6450) decreases in hepatitis C risk, with a corresponding RRs of 0.9687(95%CRI: 0.9540-0.9837) (Tables 3, 4).

Increased prevalence of hepatitis B and hepatitis C can increase the risk of hepatitis C and hepatitis B, respectively. A 1% increase in the incidence of hepatitis C was related to an increase of 2.7410% (95%CRI: 1.9610-3.4800) in the risk of hepatitis (RRs: 1.0280; 95% CRI: 1.0200,1.0350). Meanwhile, every 1% increase in the incidence of hepatitis B was related to a 0.3866% (0.2699, 0.5132) increase in hepatitis C risk, with a corresponding RRs of 1.0040(95%CRI: 1.0030-1.0050). The influence of the remaining factors on the risk of acquiring hepatitis B or hepatitis C infection was not significant (Tables 3, 4).

4 Discussion

In this study, we used Bayesian spatiotemporal hierarchy models to study the spatiotemporal heterogeneity of hepatitis B and C in China and measured the potential impact of socioeconomic factors on hepatitis B and hepatitis C in China, based on the national disease surveillance dataset from 2010 to 2018 of the Chinese Center for Disease Control and Prevention. BSTHM embeds spatiotemporal information, prior distribution and

TABLE 3 Quantified posterior mean values and relative risks (RRs) of potential driving factors of hepatitis B in China from 2010 to 2018.

Variable	Posterior mean(95%CRI)	RRs (95%CRI)
Per capita GDP	-0.0004(-0.0011, 0.0003)	1.0000(1.0000,1.0000)
Urbanization rate (%)	1.0900(-0.8540, 3.1520)	1.0110(0.9915,1.0320)
Incidence rate of hepatitis C (%)	2.7410(1.9610, 3.4800)	1.0280(1.0200,1.0350)
Illiteracy rate (%)	2.5430(0.9444, 4.2530)	1.0260(1.0090,1.0430)

TABLE 4 Quantified posterior mean values and relative risks (RRs) of potential driving factors of hepatitis C in China from 2010 to 2018.

Variable	Posterior mean(95%CRI)	RRs (95%CRI)
Per capita GDP	-0.0008(-0.0015, -0.0003)	1.0000(1.0000,1.0000)
Urbanization rate (%)	0.4989(-0.7851, 2.4170)	1.0005(0.9922,1.0240)
Incidence rate of hepatitis B (%)	0.3866(0.2699, 0.5132)	1.0040(1.0030,1.0050)
Illiteracy rate (%)	-3.1830(-4.7090, -1.6450)	0.9687(0.9540,0.9837)

spatiotemporal correlation factors, which solves the estimation bias caused by spatial structure and makes the estimation more stable and reliable (Best et al., 2005). The results showed significant spatial and temporal heterogeneity in the risk of hepatitis B and C. Over time, the risk of hepatitis B had generally shown a slow downward trend, while the risk of hepatitis C had been on the rise. Spatially, the high-risk areas of hepatitis B, were mainly distributed in the southeast and northwest regions, while the high-risk areas of hepatitis C were mainly distributed in the northern regions. In addition, for hepatitis B, illiteracy, and hepatitis C prevalence were the main contributing factors, while GDP per capita, illiteracy rate, and hepatitis B prevalence were the main contributing factors to hepatitis C.

The spatial distribution of viral hepatitis was uneven, indicating that socioeconomic conditions were strongly associated with viral hepatitis risk. For example, increased prevalence of hepatitis B and hepatitis C can increase the risk of hepatitis C and hepatitis B, respectively. The illiteracy rate of people aged 15 and above represents the local education level to some extent, and the illiteracy rate in Fujian showed an upward trend, which partly explained the high risk in Fujian. On the other hand, HBV detection was removed from routine health check-ups for new employees and students from 2010 due to population discrimination against people with hepatitis B (Cooke et al., 2019), which would also affect the diagnosis of hepatitis B and may be an important factor in the decline in the prevalence of hepatitis B. For hepatitis C, an increase of 1 yuan in per capita GDP was related to a decrease of 0.0008% in the risk of hepatitis B. High risk areas of hepatitis C were mainly distributed in the north. The per capita GDP in the north was lower than that in the south, while Beijing and Tianjin in the north were in the forefront of the country. With their high level of culture and medical care and complete infrastructure, they had a low risk of hepatitis C. Therefore, per capita GDP was a protective factor against hepatitis C. The results of this research also showed that the improvement of education level (the reduction of illiteracy rate) had increased the RRs value of hepatitis C, which might be attributed to but not limited to the following reasons: on the one hand, since 2009, the Center for Sexual AIDS Prevention and Control of the Chinese Center for Disease Control and Prevention has carried out comprehensive prevention and treatment of hepatitis C, and in 2012, the Office of Hepatitis C and STD Prevention and Control was established to explore the "Chinese experience and model" of eliminating hepatitis C, and do a good job in hepatitis C publicity and education and comprehensive intervention. The prevention and control level of hepatitis C by the population and relevant staff had been improved, and the detection rate of hepatitis C had been improved. On the other hand, with the improvement of literacy level, people recognized that hepatitis C was preventable and curable, national medical insurance and other policy measures reduce public fear of hepatitis C and discrimination against patients, improve self-protection and positive medical awareness (Li et al., 2022). At present, China's viral hepatitis control system is relatively fragmented, and at the same time, the funds clearly allocated to hepatitis C are relatively small (Chen et al., 2020), so the process of preventing and treating hepatitis C needs to enhance the top-level design to make up for the lack of financial and personnel support to a certain extent.

In addition, the research results showed that the increased prevalence of hepatitis B and hepatitis C would increase the risk of hepatitis C and hepatitis B, respectively, which to some extent indicated that people with hepatitis B or one of hepatitis C were often high-risk groups for another type of hepatitis. Relevant research showed that the incidence of co-infection of hepatitis B and hepatitis C was between 1% and 15%, while the presence of unidentified occult HBV infection might lead to the underestimated incidence (Senturk et al., 2008; Pol et al., 2017). Compared with single infection, HBV/HCV co infection will increase the severity of liver disease (Mavilia and Wu, 2018). In addition, some studies had revealed that hepatitis C treatment can reactivate hepatitis B (Blackard and Sherman, 2018; Ma and Feld, 2018). Therefore, surveillance of people who already have hepatitis C or B should be strengthened to reduce co-infection.

The study had some limitations. We used provincial data to explore population-level associations, which may inevitably lead to ecological fallacies (Jelinski and Wu, 1996), but this does not affect long-term trends in hepatitis B and C. Furthermore, the indicators used in the model are all macro-control statistics, but the elements affecting hepatitis are complex and diverse, so factors other than those considered in this study may bring some uncertainty to hepatitis B and C. Finally, there may be a delay or later between the reported number of hepatitis infections and the exact number of hepatitis infections, resulting in differences in RRs.

In short, the burden of hepatitis B and C in China remains high, and prevention and treatment faces many challenges (Wang et al., 2017), including economic development, education level, allocation of prevention and control resources, etc., which are important factors affecting hepatitis. In order to promote the prevention and control of the prevalence of hepatitis B and hepatitis C in China, we put forward the following suggestions: Firstly, China should set up special institutions to rationally allocate resources for hepatitis prevention and control (strengthen the prevention and control of hepatitis B in the southeast and northwest and hepatitis C in the north), and coordinate the cooperation among public health institutions, medical care providers,and communities to ensure the effective use of resources and expertise. Secondly, stigma and discrimination related to hepatitis B and hepatitis C are also a serious obstacle. Medical professionals should actively participate in and provide relevant publicity activities to improve public awareness and eliminate discrimination, while respecting the privacy of infected persons (Buti et al., 2022). Finally, scientific diagnostic criteria and screening technology (especially mixed infection) and advanced modeling technology are crucial for monitoring and eliminating hepatitis. Therefore, we can use GISAID, Github, and other data sharing platforms to manage, share and analyze data and promote the optimization of public prevention and control measures.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding authors.

Author contributions

Funding acquisition: CHW, WT. Data accept: JQ, LA, MY, YL. Data analysis: JQ, MY, NY, CCW. Project administration: YZ, CHW, CZ. Supervision: CHW, PH. Writing-original draft: JQ, WT, MY. Writing-review and editing: CHW, JQ, WT. All authors contributed to the article and approved the submitted version.

Funding

This research was supported by the grant of National Natural Science Foundation of China (82273691), grants from Jiangsu Social Development Plan (BE2022682, BE2017620), Jiangsu Natural Science Foundation (BK20221196).

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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frontiers | Frontiers in Cellular and Infection Microbiology

TYPE Review
PUBLISHED 15 March 2023
DOI 10.3389/fcimb.2023.1128807

OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to Virus and Host, a section of the journal Frontiers in Cellular and Infection Microbiology

RECEIVED 21 December 2022
ACCEPTED 03 February 2023
PUBLISHED 15 March 2023

CITATION
Pan Y, Xia H, He Y, Zeng S, Shen Z and Huang W (2023) The progress of molecules and strategies for the treatment of HBV infection.
Front. Cell. Infect. Microbiol. 13:1128807.
doi: 10.3389/fcimb.2023.1128807

The progress of molecules and strategies for the treatment of HBV infection

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Hepatitis B virus infections have always been associated with high levels of mortality. In 2019, hepatitis B virus (HBV)-related diseases resulted in approximately 555,000 deaths globally. In view of its high lethality, the treatment of HBV infections has always presented a huge challenge. The World Health Organization (WHO) came up with ambitious targets for the elimination of hepatitis B as a major public health threat by 2030. To accomplish this goal, one of the WHO's strategies is to develop curative treatments for HBV infections. Current treatments in a clinical setting included 1 year of pegylated interferon alpha (PEG-IFNα) and long-term nucleoside analogues (NAs). Although both treatments have demonstrated outstanding antiviral effects, it has been difficult to develop a cure for HBV. The reason for this is that covalently closed circular DNA (cccDNA), integrated HBV DNA, the high viral burden, and the impaired host immune responses all hinder the development of a cure for HBV. To overcome these problems, there are clinical trials on a number of antiviral molecules being carried out, all -showing promising results so far. In this review, we summarize the functions and mechanisms of action of various synthetic molecules, natural products, traditional Chinese herbal medicines, as clustered regularly interspaced short palindromic repeats and their associated proteins (CRISPR/Cas)-based systems, zinc finger nucleases (ZFNs), and transcription activator-like effector nucleases (TALENs), all of which could destroy the stability of the HBV life cycle. In addition, we discuss the functions of immune modulators, which can enhance or activate the host immune system, as well some representative natural products with anti-HBV effects.

KEYWORDS

HBV, molecules, HBV life cycle, inhibitors, treatment

Introduction

Hepatitis B virus (HBV), a specific small hepatotropic DNA virus, is the causative agent of hepatitis B, which is now the most common serious liver infection all over the world. In 2019, the global prevalence rate of HBV reached 4.1%, representing 316 million people living with HBV. In the same year, HBV-related diseases were the causes of approximately

555,000 deaths around the world (Sheena et al., 2022). Hepatitis B is therefore regarded as a serious threat to human life and health, despite it being preventable. To solve this public health threat by 2030, the World Health Organization (WHO) put forward an ambitious target for the elimination all types of viral hepatitis, especially hepatitis B and C, in its document *Global Health Sector Strategy on Viral Hepatitis 2016–2021. Towards Ending Viral Hepatitis.*

Typically, the treatment of hepatitis B is divided into three clinical levels (Lok et al., 2017; Suk-Fong Lok, 2019). The first level is partial treatment, which results in the detection of hepatitis B surface antigens (HBsAgs), but after this finite treatment course, HBV DNA is still persistently undetectable in a patient's serum. The second level is functional treatment, after which both HBsAg and HBV DNA can be detected in the patient's serum, but not continuously. The third level of treatment, which is the ultimate goal, entails complete removal, resulting in HBsAg being undetectable in the patient's serum, and in the eradication of HBV DNA (Lok et al., 2017; Suk-Fong Lok, 2019).

Traditionally, there have been mainly two strategies used in the treatment of chronic HBV infections in clinical settings, that is, antiviral drugs [i.e., nucleoside analogues (NAs)] and interferons (IFNs). In the 1990s, IFN alpha (IFN-α) was approved by the US Food and Drug Administration (FDA) for the treatment of HBV infections, which started a new era of antiviral therapy (Trepo, 2014). IFNs are endogenous cytokines produced by immune system cells in response to viral infection. Of note, pegylated IFN-α is not only injectable, and, therefore, usable in the treatment of HBV infections, but could also play a significant role in regulating host immunity (Ye and Chen, 2021). However, serious adverse reactions, including cytopenia, exacerbations of neuropsychiatric symptoms (i.e., depression and insomnia), and the production of thyroid autoantibodies associated with this treatment occurred on a frequent basis (Tang et al., 2018). As a result of the focus on the development of specific antiviral drugs, lamivudine was marketed and became the first NA approved by the FDA for the treatment of hepatitis B, meaning that a new therapy for HBV infection was available (Dienstag et al., 1999). It has been reported that NAs block the normal replication processes of HBV by inhibiting the activity of HBV polymerase (Pierra Rouviere et al., 2020). However, although NAs are effective in reducing viral load, they require long-term administration and frequently induce adverse effects, such as fatigue, dizziness, headache, and nausea (Bedre et al., 2016; Roediger et al., 2022). In addition, despite the capacities of both NAs and IFNs to significantly lower levels of viral DNA in the blood, functional cure rates after these therapies remained low (i.e., 3%–7% for pegylated IFNs and 1%–12% for NAs) (Feng et al., 2018; Tang et al., 2018). Therefore, there is an urgent need to develop more effective and ideal medicines or strategies to achieve higher functional cure rates, eventually developing a complete eradication of HBV. Hence, in this review, we summarize the existing therapeutic agents for HBV infections, including various synthetic molecules, natural products, traditional Chinese herbal medicines, clustered regularly interspaced short palindromic repeats and their associated proteins (CRISPR/Cas)-based systems, zinc finger nucleases (ZFNs), and transcription activator-like effector nucleases (TALENs), all of which have the ability to destroy the stability of the HBV life cycle. We also provide a summary of the existing anti-HBV immunotherapies and host immune modulators.

HBV life cycle

There are some barriers to the treatment of HBV infections. The first of these is its involvement with covalently closed circular DNA (cccDNA) and integrated HBV DNA. cccDNA is the transcriptional template for DNA replication and antigen production, and, meanwhile, integrated HBV DNA can be a template for the generation of HBsAgs. In addition, a higher viral burden, associated with increasing levels of HBV DNA and HbsAG, is also a barrier to treatment. Finally, an impaired host immune response can also prevent the treatment of HBV infections (Wong et al., 2022).

Before the specifics of the HBV life cycle are detailed, it is meaningful to describe the structure of HBV. There were three common types of HBV particles observed in patients' serum (Tsukuda and Watashi, 2020). For instance, complete HBV virions, also known as Dane particles, were in the form of spheres that were 42 nm in diameter and could routinely be detected in the blood of infected patients. In addition, the structure of Dane particles possess 3.2 kb of partially double-stranded relaxed circular DNA (rcDNA) bonded to polymerase, an inner nucleocapsid formed by the core protein hepatitis B core antigen (HBcAg), and an outer envelope generated by lipid-embedded small (SHBs), middle (MHBs), and large HBsAgs (LHBs) (Figure 1) (Kim et al., 2022). Comparatively, the incomplete particles, also known as the subviral particles of HBV, were of two major types, that is, the classical HBsAg spheres and filaments (Figure 1). In addition, there were putative particles containing HBV RNA in much lower levels than in other particles (i.e., 100- to 1,000-fold lower than in complete virions) (Roediger et al., 2022).

Initially, HBV attaches to the host hepatocyte surface by binding to specific factors, such as heparan sulfate proteoglycans (HSPGs), in a low-affinity manner (Figure 2). Subsequently, the virus makes contact with the specific receptor sodium taurocholate co-transporting polypeptide (NTCP) through the pre-S1 domain of a large viral envelope protein in a high-affinity manner. Virus–receptor interactions can also trigger HBV internalization into hepatic cells in an endocytosis-dependent manner (Tsukuda and Watashi, 2020). Of note, it has been reported that the epidermal growth factor receptor (EGFR) is a host factor that interacts with NTCP and mediates HBV internalization (Iwamoto et al., 2019). After the HBV enters the cell, the viral envelope fuses with the endosome membrane and releases the free nucleocapsid into the cytoplasm. The nucleocapsid can then utilize the microtubule network to facilitate its transition into the nucleus, which occurs *via* its interaction with motor proteins (Hayes et al., 2016). In the nucleus, the rcDNA of HBV is modified and repaired into cccDNA, and a part of the incoming HBV DNA can

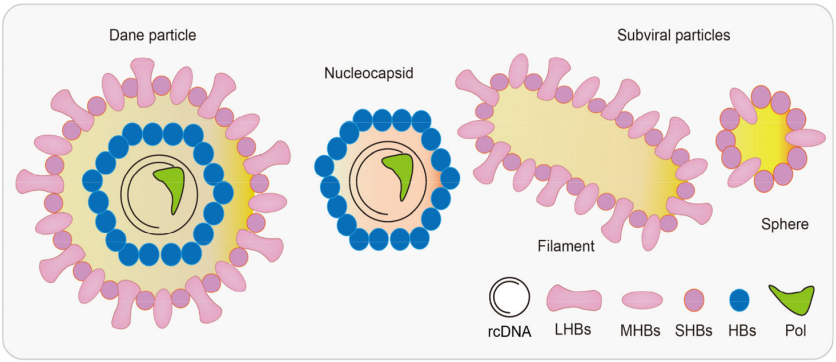


FIGURE 1
Hepatitis B virus (HBV)-associated particles. The diameter of Dane particles is about 42 nm, and their envelopes are made up of large/middle/small HBsAgs (LHBs/MHBs/SHBs). A nucleocapsid contains both viral genomes and polymerases. There are two types of subviral particles: filaments and spheres. HBV, hepatitis B virus; HBsAgs, hepatitis B surface antigens; LHBs, lipid-embedded large HBsAgs; MHBs, lipid-embedded medium HBsAgs; SHBs, lipid-embedded small HBsAgs.

also be integrated into the host's genome (Tsukuda and Watashi, 2020). Significantly, cccDNA can reinitiate infection after long-term antiviral therapy, which is typically the main reason for HBV infections not being cured completely.

The cccDNA in the nucleus functions as a template for the transcription of different lengths of messenger RNAs (mRNAs), which can thereafter be released into the cytoplasm to enable the translation of the corresponding proteins, including polymerases, HBcAgs, precore proteins, HBsAgs, and hepatitis B virus X proteins (HBxs) (Wang et al., 2020). Synthesized HBcAg monomers are initially combine to yield a dimer, which can subsequently constitute an icosahedral capsid by self-assembly. Thereafter, partial polyvalent guide RNAs (pgRNAs), along with polymerases, are encapsulated into icosahedral capsid to generate the core proteins (Tsukuda and Watashi, 2020). Subsequently, after the catalysis of the reverse transcriptase region of the HBV polymerase and HBV ribonuclease H (RNaseH) in the nucleocapsid, pgRNA serves as the template for the strand DNA. After post-translational modifications within the endoplasmic reticulum and Golgi apparatus, the mature viral particles are secreted out of the infected hepatocyte, and this occurs contemporaneously with the secretion of a large number of non-infectious particles. Finally, the mature hepatitis Be antigen (HBeAg), generated from precore polypeptides, can be released directly into circulation (Wang et al., 2020), so as to regulate the immune response to the intracellular nucleocapsid. As shown in Figure 2, every step of the HBV life cycle can be catalyzed by corresponding enzymes, and, therefore, serves as a potential drug target for the development of novel anti-HBV medicines or theoretical methods of treatment.

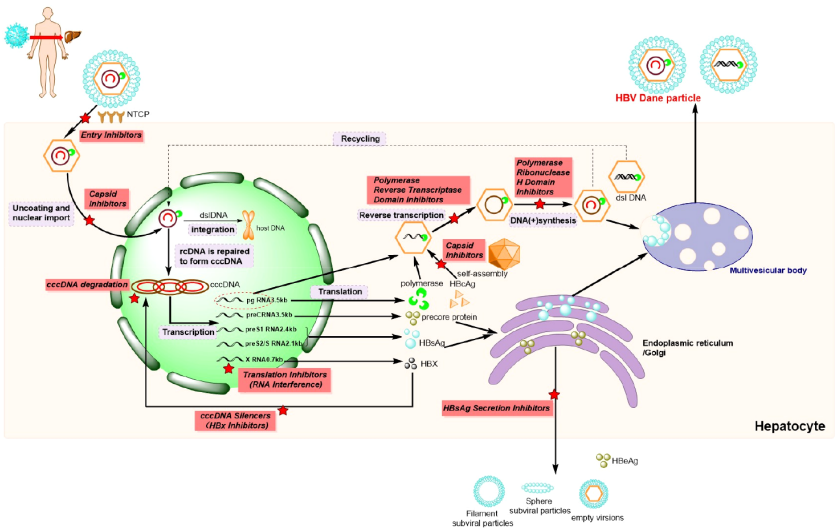


FIGURE 2
Hepatitis B virus (HBV) life cycle and potential drug targets (). HBV, hepatis B virus. NTCP, Na^+-taurocholate co-transporting polypeptide; cccDNA, covalently closed circular DNA; rcDNA, relaxed circular DNA; dslDNA, double-stranded linear DNA; pgRNA, polyvalent guide RNA; HBcAg, hepatitis B virus core antigen; HBsAg, hepatitis B surface antigen; HBX, hepatitis B-encoded X antigen; HBeAg, hepatitis Be antigen; cRNA, cytoplasmic RNA.

The development of drugs affecting the HBV life cycle

At present, there have already been drugs and active compounds developed for the treatment of HBV infection. The following mainly introduced the structures and relevant properties, including small molecules, RNAs, peptides, and NPs. The following text introduces their structures and describes the relevant properties of these molecules.

Entry inhibitors

It had been reported by Yan's group in 2012 that the NTCP could be an acceptable drug target for the treatment of HBV infection (Yan et al., 2012). Myrcludex B (Table 1), a first-in-class entry inhibitor, is a chemically synthesized polypeptide consisting of 47 amino acids, and it also has the preS1 domain of HBV large surface proteins (Uhl et al., 2016; Chen et al., 2022). Myrcludex B competes with HBV for NTCP receptor sites to prevent it from entering hepatocytes. This drug was approved in 2020 by the European Union (EU) for the treatment of chronic hepatitis D virus (HDV) and of HBV infections in phase 1/11 trials (Kang and Syed, 2020). The findings of a phase I trial indicated that treatment with Myrcludex B was well tolerated by participants, with no serious or relevant adverse reactions occurring (Cheng et al., 2021a). In addition, it also demonstrated a high potential foreffective, combined use with IFNs and NAs.

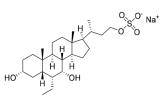
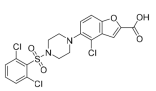
Monoclonal antibodies were also efficient as HBV entry inhibitors (Wi et al., 2017). HH-003 and HH-006 (Table 1), full human monoclonal antibodies developed by Huahui Health Ltd, targeted the preS1 domain of the HBV large surface protein (Yang and Xie, 2022). These two antibodies could suppress the binding of HBV with NTCP receptor sites to block HBV infection. To date, phase II trials of HH-003 injection have been conducted in China, and a phase I trial of HH-006 injection is currently under way in Australia.

It has also been reported that, under normal conditions, farnesoid X receptor (FXR) can down-regulate the expression of NTCP *via* the induction of small heterodimer partner (SHP) nuclear receptors (Farooqui et al., 2022). INT-767, a bile acid (BA) derivative, was also identified as a specific FXR agonist and could effectively block the entry of HBV by down-regulating the expression of NTCP (Ito et al., 2021). In chimeric mice with humanized liver, INT-767 markedly delayed the initial increase in HBsAg, hepatitis B e antigen (HBeAg), and HBV DNA levels, as well as reducing levels of cccDNA (Ito et al., 2021). After a series evaluation of pharmacokinetic (PK) and pharmacodynamic (PD) properties, INT-767 became a candidate for the treatment of HBV infections and a phase I study evaluating its possible use as a treatment for HBV was conducted (Ito et al., 2021). In addition, INT-767 was also evaluated for the treatment of non-alcoholic steatohepatitis (NASH) and intestinal ischemia reperfusion injury (IRI) because of its function as a dual FXR/Takeda G protein-coupled receptor 5 (TGR5) agonist (Anfuso et al., 2020).

A clinical study primarily evaluating the safety and antiviral effect of vonafexor, another FXR agonist with a benzofuran-2-carboxylic acid moiety, was also conducted (Erken et al., 2021). The corresponding experimental results, as well as those of a phase II trial, demonstrated that vonafexor was safe, and that its use resulted in an observable decline in the number of HBV markers observed in chronic hepatitis B (CHB) patients (Erken et al., 2021). In addition, as with INT-767, the presence of vonafexor was also identified in a phase IIa assessment of non-alcohol steatohepatitis (NASH) (Fiorucci et al., 2020).

In addition to the drugs discussed above, several marketed drugs have also been identified as efficient inhibitors of the NTCP–LHB interaction, such as the immunosuppressive agent

TABLE 1 Summary of molecules as entry inhibitors.

Structure	Target	Company	NCT number	Clinical status
GTNLSVPNPLGFFPDHQLDP AFGANSNNPDWDFNPNKDHWPEANKVG Myrcludex B	NTCP	Gilead Sciences Inc.	NCT02881008 NCT04166266 NCT02637999	Approved by the EU for the treatment of chronic HDV; phase I/II trial(s)
Undisclosed HH-003	NTCP	Huahui Health Ltd	NCT05542979	Phase I trial(s) in Australia; phase II trial(s) in China
Undisclosed HH-006	NTCP	Huahui Health Ltd	NCT05275465	Phase I trial(s)
 **INT-767**	FXR	Intercept Pharma	Undisclosed	Phase I trial(s)
 **Vonafexor**	FXR	ENYO Pharma	NCT03469583 NCT03320616 NCT04365933 NCT03272009	Phase II trial(s)

EU, European Union; FXR, farnesoid X receptor; HDV, hepatitis D virus; NCT, National Clinical Trial; NTCP, sodium taurocholate co-transporting polypeptide.

cyclosporine A and its derivatives (Nkongolo et al., 2014), angiotensin II receptor antagonist irbesartan (Ko et al., 2015), and anti-hyperlipidemia agent ezetimibe (Lucifora et al., 2013).

Inhibition of assembly or formation of the cccDNA minichromosome

Because of the key role HBV cccDNA plays in viral persistence, the removal, destruction, or inhibition of cccDNA are regarded as the keys to virus eradication. In the nucleus, HBV cccDNA is formed from rcDNA, which binds with both histones (i.e., H2A, H2B, H3, H4, and H1) and non-histone proteins (i.e., HBcs, HBxs, and host factors), and is then organized into a chromatin-like structure termed as the HBV cccDNA minichromosome (Zhang et al., 2021). Because of the Histone Acetyltransferase 1 (HAT1) regulating the HBV cccDNA minichromosome, it had been a promising target for controlling the assembly of the cccDNA (Yang et al., 2019). Nevertheless, to date, relative inhibitors have not been reported.

Notably, two disubstituted sulfonamides (DSS), namely CCC-0975 and CCC-0346 (Table 2), have been identified as the novel inhibitors of HBV cccDNA biosynthesis through a specific screening approach (Cai et al., 2012). These two compounds could interfere with the conversion of rcDNA to cccDNA, and further research into their function might ultimately change the landscape of hepatitis B treatment.

In addition to the molecules and compounds discussed above, HBV core protein allosteric modulators (CpAMs) could also efficiently inhibit HBV reproduction by modulating the assembly of capsids at a late stage (Mak et al., 2017). It has also been reported that HAP_R01 and HAP_R10, which are 4–H heteroaryldihydropyrimidine (HAP) analogues, can alter capsid integrity to affect HBV infectivity and suppress cccDNA formation by inhibiting the activity of C-terminally truncated proteins (i.e., Cp149) (Qiu et al., 2017). As a third-generation 4-H HAP inhibitor, HAP_R10 is optimized from NVR-010–001-E2 by the introduction of carboxyl groups (Figure 3A), and subsequently selected for further development as an oral anti-HBV agent (Qiu et al., 2017).

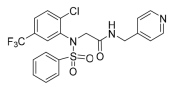
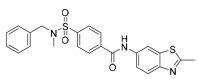
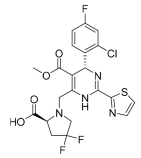
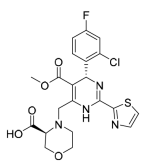
Importantly, the co-crystal structure of HAP_R01 (Table 2) with Cp149 has been determined (PDB ID: 5WRE) (Zhou et al., 2017). As shown in Figure 3B, in addition to the H-bond interaction of the parent nucleus with Thr33 and Trp102, the carboxyl group can also generate a H-bond interaction with the Ser141 side chain. In addition, thiazole moiety can also be inserted into a specific hydrophobic pocket that is surrounded by Phe23 and Phe122, thereby forming a strong hydrophobic interaction (Figure 3C). Because of these crucial interactions, HAP_R01 became a potent inhibitor and guided the further structure optimization.

Silencing cccDNA transcription

Accumulating evidence has proved that epigenetic modifications of cccDNA contribute to viral replication and chronic HBV infection (Hong et al., 2017). With the goal of silencing cccDNA in infected hepatocytes, epigenetic therapy might be a promising therapeutic strategy.

A significant curative strategy, that is the functional silencing of cccDNA, might be achieved by targeting the viral protein HBx

TABLE 2 A summary of compounds that interfere with the function of cccDNA minichromosomes.

Structure	Mechanism of action	Activity	PK/PD	Clinical status
 **CC-0975**	Interferes with cccDNA synthesis	HepDE19 cells: EC_{50} = 4.55 μM; CC_{50} > 50 μM; CC_{50}/EC_{50} > 11	Undisclosed	Preclinical
 **CC-0346**	Interferes with cccDNA synthesis	HepDE19 cells: EC_{50} = 0.35 μM; CC_{50} = 2.57 μM; CC_{50}/EC_{50} = 7.34	Undisclosed	Preclinical
 **HAP_R01**	Binds Cp149	HepG.2.2.15 cells: EC_{50} = 0.003 μM; CC_{50} = 65 μM; CC_{50}/EC_5 = 21,667	Undisclosed	Preclinical
 **HAP_R10**	BindsCp149	HepG.2.2.15 cells: EC_{50} = 0.003 μM; CC_{50} > 100 μM; CC_{50}/EC_{50} > 33,333	Mice plasma: CL (iv): 19 mL/min/kg; $t_{1/2}$ 1.5 h; F (%): 37; C_{max}: 413 μg/L; AUC (PO): 962 μg/L/h	Preclinical

Clearance (CL), Area Under Curve (AUC), Per Os (PO), Pharmacokinetics (PK), Pharmacodynamics (PD), concentration for 50% of maximal effect (EC50), 50% cytotoxicity concentrations (CC50), t1/2, half-life; Cmax, maximum concentration; Tmax, time to peak.

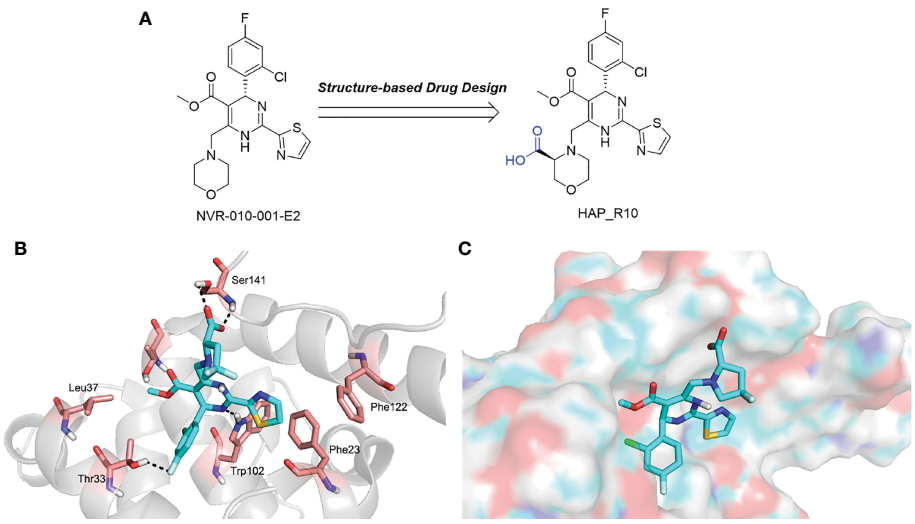


FIGURE 3
(A) The design of HAP_R10; **(B)** The interactions of HAP_R01 with Cp149; **(C)** The binding mode of HAP_R01. Cp149, the 149-residue core protein assembly domain.

because of its crucial role in stimulating the transcription of HBV cccDNA (Sekiba et al., 2022). Furthermore, HBx can enable cccDNA transcription by means of inactivating the cellular damage-specific DNA-binding protein 1 (DDB1). Notably, DDB1 contains E3 ubiquitin ligase and could degrade structural maintenance of chromosomes 5/6 (SMC5/6), which bonds with the cccDNA minichromosome (Sekiba et al., 2022). In addition, HBx prevents transcriptional repressor recruitment to the cccDNA minichromosome (Zhang et al., 2021). In brief, HBx can activate the transcription of host genes by directly interacting with nuclear transcription factors, or activating various signal transduction pathways in the cytoplasm (Hong et al., 2017). Nitazoxanide (NTZ, Figure 4), a thiazolide anti-infective agent, has been approved by the FDA for the treatment of protozoan enteritis (Sekiba et al., 2019). Interestingly, it has been discovered that NTZ efficiently inhibits the protein interaction of HBx with DDB1, which in turn leads to a significant reduction in viral transcription activity, and, therefore, the number of viral products (Sekiba et al., 2019). Recently, much more indepth research of NTZ for the treatment of HBV infection has been going on. In addition, it has been proven that an NQO1 inhibitor, dicoumarol (Figure 4), can exerts an anti-HBV influence, in that it promotes the degradation of HBx and blocks cccDNA transcription (Cheng et al., 2021b). Further experimental results demonstrate that dicoumarol is capable of potent antiviral activity that restricts the production of HBV RNAs, HBV DNA, HBsAgs, and HBc proteins in HBV-infected cells and in a humanized-liver mouse model.

Destroying the stability of cccDNA

In the destabilization process for HBV cccDNA, a number of tools for editing genomes were reported, such as CRISPR/Cas 9 mechanism systems, ZFNs, and TALENs. All of these representative gene-editing approaches can cause a nick in double-stranded DNA at a specific target region in turn changing the specific DNA sequence in said region (Bhat and Kazim, 2022).

The CRISPR/Cas9 mechanism technique is the most famous, and has garnered considerable interest because of its accessibility and versatility. By designing the guide RNA (gRNA), so that it is complementary to the target DNA sequence, the CRISPR/Cas9 mechanism can be redirected to specifically cleave any desired DNA genome, resulting in site-specific DNA double-strand breaks (DSBs). Although the CRISPR/Cas9 mechanism technique represents

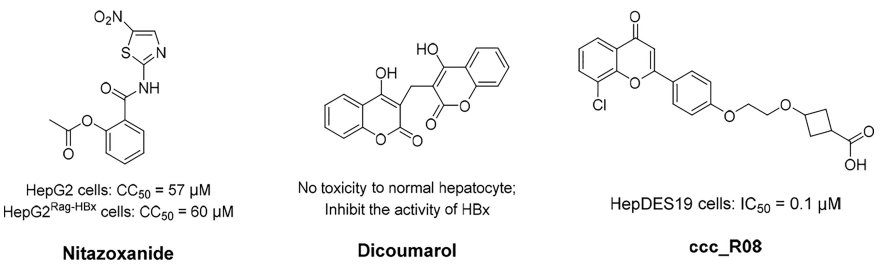


FIGURE 4
The structures of nitazoxanide, dicoumarol, and ccc_R08. Concentration for 50% of maximal effect (EC50), 50% cytotoxicity concentrations (CC50), the half maximal inhibitory concentration (IC50).

TABLE 3 Summary of molecules for RNA interference.

Name	Type	Company	Target(s)	NCT number	Clinical status
ARC-520	siRNA	Arrowhead Pharmaceuticals	Undisclosed	NCT02349126 NCT01872065 NCT01872065	Terminated during phase II trial(s)
ARB-1467	siRNA	Arbutus Biopharma Corp.	HBV S ORF; HBV X ORF	NCT02631096	Phase II trial(s) complete
AB-729	siRNA	Antios Therapeutics Inc.	HBV X protein	NCT04980482 NCT04820686 NCT04847440	Phase II trial(s)
RG-6346	siRNA	F. Hoffmann-La Roche AG	HBV S ORF	Undisclosed	Phase I trial(s)
JNJ-3989	siRNA	Integrity Bio Inc.	HBV X and HBV S domains	NCT04667104 NCT05275023 NCT04439539	Phase II trial(s)
ALG-125755	siRNA	Aligos Therapeutics Inc.	Undisclosed	NCT05561530	Phase I trial(s)
RO7062931	ASO	Roche	Conserved sequence in the shared 3′-region	NCT03505190 NCT03038113	Phase I trial(s)
GSK3389404	ASO	GSK	HBV X ORF	NCT03020745 NCT02647281	Phase I trial(s)
Bepirovirsen	ASO	GSK	Undisclosed	NCT05276297 NCT05630807 NCT05630820	Phase III trial(s)
ALG-020572	ASO	Aligos Therapeutics Inc.	HBV S ORF	NCT05001022	Phase I trial(s)

ASO, allele-specific oligonucleotide; HBV S, hepatitis B virus S; HBV X, hepatitis B virus X; HBV S ORF, hepatitis B virus S open-reading frame; HBV X ORF, hepatitis B virus X open-reading frame; NCT, National Clinical Trial.

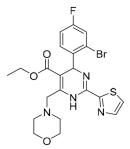
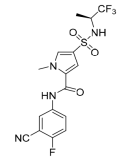
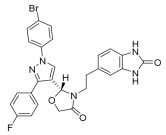
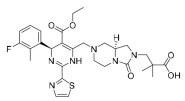
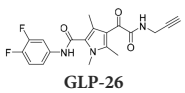
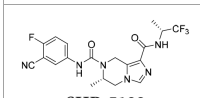
a promising therapeutic approach, there are some challenges that must be overcome before it can be applied in a clinical setting. For instance, the cleavage of integrated HBV DNA by the CRISPR/Cas9 mechanism might cause DSBs to occur in the host genome, in turn giving rise to serious safety concerns related to host genome instability and carcinogenesis. In addition, the off-target effects, the difficulty in finding conserved target of HBV sequences, and *in vivo* delivery efficiency also hinder the extensive application of the CRISPR/Cas9 mechanism (Komor et al., 2016). However, excitingly, research indicates that newly developed CRISPR-derived base editors (BEs) could permanently inactivate the HBV genome by introducing irreversible point mutations for the formation of premature stop codons, without affecting the host genome (Yang and Yang, 2022). The application of Cas9 with high fidelity and the broad protospacer adjacent motif (PAM) in the treatment of hepatitis B could also further reduce the impact [or severity] of off-target effects and increase the capacity of gRNA pools to target conserved HBV DNA sequences across different genotypes of HBV (Kleinstiver et al., 2015). As for the delivery efficiency by lipid nanoparticles *in vivo*, the non-viral delivery of Cas9 mRNA and ribonucleoproteins has considerable potential for use in liver-targeted delivery in clinics (Gillmore et al., 2021). In view of the research progress detailed above, CRISPR/Cas9 therapy might yet provide the ultimate approach to developing a cure for HBV.

Zinc-finger nucleases (ZFNs) could introduce a DSB into a desired target and significantly upregulate gene targets by activating cellular DNA repair pathways (Zakirova et al., 2020). Weber's group developed ZFNs targeting HBV polymerase, including X and core genes, and delivered them into HepAD38 cells by self-complementary adeno-associated virus vectors. Surprisingly, HBV-ZFNs could efficiently induce site-specific mutations, and the activity of these HBV-targeted ZFNs resulted in the sustained suppression of HBV DNA replication. Notably, high specificity was observed for all ZFNs, and their off-target effects were negligible (Weber et al., 2014).

Moreover, it has been shown that TALENs also possess properties similar to ZFNs (Bhardwaj and Nain, 2021). Bloom's group engineered mutagenic TALENs that targeted four HBV-specific sites within the viral genome (Dreyer et al., 2016). The researchers discovered that TALENs with cognate sequences in the S or C open-reading frames (ORFs) efficiently disrupted HBV sequences at the intended sites and suppressed the production of markers related with viral replication. To maximize this durable antiviral effect, Dreyer's group put forward a strategy that combined gene editing with homology-directed DNA recombination (HDR), to introduce HBV silencing artificial primary microRNAs (miRNAs) into HBV DNA targets (Bloom et al., 2013).

Epigenetic modification could alter the status of transcribed DNA to transcriptionally inactive without changing the nucleotide sequence. Thereafter, DNA-binding domains can predefine cccDNA sequences for targeted modifications. There are two major forms of HBV epigenetic regulation: the posttranslational modification of histone proteins associated with the cccDNA mini-chromosome, and the DNA methylation of viral and host genomes (Hong et al., 2017).

TABLE 4 Summary of CAMs.

Structure	Company	Activity	PK/PD	NCT number	Clinical status
 **GLS4**	Sunshine Lake Pharma Co., Ltd	HepG2.2.15 cells: EC_{50}: 1 nM Lamivudine-resistant and entecavir-resistant HBV strains with EC_{50} values of 10 to 20 nM	24 h: C_{max} (ng/mL): 885 $t_{1/2}$: 13.1 h AUC_{0-24} (ng × h/mL): 4,268	NCT04147208 NCT03638076	Phase I/II trial(s)
 **JNJ-56136379**	Janssen Research & Development Inc.	75-mg Asian group: HBV-DNA Mean (SD) baseline (log_{10} IU/mL): 6.90 (1.91) HBV-RNA mean (SD) baseline (log_{10}-p/mL): 5.59 (2.37)	75 mg Asian group: C_{max} (ng/mL): 1,832 T_{max}: 12 h AUC (ng × h/mL): 36,202	NCT03361956 NCT03982186 NCT04129554	Phase I/II trial(s)
 **ZM-H1505R**	Shanghai Zhimeng Biopharma, Inc.	EC_{50}: 12 nM	T_{max}: 2.5–3.5 h $t_{1/2}$: 11.98–13.00 h	NCT05484466	Phase II trial(s)
Undisclosed **QL-007**	Qilu Pharmaceutical Co., Ltd	Undisclosed	Undisclosed	NCT04157699NCT04157257	Phase II trial(s)
Undisclosed **EDP-514**	Enanta Pharmaceuticals Inc.	HepAD38 EC_{50}: 18 nM HepDE19 EC_{50}: 27 nM HepG2.2.15 EC_{50}: 17 nM	T_{max}: 2.0–3.5 h $t_{1/2}$: 15.5–26.1 h	NCT04008004 NCT04470388 NCT04783753	Phase I trial(s)
 **RG-7907**	Roche	HepG2.2.15 cells: EC_{50}: 6.1 ± 0.9 nM CC_{50}: > 100 μM	Rapid absorbed; there was no accumulation after 28 days' dosing	NCT02952924	Phase I trial(s)
Undisclosed **ABI-H3733**	Assembly Biosciences Inc.	HepAD38 cells: EC_{50}: 5 nM PHH cells: EC_{50}: 12 nM	$t_{1/2}$: 18.4–23.8 h C_{max} (ng/mL): 2,121–4,156 $AUC_{0\text{-}last}$ (h × ng/mL): 46,380–128,600	NCT04271592 NCT05414981	Phase I trial(s)
Undisclosed **ALG-000184**	Aligos Therapeutics Inc.	EC_{50}: 1.98 nM	$t_{1/2}$: 6.9–8.0 h T_{max}: 1–3.5 h	NCT04536337	Phase I/II trial(s)
 **GLP-26**	Emory University School of Medicine	EC_{50}: 3 nM	$t_{1/2}$: 0.77 h T_{max}: 0.40 h C_{max} (ng/mL): 2,513	Not available	Preclinical trial(s)
 **SHR-5133**	Shanghai Hengrui Pharmaceutical Co., Ltd	EC_{50} = 26.0 nM; CC_{50}: > 10,000 nM.	CL (mL/min/kg): 5.3 (mouse), 4.5 (rat), 1.1 (dog); *in vivo* efficacy: 2.32 log-HBV DNA reduction @ 30 mpk	Not available	Preclinical trial(s)

CAMs, capsid assembly modulators; NCT, National Clinical Trial. Clearance (CL), Area Under Curve (AUC), Per Os (PO), Pharmacokinetics (PK), Pharmacodynamics (PD), concentration for 50% of maximal effect (EC50), 50% cytotoxicity concentrations (CC50), t1/2: half-life; Cmax, maximum concentration; Tmax, time to peak.

Correspondingly, HBV DNA modifiers mainly comprised histone acetyltransferases/deacetylases (HATs/HDACs) (Belloni et al., 2009), lysine methyltransferases (Hayashi et al., 2016), protein arginine methyltransferases (Zhang et al., 2017b), and DNA methyltransferases (DNMTs) (Zhao et al., 2017), acting in cooperation with viral factors such as HBxs and HBcAgs. As a novel first-in-class molecule cccDNA destabilizer, ccc_R08 (Figure 4) can target pre-existing viral genome reservoirs and displayed a robust and sustained suppression of HBsAg, HBeAg, HBV DNA, and HBV RNA levels in patient serum (Wang et al., 2022). In addition, the reduction of cccDNA levels in the liver of an experimental mouse model was also detected. Recently, the workings of this molecule were investigated in a preclinical study.

Although gene editing technology alongside the use of small-molecule drugs could destroy cccDNA, there have been many (and there are likely still unknown) challenges associated with the implementation of these therapy methods. Hence, the goal of developing a complete cure using gene editing therapy, by which all HBV genomes can be purged, will likely be achieved only at the end of the long journey to eradicate hepatitis B.

RNA interference

After the outbreak of the coronavirus disease in 2019 (COVID-19), the mRNA vaccines have driven the research and development of nucleic acid drugs and these types of vaccines became one of the hottest research fields in the world. In comparison to traditional small-molecule drugs and antibodies, the biggest advantage of nucleic acid drugs was that they have a notably shorter development timeline (Kulkarni et al., 2021). For instance, different nucleic acid drugs could be swiftly developed by changing the specific DNA sequence. Recently, the development of small nucleic acid drugs has exponentially increased (Table 3). In general, small nucleic acid drugs possess 12–30 single or double strands of nucleotides, including allele-specific oligonucleotides (ASOs), small-interfering RNAS (siRNAS), and miRNAs (Kulkarni et al., 2021).

There are already a series of siRNAs and ASOs to be used for the treatment of HBV infections in development (Hui et al., 2022). Initially, Wooddell et al. reported the efficacy of siRNA in suppressing HBV in animal models (Wooddell et al., 2013). As the first siRNA for CHB, ARC-520 could induce a reduction in HBsAg levels of 3.0 $\log_{10}$ IU/mL and 2.7 $\log_{10}$ IU/mL in mice and chimpanzees, respectively (Wooddell et al., 2013; Wooddell et al., 2017). However, because of the mortality induced by the excipient, the development of ARC-520 was terminated (Hui et al., 2022).

A phase II trial completed in 2018 demonstrated that ARB-1467 can target the ORFs of HBV S and HBV Xdomains (Hui et al., 2022). Moreover, it was shown that AB-729 and RG-6346, subcutaneous *N*-acetylgalactosamine (GalNAc)-conjugated siRNAs, can target the HBV X and HBV S domains, respectively. Comparatively, JNJ-3989 could target both the HBV X and HBV S domains with subcutaneous injection (Hui et al., 2022). Excitingly, AB-729, RG-6346, and JNJ-3989 showed good efficacy in a clinical study. Moreover, it has been reported that AB-729 and RG-6346 could play a significant role in inducing immune reconstitution against HBV (Hui et al., 2022). In addition, ALG-125918 and ALG-125755, two siRNAs, were tested in a preclinical study. Importantly, ALG-125918 was designed with a novel 5′-cap phosphate mimic to enhance siRNA loading and cleavage efficiency.

In addition to siRNAs, four ASO agents, including RO7062931, GSK3389404, bepirovirsen and ALG-020572, were also in a clinical study. RO7062931 is a subcutaneous GalNAc-conjugated ASO targeting a highly conserved sequence in the shared 3′-region. Similarly, GSK3389404 was also a GalNAc-conjugated ASO with subcutaneous injection, and could target the HBV X ORF (Hui et al., 2022). Bepirovirsen (GSK3228836) is the unconjugated version of GSK3389404, and 300 mg of bepirovirsen, administered by injection could reduce HBsAg levels to 1.99 $\log_{10}$ IU/mL (Yuen et al., 2021). ALG-020572, targeted the HBV S ORF, was absorbed and distributed rapidly in mice and displayed an intrahepatic half-life of 12 days in non-human primates. At present, ALG-020572 is being evaluated in phase I.

Capsid assembly modulators

Hepatitis B virus (HBV) capsids have numerous functions in the HBV life cycle, such as providing sites for reverse transcription, packaging genomes, and facilitating intracellular transport (Kuduk et al., 2022). Therefore, HBV capsids could be an appropriate target for the development of active compounds. Capsid assembly modulators (CAMs) can mainly suppress HBV replication by interfering with HBV capsid assembly and the encapsidation of pgRNA. In addition, it has been found that CAMs can inhibit the establishment and replenishment of cccDNAs by interfering with capsid disassembly and the intracellular recycling of HBV nucleocapsids (Wong et al., 2022). Because of the significant role core proteins play in the HBV nucleocapsid, CAMs can also be referred to as core protein allosteric modulators (CpAMs) and promising antiviral strategy to eradicate HBV (Table 4). In addition, CAMs can be inserted into a specific hydrophobic pocket (i.e., the HAP pocket), located at the interface between core protein dimers, to disrupt capsid assembly and packaging of the HBV pgRNA and DNA polymerases (Bourne et al., 2006; Zhang et al., 2019). CAMs can be categorized into two types: type I CAMs (CAMI) and type II CAMs (CAMII). Of note is that it has been reported that CAMII can promote nucleation and the formation of empty capsids without packaging HBV pgRNA and DNA polymerases (Zhang et al., 2019).

It has been found that GLS4 (Morphothiadine), a heteroaryl dihydropyrimidine compound from Bay 41-4109, is the first HBV capsid assembly modulator (CAMI) that can inhibit HBV replication (Brezillon et al., 2011). It has been reported that GLS4 can disrupt or misdirect the assembly of the HBV capsid; however, at high doses, it has also been found to be hepatotoxic in rats (Brezillon et al., 2011). GLS4 demonstrated potent inhibitory activity in HBV HepG2.2.15 cells (EC_{50} 1 nM), and exhibited high potency against various mutated HBV polymerases, such as lamivudine and entecavir-resistant HBV strains with EC_{50} values of 10 to 20 nM (Ren et al., 2017). A preclinical trial indicated that GLS4 was well tolerated, and is not associated with serious adverse events or dose-limited toxicity. However, trial results also showed that the required concentration for effective antiviral activity could not be reached using GLS4 alone (Zhao et al., 2019). At present, a phase II trial of a novel therapeutic regimen, in which GLS4 and small doses of ritonavir are used to enhance plasma concentrations, is underway.

A phase II trial on JNJ-56136379 (bersacapavir), a novel and potent CAMII modulator, found that it could accelerate both the rate and extent of HBV capsid assembly *in vitro* (Berke et al., 2020). It can also suppress pgRNA encapsidation and the regeneration of cccDNA by interfering with capsid disassembly (Gane et al., 2022). JNJ-56136379 was well tolerated and demonstrated dose-dependent

PK properties. In more than half of patients administered with JNJ-56136379, an obvious reduction of the HBV DNA and RNA levels was observed (Zoulim et al., 2020). However, JNJ-56136379 did not have an effect on the levels of HBsAgs and HBeAgs, and viral rebound was observed after the end of treatment (Gane et al., 2022; Taverniti et al., 2022).

A phase II trial evaluating the effectiveness of ZM-H1505R, a small-molecule HBV capsid assembly modulator with a novel pyrazole moiety for the treatment of CHB, was also carried out (Jiang et al, 2023). It was reported that ZM-H1505R was safe and well tolerated, and its plasma exposure was above its effective inhibitory concentration. In addition to this, a phase II trial evaluating the effectiveness of both ZM-H1505R and QL-007 in the treatment of CHB was carried out (Jiang et al, 2023).

Currently, drug development for anti-HBV treatment is focused on CAMs. In addition to the small molecules mentioned above, there are some other small molecules currently being studied in clinical or preclinical studies, such as EDP-514 (Feld et al., 2022), RG7907 (Gonçalves et al., 2021), ABI-H3733 (Taverniti et al., 2022), ALG-000184 (Taverniti et al., 2022), GLP-26 (Amblard et al., 2021), and SHR5133 (Li et al., 2022).

Reverse transcriptase inhibitors

Nucleoside analogues (NAs) are the most commonly administered anti-HBV treatment in clinical settings worldwide. As small-molecule drugs, NAs can directly inhibit the activity of HBV DNA polymerases, resulting in reduced virion production (Pierra Rouviere et al., 2020). In addition, they also compete with natural nucleotide substrates to interrupt HBV DNA synthesis. There are some NAs that have been approved for CHB treatment, including lamivudine, adefovir dipivoxil, entecavir, telbivudine, and tenofovir (Leowattana and Leowattana, 2022). Long-term treatment with NAs could reduce numbers of cccDNA pools in HBV-infected hepatocytes by inhibiting nucleocapsid recycling (Leowattana and Leowattana, 2022). Nevertheless, NAs cannot suppress initial cccDNA formation in newly infected hepatocytes.

The first-generation NAs are lamivudine and adefovir dipivoxil (Table 5). Notably, lamivudine, which can compete with cytosine during the synthesis of viral DNA, was approved by the FDA for the treatment of CHB. However, it has been reported that lamivudine resistance occurs in approximately 70% of patients after 5 years (Jarvis and Faulds, 1999). Adefovir dipivoxil, a phosphonate acyclic NA of adenosine monophosphate, was approved in 2002. However, as with lamivudine, it was found that serious drug resistance occurred in patients receiving long-term treatment with this drug (Salpini et al., 2013).

Entecavir, telbivudine, and tenofovir are second-generation NAs with a high genetic barrier to HBV resistance. In 2005, entecavir, a selective NA against HBV, was marketed, and its EC_{50} reached 4 nM, which was much more potent than that of lamivudine and adefovir dipivoxil (Shepherd et al., 2009). Telbivudine, an acyclic NA with activity against retroviruses, was approved in 2008 (Magalhães-Costa et al., 2015). Although telbivudine resistance was relatively low, it was associated with renal toxicity and Fanconi syndrome. Recently, tenofovir, which is suitable for the treatment of CHB patients at risk of renal dysfunction, was approved as an alternative to telbivudine because it induces fewer side effects (Childs-Kean et al., 2018). In addition, besifovir, a derivative of tenofovir, was approved by the Korean Ministry of Food and Drug Safety in 2017 for CHB treatment (Song and Park, 2021), and its antiviral efficacy was found to be similar to that of entecavir. However, the side effect of L-carnitine depletion occurred in 94.1% of patients administered besifovir (Lai et al., 2014). A phase II clinical trial on another new NA, CMX157, was also recently conducted (Painter et al., 2007; Tao et al., 2020).

Except for the inhibitors acting on the active site of HBV reverse transcriptase enzymes, the compounds could bind with the allosteric pocket being reported. PDM2, a stilbene derivative acquired by applying the high-throughput screening method, was able to inhibit HBV replication with an IC_{50} of 14.4 ± 7.7 μM, and inhibited the replication of HBV, rather than blocking its entry (Nakajima et al., 2020). In the meantime, the surface plasmon resonance (SPR) analysis demonstrated a specific interaction between PDM2 and HBV reverse transcriptase enzymes. Importantly, PDM2 showed similar inhibitory activity against the replication of both wild-type HBV and lamivudine/entecavir-resistant HBV variants (Nakajima et al., 2020).

Ribonuclease H inhibitors

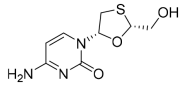
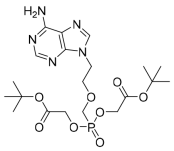
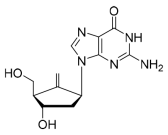
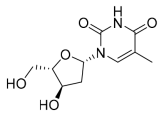
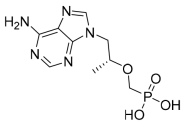
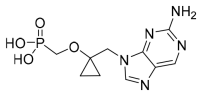
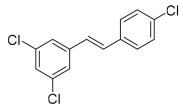
The HBV reverse transcription process is catalyzed by reverse transcriptase and ribonuclease H (RNaseH) (Shih et al., 2018). Inhibition of the activity of RNaseH causes the synthesis of strand DNA to be truncated, which in turn suppresses the formation of cccDNA (Tavis et al., 2019). Thus, RNaseH, a specific functional domain of HBV polymerase, has become a new drug target, but development is still in the primary stages.

Significantly, some compounds with a unique skeletal structures were discovered to show inhibitory activity of RNaseH. Recently, three RNaseH inhibitors with different moieties, that is, α-HT-110, HND-1073, and HPD-1133 (Figure 5), that could suppress HBV replication, were identified in a study (Chauhan et al., 2021). In HBV-infected HepG2-NTCP cells, the EC_{50} values of these three compounds were between 0.29 and 1.60 μM. Comparably, the CC_{50} values were between 16 and 100 mM in HepG2-derived cell lines, indicating the non-toxicity of these compounds in primary human hepatocytes. Notably, these compounds also exhibited behaviour similar to that of lamivudine/adefovir-resistant cell lines.

HBsAg release inhibitors

Except for complete particles, an excess of subviral particles (SVPs) were also observed. It has been found that SVPs can transport most circulating HBsAgs to [Location]: a post-ER, pre-Golgi compartment, where they interfere with innate and adaptive

TABLE 5 The summary of NAs.

Structure	Company	Activity	PK/PD	Clinical status
 **Lamivudine**	GSK	Lamivudine inhibited DHBV replication with an IC_{50} of 0.55 μM	In human: *F*% = 86%; C_{max} (μg/mL): 1.5 ± 0.5; Vd (L/kg): 1.3 ± 0.4; PPB: < 30%; CL (mL/min): 199.7 ± 56.9	Approved
 **Adefovir dipivoxil**	Gilead Sciences Inc.	In HBV transfected human hepatoma cell lines, IC_{50}: 0.2–2.5 μM	In human: *F*%: 59%; C_{max} (ng/mL): 18.4 ± 6.26; T_{max} (h): 0.58–4; AUC (h × ng/mL): 220 ± 70.0	Approved
 **Entecavir**	BMS	Entecavir inhibited HBV replication with an EC_{50} of 4 nM	In human: *F*%: 100%; PPB%: 13%; CL (mL/min): 383.2 ± 101.8	Approved
 **Telbivudine**	NVS	Telbivudine inhibited anti-compliment or second-strand DNA	PPB%: 13% *in vitro* study; CL (L/h): 7.6 ± 2.9	Approved
 **Tenofovir**	Gilead Sciences Inc.	Tenofovir inhibited HBV replication	In human: low bioavailability; C_{max} (I.V.): 2,500 ng/mL; PPB%: 7.2%; Vd (L/kg): 0.813; AUC (h × ng/mL): 4,800	Approved
 **Besifovir**	Ildong Pharmaceutical	Besifovir inhibited HBV replication and the activity was similar to that of entecavir	Undisclosed	Approved
 **PDM2**	NIID (Japan)	PDM2 inhibited HBV replication with an IC_{50} of 14.4 ± 7.7 μM	Undisclosed	Not available

HBV, hepatitis B virus; NAs, nucleoside analogues. Clearance (CL), Area Under Curve (AUC), Per Os (PO), Pharmacokinetics (PK), Pharmacodynamics (PD), concentration for 50% of maximal effect (EC50), 50% cytotoxicity concentrations (CC50), t1/2: half-life; Cmax, maximum concentration; Tmax, time to peak.

immunity, and therefore contribute to viral persistence (Ho et al., 2020) is a promising therapeutic method for the treatment of HDV (Table 6).

Notably, nucleic acid polymers (NAPs) can disrupt apolipoprotein interactions, which are involved in the assembly and secretion of SVPs (Real et al., 2017). Recently, it has also been reported that REP 2139 and REP 2165 can reduce levels of HBsAgs in the majority of CHB patients (Vaillant, 2019). Importantly, 14 out of 40 patients achieved a functional cure status with durable HBsAg seroconversion in a clinical study (Bazinet et al., 2020).

RG7834, an orally bioavailable small molecule that belongs to the dihydroquinolizinone (DHQ) chemical class, can selectively reduce the expression levels of HBsAgs and HBeAgs *in vitro* and *in vivo* (Han et al., 2018). However, it has been announced that, because of its safety profile, the development of RG7834 will be stopped. Based on RG7834, Hu's group designed and synthesized a series of dihydrobenzopyridooxazepine (DBP) derivatives, and GST-HG131 was discovered to be an appropriate clinical candidate (Hu et al., 2022). Notably, GST-HG131 exhibited an acceptable safety profile in healthy subjects at single doses ranging from 10 to 300 mg, and multiple doses (BID) ranging from 30 to 60 mg. Meanwhile, a multiple ascending dose study (on 30- and 60-mg doses) indicates that GST-HG131 meets therapeutic area under the curve (AUC) requirements. These corresponding experimental results indicate that GST-HG131 is a potential therapeutic option for CHB patients. In addition, the HBsAg release inhibitors in development also had LP-128, which was in a phase I trial (NCT05130567). This compound was shown to obviously reduce levels of HBsAgs, but the

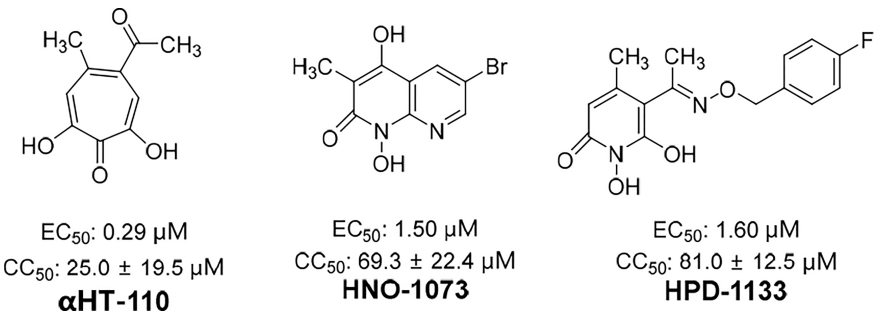


FIGURE 5
The structures of α-HT-110, HND-1073, and HPD-1133.

corresponding structure and experimental data were undisclosed.

The treatment strategies for modulating host immunity

The discussion above focused mainly on the HBV life cycle. What follows concerns treatment strategies for host immunity. A functional cure for HBV can be achieved by appropriately orchestrating the activation of antiviral immunity (Bertoletti and Le Bert, 2018). Indeed, HBV infection can be controlled and characterized by the coordinated activation of anti-HBV-specific humoral and cellular immunity might be an effective treatment strategy (Table 7).

During the early phase of viral infections, the production of pro-inflammatory cytokines and IFNs, and the activation of natural killer (NK) cells are frequently observed. On account of these findings, it was shown that HBV was detected by different types of liver cells with *in vivo* and *vitro* models. Taken together with the data obtained from recent studies, this suggests that liver cell populations, as well as circulating innate immune cells, can detect and respond to HBV infection, which in turn enables the innate immune system to detect and restrict the invading virus. Therefore, it is necessary to explore the receptors and the signaling pathways responsible for detecting HBV within infected hepatocytes or other immune cells (Liu and Zhang, 2015; Naghib et al., 2022).

There is a large number of new immunotherapeutic approaches in development, and some have already shown promising results (Figure 6). For example, it has been reported that both toll-like receptor (TLR) agonists and check-point inhibitors can restore dysfunctional HBV-specific immune responses (Gane, 2017). Similarly, vaccination is able to induce novel HBV-specific immune responses. In addition, a T-cell engineering approach has been considered as means to replace host T-cell responses (Lang et al., 2019).

Toll-like receptor agonists

Toll-like receptor (TLR) agonists are a distinct class of pattern recognition receptors that recognize both pathogen- and damage-associated molecular patterns (Tsounis et al., 2021). TLRs induce antiviral defenses through intracellular signaling pathways that induce antiviral inflammatory cytokines and IFNs use to shape adaptive immunity (Du et al., 2022).

GS-9620, an oral agonist of TLR7, was shown to induce the prolonged suppression of viral DNA and antigens in the sera of woodchuck and chimpanzee models (Lanford et al., 2013; Menne et al., 2015). Furthermore, GS-9620 was safe and well tolerated by CHB patients. In spite of on-target biomarker responses in patients being detected, no corresponding significant declines in the levels of surface antigens were observed (Janssen et al., 2018). In attempts to

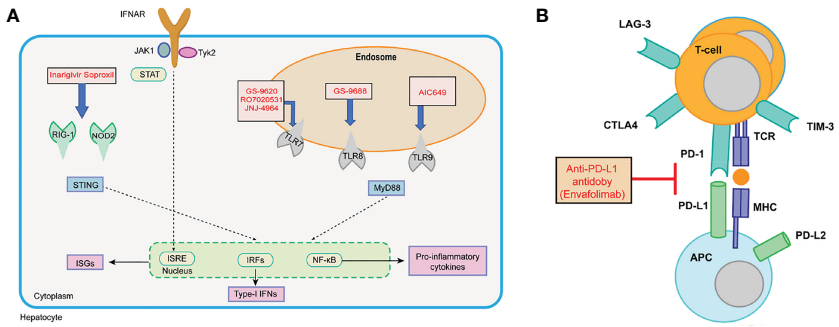
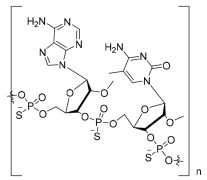
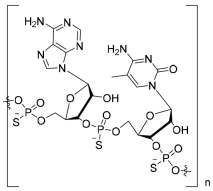
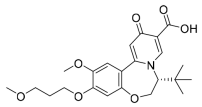
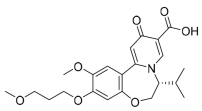


FIGURE 6
(A) Interferon and PRR agonism with IFN-α, RIG-I/NOD-2 agonists, TLR7 agonists, TLR8 agonist, and TLR9 agonists; **(B)** checkpoint inhibition with anti-PD-L1 antibodies.

TABLE 6 The summary of molecules inhibiting the release of HBsAgs.

Structure	Type	Company	Activity	NCT number	Clinical status
 **REP 2139 (*n* = 20)**	NAP	Replicor Inc.	Both HBV and HDV infection EC_{50}: 5–10 μM	NCT02233075 NCT02565719 NCT02726789	Phase II trial(s)
 **REP 2165 (*n* = 20)**	NAP	Replicor Inc.	EC_{50}: 6 μM.	NCT02565719	Phase II trial(s)
 **GST-HG131**	Small molecule	Fujian Cosunter Pharmaceutical Co., Ltd	HBV-DNA EC_{50}: 2.6 nM; HBsAg EC_{50}: 3.9 nM	NCT04499443	Phase I trial(s)
 **RG7834**	Small molecule	Roche	HBV-DNA EC_{50}: 0.8 nM; HBsAg EC_{50}: 1.1 nM	NCT02604355	Terminated

HBV, hepatitis B virus; HBsAg, hepatitis B surface antigen; HDV, hepatitis D virus; NCT, National Clinical Trial.

optimize hepatic selectivity, APR002 was utilized, which is a novel TLR-7 agonist exhibiting a better serum-to-liver ratio than GS-9620. Importantly, APR002, in combination with entecavir, over a 12-week period was shown to cause the sustained immune-mediated suppression of cccDNA by inducing antibody production and stimulating IFN gene expression (Korolowizc et al., 2019).

RO7020531, an oral double prodrug of RO7011785, was developed for the treatment of people with CHB. At doses of 100 mg or more, RO7020531 showed acceptable safety and tolerability, and up-regulated biomarkers of TLR7 activation, including INFα- and INF-stimulated gene expression levels (Luk et al., 2020). Recently, a phase II trial for this treatment was also carried out; however, the corresponding structure and activity data *in vitro* for RO7020531 are still undisclosed.

JNJ-4964 is also an oral TLR7 agonist. In healthy adults, JNJ-4964 was well tolerated, and induced cytokines with potential anti-HBV activity rapidly, that is INFα, IP-10, IL-1 RA, MCP-1, and INF-stimulated genes (ISG15, MX1, and OAS1) in patient serum (Gane et al., 2021). In addition to RO7020531 and JNJ-4964, T7-EA, another TLR-7 agonist, displays promising pharmacodynemics (PD)/pharmacokinetics (PK) characteristics, and has been developed as a component of a therapeutic vaccine (Hu et al., 2020).

In comparison with TLR7 activation, TLR8 activation was shown to promote the production of a much higher levels of proinflammatory cystokines and chemokines, whereas it was reported that the former promoted the production of a higher levels of IFNs. (Gorden et al., 2005). GS-9688 is a TLR8 agonist in clinical development (Mackman et al., 2020), and antiviral activity has been evaluated extensively *in vitro* and *vivo* in a woodchuck model (Daffis et al., 2021). Although the therapeutic efficacy of GS-9688 in combination with NAs is still to be fully evaluated in patients with CHB, it was shown to be safe and well tolerated in a phase II trial, with a reduction in the levels of HBsAgs and HBeAgs being observed in a subset of patients (Amin et al., 2021). In addition to the trial for GS-9688, a clinical trial for its derivative, GS-9620, was carried out (Niu et al., 2018).

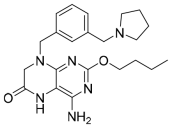
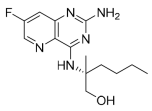
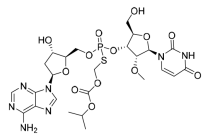
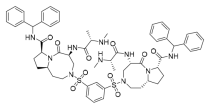
In addition to the TLR7 and TLR8 agonists, the TLR9 agonist was also developed for the treatment of HBV infection. AIC649 is an inactivated *Parapoxvirus ovis* (iPPVO) particle prepared with distinct immunological activities, including regulated cytokine release and the activation of T-cell responses. Currently, AIC649 is being investigated in a phase I clinical trial in patients with CHB (Paulsen et al., 2015).

Retinoic acid-inducible gene I, nucleotide-binding oligomerization domain-like receptors agonists

The retinoic acid-inducible gene I (RIG1) and nucleotide-binding oligomerization domain-containing protein 2 (NOD2) are two types of recognition receptors that can recognize the signature patterns of foreign RNA, resulting in the activation of the IFNα signaling pathway, and the subsequent production of ISGs and proinflammatory cytokines (Sato et al., 2015). Inarigivir soproxil is a novel and oral modulator of innate immunity that is

10.3389/fcimb.2023.1128807

TABLE 7 The summary of compounds modulating the host immune system.

Name or structure	Target	Company	NCT number	Clinical status
 **GS-9620**	TLR7	Gilead Sciences Inc.	NCT05281510 NCT02579382 NCT04364035	Phase II trial(s)
Undisclosed APR-002	TLR7	Apros Therapeutics Inc.	NCT05268198	Phase I trial(s)
Undisclosed RO7020531	TLR7	Roche	NCT04225715	Phase II trial(s)
Undisclosed JNJ-4964	TLR7	Chia Tai-Tianqing Pharmaceutical Holdings Co., Ltd	NCT04273815 NCT04180150 NCT04202653	Phase II trial(s)
 **GS-9688**	TLR8	Gilead Sciences Inc.	NCT05551273 NCT04891770	Phase II trial(s)
iPPVO (undisclosed) AIC649	TLR9	AiCuris GmBH & Co.KG	EUCTR2021–000167–69-DE	Phase II trial(s)
 **Inarigivir soproxil**	RIG-I	Spring Bank Pharmaceuticals	NCT02751996 NCT03434353	Phase II trial(s)
Antibody (undisclosed) Envafolimab	PD-1	Ascletis Pharma Inc.	NCT04465890	Phase II trial(s)
 **APG-1387**	Mimicking the endogenous Smac molecule	Ascentage Pharma Inc.	NCT04568265 NCT04643405 NCT04284488	Phase II trial(s)

NCT, National Clinical Trial.

believed to activate the viral sensor proteins, that is RIG-I and NOD-2, and effects IFN-mediated antiviral immune responses in virus-infected cells (Yuen et al., 2022). The results of a phase II trial show that 12-week inarigivir soproxil, in doses of up to 200 mg, contributed to a reduction in HBV DNA, HBV RNA, and antigen levels. However, a larger reduction in the levels of HBsAgs was observed in inarigivir soproxil-pretreated patients after switching to tenofovir, although adverse events occurred in 4.7% of patients treated with the second regimen. Further studies using this new class of agents in combination with NAs to establish antiviral efficacy and safety in CHB patients are ongoing (Yuen et al., 2022).

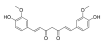
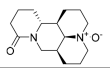
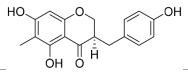
T-cell engineering

T cells are crucial players in the coordination of the adaptive immune response. It has been shown that CD4+ T cells contribute to the activation of B cells and regulate their differentiation into antibody-producing plasma cells (Zhu and Paul, 2010). In addition, they can also promote the formation and proliferation of memory CD8+ T cells so that they respond directly to pathogen-infected cells. CD4+ T cells can secrete a variety of cytokines and generate specific environmental stimuli that are responsible for the activation of APCs and the development of specific types of T effector cells (Zhu and Paul, 2010). However, the achievement of sustained activity, T-cell delivery, cell volume, and frequency of infusions are challenges that still need to be overcome. Recently, some relevant research has been published based on the findings of three clinical trials (i.e., NCT03899415, NCT02719782, and NCT02686372) (Bertoletti and Tan, 2020).

Immune checkpoint inhibitors

It has been reported that programmed death receptor 1 (PD-1), the most highly expressed inhibitory receptor in HBV-specific T cells, together with the increased expression of PD-L1 (PD-1's

TABLE 8 A summary of representative NPs.

Compound	Structure	Source	Mechanism of action
Punicalin		*Punica granatum* L.	It inhibits HBV cccDNA production *via* a dual mechanism, which entails the formation of cccDNA and the promotion of cccDNA decay
Curcumin		*Curcuma* L.	Down-regulating cccDNA-bound histone acetylation and metabolic co-activator PGC-1α
Oxymatrine		*Sophora* L.	Host Hsc70 was identified to be the target of oxymatrine
EGCG		Green tea	EGCG simulates the endocytosis and degradation of NTCP by inducing the transport of NTCP from the plasma membrane to the cytoplasm
Asiaticoside		*Hydrocotyle sibthorpioides*	HBV DNA transcription and replication are affected by inhibiting the activity of C, S, and X gene promoters
HE-145		*Taiwania cryptomerioides*	HE-145 inhibits HBV DNA replication by selectively inhibiting the activity of S gene promoters II and C gene promoters in liver cancer cells
BetA		*Pulsatilla chinensis*	BetA inhibits HBV replication by down-regulating SOD2 expression
LPRP-Et-97543		*Liriope platyphylla*	LPRP-Et-97543 inhibits HBV gene expression by interfering with the NF-κB signaling pathway in host cells

cccDNA, covalently closed circular DNA; EGCG, epigallocatechin-3-gallate; HBV, hepatitis B virus.

ligand) in HBV-infected hepatocytes, contributes to the exhaustion of T cells and therefore, high HBV replication in CHB patients (Tao et al., 2020).

Envafolimab is a single-domain antibody generated by a fusion of the PD-L1 domain with the Fc fragment of human IgG1 antibody (Markham, 2022). This chimeric molecule can bind to PD-L1 in a high-affinity manner and inhibit the PD-1/PD-L1 pathway, therefore improving T-cell function (Zhang et al., 2017a). Currently, a phase II trial evaluating the safety, tolerability, and efficacy of envafolimab in CHB patients is underway (Kim et al., 2022).

APG-1387 is a novel Smac mimetic and a highly specific antagonist of apoptosis proteins, which was independently developed by Ascentage Pharma in China (Liu et al., 2018). It degrades apoptosis proteins by mimicking the endogenous Smac molecule to induce programmed cell death or apoptosis. In a phase I trial, APG-1387 was administered intravenously at escalating dose levels (7, 12, 20, and 30 mg), followed by a 12-week observation period. Thirty patients experienced adverse events. At the end of APG-1387 treatment, a significant decline in the levels of HBV DNA, HBsAgs, and HBeAgs was observed in the cohorts administered with the 12- and 30-mg doses. In addition, APG-1387 demonstrated synergistic effect with sequential NA treatment (Rasco et al., 2019).

Natural products

NPs have a high level of molecular complexity and diversity (except for their small molecules and RNA), and this presents a great opportunity to find novel anti-HBV drugs or lead compounds with specific antiviral mechanisms. Approximately over 160 anti-HBV NPs have been found and can be classified according to their chemical classes, that is terpenes, lignans, phenolic acids, polyphenols, lactones, alkaloids, and flavonoids (Liu et al., 2020). The following discusses the properties and mechanisms of action of representative NPs (Table 8).

Hydrolyzable tannins

By screening a compound library derived from Chinese herbal remedies, punicalagin, punicalin, and geraniin were identified as novel anti-HBV agents. Hydrolyzable tannins can inhibit HBV cccDNA production and promote cccDNA decay *via* a dual mechanism (Liu et al., 2016). Therefore, hydrolyzable tannins may serve as lead compounds for the development of new agents to cure HBV infections (Liu et al., 2016).

Curcumin

Curcumin was isolated from the rhizome of *Curcuma longa* L., which exhibited antimicrobial activity against various bacteria, viruses, fungi, and parasites (Wei et al., 2017). Of note is that curcumin could inhibit HBV replication and expression through its reduction of cccDNA-bound histone acetylation (Wei et al., 2017). Accordingly, curcumin has the potential to be developed as a cccDNA-targeting antiviral agent for the treatment of hepatitis B.

Furthermore, it has been shown that siRNAs targeting HBV act synergistically with curcumin, resulting in the latter's enhanced inhibition of HBV infection (Wei et al., 2017).

Oxymatrine

It has been shown that oxymatrine (OMTR), an alkaloid extracted from the Chinese herb *Sophora alopecuroides* L., has the capacity to suppress HBV (He et al., 2016). Host Hsc70 was identified to be the target of OMTR (Wang et al., 2010). OMTR (orally for 12 months) reduced blood HBV DNA and HBeAgs levels by 96% and 70%, respectively, in CHB patients resistant to lamivudine. A liver biopsy study demonstrated that OMTR caused a decrease in Hcs70 mRNA in liver cells and a reduction in intracellular HBV DNA. In combination with lamivudine (n = 15) (orally for12 months), OMTR also demonstrated an enhanced anti-HBV effect compared with lamivudine monotherapy (n = 25) (Wang et al., 2011). OMTR has been approved for the treatment of hepatitis B infections by the China Food and Drug Administration (CFDA), and was recommended as an anti-HBV agent (He et al., 2016).

Epigallocatechin-3-gallate

It had been reported that epigallocatechin-3-gallate (EGCG), a major component of green tea, is an important transcriptional regulator of the HBV genome and can interact with Farnesoid X receptor alpha (FXRα) (Xu et al., 2016). It has also been shown that EGCG exhibits inhibitory activity against HBV infection and replication in HuS-E/2 cells (Lai et al., 2018). In addition to those discussed above, there are other NPs capable of anti-HBV activity, such as asiaticoside (Huang et al., 2013), HE-145 (Tseng et al., 2008), BetA (Yao et al., 2009), and LPRP-Et-97543 (Huang et al., 2014).

At present, a variety of NPs with novel structures and high levels of anti-HBV activity have been identified. However, the research conducted thus far is disordered and has been mainly focused on the simple isolation and identification of anti-HBV activity. More comprehensive studies about anti-HBV mechanisms and targets are relatively rare. In addition, the research carried out in most of the studies was restricted to the cellular level and, in general, there was a lack of experiments carried out with animal models. To increase the role that NPs could play in the development of anti-HBV agents, it is necessary to address these problems.

In addition to NPs, some traditional Chinese medicines (TCMs) have also demonstrated a potential ability to combat HBV infection. For example, ma-huang-tang, also known as maoto, can induce the expression of a host gene tropomyosin 2 (*TPM2*) to suppress HBV production. As the safety of ma-huang-tang has already been confirmed, it is suitable for use in the development of anti-HBV treatments (Rahman et al., 2021). *Salvia miltiorrhiza* is also a commonly used TCM and contains polyphenol (lithospermic acid). Cai's group reported that *S. miltiorrhiza* exhibited anti-HBV activity by inhibiting HBV DNA replication in HepG2.2.15 and pHBV-transfected HepG2 cells in dose- and time-dependent manners, and by reducing the levels of HBsAgs and HBeAgs in HepG2.2.15 cells, to a certain extent. In addition, *S. miltiorrhiza* reduced HBV DNA, HBsAgs/HBeAgs, and HBcAgs levels in the serum/liver tissue of HBV-HDI C57BL/6 mice during a 3-week treatment, and suppressed the withdrawal of rebound of HBV DNA and HBsAgs levels in the mice serum (Zhu et al., 2023). It has also been reported that *Iris tectorum* Maxim. (Geng et al., 2018), *Artemisia capillaris* Thunb. (Parvez et al., 2019), and *Polygonum cuspidatum* Sieb. (Sang et al., 2017) demonstrate inhibitory activity against HBV infection.

Conclusion and perspectives

Hepatitis B virus (HBV) infection is still a great healthcare burden worldwide. It is clear that HBV cccDNA is the viral persistence reservoir and the key obstacle to the development of a cure for CHB. The complete eradication of cccDNA could result in the development of a cure for HBV infection, enabling patients to lead a normal life after undergoing finite treatment without worrying about viral rebound. Most anti-HBV drugs on the market can effectively suppress viral replication, but, in the majority of patients, none can achieve the eradication of subviral particles and cccDNA. As we know about the HBV life cycle, a large number of direct antiviral agents with unique mechanisms of action that target specific steps of the HBV life cycle are being actively discovered, and the effects of some of these have already

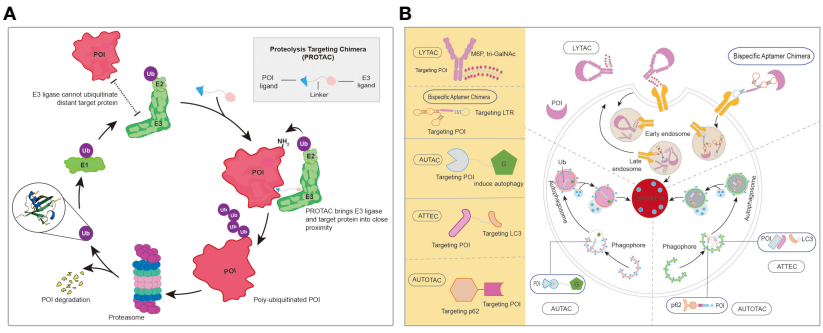


FIGURE 7
(A) The brief mechanism of PROTACs. **(B)** The representative of protein degradations by lysosome. AUTAC, autophagy-targeting chimera; LYTAC, lysosomal-targeting chimera; PROTAC, proteolysis-targeting chimera. AUTOTAC, Autophagy-targeting chimera; ATTEC, autophagosome-tethering compound; POI, protein of Interest.

been evaluated in HBV patients. This extensive list of different active compounds covers nearly the entire HBV life cycle, that is entry inhibitors, viral transcription inhibitors, viral polymerase inhibitors (both RT and RNase H domains), nucleocapsid assembly modulators, and HBsAg secretion inhibitors. Furthermore, restoring or enhancing innate immunity and inducing HBV-specific adaptive immune responses can be useful in the treatment of HBV. Thus, a number of new agents restoring host immunity in HBV infection are also in development. Although the NPs are important sources of drugs, the developing of anti-HBV agents still have difficulties to overcome.

In addition to traditional anti-HBV agents, drug designs with novel mechanisms, such as protein degradation, are also being developed. As shown in Figure 7A, the proteolysis-targeting chimera (PROTAC) technique was well developed, and more than 20 molecules were in clinical trails (Guenette et al., 2022). As for protein degradation by lysosomes, such as that occurring in the lysosomal-targeting chimera (LYTAC) and autophagy-targeting chimera (AUTAC) degraders, the development of this modality is still at a very early stage (Figure 7B) (Zhao et al., 2022). Importantly, different enzymes played significant roles during the HBV life cycle. protein degradation in the form of specialized PROTACs, could be introduced into the design of novel anti-HBV agents with the purpose of degrading the crucial enzymes or proteins involved in this specific life cycle, and might represent a promising approach to cure this disease.

Author contributions

YP and HX wrote the manuscript. YH and SZ created the figures and edited the manuscript. ZS and WH conceived of and supervised the work. All authors contributed to the article and approved the submitted version.

Funding

This research was supported by the Huadong Medicine-Joint Funds of the Zhejiang Provincial Natural Science Foundation of China under Grant No. LHDMD22H300001, Zhejiang Provincial Key Research & Development Plan (2021C03083).

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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frontiers | Frontiers in Medicine

TYPE Original Research
PUBLISHED 22 March 2023
DOI 10.3389/fmed.2023.1120083

OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to Gastroenterology, a section of the journal Frontiers in Medicine

RECEIVED 13 December 2022
ACCEPTED 24 February 2023
PUBLISHED 22 March 2023

CITATION
Tao J, Yan Z, Huang W and Feng T (2023) Seropositive for hepatitis B and C viruses is associated with the risk of decreased bone mineral density in adults: An analysis of studies from the NHANES database.
Front. Med. 10:1120083.
doi: 10.3389/fmed.2023.1120083

Seropositive for hepatitis B and C viruses is associated with the risk of decreased bone mineral density in adults: An analysis of studies from the NHANES database

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Background: Some studies had reported that patients with viral hepatitis are at increased risk of reduced bone mineral density and even osteoporosis. However, the interaction between reduced bone mineral density (BMD) and viral hepatitis remains inconclusive. Therefore, our study collected hepatitis test results and bone mineral density from respondents in the NHANES database. The aim of this study was to investigate whether there is an association between hepatitis and a decrease in bone mineral density.

Methods: The respondents with both hepatitis- and BMD-related indicators from the NHANES database in the United States from 2005–2010, 2013–2014, to 2017–2020 were collected for this study. BMD were compared between respondents who were positive and negative for respondents related to hepatitis B and C. BMD was measured using dual-energy X-ray absorptiometry of the femur and lumbar spine. Finally, multiple regression analysis was performed between hepatitis B surface antigen (HBsAg) and hepatitis C RNA (HCV-RNA) and BMD in the respondents.

Results: A total of 15,642 respondents were included in the hepatitis B surface antigen-related survey. Of these, 1,217 respondents were positive for hepatitis B surface antigen. A total of 5111 hepatitis C RNA-related responders were included. Hepatitis C RNA-positive had 268 respondents. According to the results of the multiple regression analysis, the femoral BMD was significantly lower in HBsAg (+) respondents compared to HBsAg (−) respondents: −0.018 (−0.026, −0.009) ($P < 0.01$). Moreover, spinal BMD was significantly lower in HBsAg (+) respondents compared to HBsAg (−) respondents: −0.020 (−0.030, −0.010) ($P < 0.01$). According to the results of multiple regression analysis for hepatitis C RNA, HCV-RNA (+) respondents had significantly lower BMD compared to HCV-RNA (−) respondents: −0.043 (−0.059, −0.026) ($P < 0.01$).

Conclusion: During the analysis of respondents in the NHANES database in the United States, positive tests for hepatitis B surface antigen and hepatitis C RNA were found to be associated with a reduction in BMD. Positive serology for these hepatitis indicators may increase the risk of reduced BMD. Of course, this conclusion still needs to be further confirmed by more large clinical trials.

KEYWORDS

bone mineral density, NHANES, hepatitis, hepatitis B surface antigen, hepatitis C RNA

Introduction

Osteoporosis is caused by bone loss, which is a global public health problem that can easily lead to fractures with serious consequences and even death (1–3). Osteoporosis affects ∼200 million people all over the world, with a prevalence of ∼18.3% (4). Some studies have shown that patients with hepatitis are prone to reduced BMD, which, in turn, can increase the risk of osteoporosis. Moreover, osteoporotic fractures in patients with chronic hepatitis are on the rise worldwide (5–7).

According to some current reports, osteoporosis is one of the complications of hepatitis, and the prevalence of osteoporosis may be higher in patients with liver cirrhosis (8–10). About the effect of chronic hepatitis and cirrhosis on bone mineral density, some studies had pointed out that under the influence of various chronic inflammatory factors. This series of changes gradually leads to a loss of bone mass and a decrease in bone mineral density, which, in turn, increases the risk of osteoporosis (6, 11, 12).

Hepatitis virus infection is the most common pathogenic route of hepatitis. Viruses that cause viral hepatitis commonly include hepatitis A, B, C, D, and E viruses, and viral hepatitis is often an important cause of liver cirrhosis (13–15). There is still no clear consensus on the relationship between hepatitis and the reduction in BMD. Many studies had shown that hepatitis can lead to a decrease in BMD, and a number of studies had shown a weak association between the two. For these reasons, we compared BMD levels between positive and negative respondents for hepatitis virus-related indicators from the NHANES database in the United States to analyze the relationship between the two.

Methods

Study design and population

The data of our current study were obtained from the National Health and Nutrition Examination Survey (NHANES) for the period of 1999 to 2020. This database is a nationally representative survey of the civilian, de-institutionalization population of the United States conducted by the National Center for Health Statistics (NCHS) of the Centers for Disease Control and Prevention (CDC) (16). The data in the NHANES database contain five sections, such asdemographic data, dietary data, examination data, laboratory data, and questionnaire data. Informed consent was available for the content of all respondents in the NHANES database. The disclosure of this content has been approved by the NCHS Research Ethics Review Committee (17).

In the NHANES database, we downloaded the data related to hepatitis B surface antigen, hepatitis C RNA, and BMD in respondents during the period of 2005–2010, 2013–2014, and 2017–2020. In bone imaging, there is interference in imaging, as the bones are still underdeveloped below the age of 20 years. In contrast, respondents older than 70 years of age enter the stage of senile osteoporosis and it is more prone to bone loss (18, 19), and the possible presence of senile osteoporosis can have an impact on the final results. Therefore, we selected data from respondents aged 20 years or older and younger than 70 years. After excluding data from non-compliant respondents, we ended up collecting 15,642 respondents with both hepatitis B surface antigen and BMD and 5,111 respondents with both hepatitis C RNA and BMD. The data were then collated and analyzed using R and Empower software. The BMD of serologically positive and serologically negative respondents for hepatitis B surface antigen and serologically positive and serologically negative respondents for hepatitis C RNA were compared separately to see if there were differences in BMD between positive and negative respondents. The specific inclusion and exclusion processes are shown in Figures 1, 2.

Bone mineral density levels

Bone mineral density (BMD) is measured by using a dual X-ray absorptiometry (DXA) examination. Dual-energy X-ray absorptiometry (DXA) is the most widely accepted method of measuring bone mineral density due in part to its speed, ease of use, and low radiation exposure. DXA scans of the proximal femur were administered in the NHANES mobile examination center (MEC) from 2005–2010, 2013–2014, to 2017–March 2020 (20, 21).

Hepatitis serology levels

Hepatitis B surface antigen is tested by using the VITROS HBsAg test, the VITROS HBsAg kit on the VITROS ECi/ECiQ Immunodiagnostic System and VITROS 3600 Immunodiagnostic System, and the VITROS Immunodiagnostic Product HBsAg Calibrator (22). Hepatitis C ribonucleic acid is tested by using the COBAS Amplicon HCV Monitor test. The COBAS Amplicon HCV Monitor version 2 0 (v2.0) is an *in vitro* nucleic acid amplification test for the quantification of hepatitis C virus RNA in human serum or plasma on the COBAS Amplicon analyzer (23).

Assessment of covariates

In the NHANES database, there is a column for demographic data. In this column, we collected information on the age, gender, race, income level, education level, and other relevant information of the respondents. The race is categorized as Mexican American, other Hispanic, non-Hispanic White, non-Hispanic Black, and other. The household income levels are categorized as low income, middle income, and high income; education levels are categorized as below high school, high school or equivalent, college or above, and other.

In the dietary data column, information on the diet of the respondents is recorded, and it is possible to know about the nutritional intake of the respondents. In this column, we collected information from respondents about their intake of calcium and alcohol. The examination information column contains the data on the physical examination of the respondents. In this column,

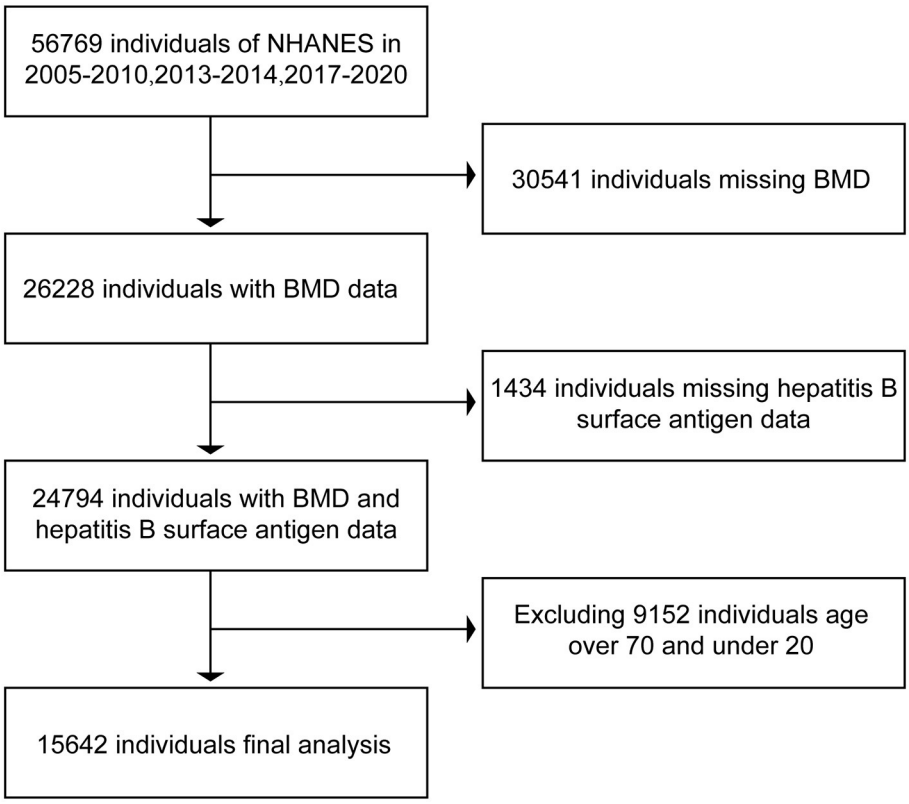

FIGURE 1
Flow chart of inclusion and exclusion of respondents associated with hepatitis B surface antigen.

we collected not only the BMD data of the respondents but also the data of the respondents' body mass index (BMI).

In the column of laboratory test data, the results of the relevance of the respondents' tests are recorded. Here, we collected the data on the hepatitis B surface antigen and hepatitis C RNA of respondents. In addition, data on liver function, HDL, uric acid, creatinine, calcium levels, and blood glucose, which are all relevant covariates that may affect the results, were also collected in this column.

Finally, we collected data about smoking and the presence of diabetes in the respondents in the questionnaire column. Respondents were considered to have a smoking habit if they had smoked more than 100 cigarettes previously.

Statistical analyses

For statistical analysis of the data, we used the R language 3.4.3 and EmpowerStats 2.0. According to the hepatitis indicators, the respondents who were positive and negative for hepatitis were divided into two different groups. In terms of data statistics, the data of categorical variables are expressed as numbers with percentages (*N*%), while the data of continuous variables are expressed as mean values with standard deviations (mean ± SD). Hepatitis as the exposure variable is a categorical variable, and BMD as the outcome indicator is a continuous variable. Therefore, the χ^2 test (categorical variable) and the linear regression model (continuous variable) were used to calculate the difference in BMD among different groups. For analyzing the differences in BMD between the groups, three different multiple regression equation models were used. No adjustment was made for Model 1; Model 2 was adjusted for age, race, and gender; and Model 3 was adjusted for age, race, gender, income level, education level, BMI, smoking, alcohol consumption, calcium intake, HDL, uric acid, creatinine, blood calcium levels, blood glucose, and diabetes.

For the relationship between serological indicators of viral hepatitis and BMD, we used multiple regression model analysis, using a smooth curve fitting. For smooth curve fitting, the variables of adjustment were similar to that of Model 3. In addition, we performed stratified analyses, according to different ages and BMI. The age was divided into three groups: <40, <60, and ≥60 years old. The BMI was divided into four groups: <18.5, 18.5–24.9, 24.9–30, and >30 kg/m^2. A *P*-value of <0.05 is considered statistically significant.

Results

Baseline characteristics of study participants

In this study, we initially collected relevant data from 56,769 respondents. A total of 31,975 respondents were excluded due

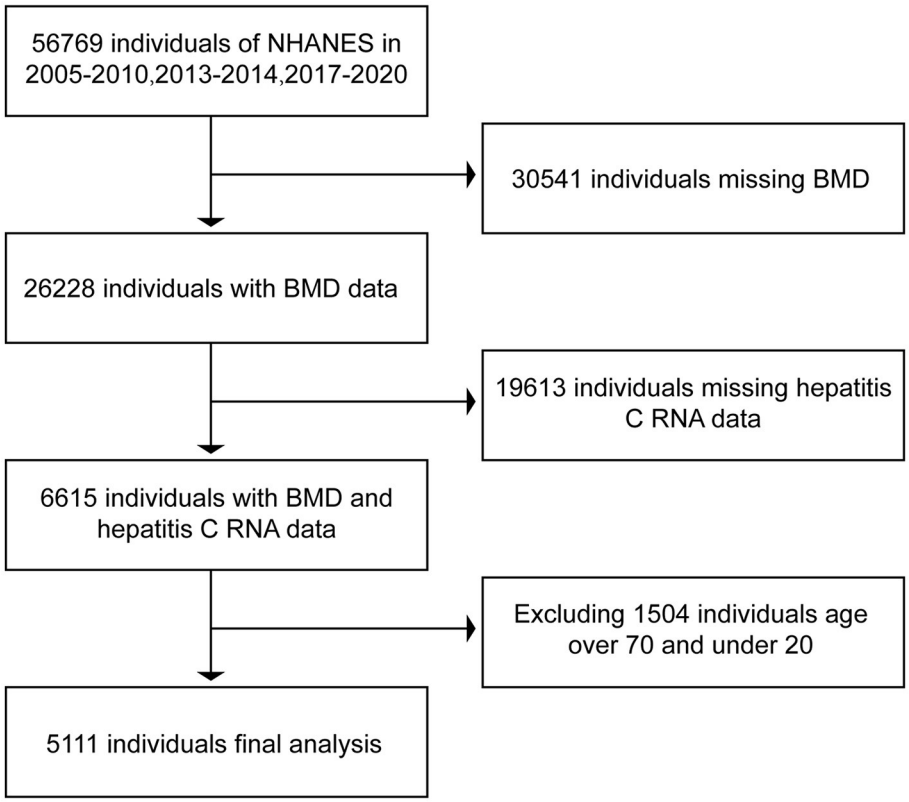

FIGURE 2
Flow chart of inclusion and exclusion of respondents associated with hepatitis C RNA.

to a lack of data related to hepatitis B surface antigen or bone mineral density. Moreover, 9,152 respondents older than 70 years of age and younger than 20 years of age were excluded. A total of 15,642 respondents were included in the final study. Of the 15,642 respondents included in the analysis, 1,217 were positive for hepatitis B surface antigen and the rest of the respondents are negative. Data related to hepatitis C RNA included a total of 56,769 respondents at first. Of these, 50,154 respondents were excluded due to lack of hepatitis C RNA or bone mineral density-related data, 1,504 respondents older than 70 years of age and younger than 20 years of age were excluded, and a total of 5,111 respondents were included in the study finally. A total of 5,111 respondents had hepatitis C RNA data, of which 268 were positive, and the rest were negative. The data relating to the included respondents are shown in Table 1.

Multiple regression analysis results

Hepatitis B-related respondents included in the population ended up with a total of 15,642 respondents, of which 1,217 have HBsAg (+) vs. 14,425 have HBsAg (–). According to the results of the multiple regression equation, we can see a significant difference in total femur BMD between HBsAg (+) and HBsAg (–) respondents: −0.022 (−0.031, −0.013) ($p < 0.01$). In Model 2, which was adjusted for age, gender, and ethnicity of respondents, there was also a significant difference in total femoral BMD between HBsAg (+) and HBsAg (–) respondents: −0.018 (−0.026, −0.009) ($P < 0.01$). In Model 3, adjusted for all covariates, there was no significant difference in total femur BMD between HBsAg (+) and HBsA (–) subjects though −0.002 (−0.010, 0.005) ($P = 0.51$). However, total femur BMD was reduced in HBsAg (+) respondents compared to HBsAg (–) respondents in Model 3, but HBsAg (+) respondents had significantly lower total femur BMD than HBsAg (–) respondents (the results of this analysis are shown in Table 2).

In the included population of HCV-RNA-associated respondents, there were 268 HCV-RNA (+) and 4,843 HCV-RNA (–) respondents. In the same way, we performed multiple regression analyses on these data. In Model 1, there was no significant difference in total femoral BMD between HCV-RNA (+) respondents and HCV-RNA (–) respondents 0.007 (−0.012, 0.025) ($p = 0.48$). In contrast, there was a significant difference in total femoral BMD between HCV-RNA (+) respondents and HCV-RNA (–) respondents in Model 2 −0.043 (−0.059, −0.026) ($P < 0.01$). In Model 3, there was no significant difference in total femur BMD between HCV-RNA (+) and HCV-RNA (–) respondents −0.015 (−0.032, 0.002) ($P = 0.07$), but HCV-RNA (+) respondents showed a significant reduction in total femur BMD. HCV-RNA (+) may also increase the risk of bone loss (the results of this analysis are shown in Table 2).

TABLE 1 Baseline characteristics of respondents with BMD according to HBsAg or HCV-RNA seropositivity.

	Hepatitis B and C related indicators					
	HBsAg (−)	HBsAg (+)	*P*-value	HCV-RNA (−)	HCV-RNA (+)	*P*-value
N	14,425	1,217		4,843	268	
Age	47.94 ± 14.06	54.51 ± 10.94	<0.01	56.87 ± 8.17	52.64 ± 9.32	<0.01
Gender			<0.01			<0.01
Male (%)	7,276 (50.44%)	699 (57.44%)		2,415 (49.87%)	182 (67.91%)	
Female (%)	7,149 (49.56%)	518 (42.56%)		2,428 (50.13%)	86 (32.09%)	
AST	25.38 ± 15.33	29.90 ± 32.14	0.02	23.56 ± 13.34	58.39 ± 41.99	<0.01
ALT	25.84 ± 19.19	29.02 ± 29.50	<0.01	23.65 ± 18.31	61.68 ± 47.92	<0.01
Creatinine	78.67 ± 37.26	81.41 ± 39.86	0.01	80.46 ± 38.60	88.12 ± 72.71	0.40
Blood.calcium	9.43 ± 0.37	9.41 ± 0.37	0.24	9.36 ± 0.36	9.39 ± 0.39	0.01
Uric.acid	5.39 ± 1.39	5.58 ± 1.42	<0.01	5.44 ± 1.41	5.72 ± 1.42	<0.01
BMI (kg/m^2)	28.70 ± 5.90	27.52 ± 5.82	<0.01	29.29 ± 6.31	27.45 ± 6.00	<0.01
Ratio.of.family.income.to.poverty	2.70 ± 1.60	2.34 ± 1.50	<0.01	2.76 ± 1.58	1.65 ± 1.26	<0.01
Calcium	938.01 ± 589.10	835.48 ± 580.48	<0.01	893.90 ± 524.96	985.56 ± 733.72	0.47
Alcohol	11.48 ± 28.94	13.09 ± 45.93	0.85	10.00 ± 26.08	29.86 ± 86.16	<0.01
Fasting.glucose	6.07 ± 1.44	6.18 ± 1.54	0.02	6.39 ± 1.58	6.31 ± 1.52	0.21
HDL	52.86 ± 16.26	53.56 ± 16.28	0.15	48.88 ± 14.87	59.33 ± 17.06	<0.01
Race			<0.01			<0.01
Mexican American (%)	2,759 (19.13%)	92 (7.56%)		634 (13.09%)	26 (9.70%)	
Other hispanic (%)	1,441 (9.99%)	144 (11.83%)		558 (11.52%)	21 (7.84%)	
Non-hispanic white (%)	6,432 (44.59%)	212 (17.42%)		1,738 (35.89%)	102 (38.06%)	
Non-hispanic black (%)	2,820 (19.55%)	435 (35.74%)		1,128 (23.29%)	110 (41.04%)	
Other race—including multi-racial (%)	973 (6.75%)	334 (27.44%)		785 (16.21%)	9 (3.36%)	
Education.level			<0.01			<0.01
Less than high school (%)	3,540 (24.54%)	354 (29.09%)		985 (20.34%)	98 (36.57%)	
High school or equivalent (%)	3,313 (22.97%)	314 (25.80%)		1,119 (23.11%)	90 (33.58%)	
College or above (%)	7,558 (52.40%)	548 (45.03%)		2,734 (56.45%)	79 (29.48%)	
Not recorded (%)	14 (0.10%)	1 (0.08%)		5 (0.10%)	1 (0.37%)	
Diabetes			<0.01			<0.01
Yes (%)	1,643 (11.39%)	175 (14.38%)		862 (17.80%)	26 (9.70%)	
No (%)	12,459 (86.37%)	998 (82.00%)		3,795 (78.36%)	237 (88.43%)	
Not recorded (%)	323 (2.24%)	44 (3.62%)		186 (3.84%)	5 (1.87%)	
Smoked			<0.01			<0.01
Yes (%)	6,696 (46.42%)	627 (51.52%)		2,201 (45.45%)	231 (86.19%)	
No (%)	7,724 (53.55%)	590 (48.48%)		2,641 (54.53%)	37 (13.81%)	
Not recorded (%)	5 (0.03%)	0 (0.00%)		1 (0.02%)	0 (0.00%)	

Data are presented as mean ± SD or n (%).

In Model 1 of the multiple regression equation for femoral neck BMD in HBsAg (+) vs. HBsAg (−) respondents, we can see a significant difference in femoral neck BMD between HBsAg (+) and HBsAg (−) respondents −0.024 (−0.033, −0.015) ($p < 0.01$). In Model 2, there was also a significant difference in femoral neck BMD between HBsAg (+) and HBsAg (−) subjects −0.010 (−0.018, −0.002) ($P = 0.01$). In Model 3, adjusted for all covariates, there was no significant difference in femoral neck BMD between HBsAg (+) and HBsAg (−) respondents 0.002 (−0.005, 0.009) ($P = 0.62$). The BMD of the femoral neck was lower in HBsAg (+) respondents

TABLE 2 β (95% CIs) for decreased bone mineral density among respondents with BMD, according to HBsAg or HCV-RNA seropositivity.

	Hepatitis B and C					
	HBsAg (−)	HBsAg (+)	*P*-value	HCV-RNA (−)	HCV-RNA (+)	*P*-value
TFB						
Model 1 β (95% CI) *P*-value	0	−0.022 (−0.031, −0.013)	<0.01	0	0.007 (−0.012, 0.025)	0.48
Model 2 β (95% CI) *P*-value	0	−0.018 (−0.026, −0.009)	<0.01	0	−0.043 (−0.059, −0.026)	<0.01
Model 3 β (95% CI) *P*-value	0	−0.002 (−0.010, 0.005)	0.51	0	−0.015 (−0.032, 0.002)	0.07
Model 4 β (95% CI) *P*-value	0	−0.013 (−0.021, −0.005)	<0.01	0	−0.027 (−0.045, −0.009)	<0.01
FNB						
Model 1 β (95% CI) *P*-value	0	−0.024 (−0.033, −0.015)	<0.01	0	0.029 (0.011, 0.046)	<0.01
Model 2 β (95% CI) *P*-value	0	−0.010 (−0.018, −0.002)	0.01	0	−0.021 (−0.037, −0.005)	0.01
Model 3 β (95% CI) *P*-value	0	0.002 (−0.005, 0.009)	0.62	0	0.000 (−0.016, 0.017)	0.96
Model 4 β (95% CI) *P*-value	0	−0.007 (−0.015, 0.000)	0.06	0	−0.009 (−0.027, 0.008)	0.29
TSB						
Model 1 β (95% CI) *P*-value	0	−0.023 (−0.033, −0.013)	<0.01	0	0.016 (−0.008, 0.040)	0.19
Model 2 β (95% CI) *P*-value	0	−0.020 (−0.030, −0.010)	<0.01	0	−0.035 (−0.058, −0.012)	<0.01
Model 3 β (95% CI) *P*-value	0	−0.008 (−0.017, 0.001)	0.08	0	−0.014 (−0.040, 0.012)	0.28
Model 4 β (95% CI) *P*-value	0	−0.015 (−0.025, −0.006)	<0.01	0	−0.019 (−0.046, 0.007)	0.15

TFB, total femur BMD; FNB, femoral neck BMD; TSB, total spinal BMD.
Model 1: Non-adjusted.
Model 2: Adjusted for age, gender, and race.
Model 3: Adjusted for age, gender, race, income level, education level, AST, ALT, BMI, smoking, alcohol consumption, calcium intake, HDL, uric acid, creatinine, blood calcium levels, blood glucose, and history of diabetes.
Model 4: Adjusted for age, gender, race, income level, education level, AST, ALT, smoking, alcohol consumption, calcium intake, HDL, uric acid, creatinine, blood calcium levels, blood glucose, and history of diabetes.

TABLE 3 Stratified analyses of bone mineral density in respondents, according to age in HBsAg or HCV-RNA seropositivity.

	Hepatitis B and C					
	HBsAg (−)	HBsAg (+)	*P*-value	HCV-RNA (−)	HCV-RNA (+)	*P*-value
TFB						
20–39	0	−0.001 (−0.023, 0.021)	0.90	–	–	–
40–59	0	−0.006 (−0.017, 0.005)	0.28	0	−0.011 (−0.032, 0.010)	0.32
60–70	0	−0.009 (−0.021, 0.003)	0.16	0	−0.012 (−0.043, 0.020)	0.47
FNB						
20–39	0	0.002 (−0.020, 0.024)	0.87	0	−0.001 (−0.072, 0.071)	0.99
40–59	0	−0.001 (−0.011, 0.010)	0.87	0	0.011 (−0.010, 0.033)	0.30
60–70	0	−0.007 (−0.019, 0.005)	0.23		−0.015 (−0.046, 0.016)	0.35
TSB						
20–39	0	0.004 (−0.017, 0.025)	0.70	0	−0.025 (−0.109, 0.059)	0.56
40–59	0	−0.012 (−0.025, 0.001)	0.07	0	0.005 (−0.026, 0.036)	0.77
60–70	0	−0.020 (−0.037, −0.003)	0.02	0	−0.050 (−0.103, 0.004)	0.07

Adjusted for gender, race, income level, education level, AST, ALT, BMI, smoking, alcohol consumption, calcium intake, HDL, uric acid, creatinine, blood calcium levels, blood glucose, and history of diabetes.

than in HBsAg (–) respondents (the results of the analysis are shown in Table 2).

In Model 1, there was a significant difference in femoral neck BMD between HCV-RNA (+) respondents and HCV-RNA (–) respondents 0.029 (0.011, 0.046) ($p < 0.01$). However, the femoral neck BMD was to be increased in HCV-RNA (+) respondents. In Model 2, there was no significant difference in femoral neck BMD between HCV-RNA (+) respondents and HCV-RNA (–) respondents −0.021 (−0.037, −0.005) ($P = 0.01$), and there was also no significant difference in

TABLE 4 Stratified analyses of bone mineral density in respondents, according to BMI in HBsAg or HCV-RNA seropositivity.

	Hepatitis B and C					
	HBsAg (−)	HBsAg (+)	*P*-value	HCV-RNA (−)	HCV-RNA (+)	*P*-value
TFB						
<18.5	0	−0.006 (−0.056, 0.044)	0.82	0	0.034 (−0.111, 0.180)	0.65
18.5–24.9	0	0.002 (−0.011, 0.015)	0.77	0	−0.022 (−0.051, 0.006)	0.13
24.9–30	0	0.000 (−0.012, 0.012)	0.98	0	−0.016 (−0.045, 0.012)	0.26
>30	0	−0.003 (−0.017, 0.011)	0.69	0	−0.015 (−0.049, 0.018)	0.37
FNB						
<18.5	0	−0.002 (−0.050, 0.046)	0.94	0	0.000 (−0.141, 0.141)	0.99
18.5–24.9	0	0.003 (−0.010, 0.015)	0.67	0	−0.012 (−0.040, 0.016)	0.40
24.9–30	0	0.006 (−0.005, 0.017)	0.31	0	−0.007 (−0.034, 0.021)	0.63
>30	0	0.000 (−0.014, 0.015)	0.96	0	0.020 (−0.014, 0.055)	0.25
TSB						
<18.5	0	−0.008 (−0.075, 0.058)	0.80	0	0.248 (0.004, 0.491)	0.06
18.5–24.9	0	−0.006 (−0.022, 0.009)	0.42	0	−0.010 (−0.057, 0.036)	0.67
24.9–30	0	−0.005 (−0.020, 0.010)	0.55	0	−0.018 (−0.060, 0.024)	0.41
>30	0	−0.012 (−0.030, 0.006)	0.19	0	−0.026 (−0.073, 0.020)	0.27

Adjusted for age, gender, race, income level, education level, AST, ALT, smoking, alcohol consumption, calcium intake, HDL, uric acid, creatinine, blood calcium levels, blood glucose, and history of diabetes.

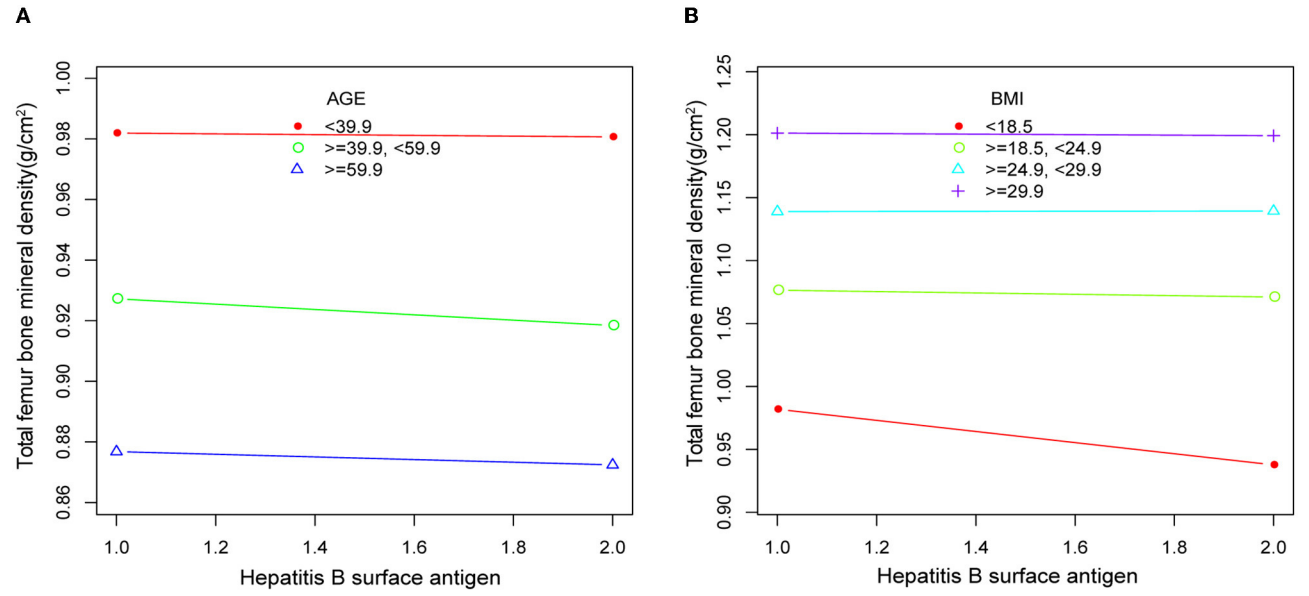

FIGURE 3
Smooth curve plots of bone mineral density in respondents according to age **(A)** and BMI **(B)** in HBsAg or HCV-RNA seropositivity. Adjusted for gender, race, income level, education level, AST, ALT, BMI, smoking, alcohol consumption, calcium intake, HDL, uric acid, creatinine, blood calcium levels, blood glucose, and history of diabetes.

femoral neck BMD between HCV-RNA (+) and HCV-RNA (−) respondents in Model 3, 0.000 (−0.016, 0.017) ($P = 0.96$). In terms of femoral neck BMD, HCV-RNA (+) respondents did not appear to receive a significant effect on femoral neck BMD (the specific results of the analysis are shown in Table 2).

In the Model 1 multiple regression analysis Modelof HBsAg and spinal BMD, there was a significant difference in spinal BMD between HBsAg (+) respondents and HBsAg (−) respondents −0.023 (−0.033, −0.013) ($P < 0.01$). In Model 2, there was also a significant difference in spinal BMD between HBsAg (+) respondents and HBsAg (−) respondents −0.020 (−0.030, −0.010)

($p < 0.01$). In Model 3, there was no statistically significant difference in spinal BMD between the two −0.008 (−0.017, 0.001) ($P = 0.08$), but there was a reduction in spinal BMD in HBsAg (+) respondents compared to HBsAg (–) respondents. This suggests that HBsAg (+) may reduce the spinal BMD of patients (the results of this analysis are shown in Table 2).

In Model 1, the spinal BMD between HCV-RNA (+) respondents and HCV-RNA (–) respondents was not significantly different by 0.016 (−0.008, 0.040) ($p = 0.19$). In Model 2, there was a significant difference in spinal BMD between HCV-RNA (+) respondents and HCV-RNA (–) respondents −0.035 (−0.058, −0.012) ($p < 0.01$). In Model 3, there was no significant difference in spinal BMD between HCV-RNA (+) and HCV-RNA (–) respondents −0.014 (−0.040, 0.012) ($P = 0.28$). However, some reduction in spinal BMD has also been seen in HCV-RNA (+) respondents in Model 3 (the results of the analysis are shown in Table 2).

Stratified analyses

We conducted separate stratified analyses for age and BMI. There were no significant differences in femoral and spinal BMD between respondents of different ages, regardless of positive or negative hepatitis B and C test results (Table 3). In multiple regression analyses with BMI groupings, there was also no significant difference in femoral and spinal BMD between respondents (Table 4).

The detection of linear relationships

Body mass index (BMI) was categorized as BMI $\leq$ 18.5, 18.5 $<$ BMI $\leq$ 25, 25 $<$ BMI $\leq$ 30, and BMI $>$ 30. Age was categorized as age $<$ 40 years, 40 $\leq$ age $<$60 years, and age $\geq$ 60 years. All covariates were included, and a smooth curve was fitted. The resulting smooth curve plots showed little variation in total femoral BMD across the same age and BMI ranges (Figure 3).

Discussion

Hepatitis, as a common infectious disease worldwide, is prone to liver cirrhosis and even liver cancer in its end stage (24, 25). Osteoporosis is a common complication in patients with hepatitis, and it is even more prevalent in patients with liver cirrhosis. However, there is still no definitive conclusion as to whether infection with the hepatitis virus directly causes a decrease in bone mass or even osteoporosis. Some studies had suggested that patients with hepatitis and liver cirrhosis are at higher risk of developing reduced bone BMD and osteoporosis (26, 27). Some studies had suggested that the long-term use of antiviral drugs in patients with hepatitis could lead to increased bone loss and impairment of renal function, which will lead to an increased risk of osteoporosis in patients with hepatitis (28, 29). However, studies on the relationship between viral hepatitis-related indicators and the decline in bone BMD are still scarce and have not been able to draw definitive conclusions. For these reasons, we collected the data related to hepatitis B surface antigen, hepatitis C RNA, femoral BMD, and spinal BMD from the NHANES database and perform a multiple regression analysis on these data. The aim of the study was to analyze whether positive serological indicators of viral hepatitis in US adults are associated with reduced BMD.

According to our final multiple regression analysis results, the BMD of HBsAg (+) and hepatitis C RNA (+) respondents was lower than serologically negative adults after combining various covariables that may affect the BMD of adults who were serologically negative, indicating that our final results are reliable. Furthermore, our smooth plots showed that the BMD of the hepatitis virus seropositive respondents was significantly lower than that of the hepatitis virus seronegative respondents, and these results largely validate the association between hepatitis virus infection and reduced BMD. Combined with the current research, bone loss in patients with hepatitis may be caused by the metabolism of the body and a series of inflammatory reactions after infection with the hepatitis virus. Because abnormal metabolism can easily lead to malnutrition, systemic inflammatory response and malnutrition can cause skeletal muscle loss, both of which are risk factors for bone loss (30–32). The interaction of various factors increases the risk of bone loss in patients with hepatitis virus infection.

In addition, the results of our analysis after removing the covariate BMI from Model 4 were statistically significant, with more significant differences in BMD between serologically positive and negative respondents. Furthermore, previous studies had suggested that BMI and age may be negatively correlated with BMD and that higher BMI and increasing age may lead to lower BMD in patients (33–36). Therefore, we conducted stratified analyses of the two covariates of BMI and age, with the aim of identifying the degree of influence of BMI and age on our analysis results. Regardless of age or BMI, the results of the stratified analysis indicated that there were no significant differences in BMD among respondents of different ages and BMIs. Moreover, the smooth curve plots of age and BMI also showed little variation in total femoral BMD across the same age and BMI ranges. Such results further validate the stability and accuracy of the results of our Model 4 analysis. We speculated that the infection with hepatitis B or C virus can cause an inflammatory response and metabolic disturbances in the body, leading to a decrease in BMD in the bones, which can increase the risk of osteoporosis.

Other studies had also suggested that hepatitis B and C virus serologically positive respondents show decreased BMD in the femur and spine compared to negative respondents, which may increase the risk of osteoporosis (37–39). In a study involving 51,144 respondents on the relationship between positive hepatitis B surface antigen and BMD in Taiwan (37), the results of their multiple regression analysis suggested a negative association between HBV positivity and BMD. HBV infection has a significant impact on the development of reduced BMD in the Taiwanese adult population. Similarly, the results from a national data study in Korea (38) suggested that serological positivity of hepatitis B is significantly associated with reduced BMD in men. In terms of hepatitis C-related studies, a meta-analysis (39) study suggested an increased risk of osteoporosis in patients with HCV infection. However, we did not believe that the results of these studies can be extrapolated to the BMD status of adults with viral hepatitis in the United States. This is because there are ethnic and lifestyle differences between countries, which may have different effects on

bone mass. Therefore, our study can more truly reflect the BMD status of American adult patients with viral hepatitis.

Of course, there are some studies suggesting that the cause of osteoporosis in patients with hepatitis or liver cirrhosis is not caused by the infection with the hepatitis virus, but rather a decline in liver function that causes abnormal bone metabolism. Abnormal bone metabolism could lead to the deceased of bone synthesis and increased bone resorption, which would further lead to a decrease in BMD and osteoporosis. In a study of subjects from several hospitals in Taiwan (40), serum BAP and CTX levels were found to be higher in patients with chronic non-cirrhotic hepatitis C. These results implied that the early bone loss in patients with chronic non-sclerotic hepatitis C may be due to increased bone resorption. Several studies had also suggested that although the current mechanism of action between hepatitis virus infection of the liver and BMD is unclear, the physiological responses grown by various inflammatory factors following infection with the virus tend to stimulate osteoclast formation. Increased osteoclast formation could lead to a decrease in bone formation along with increased bone resorption, which could further lead to a decrease in BMD in patients with hepatitis (41–43).

Compared with some previous clinical studies, the samples of our study come from the NHANES database in the United States. Due to the relatively large sample size of these data, which is representative of the sample of adult respondents related to HBB and HBC in the United States, our research results are objective to a certain extent. Moreover, the proven sample follow-up of the NHANES database can provide a reliable basis for our analytical results. These are some of the advantages that we have in this study.

Of course, our current study also has certain limitations. First, this study is a cross-sectional observational study, and it can only analyze the relationship between hepatitis-related serological indicators and bone BMD. Second, the data included in this study do not include the specific medication status of patients infected with hepatitis B and C. For example, the use of tenofovir may increase the risk of reduced BMD. However, the NHANES database lacks information on tenofovir use in patients with hepatitis B. Therefore, a possible bias of tenofovir on the results of the analysis cannot be excluded. Third, glomerular filtration rates in chronic kidney disease and cirrhosis are also strongly associated with reduced BMD, and these data are not available in the NHANES database. We also cannot rule out the possibility that glomerular filtration rate and cirrhosis may bias the results of the study. Finally, as this is a large national survey, there may be some confounding factors due to measurement error and some unmeasured variables, and these potential confounding factors may have an impact on the results of our analysis.

Conclusion

Following multiple regression analysis of hepatitis serologic indicators and BMD, we find that serologic HBsAg (+) and HCV-RNA (+) may be associated with an increased risk of reduced bone mass in patients. This suggests the importance of monitoring and preventing bone loss in our hepatitis serology-positive patients.

Data availability statement

Publicly available datasets were analyzed in this study. This data can be found here: The datasets generated and analyzed in the current study are available at NHANES website: https://www.cdc.gov/nchs/nhanes/index.htm.

Ethics statement

The protocols of NHANES were approved by the Institutional Review Board of the National Center for Health Statistics, CDC (https://www.cdc.gov/nchs/nhanes/irba98.htm). NHANES has obtained written informed consent from all participants. The patients/participants provided their written informed consent to participate in this study. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

Author contributions

TF conceived the theme and take responsibility for the article. JT and ZY are responsible for conceiving, writing the manuscript, and as well as analyzing the data. WH is responsible for the collation and collection of data and as well as the production of charts and graphs. All authors contributed to the article and approved the submitted version.

Funding

This study was supported by the Nantong Science and Technology Bureau, Social and People's Livelihood Science and Technology Program (Grant No: [2020]170) in the form of covering the consultation fees of data statistical analysis. TF received scientific funding from the Nantong Science and Technology Bureau and the grant number is [2020]170.

Acknowledgments

The authors thank the respondents of the NHANES databases.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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frontiers | Frontiers in Cellular and Infection Microbiology

TYPE Original Research
PUBLISHED 31 March 2023
DOI 10.3389/fcimb.2023.1083912

OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to Virus and Host, a section of the journal Frontiers in Cellular and Infection Microbiology

RECEIVED 29 October 2022
ACCEPTED 08 March 2023
PUBLISHED 31 March 2023

CITATION
Zhao J, Bian D, Liao H, Wang Y, Ren Y, Jiang Y, Liu S, Chen X, Hu Z, Duan Z, Lu F and Zheng S (2023) Serum HBsAg and HBcrAg is associated with inflammation in HBeAg-positive chronic hepatitis B patients. *Front. Cell. Infect. Microbiol.* 13:1083912. doi: 10.3389/fcimb.2023.1083912

Serum HBsAg and HBcrAg is associated with inflammation in HBeAg-positive chronic hepatitis B patients

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Backgrounds & aims: Liver inflammation is the main risk factor for developing liver fibrosis, cirrhosis, and even hepatocellular carcinoma in chronic hepatitis B (CHB) patients. To replace biopsy, additional non-invasive biomarkers to diagnose and grade liver necroinflammation are urgently required in clinical practice.

Method: Ninety-four CHB patients, including 74 HBeAg-positive and 20 HBeAg-negative patients, were enrolled and started entecavir or adefovir therapy. Serum HBV RNA, HBV DNA, HBsAg, hepatitis B core-related antigen (HBcrAg), ALT and AST levels, as well as intrahepatic HBV DNA and cccDNA were measured at baseline and during treatment. Liver inflammation was assessed at baseline and month 60 by liver biopsy. Inflammation regression was defined as a ≥1-grade decrease according to the Scheuer scoring system.

Results: In HBeAg-positive CHB patients, at baseline, serum HBsAg and HBcrAg levels negatively correlated with inflammation grade, while ALT and AST levels positively correlated with inflammation grade. AST plus HBsAg exhibited excellent diagnostic ability for significant inflammation with an AUROC of 0.896. After 60 months of antiviral treatment, almost all the patients' liver inflammation ameliorated to G1, and no patients had inflammation progression.

Conclusion: Besides ALT and AST, serum HBsAg and HBcrAg correlated with inflammation grade in HBeAg-positive CHB patients before NAs treatment. Moreover, the combination of HBsAg and AST exhibited excellent diagnostic ability for significant inflammation.

KEYWORDS

chronic hepatitis B, HBsAg, HBcrAg, inflammation, nucleos(t)ide analogues

Introduction

Hepatitis B virus (HBV) infection is a major public health issue affecting about 250 million individuals globally (Schweitzer et al., 2015; World Health Organization, 2017). As a noncytopathic virus, HBV leads to hepatocellular injuries mediating by the host's immune response to the inflammatory damage in hepatocytes (Liaw et al., 1983; Perrillo, 2001; Liaw, 2003). Chronic hepatic inflammation not only hinders the body from clearing HBV but also promotes the development of liver fibrosis, cirrhosis, and even hepatocellular carcinoma (HCC) (Revill et al., 2019). Thus, it is essential to evaluate the grade of liver inflammation early and effectively reverse its progression in chronic hepatitis B (CHB) patients.

Liver biopsy is still considered the gold standard to evaluate inflammation, but the invasiveness limited its application (Sarin et al., 2016). Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) are indicators of liver injury and widely used to reflect liver necroinflammation. Nevertheless, there are limitations in their accuracy in evaluating inflammation in CHB patients (Nguyen et al., 2015). Additional non-invasive biomarkers to diagnose and grade liver necroinflammation are urgently required in clinical practice.

In this study, we aimed to assess the correlation of serum and intrahepatic HBV markers, including serum HBsAg, HBcrAg, HBV DNA and HBV RNA, as well as intrahepatic HBV DNA and cccDNA, with the degree of liver inflammation according to Scheuer scoring system (Scheuer, 1991) in CHB patients before and during nucleos(t)ide analogues (NAs) therapy. Furthermore, we analyzed the performances of these makers in diagnosing significant liver necroinflammation before NAs treatment, and their dynamic changes during NAs treatment.

Material and methods

Patients and study design

This study was conducted using a cohort of 94 CHB patients receiving NAs monotherapy, who were prospectively recruited from Beijing YouAn Hospital, Capital Medical University (Beijing, China) between June 2007 and July 2008. CHB patients who were diagnosed by the American Association for the Study of Liver Diseases guideline (Terrault et al., 2016) were enrolled if they aged ≥ 16 years and were treat-naïve. The exclusion criteria were as follows: (a) co–infection with hepatitis C, hepatitis D virus, or human immunodeficiency viruses; (b) existence of the alcoholic liver disease or autoimmune liver disease; (c) with decompensated cirrhosis or HCC; (d) with a history of immunosuppressive therapy or organ transplantation; (e) pregnant or breastfeeding women.

Once recruitment, patients were given entecavir (ETV) or adefovir (ADV) and followed up. At each follow-up, serum samples were collected for HBV DNA quantification and liver function tests. The remaining serum specimens were stored at -80°C for subsequent research. At enrollment and Month 60, percutaneous liver biopsies were performed to evaluate the histology. With cryopreserved serum samples, HBsAg and HBcrAg levels at baseline, months 6 and 60, as well as HBV RNA levels at baseline, months 6, 12, 24, 36, 48, and 60 were retrospectively quantified.

This study followed the Declaration of Helsinki and was approved by the Institutional Review Board of Beijing YouAn Hospital, Capital Medical University (Beijing, China). All subjects provided written informed consent.

Assays for serum HBsAg, HBcrAg, HBV RNA and HBV DNA

Serum HBsAg was quantified by Elecsys HBsAg II Quant reagent kits (Roche Diagnostics) with a lower limit of detection (LLD) of 0.05 IU/mL. Quantitative levels of HBcrAg were determined by chemiluminescent enzyme immunoassay in an automated analyzer system (Fujirebio Inc., Tokyo, Japan) with the LLD of 1,000 IU/mL and a linear range of 3-7 log IU/mL. Serum HBV RNA was isolated with the nucleic acid extraction or purification kit (Sansure Biotech, Changsha, China) and treated with DNase I (Thermo Fisher Scientific, Waltham, MA, USA). The LLD of the assay was 200 copies/mL. Details on HBV RNA assay can be found in Supplementary Materials. The serum HBV DNA level was determined using the Cobas HBV Amplicor Monitor assay (Roche Diagnostics, Pleasanton, CA, USA) with an LLD of 50 IU/mL.

Hepatic histological evaluation

At baseline and after 60 months of NAs treatment, a percutaneous liver biopsy was performed. A minimal 18mm length of liver tissue containing at least three complete portal tracts was obtained for pathological evaluation (Colloredo et al., 2003). All liver biopsies were reviewed continuously by an experienced pathologist blinded to treatment assignment and time of biopsy. Inflammation was assessed according to the Scheuer scoring system, which is entirely based on histology results. And histologic findings of portal inflammation, interface hepatitis, and lobular inflammation are assigned a score ranging from 0 to 4 (Scheuer, 1991). G≥3 was defined as having significant inflammation. Inflammation regression was defined as a ≥1-grade decrease according to the Scheuer scoring system.

Quantitation of intrahepatic HBV DNA and cccDNA

For DNA extraction, about 30 μm formalin fixation and paraffin embedding (FFPE) liver biopsy tissue in sections of 6 μm each were

Abbreviations: ADV, adefovir dipivoxil; ALT, alanine aminotransferase; AST, aspartate aminotransferase; AUROC, area under the ROC curve; CHB, chronic hepatitis B; ETV, entecavir; HBV, hepatitis B virus; HBcrAg, hepatitis B core-related antigen; HBeAg, hepatitis B e antigen; HBsAg, hepatitis B surface antigen; cccDNA, covalently closed circular DNA; HCC, hepatocellular carcinoma; LLD, lower limit of detection; NAs, nucleos(t)ide analogues; ROC, receiver operating characteristic.

used. QIAamp FFPE DNA Mini Kit (QIAGEN, GmbH, Hilden, Germany) was used to extract DNA according to the instructions of the manufacturer. HBV rcDNA, replicative dsDNA, and ssDNA were digested using T5 Exonuclease (New England Biolabs, USA). The reaction mixture contained 100 ng extracted DNA, 0.5 μL (10 units) T5 Exonuclease, 1 μL NEBuffer 4 (10×) with Nuclease-free H2O to a final volume of 10 μL. The digestion was conducted at 37°C for 1 h, and stop the reaction with EDTA to at least 11 mM. We combined 6.42 μL of digestion product obtained in the previous step, with 7.50 μL QuantStudio™ 3D Digital PCR Master Mix, 0.06 μL of TaqMan Probe-RC-MGB (50 μM), 0.06 μL TaqMan Probe-RNAseP-VIC (50 μM), 0.24 μL primer of rc-F, 0.24 μL primer of rc-R, 0.24 μL primer of RNaseP-F and 0.24 μL primer of RNaseP-R. 15μL of this sample mix was added to each chip and loaded on ProFlex™ 2x Flat PCR System. Absolute quantification was determined with QuantStudio™ 3D Digital PCR System (Thermo Fisher Scientific Inc., Waltham, Massachusetts, USA) and analyzed using QuantStudio 3D AnalysisSuite Cloud Software. (https://china.apps.thermofisher.com/quantstudio3d/). Intrahepatic HBV cccDNA values were normalized to cell number assessed by RNase P copy number assay.

Statistical analysis

Continuous variables were expressed as medians and ranges or means and standard deviations and categorical variables as frequencies. Differences between groups were analyzed using the student t or Mann-Whitney tests for continuous parameters and chi-square or Fisher exact tests for categorical parameters, as appropriate. The receiver operating characteristic (ROC) analysis assessed the markers' diagnostic capacity for inflammation with the cut-off values determined using the Youden index. The 95% confidence interval of the area under the ROC curve (AUROC) was determined using a bootstrap method. The regression and Spearman correlation coefficients (r) were used to depict the correlation between the two variables. All analyses were performed with SPSS version 26.0, with a two-tailed p-value <0.05 considered statistically significant.

Results

Baseline characteristics of the patients

A total of 94 CHB patients who performed liver biopsies at baseline were analyzed in this study, including 74 HBeAg-positive and 20 HBeAg-negative patients. Table 1 summarizes the characteristics of this population, which was predominantly male (n=74, 78.7%) with a median age of 35.5 years and a median BMI of 23.8. Forty-two patients had available HBV genotype data, with 71.4% (30/42) genotype C. Of the 94 patients, 47.9% (45/94) were treated with ETV, and 52.1% (49/94) were treated with ADV after

TABLE 1 Baseline characteristics of the patients.

	Total (n=94)	HBeAg(+) (n=74)	HBeAg(-) (n=20)	*P*
Age (year)	35.5(16-60)	35(16-60)	41.5(24-56)	0.15
Male/Female	74/20	58/16	16/4	1.0
BMI (Kg/m^2)	23.8(16.2-32.9)	23.8(17.2-32.9)	23.8(16.2-31.5)	0.84
HBV Genotype †				0.67
C/others	30/12	25/9	5/3	
Treatment (n (%))				***0.03***
ETV/ADV	45/49	40/34	5/15	
ALT (U/L)	69.8(12.6-681.9)	68.5(36.1-113.0)	102.3(17.2-527.5)	0.38
AST (U/L)	44.1(10.9-358.8)	44.0(28.9-69.6)	56.2(16.0-200.6)	0.26
HBVDNA ($log_{10}IU/mL$)	6.27(1.70-9.28)	6.71(1.99-9.28)	4.55(1.70-8.32)	***0.004***
HBVRNA ($log_{10}copies/mL$)	5.08(1.40-8.49)	5.43(2.00-8.49)	3.71(1.40-6.10)	***<0.001***
HBV RNA/DNA ratio	0.79(0.34-1.83)	0.82(0.34-1.83)	0.69(0.38-1.61)	0.53
HBsAg ($log_{10}IU/mL$)	3.52(-0.07-4.95)	3.77(-0.07-4.95)	3.07(1.67-3.63)	***0.006***
HBcrAg ($log_{10}IU/mL$)	6.72(3.40-8.73)	7.32(4.84-8.73)	5.29(3.40-7.27)	***<0.001***
Intrahepatic HBV DNA ($log10copies/10^5$ cell)	6.44(3.88-8.50)	6.60(4.42-8.50)	5.82(3.88-6.98)	***0.02***
Intrahepatic cccDNA ($log10copies/10^5$ cell)	4.73(2.69-7.18)	4.90(2.69-7.18)	4.05(3.12-5.09)	***0.004***

†Forty-two patients with available genotype data were analyzed.
Continuous variables are expressed as medians and ranges; categorical variables are expressed as frequencies.
ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, Body Mass Index; ETV, Entecavir; ADV, Adefovir dipivoxil; HBsAg, hepatitis B surface antigen; HBcrAg, hepatitis B core-related antigen; HBeAg, hepatitis B e antigen; cccDNA, covalently closed circular DNA.
P values <0.05 are shown in bold.

recruitment. The median serum ALT and AST were 69.8 U/L and 44.1 U/L, respectively. Median levels of baseline serum HBV RNA, HBV DNA, HBsAg and HBcrAg were 5.08 log10copies/mL, 6.27 log10IU/mL, 3.52 log10IU/mL and 6.72 log10IU/mL, respectively. Median levels of intrahepatic HBV DNA and cccDNA are 6.44 and 4.73 log10 copies/10^5 cells.

Compared to HBeAg-negative CHB patients, HBeAg-positive patients have significantly higher serum HBV DNA, HBV RNA, HBsAg and HBcrAg, as well as intrahepatic HBV DNA and cccDNA.

Correlation of serum and intrahepatic HBV markers, ALT and AST with inflammation grade according to Scheuer scoring system

Spearson's correlation coefficients (r) were used to evaluate the correlation of serum and intrahepatic HBV markers, ALT and AST with inflammation grade according to the Scheuer scoring system. As shown in Figure 1, serum HBsAg and HBcrAg levels weakly negatively correlated with inflammation grade (r=-0.39, P=0.008; r=-0.34, P=0.02), while ALT and AST levels weakly positively correlated with inflammation grade (r=0.39, P<0.001; r=0.38, P<0.001) in HBeAg-positive patients at baseline. Nonetheless, after 60 months of NAs treatment, these correlations disappeared, and intrahepatic HBV DNA and cccDNA got a positive correlation with inflammation grade (r=0.29, P=0.04; r=0.29, P=0.04) (Figure 2).

In HBeAg-negative patients, all these serum and intrahepatic HBV markers, ALT and AST had no relevancy with inflammation grade neither at baseline nor after 60 months of NAs treatment (Supplementary Figures 1, 2).

Factors associated with significant inflammation at baseline

At baseline, HBeAg-positive patients with G1, G2, G3 and G4 were 29(39.2%), 25(33.8%), 17(23.0%) and 3(4.1%) respectively (Figure 3). We defined G≥3 as significant inflammation, and the associated factors are depicted in Table 2. The results showed that patients with significant inflammation had lower serum HBsAg and HBcrAg, higher serum ALT and AST, as well as older age. To evaluate the abilities of these variables to diagnose significant inflammation, ROC analyses were conducted. HBsAg performed best with the AUROC of 0.784 (95%CI 0.655-0.912; P=0.002). While the AUROCs of ALT, AST, HBcrAg and age were 0.733 (95%CI 0.601-0.865; P=0.002), 0.725 (95% CI 0.586-0.865; P=0.003), 0.728 (95%CI 0.574-0.883; P=0.02) and 0.677 (95%CI 0.541-0.812; P=0.02), respectively. Moreover, the combination of these markers would further improve the performance, with AST>101(U/L) plus HBsAg<4(log10IU/mL) having the best AUROC of 0.896 (95% CI 0.802-0.990; P<0.001), of which the sensitivity and specificity are 57.1% and 100%, respectively (Table 3).

HBeAg-negative patients with G1, G2, G3 and G4 at baseline were 4(20.0%), 11(55.0%), 5(25.0%) and 0(0.0%), respectively (Supplementary Figure 3). All these serum and intrahepatic HBV

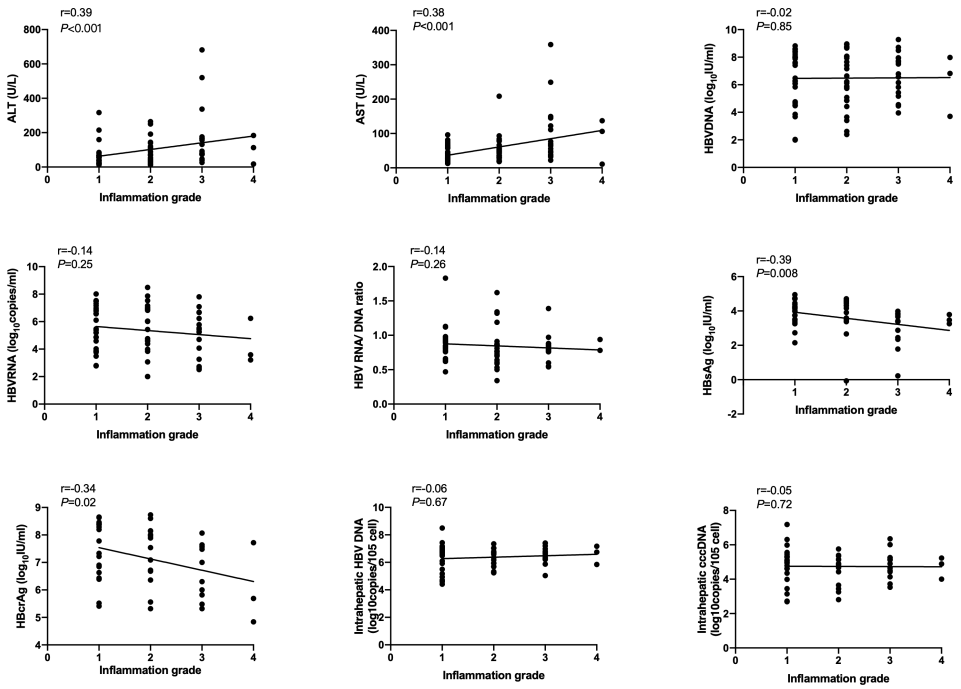

FIGURE 1
Correlation of HBV markers, ALT and AST with inflammation grade according to Scheuer scoring system in HBeAg-positive CHB patients at baseline. HBsAg, hepatitis B surface antigen; HBcrAg, hepatitis B core-related antigen.

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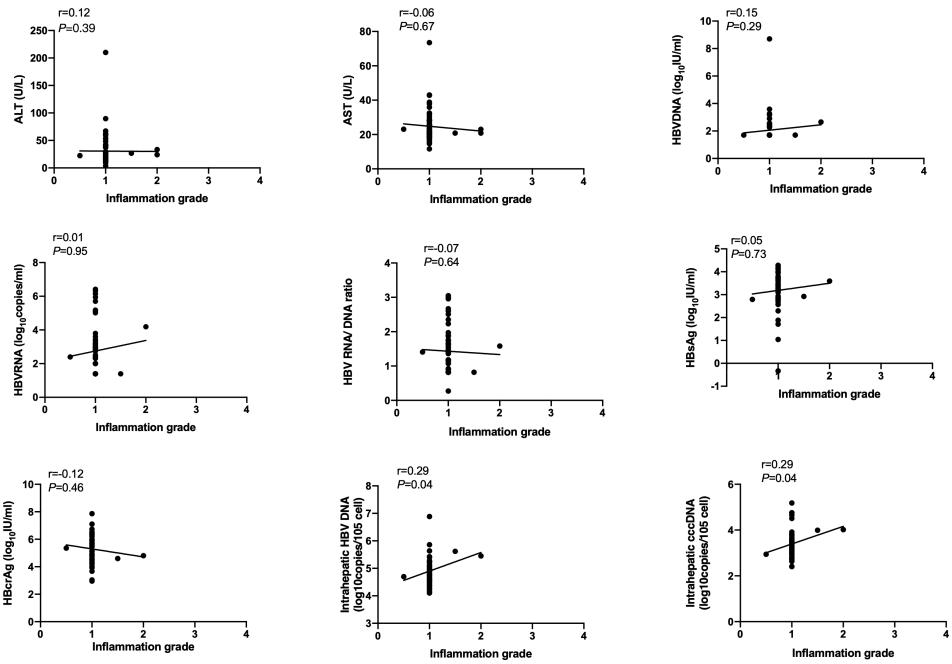

FIGURE 2
Correlation of HBV markers, ALT and AST with inflammation grade according to Scheuer scoring system in HBeAg-positive CHB patients after 60 months of NAs therapy. HBsAg, hepatitis B surface antigen; HBcrAg, hepatitis B core-related antigen.

markers, ALT and AST had no difference between patients with or without significant inflammation (Supplementary Table 1).

Inflammation changes after 60 months of NAs therapy

Figure 3 shows 59 out of 74 HBeAg-positive patients have undergone liver biopsies after 60 months of NAs therapy, with 94.9% (56/59) of G1 and 5.1% (3/59) of G2. Among the 59 patients, 32 (54.2%) achieved inflammation regression at month 60, including 17 (28.8%) had 1- grade regression, 12 (20.3%) had 2- grade regression, and 3 (5.1%) had 3-grade regression (Table 4). No patients had inflammation progression.

17 out of 20 HBeAg-negative patients have undergone liver biopsies at Month 60, with 94.1% (16/17) of G1 and 5.9% (1/17) of G2 (Supplementary Figure 3). Among the 17 patients, 14 (82.4%) achieved inflammation regression, including 10 (58.8%) had 1- grade regression and 4 (23.5%) had 2- grade regression (Supplementary Table 2). No patients had inflammation progression.

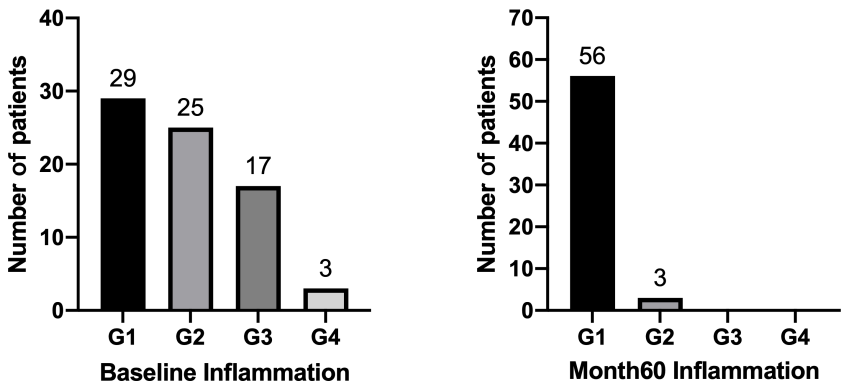

FIGURE 3
Inflammation changes after 60 months of NAs therapy in HBeAg-positive CHB patients.

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TABLE 2 Variables associated with significant inflammation (G ≥ 3 according to the Scheuer scoring system) in HBeAg-positive CHB patients at baseline.

	Total (n=74)	Liver inflammation grade at baseline		
		G <3 N= (54)	G ≥ 3 N= (20)	*P*
Age (year)	35(16-60)	32.5(16-53)	40(25-60)	***0.02***
Male/Female	58/16	40/14	18/2	0.21
BMI (Kg/m^2)	23.8(17.2-32.9)	23.6(17.2-32.9)	24.6(21.9-30.1)	0.18
HBV Genotype †				0.40
C/others	25/9	18/8	7/1	
Treatment (n (%))				0.30
ETV/ADV	40/34	27/27	13/7	
ALT (U/L)	68.5(36.1-113.0)	58.9(12.6-317.3)	102.5 (17.4-681.9)	***0.04***
AST (U/L)	44.0(28.9-69.6)	36.7(12.9-208.6)	67.1(10.9-358.8)	***0.02***
HBVDNA (log_{10}IU/mL)	6.71(1.99-9.28)	6.56(1.99-8.96)	6.80(3.70-9.28)	0.65
HBVRNA (log_{10}copies/mL)	5.43(2.00-8.49)	5.45(2.00-8.49)	5.26(2.51-7.80)	0.23
HBV RNA/DNA ratio	0.82(0.34-1.83)	0.84(0.53-1.83)	0.78(0.34-1.19)	0.10
HBsAg (log_{10}IU/mL)	3.77(-0.07-4.95)	4.12(-0.07-4.95)	3.40(0.22-3.98)	***0.02***
HBcrAg (log_{10}IU/mL)	7.32(4.84-8.73)	7.66(5.32-8.77)	6.31(4.84-8.07)	***0.02***
Intrahepatic HBV DNA (log10copies/105 cell)	6.60(4.42-8.50)	6.55(4.42-8.50)	6.67(5.03-7.41)	0.19
Intrahepatic cccDNA (log10copies/105 cell)	4.90(2.69-7.18)	4.89(2.69-7.18)	4.90(3.53-6.35)	0.67

†Thirty-four patients with available genotype data were analyzed.
Continuous variables are expressed as medians and ranges; categorical variables are expressed as frequencies.
ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, Body Mass Index; ETV, Entecavir; ADV, Adefovir dipivoxil; HBsAg, hepatitis B surface antigen; HBcrAg, hepatitis B core-related antigen; HBeAg, hepatitis B e antigen; cccDNA, covalently closed circular DNA.
P values <0.05 are shown in bold.

TABLE 3 Performance of variables to diagnose significant inflammation (G ≥ 3 according to the Scheuer scoring system) in HBeAg-positive patients at baseline.

	AUROC	*P*	Cut-off	Sensitivity (%)	Specificity (%)
Age(year)	0.677(0.541-0.812)	***0.02***	33.5	85.0	51.9
ALT (U/L)	0.733(0.601-0.865)	***0.002***	73.4	80.0	72.2
AST(U/L)	0.725(0.586-0.865)	***0.003***	101.1	40.0	98.1
HBsAg (log_{10}IU/mL)	0.784(0.655-0.912)	***0.002***	3.98	100.0	57.6
HBcrAg (log_{10}IU/mL)	0.728(0.574-0.883)	***0.02***	7.75	92.3	50.0
HBsAg>4(log_{10}IU/mL)&HBcrAg >7(log_{10}IU/mL)	0.758(0.624-0.891)	***0.006***		100.0	51.5
ALT>73(U/L) &AST>101(U/L)	0.804(0.681-0.927)	***<0.001***		40.0	98.1
ALT>73(U/L) &HBsAg<4(log10IU/mL)	0.872(0.772-0.973)	***<0.001***		78.6	84.8
AST>101(U/L) &HBsAg<4(log10IU/mL)	0.896(0.802-0.990)	***<0.001***		57.1	100.0
ALT>73(U/L) &HBcrAg<7(log10IU/mL)	0.864(0.752-0.976)	***<0.001***		76.9	78.1
AST>101(U/L) &HBcrAg<7(log10IU/mL)	0.691(0.538-0.844)	***0.01***		40.0	98.1

AUROC, area under the ROC curve; ALT, alanine aminotransferase; AST, aspartate aminotransferase; HBsAg, hepatitis B surface antigen; HBcrAg, hepatitis B core-related antigen; cccDNA, covalently closed circular DNA.
P values <0.05 are shown in bold.

10.3389/fcimb.2023.1083912

TABLE 4 Inflammation changes in HBeAg-positive patients received 60 months of NAs therapy according to Scheuer scoring system.

Inflammation changes at month 60	Baseline inflammation grade (n=74)			
	G1(n=29)	G2(n=25)	G3(n=17)	G4(n=3)
No biopsy(n=16)	3	9	3	0
No change in inflammation(n=26)	26	1	0	0
Improvement in inflammation				
1-grade(n=17)	0	15	2	0
2-grade(n=12)	0	0	12	0
3-grade(n=3)	0	0	0	3

Dynamics of serum and intrahepatic HBV markers, ALT and AST during NAs treatment stratified by inflammation change

The dynamic changes of serum and intrahepatic HBV markers, ALT and AST from baseline to Month 60 were investigated. All these markers, including serum HBV DNA, HBV RNA, HBsAg and HBcrAg, intrahepatic HBV DNA and cccDNA, as well as ALT and AST, declined in both patients with or without inflammation regression. Moreover, serum ALT, AST and HBV DNA declined most quickly in the first 6th months after initiating NAs therapy (Figure 4).

Similar findings were observed in HBeAg-negative patients (Supplementary Figure 4).

Discussion

This study aimed to recognize factors correlated with inflammation grade and capable of diagnosing significant inflammation before NAs treatment. We found that serum HBsAg, HBcrAg, ALT, and AST levels correlated with inflammation grades. The combination of HBsAg and AST exhibited excellent diagnostic ability for significant inflammation in HBeAg-positive CHB patients before NAs treatment. Furthermore, we discovered that after 60

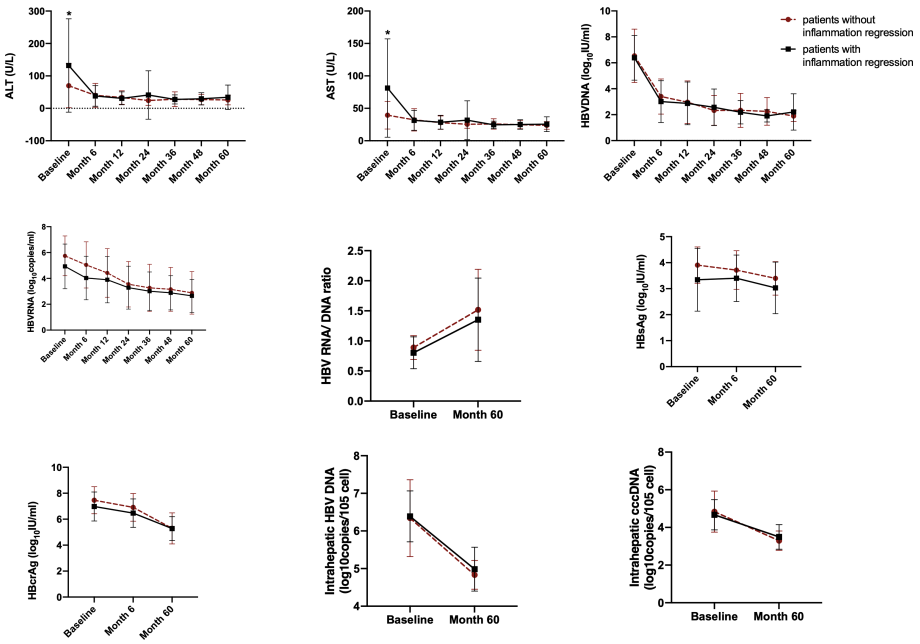

FIGURE 4
The dynamic changes of HBV markers, ALT and AST in HBeAg-positive CHB patients during NAs treatment. Variables are expressed as means and standard deviations. HBsAg, hepatitis B surface antigen; HBcrAg, hepatitis B core-related antigen; cccDNA, covalently closed circular DNA. *Significant difference between patients with inflammation regression and patients without inflammation regression.

months of NAs treatment, almost all the patients' liver inflammation ameliorated to G1 according to the Scheuer scoring system.

Hepatic inflammation drives the accumulation of extracellular matrix and causes fibrosis. It is critical to detect inflammation and initiate antiviral treatment timely (Kisseleva and Brenner, 2021). Various guidelines use the ALT level to reflect inflammation; nevertheless, there is evidence that patients with normal ALT have observable liver inflammation, which reveals the limitation of ALT levels in predicting chronic hepatic inflammation (Dufour et al., 2000; Lai et al., 2007; Kumar et al., 2008; Nguyen et al., 2015). Since inflammation eventually drives the development of hepatic fibrosis, potential serum markers that predict hepatic fibrosis may be used to grade inflammatory activity. In this study, we found that serum HBsAg and HBcrAg levels negatively, as well as ALT and AST levels, positively correlated with inflammation grade in HBeAg-positive patients at baseline. While after 60 months of NAs treatment, these correlations disappeared. Consistent with our findings, one previous research showed that HBeAg-positive chronic HBV infection patients with inflammation have a significantly lower HBsAg value than those without inflammation, and HBsAg value was a predictive factor for inflammation (Zeng et al., 2022). Further, Zhang's paper reported that HBcrAg could predict severe necro-inflammation in both HBeAg-positive and HBeAg-negative patients (Zhang et al., 2016). However, in our study, the relationship between HBV markers and inflammation was not found in HBeAg-negative patients. After 60 months of NAs treatment, intrahepatic HBV DNA and cccDNA correlated with inflammation grade in HBeAg-positive patients, which agrees with a published study that indicated that baseline HBV cccDNA is an independent predictor of liver inflammation (Liang et al., 2016).

The ability of these variables to diagnose significant inflammation in CHB patients before treatment was also explored. And we found that older age, lower serum HBsAg and HBcrAg levels, as well as higher serum ALT and AST levels, were associated with significant inflammation. The ROC analyses revealed that all these variables could diagnose significant inflammation, in which HBsAg had the best performance with an AUROC of 0.784. Furthermore, combining HBsAg and AST improved this classification ability with an AUROC of 0.896.

At baseline, 60.8% of HBeAg-positive and 80.0% of HBeAg-negative patients exhibited inflammation ≥G2. After 60 months of NAs therapy, almost all the patients' liver inflammation ameliorated to G1, irrespective of the HBeAg status. Therefore, the benefit of antiviral treatment on histology is apparent. Consistent with a previous study (Wang et al., 2019), serum levels of HBV RNA, HBV DNA, HBsAg, HBcrAg, AST and ALT, as well as intrahepatic HBV DNA and cccDNA, declined after initiating antiviral treatment. Moreover, serum ALT, AST and HBV DNA decreased most quickly in the first 6th months.

This study showed that combining ALT and HBsAg offers an attractive alternative to biopsy for assessing inflammation in HBeAg-positive patients and may help make treatment decisions in the clinical setting. Future studies could further explore whether the combination of serum markers could evaluate inflammation in different stages of CHB. This study has several limitations. First, the single-center design and limited sample size may bias the study. Second, this study employed a cohort recruited long ago. The quantification of HBV RNA using cryopreserved serum samples may result in a bias due to the degradation of HBV RNA over time. Besides, only Chinese patients were recruited; thus, the results should be carefully extrapolated to other ethnic groups. Future multi-center studies with a large sample size are needed to confirm the results of this study.

In conclusion, in HBeAg-positive CHB patients, serum HBsAg, HBcrAg, ALT, and AST levels correlated with inflammation grade, and the combination of HBsAg and AST exhibited excellent diagnostic ability for significant inflammation before NAs treatment.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The studies involving human participants were reviewed and approved by the Institutional Review Board of Beijing YouAn Hospital, Capital Medical University (Beijing, China). The patients/participants provided their written informed consent to participate in this study.

Author contributions

SZ, FL, ZH, ZD, and XC contributed to conception and design of the study. JZ, DB, YW, YR, SL, HL, and YJ organized the database. JZ, DB, and YW performed the statistical analysis. JZ and DB wrote the first draft of the manuscript. YJ, YR, and HF wrote sections of the manuscript. SZ takes responsibility for the integrity of the work as a whole, from inception to published article. All authors contributed to manuscript revision, read, and approved the submitted version.

Funding

This study was supported in part by National Science and Technology Key Project on "Major Infectious Diseases such as HIV/AIDS, Viral Hepatitis Prevention and Treatment" (2017ZX10302201-004, 2017ZX10202203-006); Beijing Municipal Administration of Hospitals Clinical medicine Development of special funding support (ZYLX202125); Beijing Natural Science Foundation (No. 7222093); High-level public health technical talents construction project of Beijing Municipal Health Commission (Academic Leader -02-14); and National Key Research and Development Program of Ministry of Science and Technology, (2022YFC2304400).

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2023.1083912/full#supplementary-material

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frontiers | Frontiers in Public Health

TYPE Original Research
PUBLISHED 05 April 2023
DOI 10.3389/fpubh.2023.1137738

OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to Infectious Diseases: Epidemiology and Prevention, a section of the journal Frontiers in Public Health

RECEIVED 04 January 2023
ACCEPTED 17 March 2023
PUBLISHED 05 April 2023

CITATION
Li S, Li Z, Du H, Zao X, Gan D, Yang X, Li X, Xing Y and Ye Y (2023) Identification of pseudo-immune tolerance for chronic hepatitis B patients: Development and validation of a non-invasive prediction model.
Front. Public Health 11:1137738.
doi: 10.3389/fpubh.2023.1137738

Identification of pseudo-immune tolerance for chronic hepatitis B patients: Development and validation of a non-invasive prediction model

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Background and aims: Patients with chronic hepatitis B (CHB) in the immune tolerant (IT) phase were previously thought to have no or slight inflammation or fibrosis in the liver. In fact, some CHB patients with normal ALT levels still experience liver fibrosis. This study aimed to develop and validate a non-invasive model for identifying pseudo-immune tolerance (pseudo-IT) of CHB by predicting significant liver fibrosis.

Methods: This multi-center study enrolled a total of 445 IT-phase patients who had undergone liver biopsy for the training cohort (n=289) and validation cohort (n=156) during different time periods. A risk model (IT-3) for predicting significant liver fibrosis (Ishak score≥3) was developed using high-risk factors which were identified using multivariate stepwise logistic regression. Next, an online dynamic nomogram was created for the clinical usage. The receiver operating characteristic (ROC) curve, net reclassification improvement and integrated discrimination improvement were used to assess the discrimination of the IT-3 model. Calibration curves were used to evaluate the models' calibration. The clinical practicability of the model was evaluated using decision curve analysis and clinical impact curves.

Results: 8.8% (39 of 445) patients presented with significant liver fibrosis in this study. Aspartate aminotransferase (AST), hepatitis B e-antigen (HBeAg), and platelet (PLT) were included in the prediction model (IT-3). The IT-3 model showed good calibration and discrimination both in the training and validation cohorts (AUC=0.888 and 0.833, respectively). The continuous NRI and IDI showed that the IT-3 model had better predictive accuracy than GPR, APRI, and FIB-4 (p<0.001). Decision curve analysis and clinical impact curves were used to demonstrate the clinical usefulness. At a cut-off value of 106 points, the sensitivity and specificity were 91.7 and 70.2%, respectively.

Conclusion: The IT-3 model proved an accurate non-invasive method in identifying pseudo-IT of CHB, which can help to formulate more appropriate treatment strategies.

KEYWORDS
chronic hepatitis B, liver fibrosis, immune tolerant, liver biopsy, nomogram

Introduction

Hepatitis B virus (HBV) infection is a serious public health problem worldwide which affects approximately 240 million individuals (1, 2). It is estimated that there are more than 50 million people in the immune tolerant (IT) phase. Previous studies (3–6) thought that IT-phase patients had slow disease progression due to little inflammation or fibrosis in liver. IT-phase patients still had poor rates of seroconversion after receiving antiviral therapy, and they were more likely to develop treatment resistance (7). Therefore, most international clinical guidelines (8–10) recommend that treatment in the IT phase be primarily based on regular monitoring instead of using nucleoside analogs or interferons. However, progression of the disease was observed in IT-phase patients during long-term follow-up, eventually resulting in cirrhosis, liver cancer, and other adverse outcomes (11). The definition of the IT phase was usually based on three main criteria: the serum HBV DNA level, the serum ALT level and the histological features of the liver. In fact, the levels of ALT were not fully representative of the extent of liver damage. Several studies (12, 13) showed that a proportion of HBeAg-positive patients with normal ALT levels actually had significant liver inflammation and fibrosis. The normal ALT levels were most likely just a false appearance of immune tolerance, as significant liver fibrosis suggested that immune responses had already occurred.

Additionally, the definition and management of IT-phase patients were not completely consistent in the clinical guidelines published by the EASL (8), AASLD (9), and APASL (10). The main differences were reflected in age, the ULN of ALT, and HBV DNA load. These differences made clinical stage and treatment ambiguous and might lead to inappropriate treatment for a certain group of patients. In order to provide accurate and individualized treatments, it was essential to identify pseudo-immune tolerance (pseudo-IT) patients from those with normal ALT. Due to the dynamic reciprocal process between immune tolerance and immune clearance, patients are at risk of developing liver fibrosis during the progression of CHB, even if they were previously diagnosed as immune tolerant. However, these patients were frequently neglected for treatment due to normal ALT levels. Histological evidence of liver is a breakthrough in identifying the pseudo-immune tolerance. Although liver biopsy was the gold standard for determining liver histology, it was impractical to use it on a regular basis because of its invasiveness. There is an urgent clinical need for a non-invasive diagnostic method to assess liver fibrosis in IT-phase patients.

In this study, we explored risk factors for liver fibrosis and developed a non-invasive nomogram model for identifying pseudo-IT of CHB from a large retrospective, biopsy-based, multi-center cohort study.

Methods

Study design

The patients were screened from 18 medical centers in different areas of China (Supplementary Table S1). We followed the TRIPOD guideline (14) (transparent reporting of a multivariable prediction model for individual prognosis or diagnosis) for training, validation and reporting of the proposed nomogram. This study was approved by the Ethics Committees of the Dongzhimen Hospital, Beijing University of Chinese Medicine. Written informed consent was provided by all patients.

Patients

The following inclusion criteria were listed (8–10) (1) positive serum HBeAg; (2) HBsAg present for ≥6 months; (3) HBV DNA > 10^6 IU/mL; (4) age > 18 years old; (5) persistently ALT <40 U/L at least 3 times in 12 months. Exclusion criteria included the following: (1) presence of other etiologies of liver diseases (e.g., viral coinfection, autoimmune hepatitis, alcoholic liver disease, nonalcoholic fatty liver disease); (2) taking antiviral drugs 6 months before enrollment; (3) liver cirrhosis or carcinoma; (4) patients with systemic diseases affecting the liver (e.g., HIV infection, heart failure, or thyroid).

A total of 670 eligible patients were retrospectively screened for this study. According to the exclusion criteria, 225 (33.6%) patients were excluded. 289 patients were in the training cohort (from May 2009 to May 2016), whereas the validation cohort included 156 patients (from May 2016 to May 2019) (Figure 1).

Definition

Assessment of liver fibrosis using the Ishak's system (15). The fibrosis stage was graded from stage 0–6. Stage 0–2 indicated no or minimal liver fibrosis, and stage 3–6 indicated significant liver fibrosis.

Collection of clinical and pathological data

We collected baseline clinical and pathological data of 445 patients, including age, gender, body mass index (BMI), histological assessment, blood routine, hepatic and renal function, serological markers of HBV, and HBV DNA load from their electronic medical records.

The formula for calculating aspartate aminotransferase to platelet ratio index (APRI) (16), fibrosis index based on the four factors (FIB-4) (17) and gamma-glutamyl transpeptidase to platelet ratio (GPR) (18) was as previously described:

$$\text{APRI} = \left(\text{AST / its ULN}\right) / \text{platelet count} \times 100$$

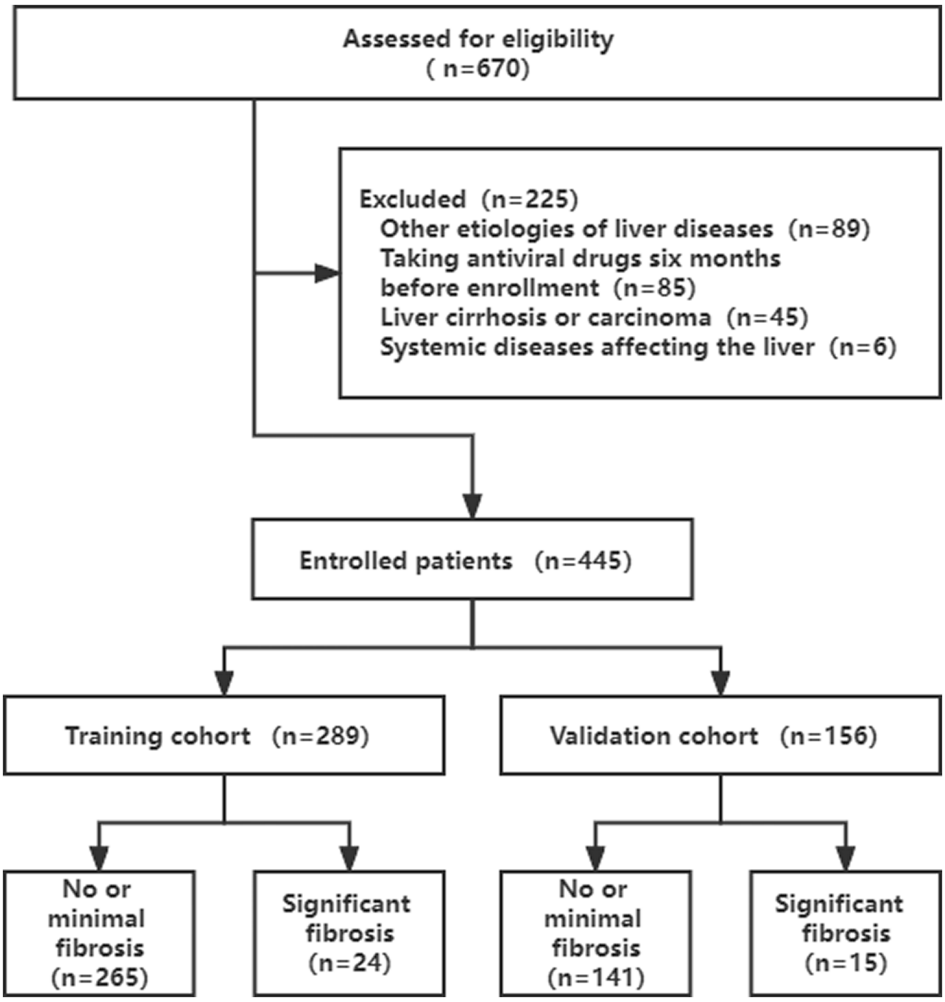

FIGURE 1
Flow chart presenting the study subjects.

$$\mathrm{FIB}-4=\left(\mathrm{age}\times\mathrm{AST}\right)/\left(\mathrm{platelet\ count}\times\left(\mathrm{ALT}\right)^{1/2}\right)$$

$$\mathrm{GPR}=\left(\mathrm{GGT}\ /\ \mathrm{its\ ULN}\right)/\mathrm{platelet\ count}\times 100$$

Histological assessment

Ultrasound-guided percutaneous liver biopsies were performed in all enrolled patients using 16-G tru-cut biopsy needles (Menghini, Bard Company of America). Following formalin fixation and paraffin embedding, the samples were stained with hematoxylin–eosin and reticular fibers. Two experienced pathologists assessed the samples while concealing the clinical information of participants. The stage of fibrosis was determined using the Ishak fibrosis score (IFS) (15) and hepatic inflammation was assessed using the modified Ishak histologic activity index (HAI) (19).

Statistical analyses

Data analyses were performed using SPSS (version 26.0, IBM, NY) and R (version 4.2.0, Vienna, Austria). A two-tailed $p<0.05$ was considered statistically significant. Continuous variables were compared using the Student t-test (normal distribution) and Mann–Whitney *U* test (skewed distribution), which were presented as mean ± standard deviation and median (interquartile range, IQR), respectively. Categorical variables were presented as number (percentage) and compared by the chi-square test or Fisher's exact test. The high-risk factors for significant fibrosis were determined through univariate and multivariate logistic regression. The variables with a value of $p<0.05$ in univariate analysis were subsequently selected and entered into multivariable logistic regression with the backward stepwise method (threshold = 0.1).

The nomogram was constructed based on proportionally converting each regression coefficient in multivariate logistic regression to a 0-to-100-point scale by using the "regplot" package in R. The area under the receiver operating characteristic curves (AUC) were used to assess the discrimination of nomogram. The continuous

TABLE 1 Baseline characteristics of patients in the training and validation cohorts.

Variable	All patients (*n*=445)	Training cohort (*n*=289)	Validation cohort (*n*=156)	*p*
Age(years)[a]	32.0 (30.0, 37.0)	32.0 (30.0, 36.0)	32.0 (30.0, 37.8)	0.830
Male sex[b]	280 (62.9)	185 (64.0)	95 (60.9)	0.516
BMI (kg/m^2)[a]	21.7 (20.1, 23.4)	21.7 (20.2, 23.5)	21.6 (19.7, 23.4)	0.444
WBC (10^{12}/L)[a]	5.6 (5.0, 6.6)	5.6 (5.0, 6.5)	5.6 (5.0, 6.7)	0.862
PLT (10^9/L)[a]	189.0 (159.5, 216.5)	185.0 (158.5, 218.5)	192.5 (161.2, 214.0)	0.694
ALT (U/L)[a]	27.0 (21.0, 35.0)	28.0 (22.0, 36.1)	26.1 (20.0, 32.6)	0.063
AST (U/L)[a]	24.0 (20.0, 29.6)	25.0 (20.0, 30.0)	23.0 (20.0, 28.0)	0.069
GGT (U/L)[a]	19.0 (14.0, 27.6)	19.3 (13.9, 28.7)	19.0 (14.0, 26.0)	0.498
BUN (mmol/L)[a]	4.9 (4.1, 5.9)	4.8 (4.1, 6.0)	4.9 (4.2, 5.9)	0.158
Cr (umol/L)[a]	75.2 (63.0, 86.0)	74.5 (62.1, 85.0)	76.0 (64.0, 87.7)	0.195
HBV-DNA (log_{10} IU/ml)[a]	8.3 (7.9, 8.7)	8.3 (7.9, 8.8)	8.2 (7.8, 8.7)	0.092
HBsAg (log_{10} IU/ml)[a]	4.8 (4.5, 5.0)	4.8 (4.5, 5.0)	4.8 (4.6, 5.0)	0.106
HBeAg (S/CO)[a]	1245.2 (1089.0, 1365.8)	1237.6 (1084.2, 1356.0)	1265.7 (1124.8, 1397.2)	0.064
HBcAb (S/CO)[a]	11.7 (10, 12.9)	11.8 (10.1, 13.0)	11.4 (9.8, 12.9)	0.270
GPR[a]	0.2 (0.2, 0.3)	0.2 (0.2, 0.3)	0.2 (0.2, 0.3)	0.225
APRI[a]	0.3 (0.2, 0.4)	0.3 (0.2, 0.5)	0.3 (0.2, 0.4)	0.052
FIB-4[a]	0.8 (0.7, 1.1)	0.8 (0.7, 1.1)	0.8 (0.6, 1.1)	0.090
IFS ≥3 points[b,c]	39 (8.8)	24 (8.3)	15 (9.6)	0.641
HAI ≥4 points[b,c]	157 (35.3)	97 (33.6)	60 (38.5)	0.302

ALT, alanine aminotransferase; AST, aspartate transaminase; APRI, aspartate aminotransferase-to-platelet ratio index; BMI, body mass index; BUN, blood urea nitrogen; Cr, creatinine; FIB-4, fibrosis index based on the four factors; GGT, gamma-glutamyltransferase; GPR, gamma-glutamyl transpeptidase to platelet ratio; HAI, histology activity index; HBcAb, anti-hepatitis B core antigen; HBeAg, hepatitis B e-antigen; HBsAg, hepatitis B surface antigen; IFS, Ishak fibrosis score; PLT, platelet; RBC, red blood cell; WBC, white blood cell. [a]Data are presented as median (interquartile range, IQR), *p* values were estimated by Mann–Whitney *U* test.
[b]Data are shown as case number (percentage), *p* values were estimated by chisquare test.
[c]Defined when HAI ≥ 4 points as significant inflammation and IFS ≥ 3 points as significant fibrosis.
p, compared the training cohort with the validation cohort.

net reclassification improvement (NRI) and integrated discrimination improvement (IDI) were computed in order to evaluate the improvement and applicability of the new model in reclassification. Confidence intervals for NRI and IDI were generated with the bootstrap method with 1,000 replications. The calibration curve was used to evaluate the predictive performance of the model. A 1000-time bootstrap resampling was used to assess the stability of the model. Decision curve analysis (DCA) and clinical impact curve (CIC) analysis were used to assess the clinical utility of the models.

Results

Baseline characteristics

As shown in Table 1, a total of 445 patients were enrolled in the current study. The median age of participants was 32 years (IQR = 30–37), and 62.9% (280 of 445) were male. All the patients were divided into two sets, with 289 patients (64.9%) assigned to the training cohort and 156 patients (35.1%) assigned to the validation cohort, according to different enrollment periods. Among them, 39 patients (8.8%) showed significant liver fibrosis (IFS score ≥ 3). All the baseline characteristics were not statistically different between the training and validation cohorts ($p > 0.05$).

Univariate and multivariate logistic regression analyses

Univariate and multivariate logistic regression analyses were performed to confirm the potential predictors in the training cohort (Table 2 and Supplementary Figure S1). Based on the results of stepwise regression, three predictors were finally identified: PLT (OR, 0.990; 95% CI, 0.980–1.001; $p = 0.084$), AST (OR, 1.084; 95% CI, 1.010–1.164; $p = 0.025$) and HBeAg (OR, 0.997; 95% CI, 0.996, 0.998; $p < 0.001$).

Nonivasive nomogram development

Based on the logistic stepwise regression analysis, a nomogram was developed to predict the significant liver fibrosis for IT-phase patients and was named the IT-3 model (Figure 2). A total score was calculated by summing all predictors scores. The higher score suggests a higher risk of significant liver fibrosis. In addition, we created an online dynamic nomogram (Supplementary Figure S2).[1]

1 https://nomogramit3.shinyapps.io/IT3model/

TABLE 2 Univariable and multivariable analysis in the training cohort.

	Univariable		Multivariable[a]	
	OR (95% CI)	*p* value	OR (95% CI)	*p* value
Age (years)	0.972 (0.901, 1.048)	0.972		
Male sex	0.933 (0.393, 2.213)	0.874		
BMI (kg/m^2)	0.959 (0.837, 1.098)	0.542		
WBC(10^{12}/L)	1.009 (0.875, 1.164)	0.900		
PLT(10^9/L)	0.988 (0.978, 0.997)	0.013	0.990 (0.980, 1.001)	0.084
ALT(U/L)	1.086 (1.023, 1.153)	0.011	–	–
AST(U/L)	1.105 (1.039, 1.175)	0.002	1.084 (1.010, 1.164)	0.025
GGT(U/L)	1.037 (1.005, 1.071)	0.025	–	–
BUN(mmol/L)	0.900 (0.648, 1.249)	0.529		
Cr (umol/L)	0.987 (0.960, 1.014)	0.335		
HBV-DNA ($\log_{10}$ IU/mL)	0.659 (0.398, 1.093)	0.106		
HBsAg ($\log_{10}$ IU/mL)	0.271 (0.136, 0.540)	<0.001	–	–
HBeAg (S/CO)	0.997 (0.996, 0.998)	<0.001	0.997 (0.996, 0.998)	<0.001
HBcAb (S/CO)	1.002 (0.964, 1.042)	0.903		

CI, confidence interval; OR, odds ratio. [a]Variables found to be significant ($p < 0.05$) by univariate analysis were entered into multivariate logistic regression analysis with backward stepwise method (threshold = 0.1).

IT-3 model evaluation

We evaluated the IT-3 model through discrimination, calibration, and clinical decision benefit. In the training cohort, IT-3 had a higher AUROC [0.888 (0.813–0.962)] than GPR [0.731 (0.641–0.821), $p = 0.007$], APRI [0.74 (0.646–0.834), $p = 0.001$], and FIB-4 [0.645 (0.546–0.743), $p < 0.001$]. In the validation cohort, IT-3 had a higher AUROC [0.833 (0.695–0.970)] than GPR [0.731 (0.641–0.821), $p = 0.147$], APRI [0.616 (0.453–0.779), $p = 0.009$], and FIB-4 [0.631 (0.484–0.777), $p = 0.050$] (Table 3 and Figure 3A). The continuous NRI and IDI showed that the IT-3 model had better predictive accuracy than GPR, APRI, and FIB-4 ($p < 0.001$, Table 3). Using a cutoff value of 106 points, the sensitivity was 91.7% and the specificity was 70.2% in the training cohort. In the validation cohort, the sensitivity was 80.0%, and the specificity was 83.0%. The IT-3 model was validated in the 1,000-time bootstrap resampling with an AUC of 0.888 (95% CI 0.810–0.947) in the training cohort and 0.833 (95% CI 0.687–0.950) in the validation cohort. The IT-3 model also showed good accuracy after 1,000-time bootstrap resampling (Table 4).

The calibration curve showed good agreement between the predicted and observed probabilities in the training and validation cohorts (brier score was 0.06 and 0.06, respectively) (Figure 3B and Table 3). The DCA of the IT-3 model demonstrated a greater net benefit with a wider range of threshold than the other non-invasive models in the training and validation cohorts (Figure 3C). The results of the clinical impact curves showed that the IT-3 model predictions had better agreement with the true positive rates. As the risk threshold increased, there was a decrease in unnecessary treatment and an increase in net clinical benefit (Figure 3D). The risk scores of patients were evaluated based on the IT-3 model were significantly correlated with the extent of liver inflammation or fibrosis ($p < 0.001$) (Figure 3E).

Relationship between serological indicators and liver fibrosis and inflammation

According to the stage of liver fibrosis, patients were divided into different groups (IFS 0, 41.8%; IFS 1–2, 49.4%; IFS 3–4, 7.9%; IFS 5–6, 0.9%). A strong association was noted between serological indicators and the extent of fibrosis (Figure 4A). Significant fibrosis was associated with increasing levels of ALT (p for trend<0.001; K-W test $p < 0.001$) and AST (p for trend<0.001; K-W test $p < 0.001$), although the levels of transaminase were within the normal range. Significant fibrosis was associated with decreasing levels of HBsAg (p for trend <0.001; K-W test $p < 0.001$), HBeAg (p for trend <0.001; K-W test $p < 0.001$) and HBV-DNA (p for trend<0.001; K-W test $p = 0.002$). There was a similar trend when patients were grouped by liver inflammatory activity (HAI 0, 11.2%; HAI 1–4, 60.0%; HAI 5–8, 24.5%; HAI 9–18, 4.3%), although no statistically significant differences were observed in HBV DNA (Figure 4B). Then, patients were stratified according to different levels of virological indicators and found that both HAI and IFS tended to decrease as the virological indicators increased (p for trend <0.001; K-W test $p < 0.001$) (Figure 4C).

Discussion

Due to the disease dynamics, it was important for IT-phase patients to monitor the liver histology in order to initiate antiviral treatments on time. In this study, we analyzed 445 IT-phase patients from 18 hospitals and developed a prediction model (IT-3) based on three non-invasive factors from a training cohort of 289 cases and validated in an external validation cohort of 156 cases. We found that lower HBeAg, higher AST, and lower PLT were high-risk factors for

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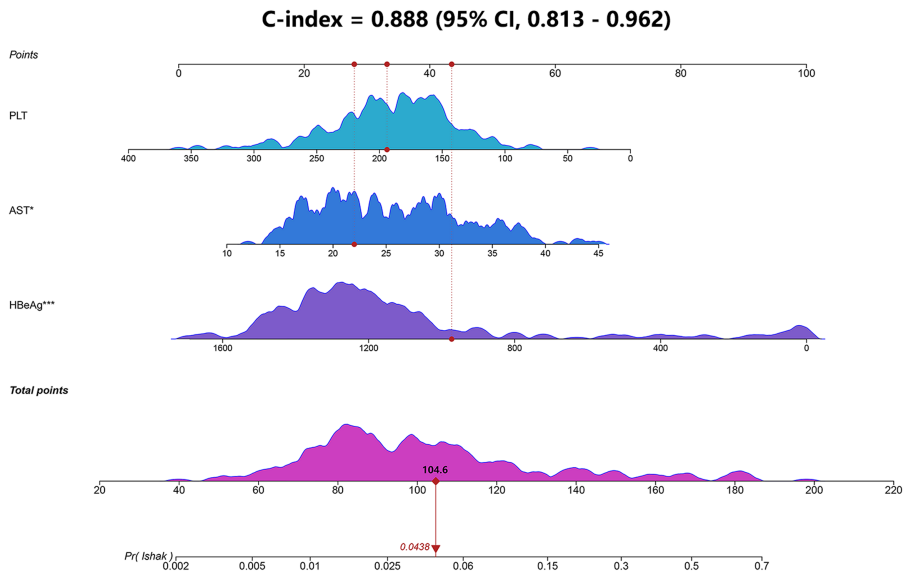

FIGURE 2
Nomogram (IT-3) for predicting liver fibrosis in IT-phase patients. The IT-3 model was developed using the training cohort and discrimination was evaluated by concordance index (Cindex). AST, aspartate transaminase; HBeAg, hepatitis B e-antigen; PLT, platelet.

TABLE 3 Discrimination of the IT-3 model and other non-invasive models.

	AUC (95%CI)	*p*-value[a]	NRI (95%CI)[b]	*p*-value	IDI (95%CI)[c]	*p*-value
Training cohort						
IT-3	0.888 (0.813–0.962)	–	–	–	–	–
GPR	0.731 (0.641–0.821)	0.007	1.27 (0.938–1.610)	<0.001	0.21 (0.124–0.302)	<0.001
APRI	0.740 (0.646–0.834)	0.001	1.36 (1.023–1.691)	<0.001	0.22 (0.134–0.302)	<0.001
FIB-4	0.645 (0.546–0.743)	<0.001	1.40 (1.086–1.704)	<0.001	0.23 (0.142–0.317)	<0.001
Validation cohort						
IT-3	0.833 (0.695–0.970)	–	–	–	–	–
GPR	0.669 (0.522–0.815)	0.147	1.21 (0.750–1.672)	<0.001	0.29 (0.145–0.433)	<0.001
APRI	0.616 (0.453–0.779)	0.009	0.97 (0.466–1.480)	<0.001	0.30 (0.153–0.441)	<0.001
FIB-4	0.631 (0.484–0.777)	0.050	1.00 (0.496–1.507)	<0.001	0.29 (0.149–0.426)	<0.001

APRI, aspartate aminotransferase-to-platelet ratio index; AUC, the area under curve; CI, confidence interval; FIB-4, fibrosis index based on the four factors; GPR, gamma-glutamyl transpeptidase to platelet ratio; IDI, integrated discrimination improvement; NRI, net reclassification improvement.
[a] Compared with the IT-3 model.
[b,c] NRI or IDI > 0 indicated the new model (IT-3) had better prediction performance than reference model (GPR, APRI or FIB-4). Cut-off of NRI: 0.2, 0.4.

significant liver fibrosis in these patients. Base on the ROC, NRI and IDI analysis, the IT-3 model showed good prediction performance in predicting significant liver fibrosis and outperformed conventional models (APRI, GPR and FIB-4) in both training and validation cohorts. We demonstrated its good reliability and robustness by using advanced statistical methods (brier score and 1,000-time bootstrap validation). The risk scores calculated by the IT-3 model and the histology scores obtained from liver biopsies were in good agreement, indicating the ability of our model in assessing liver fibrosis. We also developed an online dynamic nomogram to make it easier to apply in clinical practice.

APRI (16), FIB-4 (17), and GPR (18) were non-invasive models commonly used for liver fibrosis assessment. However, we found that these ratio models did not show excellent performance in IT-phase patients. It might be attributed to the fact that the indicators used for prediction in the IT phase were almost entirely within the normal range, which limited the ability to assess of these ratio models. Therefore, the inclusion of virological indicators was necessary for liver fibrosis assessment in IT-phase patients. Several studies (20, 21) constructed non-invasive models to predict the risk of liver fibrosis for IT-phase patients, but the number of cases in the training cohorts was relatively small. Beyond this, external validation, model calibration, and decision curve analysis were not performed in these studies. Our model addressed these deficiencies and showed better discrimination. In comparison to the fibrosis staging diagnostic model developed by Wu et al. (22), our study also showed better discrimination, sensitivity and specificity in predicting significant liver fibrosis.

AST and HBeAg were independent predictors of liver fibrosis in IT-phase patients. ALT and AST were found in the cytoplasm

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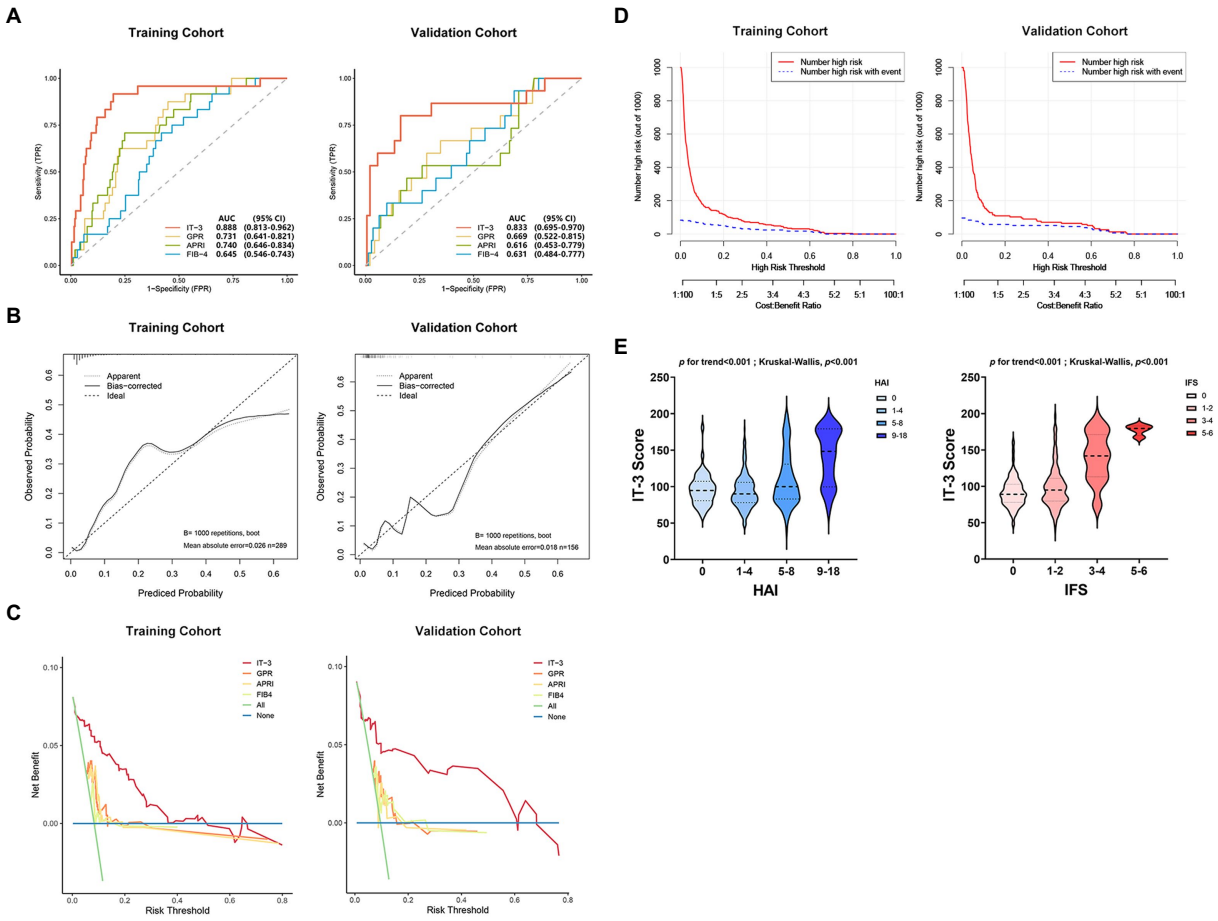

FIGURE 3
The IT-3 model performance evaluation. **(A)** Comparison of the area under the curve (AUC) between the IT-3 model and other nonivasive models. **(B)** Calibration curves. **(C)** Comparison of the decision curve analysis between the IT-3 model and other nonivasive models. **(D)** Clinical impact curves. **(E)** Relationship between IT-3 scores and liver pathology. APRI, aspartate aminotransferaseto-platelet ratio index; AUC, the area under the curve; FIB-4, fibrosis index based on the four factors; GPR, gamma-glutamyl transpeptidase to platelet ratio; HAI, histology activity index; IFS, Ishak fibrosis score.

TABLE 4 Performance and stability of the IT-3 model.

	Training cohort (*n*=289)	Validation cohort (*n*=156)
Brier score	0.06	0.06
Sensitivity (%)	91.7	80.0
Specificity (%)	70.2	83.0
1,000-time bootstrap AUC (95% CI)	0.888 (0.810–0.947)	0.833 (0.687–0.950)
1,000-time bootstrap accuracy (%)	90.9	91.3

AUC, the area under curve; CI, confidence interval.

and mitochondria, respectively. Thus, the rise in AST implied a deeper extent of liver injury and a greater likelihood of inflammatory infiltrates and desmoplasia, which might explain why AST, but not ALT, was an independent predictor in this study. Another important finding was that HBeAg levels were inversely correlated with the extent of liver fibrosis. HBeAg is an important indicator of viral replication and activity. However, when it was at a low level in IT-phase patients who were not receiving antiviral treatment, a possible explanation was the presence of immune-mediated viral clearance in the liver and it was the immunological reaction results in liver fibrosis. In fact, it was inaccurate to determine pathological status only based on the upper limit of normal (ULN) of transaminase. We observed that ALT and AST showed an increasing trend with increasing liver fibrosis, although the transaminases were within normal ranges. These findings suggested that it might be more beneficial for IT-phase patients to start antiviral therapy at a lower ULN, no longer using 40 U/L as the ULN for ALT, which was also consistent with some guidelines and opinions (9, 23). We also discovered that patients with significant fibrosis had lower levels of HBsAg and HBV DNA than patients with no or minor fibrosis, which was in line with previous studies (24–26) that found a negative correlation between these virological indicators and the stage of fibrosis in HBeAg-positive CHB patients.

There were some limitations to our study. Although this study was a multi-center study, the participants were all Chinese, and the majority of patients were of Asian ethnicity with genotypes B or

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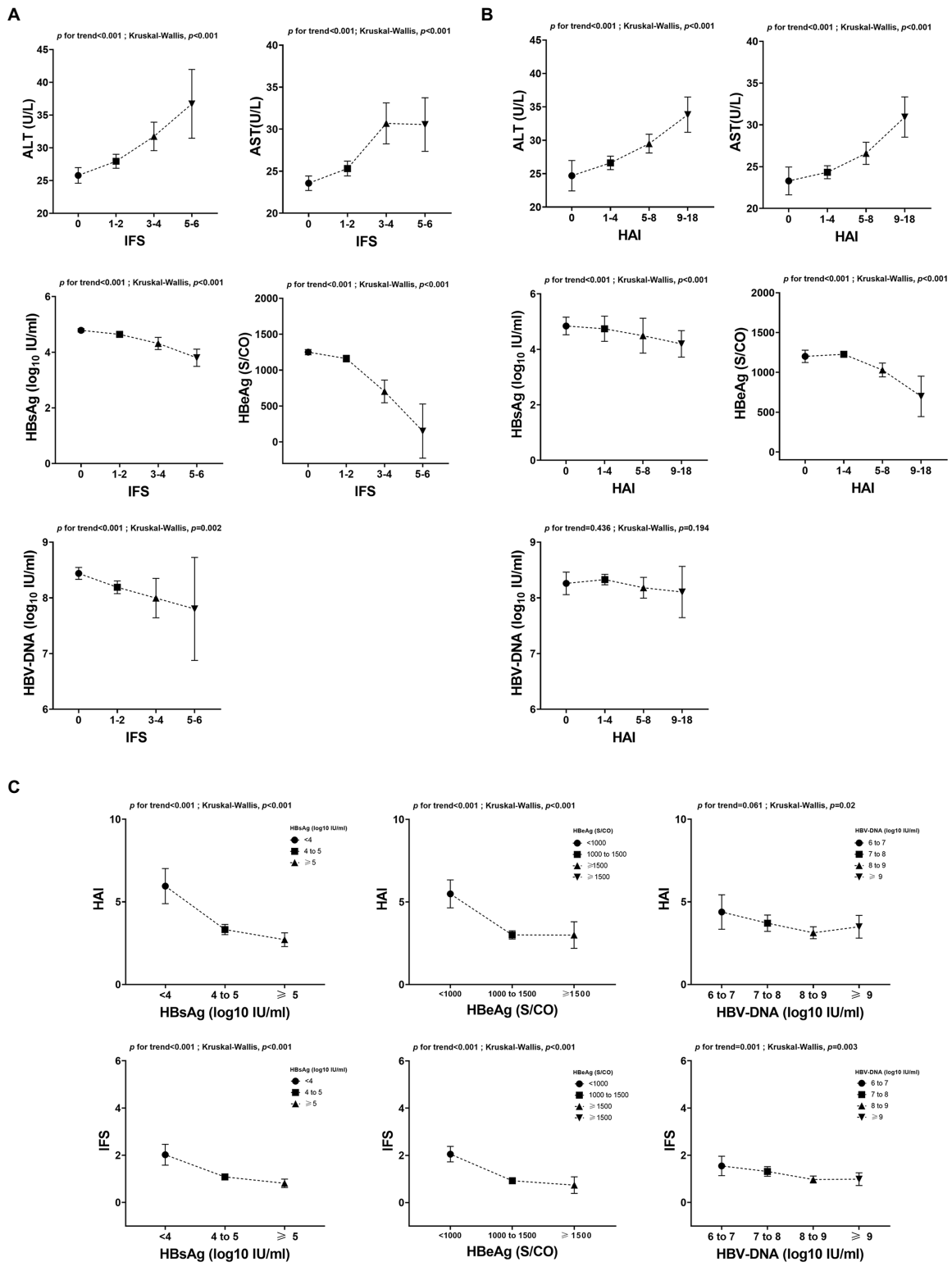

FIGURE 4
Relationship between serological indicators and liver pathology. **(A)** Relationship between serological indicators and the extent of fibrosis. **(B)** Relationship between serological indicators and the extent of inflammation. **(C)** Relationship between liver pathology and different levels of virological indicators. ALT, alanine aminotransferase; AST, aspartate transaminase; HAI, histology activity index; HBeAg, hepatitis B e-antigen; HBsAg, hepatitis B surface antigen.

C. The efficacy of this model for other races and genotypes remains to be validated. Second, the individuals in this study were all older than 18 years, which might limit the applicability in pediatric IT-phase patients. Third, we did not include transient elasography as a predictor variable when developing our model due to limited availability in China.

In conclusion, this study has developed a non-invasive and accurate model to predicting liver significant fibrosis for pseudo-immune tolerance patients and to provide more suitable therapeutic treatment regimens.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The studies involving human participants were reviewed and approved by the Dongzhimen Hospital affiliated to Beijing University of Chinese Medicine. The patients/participants provided their written informed consent to participate in this study. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

Author contributions

YY and YX: study concept and design. YX: acquisition of data. SL and ZL: analysis and interpretation of data and drafting of the manuscript. XL, HD, DG, XZ, and XY: critical revision of the manuscript for important intellectual content. XL and YY: study supervision. All authors read and approved the final version of the manuscript.

Funding

This work was supported by grants from the National Major Science and Technology Projects of China (No. 2018ZX10725505), the National Natural Science Foundation of China (No. 82174341), and the Beijing University of Chinese Medicine Major Project (No. 2020-JYB-ZDGG-115).

Acknowledgments

We want to give special thanks to Weiwei Li (Medical Examination Center, The Chronic Disease Hospital of Shandong Province) and Roger Marshall (School of Population Health, The University of Auckland) for advice on data analysis.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpubh.2023.1137738/full#supplementary-material

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frontiers | Frontiers in Medicine

TYPE Review
PUBLISHED 12 April 2023
DOI 10.3389/fmed.2023.1147782

OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to Gastroenterology a section of the journal Frontiers in Medicine

RECEIVED 26 January 2023
ACCEPTED 17 March 2023
PUBLISHED 12 April 2023

CITATION
Shi H, Dai H, Sun Q, Wang S and Chen Y (2023) CD73, a significant protein in liver diseases. *Front. Med.* 10:1147782. doi: 10.3389/fmed.2023.1147782

CD73, a significant protein in liver diseases

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Purine adenosine pathway exists widely in the body metabolism, and is involved in regulating various physiological processes. It is one of the important pathways of environmental regulation in human body. CD73 is essentially a protease that catalyzes further dephosphorylation of extracellular adenine nucleotides, hydrolyzing extracellular AMP to adenosine and phosphate. CD73 is an important part of the adenosine signaling pathway. Studies have shown that CD73-mediated adenosine pathway can convert the inflammatory ATP into the immunosuppressant adenosine. This paper aims to summarize the relevant effects of CD73 in the occurrence, development and prognosis of liver diseases such as viral hepatitis, highlight the important role of CD73 in liver diseases, especially in viral hepatitis such as HBV and HCV, and explore new clinical ideas for future treatment targets of liver diseases.

KEYWORDS

CD73, adenosine pathway, liver diseases, HBV, HCV

1. Introduction

As of late, the frequency of liver diseases has expanded decisively year by year, and liver diseases have turned into a significant clinical issue on the planet. It is estimated that 1.5 billion individuals worldwide suffer from the ill effects of persistent chronic liver diseases (1). The Asia-Pacific region accounted for 62.6 percent of liver disease deaths globally in 2015, as per the Lancet Commission on Gastroenterology and Hepatology. Hepatitis virus infection, particularly the transmission of hepatitis B virus (HBV), is the essential driver of death in more than half part of patients with cirrhosis (2). In 2017, the World Health Organization announced that there are 324 million people with viral hepatitis around the world, and 1.34 million individuals pass away from the infection every year. In this way, it is of extraordinary importance to investigate the inward physiological system and biomolecular focuses of liver diseases, especially for viral hepatitis, to work on the better quality of human life.

Purinergic signaling was first proposed and laid out by the notable scientist Jeffrey Bernstock (3). It is basically composed of adenosine triphosphate (ATP), adenosine diphosphate (ADP), adenosine monophosphate (AMP), various adenosine kinases and receptors. It is ubiquitous in the entire body metabolism (4) and has been widely studied and discussed in various diseases. As a major adenosine kinase, CD73 is a critical component of the extracellular adenosine pathway and can be expressed and labeled on an assortment of cell surfaces. CD73 is generally present in liver tissues and profoundly expressed in liver pathology, which indicates that CD73 assumes an important part in liver diseases. This paper aims to summarize the relationship between CD73 and liver physiological and pathological phenomena, highlight the significance

of CD73 in liver diseases, in order to reveal potential biological links and provide new ideas for clinical treatment strategy innovation.

2. Manuscript formatting

2.1. Biological characteristics of CD73

CD73, complete name extracellular-5′-nucleotide enzyme, is a 70-kD glycoylphosphatidylinositol (GPI), a multifunctional transmembrane glycoprotein anchored to the surface of cell membranes by 523 amino acids encoded by NT5E gene (located at 6q14-21). As an exonucleotide enzyme, CD73 has enzyme-induced and non-enzymatic functions in cells, which can catalyze further dephosphorylation of extracellular adenine nucleotides and hydrolyze extracellular AMP into adenosine and phosphate (5), acting an important role in purinergic signaling pathways (Table 1).

As an important downstream piece of the extracellular adenosine pathway, CD73 can convert AMP from upstream extracellular ATP and ADP hydrolyzed by cell surface enzymes such as CD39, ALP and NTPases into adenosine (ADO). Including the binding of four G-protein-coupled receptors (GPCR) subtypes A1, A2A, A2B, and A3 adenosine receptors (ARs) (6), activating a multi-step coordinated cascade of intracellular signaling pathways (7), and regulating the aggregation and dispersion of proteins all through cells by changing the CD73 enzyme activity in purine metabolism (8). Jointly play anti-inflammatory, dilated blood vessels and many other functions (Figure 1).

CD73 is expressed in a grouping of cell types, including leukocytes, myofibroblasts, endothelial cells and epithelial cells, and so on (9), particularly in tumor, immune and other related cells (10), such as macrophages, myofibroblasts, dendritic cells and NK cells (11), etc. CD73 is also expressed in neutrophils to a certain extent, which can propel liver regeneration and regulate inflammation (12). In ongoing years, the mechanism of adenosine generating enzyme CD73 has been preliminarily researched in liver diseases, including viral hepatitis, hepatic steatosis, hepatic fibrosis and hepatocellular carcinoma. Continuous examinations have shown that under the influence of multiple factors, CD73-mediated adenosine metabolism is immovably connected with the liver and dynamically regulates various pathological manifestations such as liver steatosis, inflammation, fibrosis and primary tumor (13), prompting the occurrence of all kinds of liver diseases.

2.2. CD73 and viral hepatitis

As a universally prevalent chronic liver disease, viral hepatitis is a typical common infectious disease caused by a variety of hepatotropic viruses. It can cause intense and persistent liver inflammation in people, and then develop into cirrhosis and liver cancer, which seriously endangers human wellbeing globally. Purinergic signaling is firmly related to the generation and progression of liver inflammation (14). As a perilous signal that promotes inflammation, ATP has an invigorating unstable quality (15). On the contrary, ADO has anti-inflammatory effects on immune cells and can safeguard tissue integrity (16). As a key component of the adenosine pathway, CD73 is one of the cell surface compounds that decompose extracellular ATP into ADO, promoting the transformation of the body from the pro-inflammatory environment stimulated by ATP to the anti-inflammatory environment directed by adenosine (13).

After the liver is hit by the virus infection and produces inflammation, the level of ATP which is a dangerous signal of extracellular inflammation increases significantly (17), stimulates and induces neutrophils to aggregate and produce chemokines (18), and recruit immune cells with high expression of CD73 (19), deplete extracellular ATP to produce adenosine, inhibit the activation of immune cells, and control the inflammatory response (10). Simultaneously, activation of adenosine receptors can increment intracellular AMP concentration, structure a partition hindrance,

TABLE 1 English abbreviation comparison table.

Full name	Abbreviation	Full name	Abbreviation
Ecto-50-Nucleotidase	CD73	Primary biliary cirrhosis	PBC
Adenosine triphosphate	ATP	Primary sclerotic cholangitis	PSC
Adenosine diphosphate	ADP	Non-alcoholic fatty liver disease	NAFLD
Adenosine monophosphate	AMP	Bile duct ligation	BDL
Cyclic adenosine monophosphate	cAMP	T1 helper cells	Th1
Adenosine	ADO	T17 helper cells	Th17
T regulatory cell	Treg	T helper cells	Th
Hepatitis B virus	HBV	Aatural killer T cells	NKT
Hepatitis C virus	HCV	Hepatic stellate cells	HSC
Hepatitis E virus	HEV	Thioacetamide	TAA
Chronic hepatitis B	CHB	Alcoholic liver fibrosis	ALF
Chronic hepatitis C	CHC	Tumor microenvironment	TME
T Effector cell	Teff	Myeloid derived suppressor cells	MDSC
Autoimmune liver disease	AILD	Hepatocellular carcinoma	HCC
Autoimmune hepatitis	AIH	Vascular endothelial growth factor	VEGF

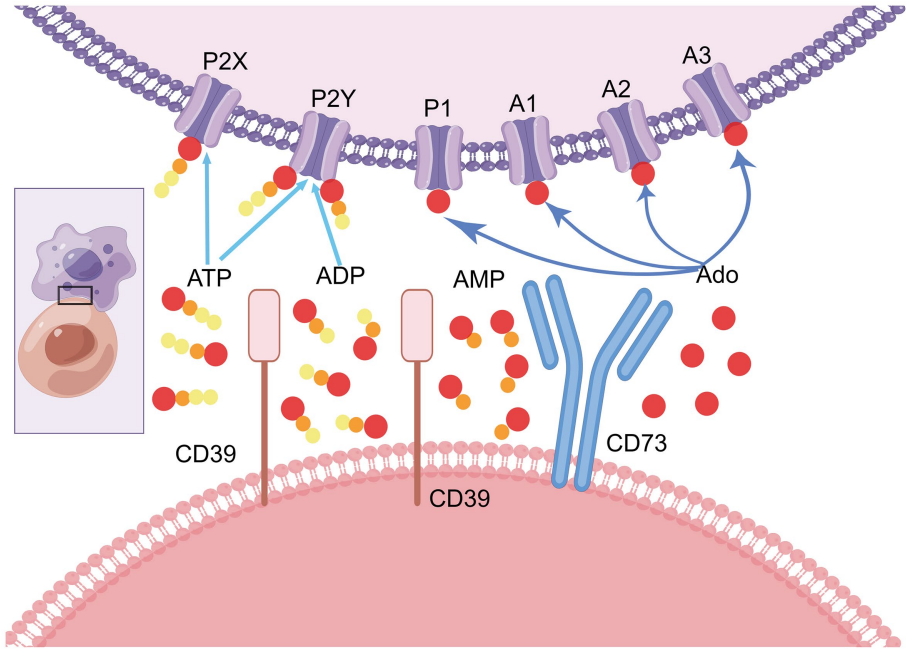

FIGURE 1
Adenosine signaling mediated by CD73. Affected by various environmental factors, extracellular pro-inflammatory risk factor ATP can bind to receptors such as P2X and P2Y to induce intense inflammation in the human body. Under the guidance of extracellular inflammation and energy balance mechanism, excessive pro-inflammatory ATP is gradually dephosphorylated to form AMP with the help of cell surface enzymes such as CD39, ALP and NTPases. AMP is further dephosphorylated by CD73 to produce adenosine which has immunosuppressive effects. Adenosine can tie to various adenosine receptors on cell surface like P1, A1, A2 and A3, and participate in various physiological and pathological reactions of the body, playing different functions and roles. CD73 is the absolute most significant hydrolytic protease for the conversion of AMP to adenosine. It is a critical step before adenosine binds to adenosine receptors and plays a vital role in the regulation and conduction of purine signaling pathways (by figdraw, export ID: YIYRA26629).

block intercellular substance trade, and avoid the abnormal accumulation of immune cells to stimulate inflammation (20). CD73-intervened adenosine pathway takes immune cells such as B cells and Tregs as the principal carriers to promote adenosine production and suppress immunity, which plays an important role in the pathogenic process of several common hepatitis viruses and is considered as a new possible therapeutic target for liver viral inflammatory diseases (21).

In patients with chronic hepatitis B (CHB), when liver inflammation occurs, CD73 on the surface of B cells is diminished, the amount of extracellular adenosine production is decreased, and the activation of B cells is enhanced, prompting the progression of inflammation (22). Blocking CD73 activity in CHB patients can lead to impaired IgG conversion of B cells, temporarily slowing down humoral immune inflammation, but further aggravating later inflammation in the long term (23). As of now, there is no clear report on the internal mechanism of CD73 increase in Treg cells of patients with hepatitis B, but some studies have shown that CD73 is an important regulator of Treg cells to restrain intracellular environmental inflammation, and the author conjectures that it may be related to the independent expression of CD73 in Treg cells under inflammatory environment and the immunosuppressant effect mediated by adenosine (24). In addition, studies have found that the expression level of CD73 decreases with the increase of HBV-DNA load and liver inflammatory response. In patients with complete antiviral response, the amount of CD73 can be gradually recovered with the transformation of serum HBeAg or the reduction of HBsAg, yet after effective antiviral treatment, the expression of CD73 does not increase (22).

In addition to hepatitis B, TOX+ HCV-specific CD8 + T cells in patients infected with hepatitis C virus (HCV) express a mass of CD73 characteristic memory phenotype (25), especially in activated Treg cells (26), playing an important role in inflammation in patients with chronic hepatitis C (CHC). ZhiqinLi believes that during antiviral treatment, the overall number of Treg cells expressing higher levels of CD73 in CHC patients show a downward trend, which also explain the reason why NatashaT. Snider found in the study that the liver CD73mRNA level of CHC patients is significantly reduced (9). This phenomenon is more obvious in liver fibrosis caused by HCV (10).

In patients with chronic hepatitis E, the expression of CD73 in different cells is also significantly different. For example, studies found that the expression of CD73 on the surface of Tregs and effector T cells (Teff) in patients infected with hepatitis E virus (HEV) was increased. Moreover, the inhibitory ability of Treg cells in patients with acute hepatitis E is obviously higher than that in recovered individuals (27), which might be influenced by CD73-mediated adenosine pathway during the course of the disease. However, the activity and function of CD73 on B cell surface under HEV invasion need to be further investigated.

As for hepatitis A and hepatitis D, few similar articles have mentioned the effect of CD73 on hepatitis A. Undeniably, we found that the infection of hepatitis D depends on the replication of hepatitis B virus itself, and adenosine receptors are the necessary proteins for human hepatocytes to infect two viruses (28). However, no studies

have discussed the effect of CD73 on hepatitis D through adenosine pathway, so the intervention of CD73 on the pathogenesis of hepatitis A and hepatitis D needs to be further explored.

2.3. CD73 and other liver diseases

With the improvement of modern living standards, coupled with unhealthy diet and hygiene habits, the incidence of various liver diseases increases year by year (29). Various chronic liver diseases persist and are prone to progress to irreversible end-stage liver disease, affecting normal metabolism of the body and eventually leading to death (30).

Studies have proven that CD73 is expressed at a high level in liver tissues (31), which is mainly distributed in the apical membrane of hepatocytes and endothelial cells of hepatic sinuses and bile ducts, and is expressed in bands in pericentral hepatocytes near the central vein of the liver (32). In CD73-deficient hepatocytes, AMP-dependent protein kinases are affected, resulting in the destruction of liver homeostasis and unexpected liver injury (33). What's more, the expression of CD73 is highly regulated in chronic liver diseases (9). In terms of current research progress, CD73 can inhibit the progression of viral hepatitis to a certain extent, promote the formation of fatty liver, delay the progression of steatohepatitis, and promote the progression of liver fibrosis and liver cancer through the adenosine pathway.

2.3.1. CD73 and autoimmune liver disease

Autoimmune liver disease (AILD) is a chronic, progressive immune-related liver disease caused by the activation of the body's immune system function due to various unknown reasons. As the final rate-limiting enzyme of adenosine production, CD73 mediates purine signaling pathway, plays an important part in regulating adenosine and immune diseases (25). Tregs act an irreplaceable role in AILD (34). Studies indicate that autoimmune hepatitis (AIH) and primary biliary cirrhosis (PBC) are closely related to adenosine pathway mediated by CD73 on Treg surface.

AIH, as a chronic liver disease caused by abnormal activation of immune cells leading to interfacial hepatitis (35), is a common clinical AILD type (36). CD73-mediated adenosine pathway is immunosuppressive toward AIH, and Treg impairment and Teff subgroup activation are typical manifestations of AIH pathogenesis (13). CD73 around the surface of normal Treg cells mediates the production of immune-suppressing adenosine (24), blocks cell communication, inhibits overimmunity, and simultaneously upregates CD73 expression (10). However, the level of CD73 on the damaged Treg surface was down-regulated (37), the secretion of TGF-β and other anti-inflammatory factors was reduced (38), and the immunosuppressive function of Treg was defective (39), leading to the occurrence of AIH. On the other hand, IL-6 and TGF-β can induce the expression of CD39 and CD73 in helper T17 cells (Th17), stimulate the production of adenosine, inhibit the transformation of naive T cells into Th (40), along with reducing the production of pro-inflammatory cytokines (41), which can be charactered as immune deficiency. On the contrary, low level of CD73 stimulates the production of pro-inflammatory factors in liver cells, and the immune effect of Th17 cells continue (42), leading to the continuous progression of chronic liver inflammation.

PBC is an immune-related liver disease characterized by diffuse destruction of small bile ducts in the liver (43). The literature indicates that there may be significant individual differences in the mechanism of PBC effect (44). There are few studies on the progression of CD73 in PBC disease. Due to immune deficiency, CD73 expression in dnRIITreg from PBC mice is significantly reduced compared with WTTreg. Studies found that there are certain differences in the expression profiles of CD39 and CD73 on Tregs, which can form energy circle outside immune cells and mediate a series of immune responses, which may be close to the pathogenesis of PBC, and its internal mechanism is worthy of further lucubrating (37).

Unlike PBC, the lesions of primary sclerosing cholangitis (PSC) are mainly in the bold ducts inside and outside the liver. At present, relevant studies on the pathogenesis of CD73 in PSC are still lacking, but previous studies have shown that the loss of CD39 in the adenosine pathway can stimulate the increase of intestinal endocrine ATP, activate dendritic cells and CD8 + T cells and transport them to the liver. Damage to biliary epithelial cells induces PSC (45), which also proves that adenosine pathway is closely connected to the progression of PSC disease. Therefore, the role of CD73 in the course of PSC disease needs to be further analyzed.

2.3.2. CD73 and fatty liver disease

Liver is an important organ for ethanol metabolism, and a large amount of ethanol metabolism tend to have toxic effects on the liver, resulting in hepatocyte damage (46), and then abnormal accumulation of metabolism-related fat, leading to hepatic steatosis. Studies have confirmed that CD73 activity is associated with ethanol-induced hepatic steatosis, mice lacking CD73 show less cell expansion and steatosis, significantly reducing the incidence of fatty liver (47). Wang, Ping et al. hypothesized that CD73-deficient mice may reduce adenosine-mediated extracellular matrix deposition through hepatic stellate cells, thereby protecting mice from ethanol induced fatty liver (10). Under chronic alcohol stimulation, AMP is released in the liver (48) and phosphorylated to adenosine catalyzed by high expression of CD73 on the cell surface, adenosine A1 and A2B receptors are activated and promote lipid depositional degeneration (47). At the same time, ethanol absorption also reduces the influx of nucleoside transporter (49), inhibits intracellular adenosine uptake and increases extracellular adenosine concentration (50), promoting the progression of fatty liver. Therefore, blocking the expression of CD73, A1 or A2B receptors in the liver can effectively reduce the accumulation of liver lipids caused by alcohol and delay the course of fatty liver disease (26).

At the same time, non-alcoholic fatty liver disease (NAFLD) is another important cause of hepatic steatosis in modern society (51). Non-alcoholic steatohepatitis (NASH), as a typical inflammatory disease of NAFLD, is closely related to the adenosine pathway. As a hydrolytic product of CD73, adenosine can perform cell protective and immunosuppressive functions through P1 receptors, thus terminating liver inflammation and promoting liver regeneration. In addition, CD73 can block the TLR4/MyD88/NF-κB signaling pathway (52), reduce the secretion of IL-6 and IL-1β, and delay the inflammatory process. In liver biopsies of NAFLD patients, CD73mRNA levels were significantly reduced (9), so CD73 knockout mice rarely developed fatty liver disease, or even progressed to steatohepatitis (13). However, under chronic inflammatory stimulation, damaged inflammatory liver cells can induce the approach of extracellular immune cells with high expression of CD39

and CD73, clear extracellular ATP, generate adenosine negative feedback to regulate endothelial cells and immune cells, inhibit white blood cell recruitment, and reduce the inflammatory response. The study on the connection between CD73-related adenosine metabolism pathway and hepatic fatty lesions is worth further exploration.

2.3.3. CD73 and liver cirrhosis

Hepatic fibrosis is a reaction of repeated prolongation of various chronic liver lesions, causing liver self-limiting healing (53), which can lead to the formation of cirrhosis. CD73, as the final rate-limiting enzyme produced by adenosine (25), has an important place in the process of liver fibrosis. CD73 is weakly expressed in normal hepatic stellate cells and portal vein fibroblasts, while its activity is enhanced in hepatic fibrosis (54) and significantly increased in cirrhosis (55). Among them, hepatic stellate cell (HSC) activation is a pivotal feature of hepatic fibrosis, CD73 and HSC activation interact with each other to jointly promote the process of hepatic fibrosis (56). For example, the CD73-adenosine-A1R axis regulates HSC activation and apoptosis through the PLC-IP3-Ca2+/DAG-PKC signaling pathway (57). CD73-deficient mice are resistant to the development of liver fibrosis (58) and protect liver cells from the risk of CCl4 and thioacetamide (TAA) inducing liver fibrosis (59). After CD73 deletion, adenosine production is reduced, resulting in the suppression of HSC activation and proliferation mediated by p2 receptor and decreased collagen expression, inhibiting the production of liver fibrosis. Meanwhile, inhibition of CD73 can promote HSC apoptosis and alleviate alcohol-induced liver fibrosis (52). Similarly, A2A adenosine receptor deficient mice were also protected from the effects of liver fibrosis by blocking the adenosine pathway (60). Just the opposite, after alcohol intake, CD73 is activated in acetaldehyde induced HSC, and the expressions of pro-fibrotic cytokines TGF-β, α-SMA and type I and III collagen are increased (61), promoting the generation of liver fibrosis. Activated HSC can up-regulate CD73 expression through specific SP1 and SMAD promoter elements (58). These studies have verified the intrinsic influence of CD73 expression and HSC activation and their co-promoting effect on liver fibrogenesis. Therefore, blocking CD73 expression may be an important approach for the treatment of hepatic fibrosis.

2.3.4. CD73 and hepatocellular carcinoma

Hepatocellular carcinoma (HCC) is one of the most common causes of cancer-related death and is one of the most frequent malignant lesions of digestive tract in the world (62). A large number of literatures have elaborated that CD73 is an important regulatory protein in the progression of various malignant tumors and is highly expressed in cancer tissues (63). CD73 was significantly increased in HCC patients and negatively correlated with overall survival (64). Studies have stated that CD73 is highly expressed in about 50% of HCC samples (55), which promotes the progression and metastasis of tumors, and can be used as a reference indicator for poor prognosis of HCC clinical outcomes (65). Recent studies claimed that tumor microenvironment (TME), as the basis for tumor survival, provides the driving force for tumor proliferation and metastasis (64). Purinergic signaling pathway is the main immunosuppressive mechanism of TME (66). As one of the core enzymes of adenosine pathway, CD73 is expressed on the surface of tumor endothelial cells, regulatory T cells (Treg), NK cells, medullogenic suppressor cells (MDSC), tumor-associated macrophages and other cells of TME. Regulated by epidermal growth factor receptor (HER) and other molecules (67), TME can induce immune escape to improve intracellular AMP level and start downstream signaling pathways, such as promoting proliferation induction of Tregs (68), blocking Teff aggregation and invasion (69), reducing NK cytotoxicity (70), and stimulating MDSC and macrophage polarization (71). Inhibit the production of cytokines, reduce the antigen-presenting effect of tumor (72), and inhibit anti-tumor response. CD73 has also been verified to motivate the increase of tumor vascular endothelial growth factor (VEGF), promote angiogenesis (73) and help tumor cells survive (74). Additionally, CD73 may also be in relation to inflammatory cancer signal transduction in liver cancer. Through A1R, A2AR, A2BR, and A3 adenosine receptor signaling pathways, CD73 stimulates inflammation, provides a suitable pro-inflammatory environment for tumor cells to survive, and plays an important role in the occurrence, development and metastasis of hepatocellular carcinoma. As mentioned above, inflammatory cells generally have low expression of CD73, while HCC cells with high expression of CD73 release a large number of inflammatory factors, which may be due to the fact that adenosine pathway is not the main pathway for the generation of inflammation in tumor cells. The influence of CD73 on the relationship between inflammatory and carcinoma transformation needs to be further explored (Tables 2, 3).

3. Conclusion

The latest World Health Organization figures for 2020 show that 325 million people worldwide are living with viral hepatitis B and C. Each year there are 900,000 deaths due to hepatitis B virus infection. Back in 2016, the World Health Organization set a goal of eliminating hepatitis B as a public health threat by 2030. Hepatitis virus infection is a serious harm to human health, and it is still a global public health problem worthy of attention.

The incidence of liver virus infection is related to the number of virus replication and the strength of the body's immunity. The progression of the disease is due to the continuous replication of the virus and the poor immunity of the body, resulting in progressive damage to the liver cells, causing a series of serious consequences. At present, antiviral therapy is difficult to achieve the ideal state of completely removing virus from the body. Therefore, the focus of treatment of viral hepatitis is to regulate the immune function of the body and weaken the damage of virus metabolism to the liver.

Studies have confirmed that the CD73-mediated adenosine pathway has a profound effect on the activated immune response of B cells. The activation of B cells induced by low expression of CD73 is an essential part of the effective immune response against HBV infection and a reference idea for the future treatment of viral liver inflammation and restoration of liver immune homeostasis. On the other hand, CD73 content increases, inducing adenosine-mediated immunosuppression. Whether this is also influenced by liver pathological microenvironment such as hepatitis B viral load, liver inflammation and antiviral intervention, and the role of CD73 in cellular immunity needs to be further studied.

Similarly, under the stimulation of hepatitis C and hepatitis E virus, the expression of CD73 on the surface of activated Treg increased, but the number of Treg cells in patients with hepatitis C decreased, so the overall content of CD73 decreased, inducing immune inflammation in the liver. However, there is still a lack of

TABLE 2 List of related mechanisms of CD73 in liver diseases.

Type of liver disease	Correlation with CD73 and relevant mechanism of action	References
Viral hepatitis	The virus invades the liver, significantly increases ATP levels, stimulates and induces neutrophils to produce chemokines, recruits immune cells with high expression of CD73, consumes extracellular ATP to produce adenosine, inhibits immune cell activation, and controls inflammation	(10, 17–19)
	Activation of adenosine receptors increases intracellular AMP concentration, forming a barrier that blocks the exchange of substances between cells and prevents the abnormal accumulation of immune cells from stimulating inflammation	(20)
	In CHB patients, CD73 and extracellular adenosine production on the surface of B cells at the site of liver inflammation were decreased, and the activation of B cells was enhanced through IgG conversion, inducing inflammation progression	(22, 23)
	CD73 expression decreased with the increase of HBV-DNA and liver inflammatory response. In patients with complete antiviral response, CD73 levels recovered gradually with serum HBeAg conversion or HBsAg reduction, and CD73 did not increase after effective antiviral therapy	(22)
	Activated Treg cells in CHC patients showed high expression of CD73, but the number of Treg cells decreased, resulting in a significant decrease in overall CD73 levels	(9, 25)
	The expression of CD73 on the surface of Treg and Teff cells was increased in patients with hepatitis E, but the inhibition ability of Treg cells was also increased	(26)
Autoimmune hepatitis	The level of CD73 on the surface of AIH-damaged Treg cells was down-regulated, and the secretion of TGF-β and other anti-inflammatory factors was reduced, leading to the deficiency of immunosuppressive function of Treg	(36–38)
	IL-6 and TGF-β induced decreased CD73 expression in Th17 cells, blocked adenosine production, stimulated more naive T cells to transform to Th, produced pro-inflammatory cytokines, and increased inflammation	(39, 40)
	Low expression of CD73 stimulates the production of pro-inflammatory factors in hepatocytes, and low level of A2A receptor on Th17 cell surface activates, and the immune effect continues, leading to chronic liver inflammation	(41)
Primary biliary cirrhosis	Due to immune deficiency, the expression profiles of CD39 and CD73 on Treg are different to some extent, which can form "purinergic halo" outside immune cells and mediate a series of immune responses, which may be closely related to the pathogenesis of PBC	(36, 43)
Primary sclerosing cholangitis	The deletion of CD39 can stimulate the increase of intestinal endocrine ATP, activate dendritic cells and CD8 + T cells and transport them to the liver, and damage biliary epithelial cells to induce PSC. The correlation with CD73 remains to be studied	(44)
Fatty liver disease	CD73-deficient mice may protect against etho-induced fatty liver by reducing adenosine-mediated extracellular matrix deposition through hepatic stellate cells	(10)
	Under chronic alcohol stimulation, AMP is released in the liver and phosphorylated to adenosine catalyzed by high expression of CD73, which binds to cell surface adenosine A1 and A2B receptors and promotes lipid depositional degeneration	(46, 47)
	Alcohol intake reduces the influx of nucleoside transporters, inhibits intracellular adenosine uptake, and leads to increased extracellular adenosine concentration, promoting the development of fatty liver	(48, 49)
	Overexpression of CD73 can block TLR4/MyD88/NF-κB signaling pathway, reduce alcohol-induced liver injury, reduce the secretion of IL-6, IL-1β and other inflammatory cytokines, delay the inflammatory process, promote cell proliferation and inhibit cell apoptosis	(51)
	Endogenous A1A adenosine receptor activation which is mediated by CD73 alleviates ethanol-induced acute liver injury by reducing oxidative stress and lipid accumulation	(25)
Liver cirrhosis	CD73 activity is enhanced when liver fibrosis occurs and expression is significantly elevated in cirrhosis	(53, 54)
	The CD73-adenosine-A1r axis regulates HSC activation and apoptosis through the PLC-IP3-Ca2+/DAG-PKC signaling pathway	(56)
	CD73 defects are resistant to the development of liver fibrosis and protect mice from CCl4 and TAA induced liver fibrosis	(57, 58)
	Absence of CD73 and reduced adenosine production can lead to p2 receptor-mediated HSC activation and proliferation and decreased collagen expression, and inhibit the production of liver fibrosis	(10)
	Mice with deficient A2A adenosine receptors were also protected from liver fibrosis by blocking the adenosine pathway	(59)
	Extracellular ATP stimulation in acetaldehyde-induced HSC induced increased CD73 activation and the expression of TGF-β, α-SMA and type I and III collagen, promoting liver fibrosis	(60)

(Continued)

TABLE 2 (Continued)

Type of liver disease	Correlation with CD73 and relevant mechanism of action	References
	Inhibition of CD73 can promote HSC apoptosis and reduce liver fibrosis induced by alcohol	(51)
	Activation of HSC can promote fibrosis progression by up-regulating CD73 expression through specific SP1 and SMAD promoter elements	(57)
	The knockdown CD73 can significantly increase cell migration and collagen I expression of HSC line, and promote fibrosis process	(60)
Hepatocellular carcinoma	The up-regulation of CD73 expression induces the gradual transformation of immune-activating and tumor-inhibiting ATP into immune-inhibiting adenosine, accumulates and activates adenosine receptors, promotes the proliferation and induction of Treg, blocks Teff aggregation, and inhibits the anti-tumor response of the immune system	(67, 68)
	The up-regulation of CD73 expression stimulates adenosine production, plays an immunosuppressive role, reduces the toxicity of NK cells, reduces the killing cleavage of tumor cells, and enables tumor survival	(69)
	High expression of CD73 induces adenosine generation and activation of adenosine receptors, increases intracellular AMP level, stimulates MDSC and macrophage polarization, inhibits cytokine production, weakens tumor antigen presentation, and inhibits anti-tumor response	(70)
	Under CD73-mediated adenosine activation, A2A receptor is highly expressed on the surface of tumor endothelial cells, stimulating VEGF production and significantly enhancing tumor angiogenesis, especially in early liver cancer	(72, 73)

experimental studies on the effect of CD73 on humoral immunity under hepatitis C and hepatitis E virus infection in the existing literature, which needs to be improved.

As an important link in the adenosine pathway, CD73 co-conducts with upstream ATP and AMP and downstream adenosine and adenosine receptors, and is involved in the occurrence and development of viral hepatitis and other liver diseases. In view of the important role of CD73 and its related metabolic pathways in liver diseases, Targeted intervention of CD73 can be one of the key breakthroughs in the future treatment of liver diseases and restoration of environmental homeostasis in the liver, which has a good application prospect. At present, there have been experimental or clinical reports on related drugs, such as metformin, which can inhibit CHB immune-related pathogenesis by regulating CD73 adenosine pathway (22). In addition, some studies have also found that CD73-related pathways in the field of traditional Chinese medicine. For example, cordycepin, as an adenosine analogue, can specifically activate adenosine receptors and improve chronic inflammation caused by liver fat accumulation in an immunosuppressive environment with high CD73 expression (75). Curcumin can inhibit the carcinogenic effect of aflatoxin on liver to a certain extent through CD73-mediated purine pathway (76), induce the differentiation of bone marrow mesenchymal stem cells, and promote liver regeneration.

Recent studies have proved that there are certain differences in the expression levels of CD73mRNA and protein in two different kinds of mice (9). This also means that the intrinsic influence of species genes on CD73 expression, including viral metabolism in the liver, needs to be further confirmed by further studies.

As an important part of the adenosine pathway, CD73 is the key to the transition from AMP to Ado, which implies partial intervention in energy metabolism and immune regulation. For CD73 itself, it exists as a protein widely spread in various cells of the body, is one of the components of human gene expression. For example, as for liver cancer, CD73 not only acts as an intermediary to help tumor cells evade immune monitoring and regulate internal environmental inflammation, but also provides nutritional support and metastasis pathway to tumor cells by promoting angiogenesis. Therefore, following studies should explore more value of CD73 affecting human metabolism and pathological changes from different new ideas and perspectives.

With the continuous development of more and more experimental studies based on protein CD73, many drugs targeting CD73 have been introduced into the clinic, which can be widely used in the field of liver disease in the future, especially providing new treatment options for patients with chronic hepatitis B and chronic hepatitis C, delaying the onset of cirrhosis and liver cancer, and hopefully improving the quality of life of patients with liver diseases.

Author Contributions

YC conceived and guided the study. HS, HD and QS completed the main part of this work, SW is responsible for the submitting of manuscripts. All authors contributed to the article and approved the submitted version.

Funding

This research was funded by the National Natural Science Foundation of China (NSFC) 81903974 to HS and 82070804 to YC, the Natural Science Foundation of Jiangsu Province BK20221421 to HS, and the Natural Science Foundation of Nanjing University of Chinese Medicine XZR2021022 to HS.

Acknowledgments

This study is related to the project National Natural Science Foundation of China (NSFC) 81903974 to HS and 82070804 to YC. We also appreciate the Natural Science Foundation of Jiangsu Province BK20221421 to HS, and the Natural Science Foundation of Nanjing University of Chinese Medicine XZR2021022 to HS for their financial support of this study.

TABLE 3 List of different effects of CD73 in liver diseases.

Liver diseases		The expression of CD73	Influence of CD73 on various liver diseases	Effects of CD73 blocking in different liver diseases	References
Viral hepatitis	Viral hepatitis B	The expression of CD73 on B cells surface is decreased	The amount of extracellular adenosine production is decreased, and the activation of B cells is enhanced, inducing the progression of inflammation	Blocking CD73 activity can lead to impaired IgG conversion of B cells, temporarily slowing down humoral immune inflammation, but further inducing later inflammation	(22, 23)
		The expression level of CD73 is increased in Treg cells	Treg cells independently express of CD73 under inflammatory environment and mediate the adenosine immunosuppressant effect		(24)
	Viral hepatitis C	The total expression of CD73 content of Treg cells decreases	TOX+ HCV-specific CD8 + T cells, especially activated Treg cells, increased expression of CD73, but the overall number of Treg cells decreased, and therefore overall CD73 content decreased		(25)
	Viral hepatitis E	The expression level of CD73 is increased on cell surface	CD73 expression on Treg and Teff surfaces both increases		(26)
Autoimmune liver disease	Autoimmune hepatitis (AIH)	The expression level of CD73 on Treg cells is down-regulated	Tregs are damaged and CD73 levels on the cell surface are down-regulated, the secretion of TGF-β and other anti-inflammatory factors is reduced, and the immunosuppressive function of Treg is defective		(36–38)
		Teff subgroup is activated and CD73 expression increased	IL-6 and TGF-β can induce the expression of CD73 in Th17, induce the production of adenosine, inhibit the transformation of naive T cells to Th and reduce the production of pro-inflammatory cytokines, showing the characteristics of immune deficiency		(39, 40)
	Primary biliary cirrhosis (PBC)	CD73 expression in Treg is significantly decreased	Compared with WTTreg, CD73 expression in dnRIITreg of PBC mice is significantly decreased		(36)
Fatty liver disease	Alcoholic fatty liver	CD73 is highly expressed on the surface of liver cells	Under chronic persistent alcohol stimulation, AMP is released in the liver and phosphorylated to adenosine catalyzed by CD73, which binds to cell surface adenosine A1 and A2B receptors and promotes degeneration of hepatic lipid deposition	CD73-deficient mice relatively exhibite less liver cell dilatation and steatosis, and the incidence of fatty liver is significantly reduced. Blocking the adenosine pathway in CD73 knockout mice can reduce adenosine-mediated extracellular matrix deposition through hepatic stellate cells, thereby protecting mice from ethanol-induced fatty liver. Blocking the expression of CD73, A1 or A2B receptors in liver can effectively reduce the accumulation of liver lipids caused by alcohol and delay the course of disease	(10, 25, 46, 47)

(Continued)

TABLE 3 (Continued)

Liver diseases		The expression of CD73	Influence of CD73 on various liver diseases	Effects of CD73 blocking in different liver diseases	References
	Nonalcoholic fatty liver disease (NAFLD)	The level of CD73mRNA is significantly decreased	CD73 can improve ETOh-induced liver injury and inflammatory response. Adenosine, as a hydrolytic product of CD73, plays a role in cell protection and immunosuppression through P1 receptors, thus terminating liver inflammation. CD73 can block the TLR4/MyD88/NF-κB signaling pathway, reduce the secretion of IL-6, IL-1β and other cytokines, and delay the inflammatory process. Under chronic inflammatory stimulation, damaged inflammatory liver cells can induce the approach of immune cells with high expression of CD39 and CD73, clear extracellular ATP, generate adenosine negative feedback to regulate endothelial cells and immune cells, inhibit leukocyte recruitment, and reduce inflammatory response	The mice with CD73 knockout rarely developed fatty liver, or even progressed to steatohepatitis	(9, 10, 13, 51)
Liver cirrhosis		CD73 expression increases and the activity of CD73 is enhanced	Acetaldehyde induces activation of CD73 in HSC, and induces the increased expression of pro-fibrotic cytokines TGF-β, α-SMA and type I and III collagen, promoting the generation of liver fibrosis. Activated HSC can up-regulate CD73 expression by specific SP1 and SMAD promoter elements	CD73-deficient mice are resistant to the development of liver fibrosis and can protect liver cells from the risk of inducing cirrhosis by CCl4 and thioacetamide (TAA). After CD73 deletion, adenosine production is reduced, leading to p2 receptor-mediated HSC activation proliferation and collagen expression decrease, and inhibiting the production of liver fibrosis. Inhibition of CD73 can promote HSC apoptosis, alleviate alcohol-related liver fibrosis. A2A adenosine receptor deficient mice were also protected from liver fibrosis due to the blocking of adenosine pathway	(10, 51, 57, 59, 60)
Hepatocellular carcinoma		The expression of CD73 is significantly elevated in HCC patients	CD73 enhances intracellular AMP by inducing immune escape in TME, initiates a variety of downstream signaling pathways, and inhibits antitumor response. CD73 can induce the increase of tumor vascular endothelial growth factor (VEGF), promote angiogenesis and help tumor cells survive. CD73 may be related to inflammatory signaling in liver cancer, stimulating inflammation through adenosine receptor signaling pathway and providing a suitable pro-inflammatory environment for tumor cells to survive		(13, 67–73)

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The handling editor MY declared a shared parent affiliation with the author YC at the time of review.

Publisher's note

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frontiers | Frontiers in Cellular and Infection Microbiology

TYPE Case Report
PUBLISHED 14 April 2023
DOI 10.3389/fcimb.2023.1143346

OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to Virus and Host, a section of the journal Frontiers in Cellular and Infection Microbiology

RECEIVED 27 January 2023
ACCEPTED 24 March 2023
PUBLISHED 14 April 2023

CITATION
Zaltron S, Cambianica A, Di Gregorio M, Colangelo C, Storti S, Tiecco G, Castelli F and Quiros-Roldan E (2023) Case report: An occult hepatitis B virus infection reactivation in an HIV/HCV coinfected patient during an immune reconstitution inflammatory syndrome.
Front. Cell. Infect. Microbiol. 13:1143346.
doi: 10.3389/fcimb.2023.1143346

Case report: An occult hepatitis B virus infection reactivation in an HIV/HCV coinfected patient during an immune reconstitution inflammatory syndrome

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The natural history of occult hepatitis B virus infection (OBI) and the mechanism involved in HBV reactivation are only partially understood. As regards people living with HIV (PLWH), HBV reactivation is estimated to occur with an incidence ratio of 0.019 cases per 100 person-year. Here we report the case of OBI reactivation in a HIV/HCV co-infected patient followed for 25 years at our Infectious Diseases Unit, but, unfortunately, lost to follow-up about 19 months after Direct-acting antivirals (DAAs) treatment. At re-engagement, blood tests showed high replication of plasmatic HIV-RNA along with severe immunosuppression and normal levels of liver enzymes. However, 3 months after ART reintroduction, an immune reconstitution inflammatory syndrome (IRIS) was diagnosed with high detectable HBV-DNA load and transaminase elevation. Our case report shows how the balance between the virus and the host immune system is quite a dynamic process that might significantly impact the course of the disease. The aim of this case report is to bring to the attention of physicians that, although OBI reactivation is a rather rare occurrence, even amongst PLWH, its potential consequences compel to a high alertness on the matter. Therefore, especially in patients with an impaired immune system and on a tenofovir or lamivudine-sparing regimen, HBV serological and virological markers should always be strictly monitored, even in the absence of a hepatitis flare.

KEYWORDS

occult HBV, reactivation, HBV/HIV coinfection, immune reconstitution inflammatory syndrome (IRIS), hepatitis B virus

Introduction

Hepatitis B virus (HBV), a partially double-stranded hepatotropic DNA virus, is the etiological agent of acute and chronic hepatitis B in humans. Chronic HBV infection prevalence among the Italian population is estimated to be 0.7% (Istituto superiore di Sanità, 2022; Sheena et al., 2022). Coinfection with Human Immunodeficiency Virus (HIV) and Hepatitis C Virus (HCV) is common among HBV-infected individuals due to shared routes of transmission, and it is often associated with worse immunological control and advanced liver disease (Puglia et al., 2016; Zhou et al., 2020). After acute HBV infection the outcome varies widely between subjects, and encompasses resolution, asymptomatic carriage and chronic active hepatitis. The most important determinants of chronicity are the immune status of the host and the age at the time of infection. Diagnosis of HBV infection status requires a plethora of serological and virological tests (Lampertico et al., 2017). Occult HBV infection (OBI) is defined as the presence of replication-competent HBV DNA (i.e. episomal HBV covalently closed circular DNA [cccDNA]) in the liver and/or HBV DNA in the blood of people who test negative for hepatitis B surface antigen (HBsAg) by currently available assays. Detection of serum anti-HBc is often used as a surrogate for the diagnosis of OBI (Raimondo et al., 2019). However, its precise prevalence is yet to be clearly defined (Im et al., 2022). Under long-term immunosuppression, i.e., during chemotherapy, hematological malignancies, steroid therapy or Acute immunodeficiency syndrome (AIDS), HBV reactivation might occur as consequence of the presence of replication-competent HBV DNA in the liver's hepatocytes. Reactivation is characterized by a sudden increase in serum HBV DNA levels, HBsAg reappearance and is often associated with a hepatitis flare (Perrillo et al., 2015). HBV infection prevalence among HIV-infected patients is now decreasing thanks to vaccination campaigns and the new antiretroviral regimens (ART) allow good immunological status in people living with HIV (PLWH): drugs such as tenofovir, which proved to be a potent HBV inhibitor, are often part of them: thanks to all these factors HBV-related chronic disease generally is not an issue and OBI reactivation is now rather infrequent among PLWH (Huang and Núñez, 2015; Soriano et al., 2019). HCV infection can be associated with HBV, and OBI is particularly common among patients affected by chronic hepatitis C. This may be related to HCV dominance over HBV, which determines HBsAg loss (Witt et al., 2013). HBV reactivation has been reported in patients starting HCV therapies who are not on active HBV agents. Typically, HBV reactivation occurs 4 to 8 weeks after direct-acting antivirals (DAAs) initiation. Because of that, International Guidelines recommend that all patients initiating DAAs be screened for HBV, and HBsAg negative, anti-HBc positive patients be monitored for HBV reactivation (Chang et al., 2018). Here we present a case of OBI reactivation in an HIV/HCV co-infected patient with severe immunodeficiency after self-suspension of ART and HCV eradication, following reintroduction of antiretroviral therapy.

Case description

A 64-year-old Caucasian man with an history of drug abuse came to the attention of our Infectious Diseases Unit solely in 1991 for HIV infection and non-A non-B hepatitis starting an irregular follow-up. HIV infection was first diagnosed in 1984. ART was not immediately started due to his good immunological status (CD4 cell count: 976 cell/mm3) and the ongoing use of recreational drugs. During the monitoring laboratory tests, isolated anti-HBc seropositivity was identified and chronic HCV hepatitis was diagnosed. In 1997, he initiated a drug rehabilitation program and, due to the worsening of his immunological status (CD4 cell count: 198 cell/mm3), an antiretroviral stavudine/lamivudine-containing regimen was promptly started. Although at first an irregular follow-up was started due to the poor adherence, the patient begun to regularly follow the scheduled visits thanks to the rehabilitation program. Several antiretroviral regimens were switched according to the over-time changing guidelines always reaching an optimal and maintained viro-immunological response. As regards his HCV infection, in 2016, he started a ledipavir/sofosvubir/ribavirin combination therapy for 24 weeks reaching sustained viral response (SVR). No changes were reported in the HBV serology with both anti-HBs and HBsAg persistently negative as shown in Table 1. Unfortunately, in early 2018, the patient was lost to follow up.

In June 2021, he resumed the follow-up visits showing an alarming viro-immunological profile: high levels of HIV-RNA were detectable and the CD4 cell count was less than 200 cells/mm3. ART and the opportunistic infection prophylaxis were promptly started. Few months later, although HIV-RNA went back to be undetectable together with an improved immunological profile, HBsAg turned positive with a detectable and high-level HBV DNA viraemia associated with an increase in transaminases. A reactive switch to an integrase inhibitor (INI)-based regimen containing tenofovir (emtricitabine/tenofovir-alafenamide/bictegravir) was started. At the end of 2022, hepatic indexes normalized, and HBV-DNA was stably undetectable.

Discussion

The case of OBI reactivation in a HIV/HCV co-infected patient presents several aspects that deserve to be discussed. A 64-year-old patient with HIV/HCV/OBI and cirrhosis followed for 25 years at our clinic was lost to follow-up about 19 months after DAAs treatment. Blood tests performed before loss of care showed an optimal immunological status with suppressed HIV viral load, isolated anti-HBc seropositivity and a sustained viral response (SVR) for HCV. At re-engagement (42 months after the last visit) blood tests showed high replication of plasmatic HIV-RNA (73142 cp/mL) along with severe immunosuppression and normal levels of liver enzymes. Three months after ART reintroduction, both CD4 and CD8 counts increased, and transaminases (ALT/AST) were slightly elevated.

10.3389/fcimb.2023.1143346

TABLE 1 HIV and HBV viral and immunological markers before the loss of care and after the re-engagement.

Date	CD4 (cell/mm3)	CD8 (cell/mm3)	% CD4	% CD8	CD4/CD8	HIV-RNA (cp/mL)	HBV-DNA (UI/mL)	AST (U/L)	ALT (U/L)	HBsAg quant (UI/L)	HBsAg qual
01/17	1074	1799	25	41,9	0,6	< 20	NA	19	21	NA	negative
04/17	1234	1953	26,3	41,6	0,6	< 20	NA	20	18	NA	negative
01/18	1154	1807	26,8	42	0,6	< 20	NA	18	22	NA	NA
06/21	104	1045	8	79,9	0,1	73142	NA	40	21	NA	positive
09/21	186	2241	6,7	80,4	0,08	< 20	63200	57	53	NA	NA
03/22	184	1585	8,1	69,3	0,12	< 20	NA	105	143	1589	positive
10/22	118	1035	7,4	64,3	0,11	< 20	< 10	27	16	NA	NA
12/22	195	1411	9,4	67,7	0,14	< 20	< 10	22	11	NA	negative

NA, Not Applicable; Quant, Quantitative; Qual, Qualitative.

Currently, occult HBV infection management and HIV management guidelines (EACS) indicate close monitoring for OBI/HIV coinfection, especially if the HIV drug regimen of choice does not include drugs with activity against both HIV and HBV (European AIDS Clinical Society, 2021). Moreover, particular attention is required for patients switching to a drug sparing regimen that does not involve either lamivudine or tenofovir. Rouphael et al, described one of the largest series of HBV reactivation in PLWH, estimating an incidence ratio of 0.019 cases per 100 person-year (Rouphael et al., 2007). Mican et al. more recently described an episode of HBV reactivation associated with a severe hepatitis flare in an HIV-infected patient with a resolved hepatitis B, three months after removing TDF from his regimen, as part of a simplification strategy (Mican et al., 2021). HBV reactivation mostly occurs in the setting of worsening CD4 cell counts or with a CD4 count <100 cells/μL. Also, HBV-related hepatic flare may occur in HIV-infected patients during an immune reconstitution syndrome (IRIS) after the initiation of an active antiretroviral treatment (Iannetta et al., 2022). This shows how the balance between the virus and the host immune system is quite a dynamic process that may significantly impact the course of the disease.

The natural history of OBI and the mechanism involved in HBV reactivation are only partially understood (Raimondo et al., 2019). OBI status is associated with an anti-HBV immune response that can exert a strong suppression over viral replication. OBI reactivation depends both on the specific virus characteristics and on several host factors such as older age, underlying diseases and the use of immunomodulant therapies (Shih and Chen, 2021).

HCV/HBV coinfection deserves further considerations. Co-transfection studies revealed that the HCV core protein may be involved in the inhibition of HBV replication (Shih et al., 1993). Therefore, quick, and effective DAA suppression of HCV replication could result in increased HBV viral replication or, in case of an OBI, even reactivation. A recent review and meta-analysis estimated a minimal risk of HBV reactivation and seroreversion in patients with OBI. Nonetheless, follow-up of HBV serological and virological markers during 24 weeks after treatment with DAA is generally advised (Mücke et al., 2018). Regarding our patient, however, the time elapsed between DAA therapy and OBI reactivation allows us to exclude HCV eradication as a possible cause of HBV reactivation.

Regarding HIV/HBV coinfection, OBI reactivation is rather rare nowadays due to the widespread use of effective antiretroviral regimens that include anti-HBV agents and suppress HBV replication (Soriano et al., 2019). Nonetheless, HBV reactivation might occur in patients with HIV/HBV coinfection when antiretroviral regimens are modified and HBV active drugs are withdrawn (i.e., dual therapy with tenofovir or lamivudine sparing regimens) (Raimondo et al., 2019).

Several hypotheses have been formulated to explain OBI reactivation and HBV-related disease in our patient. Firstly, the failure of immune surveillance and the following immune reconstitution-induced inflammatory syndrome (IRIS) after ART reintroduction. Secondly, reinfection cannot be excluded but is rather unlikely because immunity to HBV is developed against several epitopes and should be protective against other possible HBV subtype infections (Wang et al., 2021).

The outcomes of HBV infection depend on ever-evolving and dynamic interaction and balance between HBV replication and the host immune response, which involves humoral and cellular immunity with effectors T cells (CD4 cells, CD8 cells and T-Reg), B-cells and neutralizing antibodies (Gherlan, 2022).

CD8 cells have a pivotal role in the viral clearance and pathogenesis during acute HBV infection as well as the chronic phase because they recognize and eliminate infected hepatocytes (Thimme et al., 2003). HBV-specific CD8 functional exhaustion or clonal deletion are responsible for viral persistence during the chronic infection (Heim et al., 2019; Zhang et al., 2022). CD4 cells also play an important role in the immune response against HBV by activating innate immune cells, priming CD8 cells, maintaining CD8 cell effector function aimed to cell-mediated HBV clearance and inducing B cells to produce antibodies (Yang et al., 2010; Buschow and Jansen, 2021). An impaired HBV-specific

CD4 cell response has been associated with HBV persistence even in presence of HBV-specific CD8 cells in HBV/HCV co-infected patients (Urbani et al., 2005).

Wang et al. described a positive relation between frequency of HBV-specific CD4 cells (mainly HBV core-specific TNF-α producing CD4 cells), hepatitis B flares and liver damage, indicating robust on-going T cell responses in patients with HBV chronic infection (Wang et al., 2021). A further differentiation of those HBV-specific TNF-α producing CD4 cells into IFN-γ producing CD4 cells favors HBV viral clearance.

The incidence of HBV hepatitis flare in rheumatic patients with OBI receiving rituximab is rather rare (3.4 per 1,000 person-years) and lower than the incidence of HBV reactivation confirming that the cooperation among all immune cells is necessary to effective immune response after HBV reactivation (Lan et al., 2022).

B-cell depleting agents such as rituximab or ofatumumab are associated with the highest risk for HBV reactivation (>10%). All patients exposed to HBV, regardless of the HBsAg status, should start antiviral prophylaxis before initiating B-cell depleting therapies (Koffas et al., 2018). In HBsAg-negative and anti-HBc positive subjects with moderate (< 10%) or low (< 1%) risk of HBV reactivation, pre-emptive therapy, instead of prophylactic therapy, is generally recommended. It should be noted, however, that specific measures for PLWH with the same serological pattern are not provided by International Guidelines. Pre-emptive therapy consists in starting antiviral therapy in case of detectable HBV-DNA or HBsAg sero-reversion during follow up (Koffas et al., 2018; Papatheodoridis et al., 2022).

At re-engagement, our patient showed no signs of a hepatitis flare, probably due to his severe immunodeficiency and low CD4 cells count. It was only when CD4 cells improved, following the antiretroviral therapy reintroduction, that a slight elevation in transaminases was described, concomitantly to a significant increase of CD8 cells. This was suggestive of IRIS occurring after ART reinstatement. It has hypothesized that IRIS could contribute to HBsAg loss, especially when liver inflammation ensues early after ART introduction (Yoshikawa et al., 2021). The recovery of HBV-specific cytotoxic CD8 cells, has been speculated as the cause of this phenomena (Yoshikawa et al., 2021; Iannetta et al., 2022). Ultimately, the eminent hepatic flare was likely due to IRIS in our case because IRIS diagnostic criteria were present (Brust et al., 2021). Also, the hepatitis flare was associated with seroreversion for HBsAg. Fortunately, the prompt initiation of specific antiviral treatment with tenofovir alafenamide as part of the ART regimen, quickly resolved the hepatic flare and the plasmatic HBV-DNA became negative once again.

Conclusion

Hepatic damage and HBV clearance are mediated by the host immune response, which can suppress viral replication to minimal levels, achieving control of the infection and, ultimately, the OBI status. However, when multiple factors associated with a low chance of OBI reactivation are concomitantly present, the risk of this complication may exponentially increase. The aim of this case report was to bring to the attention of physicians that, although OBI reactivation is a rather rare occurrence, even amongst PLWH, its potential consequences compel to a high alertness on the matter. Therefore, especially in patients with an impaired immune system and on a tenofovir or lamivudine-sparing regimen, HBV serological and virological markers should always be strictly monitored, even in the absence of a hepatitis flare.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding author.

Ethics statement

Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

Author contributions

Conceptualization: EQ-R. Writing–original draft preparation: SZ, AC, MD, CC, SS, GT, FC, and EQ-R. Methodology: SZ, and EQ-R. Data curation: SZ, AC, MD, CC, SS, GT, FC, and EQ-R. Writing—review and editing: SZ, AC, MD, CC, SS, GT, FC, and EQ-R. Visualization: SZ, AC, MD, CC, SS, GT, FC, and EQ-R. Supervision: SZ, and EQ-R. All authors have read and agreed to the submitted. All authors contributed to the article and approved the submitted version.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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frontiers | Frontiers in Public Health

TYPE Original Research
PUBLISHED 30 May 2023
DOI 10.3389/fpubh.2023.1149694

OPEN ACCESS

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RECEIVED 22 January 2023
ACCEPTED 12 May 2023
PUBLISHED 30 May 2023

CITATION
Meyer ED, Dudareva S, Kollan C, Mauss S, Wedemeyer H, Schmidt D and Zimmermann R (2023) Additional challenges in reaching hepatitis C elimination goals in Germany due to the COVID-19 pandemic - descriptive analysis of drug prescription data from January 2018 to June 2021. *Front. Public Health* 11:1149694. doi: 10.3389/fpubh.2023.1149694

Additional challenges in reaching hepatitis C elimination goals in Germany due to the COVID-19 pandemic - descriptive analysis of drug prescription data from January 2018 to June 2021

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Effectively treating hepatitis C viral (HCV) infections prevents sequelae and onward transmission. In Germany, HCV drug prescriptions have declined since 2015. During the COVID-19 pandemic, lockdowns impacted the access to HCV care services and HCV treatment. We assessed if the COVID-19 pandemic further decreased treatment prescriptions in Germany. We built log-linear models with monthly HCV drug prescription data from pharmacies from January 2018 - February 2020 (pre-pandemic) to calculate expected prescriptions for March 2020-June 2021 and different pandemic phases. We calculated monthly prescription trends per pandemic phase using log-linear models. Further, we scanned all data for breakpoints. We stratified all data by geographic region and clinical settings. The number of DAA prescriptions in 2020 (n = 16,496, −21%) fell below those of 2019 (n = 20,864) and 2018 (n = 24,947), continuing the declining trend from previous years. The drop in prescriptions was stronger from 2019 to 2020 (−21%) than from 2018 to 2020 (−16%). Observed prescriptions met predictions from March 2020 to June 2021, but not during the first COVID-19 wave (March 2020–May 2020). Prescriptions increased during summer 2020 (June 2020-September 2020) and fell below the pre-pandemic numbers during the following pandemic waves (October 2020 – February 2021 and March 2021 – June 2021). Breakpoints during the first wave indicate that prescriptions plummeted overall, in all clinical settings and in four of six geographic regions. Both, outpatient clinics and private practices prescribed overall as predicted. However, outpatient hospital clinics prescribed 17–39% less than predicted during the first pandemic wave. HCV treatment prescriptions declined but stayed within the lower realms of predicted counts. The strongest decline during the first pandemic wave indicates a temporary HCV treatment gap. Later, prescriptions matched predictions despite of pronounced decreases during the second and third waves. In future pandemics, clinics and private practices need to adapt more rapidly to maintain a continuous access to care. In addition, political strategies

should focus more on continuously providing essential medical care during periods of restricted access due to infectious disease outbreaks. The observed decrease in HCV treatment may challenge reaching the HCV elimination goals in Germany by 2030.

KEYWORDS

hepatitis C, COVID-19, delivery of health care, Agenda 2030, antiviral agents - therapeutic use

1. Introduction

Hepatitis C virus (HCV) infections are a main cause of chronic viral hepatitis, liver cirrhosis and hepatocellular carcinoma (1–3). In the European Union and the European Economic Area, ∼35,000 deaths were attributable to HCV infections in 2015 (4). In Germany, it was estimated, that 9,528 deaths were attributable to a hepatitis B or C infection in 2015 (4). Germany is considered a low-prevalence country for HCV due to a low prevalence in the general population (5). However, the prevalence is disproportionately higher among risk groups: the prevalence ranges between 9.6% for men who have sex with men (6) and 68.0% among people who inject drugs (5). As HCV infections account for a high burden of disease and deaths world-wide, the WHO adopted a global strategy to eliminate hepatitis B and C as a public health threat by 2030 as part of Agenda 2030 (7). To reach elimination, 90% of people living with an HCV infection should be diagnosed, and 80% of the diagnosed should be cured by 2030 (8). Germany adopted the elimination goal in the strategy BIS2030 (9).

The introduction of direct acting antivirals (DAAs) in 2014 lay the foundation for reducing the morbidity and mortality of HCV infections, e.g., by reducing liver decompensation events among hospitalized patients diagnosed with hepatitis C (10). Several DAA substances have been registered and approved in Germany, and since 2017 single-tablet pan-genotypic combination treatments are available (11). Standard treatment duration with these regimens ranges between 8 and 12 weeks. The aim of the treatment is viral clearance, defined as sustained virologic response 12 weeks after the end of the treatment, which is achieved in ∼95% of treated patients (12).

HCV clinical care and treatment in Germany is mostly provided by specialist physicians, from either specialist practices for infectious diseases or gastroenterology or from specialized outpatient departments of tertiary care hospitals. From 2014 to 2020 the treatment indication for patients with statutory health insurance only included those with chronic HCV infections in Germany. As of November 2020 all viremic patients without clinical signs of acute HCV infection, are eligible for treatment with DAAs - irrespective of a proven chronic stage of the disease (12). The count of monthly DAA prescriptions increased between January 2014 and March 2015, but has decreased continuously, together with the number of treated patients, between March 2015 and December 2020 (13, 14). In recent years, treatment of HCV infection has become easier with the expanded use of pan-genotypic DAAs with a standardized treatment length. While high treatment costs were an initial barrier to prescribing treatment (13), average treatment regimen costs decreased over time from 91,000€ (2014) to 35,000€ (2018) and 30,700€ (2020) (14). For the combination of Elbasvir + Grazoprevir, the 12-week treatment costs less, ∼26,000€ (15).

Globally, a ten-fold increase of HCV treated patients has been observed between 2015 and 2020, leading to a reduction in deaths attributable to HCV (16). However, HCV care deteriorated globally during the COVID-19 pandemic: non-pharmaceutical control interventions, e.g., lockdowns, physical distancing requirements and movement restrictions, but also disrupted supply chains and health services potentially slowed down or reversed the progress from previous years (16, 17). In Germany, the COVID-19 pandemic was divided into different waves (Supplementary material 1). According to Hüppe et al. (18), the first pandemic wave negatively influenced patient care: About 60% of all liver patient consultations in Germany between March and May 2020 were impeded because of reduced service availability and cancellations of consultations by patients (18). It was estimated that the diagnosis of liver decompensation and liver cancer was delayed by 22 and 9.4%, respectively (18).

We assessed the number of DAA prescriptions and treated HCV patients in Germany between January 2018 and June 2021, including whether DAA prescriptions between March 2020 and June 2021 were impacted by the COVID-19 pandemic. Based on this assessment, we discuss if Germany can reach the HCV elimination goals by 2030.

2. Materials and methods

2.1. Data

Insight Health^{TM} provided monthly prescription data for DAA to treat HCV-infected patients (19)The data are based on prescriptions redeemed by patients with statutory health insurance [88% of Germany's population (19)] between June 2018 to June 2021. The database comprises data from ∼95% of German pharmacies. The DAAs comprised the following substances and combinations: DAA combinations effective against all genotypes (=pan-genotypic drugs) (Glecaprevir+Pibrentasvir, Velpatasvir + Sofosbuvir and Voxilaprevir + Velpatasvir + Sofosbuvir), DAA

Abbreviations: COVID-19, Coronavirus disease of 2019; DAA, Direct acting antiviral; EASL, European Association for the Study of the Liver; HCV, Hepatitis C Virus; PWID, People Who Inject Drugs; WHO, World Health Organization.

combinations effective against genotypes 1 and 4 (Elbasvir + Grazoprevir, Ledipasvir + Sofosbuvir) and others (Daclatasvir, Sofosbuvir, Dasabuvir, Ombitasvir + Paritaprevir + Ritonavir). Prescribed Ribavirin as potential part of some regimens was ignored, since it is only used in combination with DAAs in German treatment guidelines. All DAA are approved for treating adults; Glecaprevir + Pibrentasvir, Ledipasvir + Sofosbuvir are approved for treating children aged 12 years or older., We grouped the place of prescription in geographical regions: North (Lower Saxony, Bremen, Hamburg, Schleswig-Holstein, Mecklenburg-Vorpommern), South (Bavaria), Southwest (Baden-Württemberg, Saarland, Rhineland Palatinate, Hesse), East (Thuringia, Saxony, Saxony-Anhalt, Brandenburg, Berlin) and West (North Rhine, Westphalia-Lippe). Clinical settings were aggregated in outpatient clinics (for prescriptions that were not assigned to a specific independent physician) and private practices (for prescriptions from all specialized independent physicians).

We divided the observation period into pre-pandemic and pandemic periods based on a retrospective classification of the COVID-19 pandemic in Germany (20): Pre-pandemic period (January 2018–February 2020), first wave (March 2020–May 2020), summer 2020 (June 2020–September 2020), second wave (October 2020–February 2021) and third wave (March 2021–June 2021).

2.2. Data analysis

To calculate the number of treated HCV patients, the DAA prescription data were analyzed according to treatment regimen (12, 21). Based on the average treatment duration of different regimen, the number of patients treated with DAA and with statutory health insurance per year was estimated with weighted calculations of standardized treatment durations: with a standard regimen of 12 weeks for all DAA except for Glecaprevir + Pibrentasvir. For Glecaprevir + Pibrenasvir it was assumed that ∼90% of the patients received DAA for 8 weeks and 10% for 12 weeks, similar to the 8.4 weeks published as the average treatment duration for Glecaprevir + Pibrenasvir by the German Hepatitis C Registry (22).

Based on pre-pandemic monthly data (January 2018–February 2020), we built a log-linear model with months as singly explanatory variable, with one unit representing 1 month, and predictions as the outcome to calculate the 80% prediction interval (23) for monthly DAA prescription counts. We considered this prediction interval to be more sensitive in detecting unexpected changes than a 95% prediction interval. We calculated the prediction intervals for the entire study period (March 2020–June 2021, start of first to end of third pandemic wave) and separately for each pandemic period. Observed prescription counts within the prediction interval were assigned a difference of 0 (0%). If observed prescription counts were below or above the prediction interval, we calculated the difference as counts and percent to the lower and upper 80% prediction interval bound.

To calculate monthly trends (changes in drug prescription in % per month) and their 90% confidence intervals, we built log-linear models based on data of the corresponding period. We chose a 90% confidence interval to increase the sensitivity in detecting changes from previous trends (24); as some pandemic waves lasted few months only, changes from trends might have been missed when using larger confidence intervals.

We scanned the log-linear models of the DAA prescription data from January 2018 to June 2021, independent from the pandemic periods, for structural breakpoints (25): a maximal number of ten structural breakpoints were assessed to detect all potentially relevant breakpoints. No other specific settings were used. The identified breakpoints were tested for significance with a Chow-Test and only those reaching a significance level of $\alpha \leq 0.05$ are presented.

We used R.4.0.5. (26) with the packages tidyverse, zoo, broom, MASS, janitor and strucchange (25) for analyzing the data.

2.3. Ethical statement

DAA prescription data are not individual data but monthly aggregated from pharmacies' billing data. No ethical clearance is needed for the analysis.

3. Results

3.1. Overview of DAA prescriptions in Germany from January 2018 to June 2021

During the study period, the total monthly DAA prescriptions halved from 2,293 in January 2018 to 1,108 in June 2021. Monthly DAA prescriptions ranged between 2,404 (March 2018) and 1,070 (January 2021). The decline in yearly prescriptions was more pronounced from 2019 to 2020 than from 2018 to 2019 (Table 1). During the first pandemic year, the number of DAA prescriptions decreased by 23% from 20,309 (March 2019–February 2020) to 15,699 (March 2020-February 2021). The decrease in DAA prescriptions from the first half of 2020 to the first half of 2021 was lower than previous yearly declines, except for the Western and Southern regions (Table 1). The estimated number of patients treated per year decreased from ∼9,900 (2018) over ∼8,100 (2019, −18%) to ∼6,500 (2020, −20%).

Prescriptions of pan-genotypic DAA combinations increased: from 73% of all prescribed DAA in January 2018 to 84% in June 2021 (Figure 1, Supplementary material 3). DAA that are effective against genotypes 1 and 4 played a minor role and their use decreased over time from 27% (January 2018) to 16% (June 2021). Other DAA (Daclatasvir, Dasabuvir, Paritaprevir + Ombitasvir + Ritonavir) were no longer prescribed after March 2019.

Between January 2018 and February 2020, the total monthly prescriptions declined by 2%. During the first wave, the total monthly prescriptions declined further (−19%); during summer 2020, a temporary increase was observed (+2%); followed by decreases during the second (−5%) and third (−10%) pandemic wave (Table 2).

10.3389/fpubh.2023.1149694

TABLE 1 Yearly count of hepatitis C drug prescriptions in Germany from January 2018 to June 2021.

		2018	2019		2020		2021 (Jan–Jun)	
		Prescriptions n	Prescriptions n	Difference to previous year n (%)	Prescriptions n	Difference to previous year n (%)	Prescriptions n	Difference to previous Jan–Jun n (%)
Total prescriptions in Germany		24,947	20,864	−4,083 (−16%)	16,496	−4,368 (−21%)	7,356	−1,029 (−12%)
Provider	Private Practice	20,373	17,014	−3,359 (−16%)	13,715	−3,299 (−19%)	6,120	−945 (−13%)
	Outpatient clinics	4,574	3,850	−724 (−16%)	2,781	−1,069 (−28%)	1,236	−84 (−6%)
Region	North[1]	4,456	3,875	−581 (−13%)	3,024	−851 (−22%)	1,449	−65 (−4%)
	East[2]	2,903	2,413	−490 (−17%)	1,967	−446 (−18%)	971	−45 (−4%)
	West[3]	5,891	5,168	−723 (−12%)	4,347	−821 (−16%)	1,863	−353 (−16%)
	South[4]	4,036	3,337	−699 (−17%)	2,435	−902 (−27%)	942	−320 (−25%)
	Southwest[5]	7,629	6,058	−1,571 (−21%)	4,716	−1,342 (−22%)	2,131	−239 (−10%)

[1]Lower Saxony, Bremen, Hamburg, Schleswig-Holstein, Mecklenburg-Vorpommern. [2]Thuringia, Saxony, Saxony-Anhalt, Brandenburg, Berlin. [3]North Rhine, Westphalia-Lippe. [4]Bavaria. [5]Baden-Württemberg, Saarland, Rhineland Palatinate, Hesse.

3.2. DAA prescription trends during the pre-pandemic period

During the pre-pandemic period (January 2018–February 2020), the total monthly prescriptions declined by 2% (Table 2). The same trend was observed when stratifying for clinical settings and regions, except for the Western region (1%). In the South and East, prescriptions increased abruptly as of September 2018 with breakpoints of trends ($p = 0.04$ and $p = 0.049$, respectively (Figure 3).

3.3. Predicted and observed DAA prescriptions and their trends during the COVID-19 pandemic

Between the start of the first and the end of the third pandemic wave, the drug prescriptions in total stayed within the lower realms of predictions. Similarly, in most regions the observed prescriptions matched predictions (Table 3). In the Southern region, less DAA were prescribed than predicted. When stratifying for the clinical settings, the observed prescriptions matched predicted numbers (Table 3).

During the first pandemic wave, the total observed prescriptions stayed below predictions in total count. When stratifying for the clinical settings, prescriptions met predictions in private practices, but remained below predictions in hospital-based outpatient clinics. Stratified analyses by region resulted in prescriptions remaining below predictions in the Western and Southern region (Table 3, Figure 2). Similarly, the monthly trends of total prescriptions dropped most during the first pandemic wave compared to all other pandemic phases. In both clinical settings and in all region, the monthly trend decreased the most during this period (Table 2, Figure 3). These drops in prescriptions correlated with breakpoints that were found in March 2020 [total prescriptions data ($p < 0.01$)], in outpatient clinics ($p < 0.01$), in private practices ($p = 0.03$), in the Northern ($p = 0.02$), Western ($p < 0.01$) and Southwestern regions ($p < 0.01$) (Figure 3). Additional breakpoints were observed in April 2020, when monthly prescription trends reversed from decreasing (during the first wave) to increasing (during Summer 2020) in the Eastern ($p = 0.053$) and Southwestern region ($p = 0.01$).

During summer 2020, total prescriptions met the predictions. When stratifying for regions or for clinical settings, DAA prescriptions were also within the prediction interval (Table 3, Figure 2). During this period, the monthly trends increased for the total DAA drug prescriptions. When stratifying for clinical settings, the monthly trends increased for private practices and outpatient clinics. When stratifying for regions, the trends in the Western and Southwestern region increased, while no monthly changes were observed in the Northern, Southern and Eastern regions. Before, the monthly trends had always decreased since January 2018 (Table 2, Figure 3). Prescriptions no longer increased after the end of summer 2020 which resulted in a breakpoint ($p = 0.02$) in the Northern region in September 2020.

During the second wave, total prescriptions met predictions. When stratifying for regions, prescriptions in the North, East and

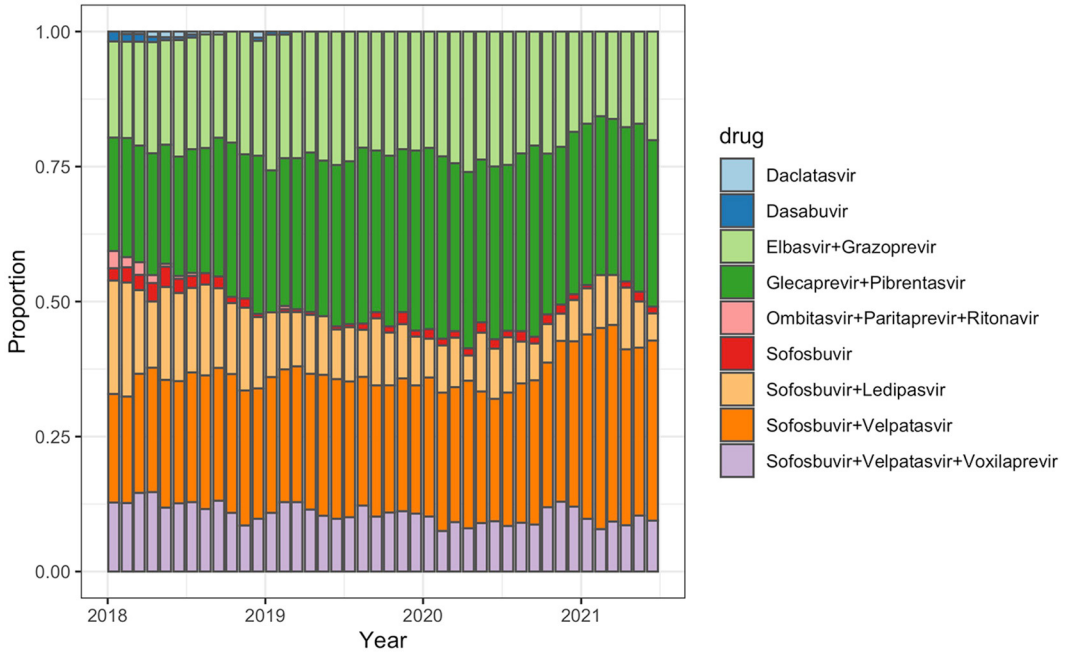

FIGURE 1
Monthly proportion of selected prescribed hepatitis C treatment regimens among all prescribed regimens in Germany between January 2018 and June 2021.

Southwest met predictions (Table 3, Figure 2) but prescriptions in the Western and Southern region dropped below the prediction interval. When stratifying for clinical settings, DAA prescriptions stayed within the prediction interval, however, in outpatient clinics they fell within the lower realms of predictions. Similar to the first pandemic wave, the monthly prescriptions trends decreased more during the second wave than during the pre-pandemic phase, however to a less severe extent. The same was found when stratifying for regions: in all regions a strong negative monthly trend was observed. Equally in all clinical settings, the monthly decrease was stronger than before the pandemic (Table 2, Figure 2). However, trends fell less than during the first wave. No breakpoints were found, indicating that trends did not change abruptly.

During the third wave, the total prescriptions fell within the prediction interval (Table 3, Figure 2), despite high COVID-19 case numbers during the third wave (Supplementary material 1). When stratifying for clinical settings, DAA prescriptions met predictions. When stratifying for the region, prescriptions were within the prediction interval except for the Southern region. In the Eastern region, prescriptions even exceeded predictions. However, monthly prescription trends reached their second lowest point throughout the study period (Table 2, Figure 3). In the Northern and Southern region, monthly trends were nearly as low as during the first wave. No breakpoints were found, indicating that even though trends decreased strongly no sudden changes occurred.

4. Discussion

We observed a declining trend of HCV antiviral drug prescriptions from 2015 to 2021. A general decline of the drug prescriptions, even before the COVID-19 pandemic, could have affected DAA prescriptions. However, the decrease in yearly treated patients from 2019 to 2020 surpassed the decline from 2018 to 2019, indicating an additional effect of control measures to contain the COVID-19 pandemic. However overall, the number of DAA prescriptions in Germany decreased during the COVID-19 pandemic but stayed within the lower realms of predictions except for the first pandemic wave. This may be due to some specifics of the German health care system where specialist care for all patients is provided by hospital-based outpatient clinics and the private sector as a second tier.

4.1. Hepatitis C care between March 2020 and June 2021

A total interruption of hepatitis C care was expected and assumed in some models (17). Instead, treatment prescriptions for hepatitis C continued in Germany during all pandemic months (Figure 2). Despite a decrease in hepatitis C case notifications compared to pre-pandemic years (14, 27), HCV infections were diagnosed and notified every month throughout all pandemic periods: While hepatitis C diagnosis and treatment were impacted, they were never fully interrupted at a monthly level in Germany at any point in time during the pandemic.

Yet, our assessment confirms other observations of impaired clinical services for hepatitis C in many countries, including Germany, with the highest impact occurring during the first pandemic wave, February to May 2020 (16, 18, 28). During the first pandemic wave, prescriptions in Germany even fell

TABLE 2 Mean monthly changes in the number of hepatitis C antiviral treatment prescriptions (in percentage to the respective previous month) in Germany between January 2018 and June 2021, stratified by pandemic phases.

		Pre-pandemic[6] % (90% CI[11])	First wave[7] % (90% CI)	Summer 2020[8] % (90% CI)	Second wave[9] % (90% CI)	Third wave[10] % (90% CI)
Total prescriptions in Germany						
		−2% (−2 to −1%)	−19% (−32 to −3%)	+2% (−7 to +12%)	−5% (−11 to +2%)	−10% (−15 to −4%)
Clinical setting	Practice	−2% (−2 to −1%)	−17% (−19 to −15%)	+2% (−4 to +9%)	−5% (−10 to +2%)	−9% (−16 to −2%)
	Outpatient clinics	−2% (−2 to −1%)	−27% (−84 to + 341%)	+1% (−20 to +28%)	−7% (−15 to +2%)	−11% (−16 to −5%)
Region	North[1]	−2% (−2 to −1%)	−18% (−50 to + 35%)	±0% (−20 to −25%)	−9% (−20 to +4%)	−17% (−32 to +3%)
	East[2]	−2% (−2 to −1%)	−16% (−23 to +3%)	±0% (−16 to + 20%)	−1% (−9 to +8%)	−9% (−29 to + 18%)
	West[3]	−1% (−1 to −1%)	−20% (−17 to +292%)	+3% (±0 to +7%)	−7% (−17 to +4%)	−10% (−15 to −5%)
	South[4]	−2% (−2 to −1%)	−18% (−50 to + 35%)	±0% (−20 to −25%)	−9% (−20 to +4%)	−17% (−32 to +3%)
	Southwest[5]	−2% (−2 to − 1%)	−19% (−38 to +5%)	+4% (−11 to + 22%)	−5% (−16 to + 8%)	−6% (−17 to +6%)

[1]Lower Saxony, Bremen, Hamburg, Schleswig-Holstein, Mecklenburg-Vorpommern. [2]Thuringia, Saxony, Saxony-Anhalt, Brandenburg, Berlin. [3]North Rhine, Westphalia-Lippe, [4]Bavaria, [5]Baden-Württemberg, Saarland, Rhineland Palatinate, Hesse, [6]January 2018 to February 2020. [7]March 2020 to May 2020, [8]June 2020 to September 2020, [9]October 2020 to February 2021, [10]March 2021 to June 2021; [11]Confidence interval.

below predictions (Table 3). The restrictions during the first pandemic wave in Germany also impacted on services in places where key populations, e.g., PWID, could access HCV testing: a survey among low threshold drug services showed that they were disproportionately disrupted during the first wave, and recovered only slowly (29). During the second and third pandemic wave, prescription trends differed from the pre-pandemic trend, indicating that services may still have been affected by the pandemic and related restrictions, but they were not completely shut down. This implies that patient care had not completely recovered to the pre-pandemic situation, even though physicians and patients had developed some contingency measures, e.g. video consultations replacing face-to-face visits (18). However, the monthly trends were nearly as low as in the first pandemic wave, suggesting that a longer duration of the second and third pandemic waves could have made DAA prescriptions fall below predictions.

In Germany, the COVID-19 pandemic reduced DAA prescriptions in hospital-based outpatient services more than in practices, also mainly during the first pandemic wave. It is likely that a number of different factors affected the services in outpatient clinics, leading to lower treatment prescriptions than expected and contributing to a world-wide decrease in the number of treated hepatitis C patients (28, 30): Outpatient clinics limited their service availability (18); politically imposed restrictions limited the accessibility of hospital-based outpatient care, e.g. by imposing SARS-CoV-2 PCR-tests before visits or travel restrictions; patients' fear and anxiety of contracting a SARS-CoV-2 infection in health facilities reduced their health-care seeking behavior (18). Further, the delay of elective in-patient care and limited access to in-patient care (31) possibly also reduced the service availability and access to outpatient clinics based in hospitals. In Germany, the primary care, such as private practices, played an important role in sustaining access to HCV treatment because services were less affected than in outpatient clinics.

Most other countries reported stronger reductions in hepatitis C services and DAA prescriptions than observed in Germany. A recent survey by the European Association for the Study of the Liver (EASL) (32) among clinical centers in European and non-European countries showed the impact of COVID-19 on newly initiated HCV antiviral treatments: Twenty-nine of 31 centers (94%) reported a reduction of 52% antiviral treatments in 2020 compared to 2019. In Germany, the number of initiated HCV treatments decreased only by 20% from 2019 to 2020. In the United States of America (USA), depending on the data source, the number of DAA prescriptions decreased by 22.7% (33) or 31.0% (34) in April 2020 and by 39.6% in May–July 2020 compared to the average of the same periods in 2018 and 2019 (33). During the first pandemic year (March 2020–February 2021), the number of DAA prescriptions decreased by 25.8% compared to March 2019-February 2020 (35). In Germany, prescriptions decreased by 35% in April 2020 and by 34% in May–July 2020, compared to the respective average of the years 2018 and 2019. During the first pandemic year, the number of DAA prescriptions decreased by 23%. Antiviral treatment during the pandemic declined less in Germany than in most of the countries that were represented in the EASL survey.

At a regional level, we observed that prescriptions remained below predictions in the Southern and Western region during the first and second pandemic waves. The regional 7-day COVID-19 incidence peaked higher in these two regions during the first pandemic wave (Supplementary material 2) which could have impacted the health-care seeking behavior and the HCV care availability. However, the prescriptions in the Southwestern region

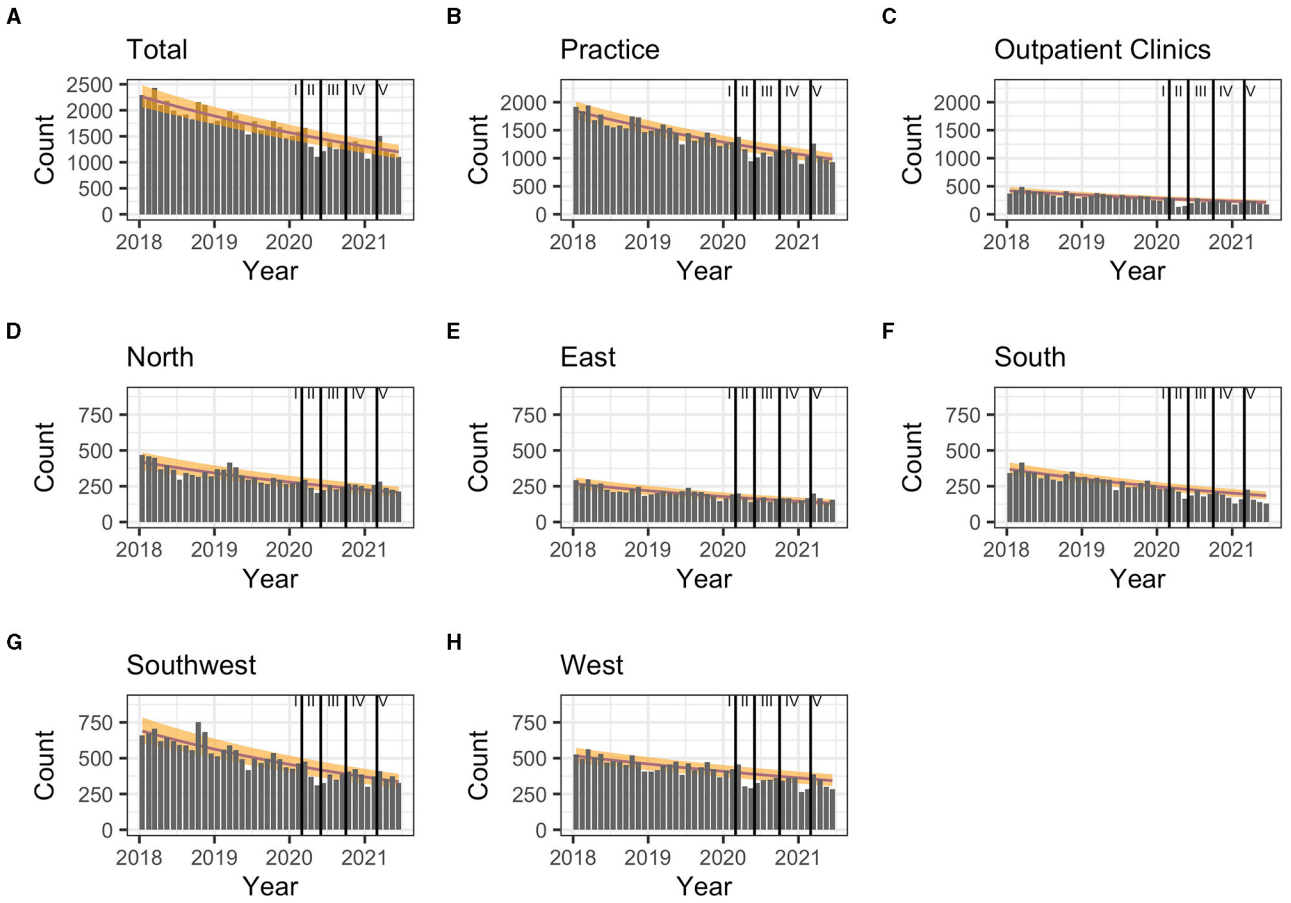

FIGURE 2
(A–H) Comparison of predicted (yellow band) and observed (gray bars) hepatitis C treatment prescriptions in Germany between March 2020 and January 2021 and by pandemic phase. 80% prediction interval based on pre-pandemic data (January 2018-February 2020). Regions: South: Bavaria; Southwest: Baden-Württemberg, Saarland, Rhineland Palatinate, Hesse; West: North Rhine, Westphalia-Lippe; North: Lower Saxony, Bremen, Hamburg, Schleswig-Holstein, Mecklenburg-Vorpommern; East: Thuringia, Saxony, Saxony-Anhalt, Brandenburg, Berlin. Pandemic phases: I: Pre-pandemic (January 2018 to February 2020), II: First pandemic wave (March 2020 to May 2020), II: Summer 2020 (June 2020 to September 2020), IV: Second wave (October 2020 to February 2021), V: Third wave (March 2021 to June 2021).

fell within the lower realms even though the peak of the regional 7-day COVID-19 incidence fell between the peaks observed in the Southern and Western regions. Additional factors apart from the regional 7-day COVID-19 incidence likely have affected the prescription numbers.

4.2. HCV elimination in Germany

Germany has committed to the international WHO 2030 elimination targets set by WHO, defined by 80% of the diagnosed to be successfully treated. Nonetheless, treatment prescriptions have steadily declined since 2015. We assume that all patients from former waiting-lists were treated, when DAAs came on the market. Even though the HCV treatment numbers before the pandemic (2014-20: $n = 76{,}400$) exceeded largely the number of notified newly diagnosed HCV cases ($n = 36{,}514$) (14), we have to assume that transmission is ongoing as new patients are diagnosed each year, and not all prevalent, longstanding infections are diagnosed. Therefore, the decline in DAA prescriptions since 2015 with an aggravation during the pandemic threatens the goal to eliminate HCV by 2030 in Germany.

According to an international modeling study (36), Germany can achieve the elimination goals if on average 9,900 are treated per year as of 2020. However, on average only 66% of the targeted patients were treated in 2020 (total treated: 6,500) (14) and only 57% in 2021 (total treated: 5,600) (37). Those 2 years with less patients treated than needed, leaves a treatment gap of 7,700 patients. To treat those patients missed in 2020 and 2021 in order to reach the treatment goals by 2029 as anticipated (36). Some additional cases might have been treated in prisons or through social welfare, private health care insurance and thus missed by our assessment. However, these were likely not enough to reach the estimated 9,900 treated cases per year necessary for HCV elimination. Other studies confirm that Germany may not reach the elimination goals by 2030: One study (38) predicts that Germany will reach HCV elimination goals by 2033, extrapolated with data from 2020. A third study (39) underlines that the declining trend of DAA prescriptions reduced the possibility that Germany reaches elimination already before the data presented

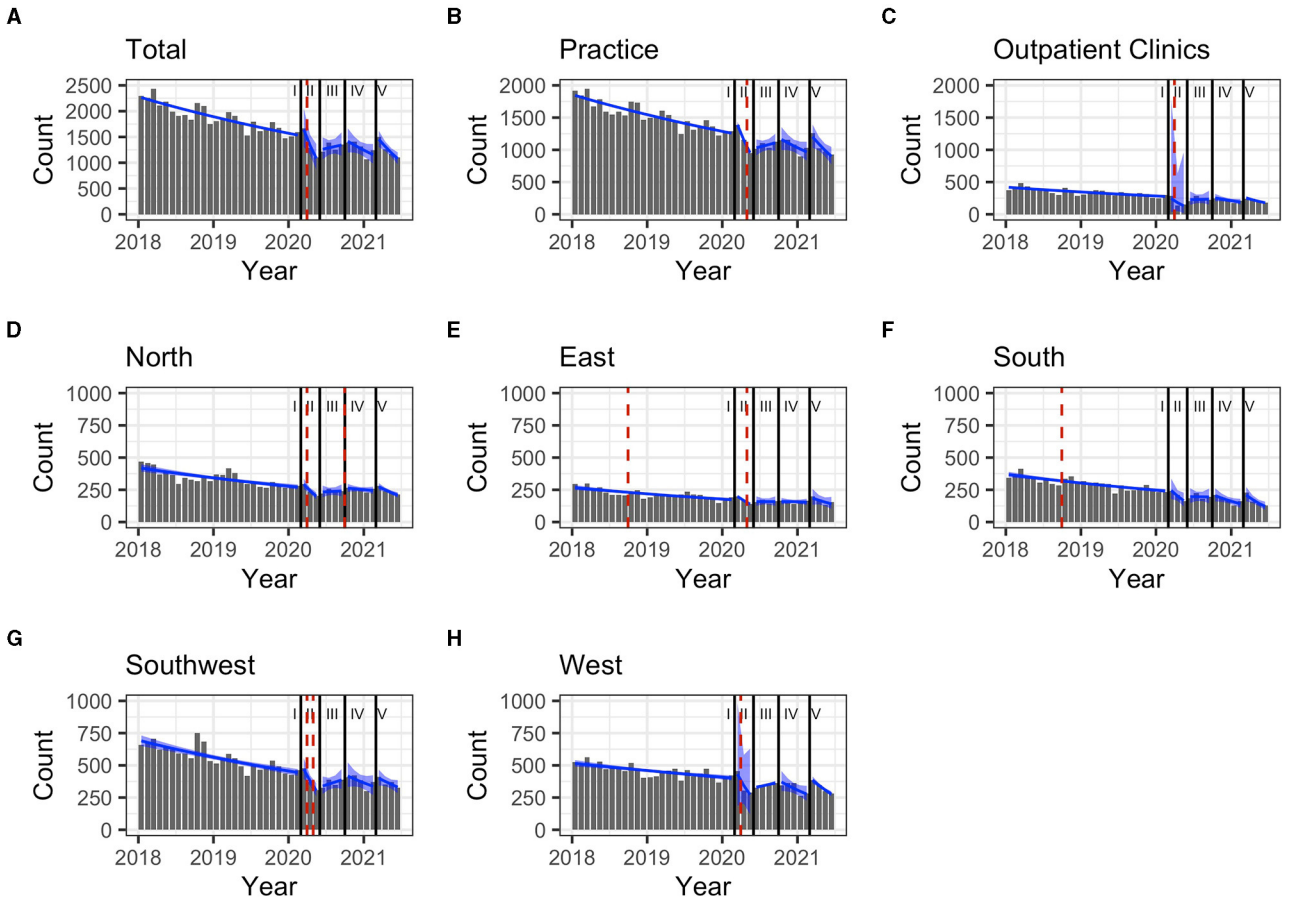

FIGURE 3
(A–H) Monthly prescription trends (90% confidence interval as blue band) and breakpoints (red dashed line) for Hepatitis C treatment prescriptions in Germany between January 2018 and June 2021, stratified by pandemic periods Regions: South: Bavaria; Southwest: Baden-Württemberg, Saarland, Rhineland Palatinate, Hesse; West: North Rhine, Westphalia-Lippe; North: Lower Saxony, Bremen, Hamburg, Schleswig-Holstein, Mecklenburg-Vorpommern; East: Thuringia, Saxony, Saxony-Anhalt, Brandenburg, Berlin. Pandemic phases: I: Pre-pandemic (January 2018 to February 2020), II: First pandemic wave (March 2020 to May 2020), II: Summer 2020 (June 2020 to September 2020), IV: Second wave (October 2020 to February 2021), V: Third wave (March 2021 to June 2021).

here from the COVID-19 pandemic. The set-back was further aggravated by the pandemic in 2020 (39). Despite expanding the German DAA treatment indication in November 2020, which allows to prescribe DAA to all viremia patients irrespective of clinical signs of acute or chronic HCV infection (12), no trend change occurred at this point, nor did the decreasing trend flatten. It seems likely that the negative pandemic impact potentially overlaid the positive effect of extending the treatment indication. We might need more data on DAA prescription over a longer period of time to see the actual effect of the broader treatment indication. Additionally, more measures are necessary to increase treatment numbers.

We assume that the declining group of hepatitis C patients already engaged in medical care explains most of the negative trend since the peak of DAA prescriptions in 2015. Studies show that there is still a large pool of more difficult to reach infected patients, in particular among key populations (5, 40, 41), with a high proportion of long-standing chronic infections, and ongoing transmission. Possibly, the established HCV treatment services do not reach these populations effectively. In addition the pandemic caused a temporary disruption of medical services, but also of low threshold HCV prevention and care services for PWID with long standing effects (18, 29). Monthly DAA prescription trends dropped below the pre-pandemic period. Targeted interventions are needed to reduce barriers and to improve access to HCV testing and linkage to treatment for groups with a higher risk of HCV infection, e.g., PWID, people in prison, homeless people, people without health insurance.

The decrease in DAA prescriptions resulted in a delay in treating HCV infections with major implications for the patients' health and the health-care systems. A modeling study (42) estimated that the decrease in DAA prescriptions during the pandemic in 2020 increased the number of cases of liver-related deaths, hepatocellular carcinoma and decompensated cirrhosis by the factor 1.4, 1.2 and 1.5 in Spain, causing high additional costs for treatment of late-stage liver disease and cancer. It was estimated that treating the additional number of patients with a decompensated cirrhosis or cancer are associated with costs of ~4.8 million euros (42). The authors advocate for reinforcement and screening programs to reduce the excess

TABLE 3 Differences between observed and predicted Hepatitis C treatment prescriptions in Germany between March 2020 and June 2021 and stratified by pandemic phase with 80% prediction interval, based on the pre-pandemic prescription data.

		March 2020 – June 2021	First pandemic wave[6]	Summer 2020[7]	Second pandemic wave[8]	Third pandemic wave[9]
Total prescriptions in Germany	Observed	20,750	4,065	5,214	6,426	5,045
	Predicted 80% PI[10]	19,540-23,867	4,067 - 4,908	5,121 – 6,217	5,942-7,282	4,409-5,461
	Difference n (%)	**±0**	**–2 to –843 (-0.1 to -17%)**	**±0**	**±0**	**±0**
Practice	Observed	17,281	3,494	4,281	5,317	4,189
	Predicted 80% PI	16,121-19,486	3,350-4,002	4,222 – 5,073	4,905-5,947	3,644-4,464
	Difference n (%)	**±0**	**±0**	**±0**	**±0**	**±0**
Outpatient clinics	Observed	3,469	571	933	1,109	856
	Predicted 80% PI	3,281 – 4,572	690-941	864-1,191	995-1,394	733-1,045
	Difference n (%)	**±0**	**–119 to –370 (–17% to –39%)**	**±0**	**±0**	**±0**
North[1]	Observed	3,919	737	950	1,271	961
	Predicted 80% PI	3,201 – 4,434	676-918	844 – 1,158	969 – 1,350	711 – 1,008
	Difference n (%)	**±0**	**±0**	**±0**	**±0**	**±0**
East[2]	Observed	2,582	505	635	789	653
	Predicted 80% PI	2,043 – 2,842	431 – 588	539 – 742	619 – 866	454 - 647
	Difference n (%)	**±0**	**±0**	**±0**	**±0**	**+6 to +199 (+1 to +44%)**
West[3]	Observed	5,372	1,052	1,386	1,616	1,318
	Predicted 80% PI	5,290 – 6,629	1,064 – 1,315	1,366 – 1,699	1,625 – 2,042	1,235-1,572
	Difference n (%)	**±0**	**–13 to –263 (-1 to -20%)**	**±0**	**–9 to –426 (-1 to -21%)**	**±0**
South[4]	Observed	2,913	613	786	861	653
	Predicted 80% PI	2,938-3,832	618-793	774-1,001	891-1,167	656 - 871
	Difference n (%)	**–25 to–919 (–1 to–24%)**	**–5 to –180 (–1 to–-23%)**	**±0**	**–30 to –306 (–3 to –26%)**	**–10 to –209 (–0.5 to –25%)**
Southwest[5]	Observed	5,958	1,155	1,454	1,889	1,460
	Predicted 80% PI	5,350 – 7,065	1,130-1,467	1,411-1,848	1,620-2,149	1,188-1,600
	Difference n (%)	**±0**	**±0**	**±0**	**±0**	**±0**

[1]Lower Saxony. Bremen. Hamburg, Schleswig-Holstein, Mecklenburg-Vorpommern. [2]Thuringia. Saxony, Saxony-Anhalt, Brandenburg, Berlin. [3]North Rhine. Westphalia-Lippe. [4]Bavaria. [5]Baden-Württemberg, Saarland, Rhineland Palatinate, Hesse. [6]March-May 2020. [7]June 2020-September 2020. [8]October 2020-February 2021. [9]March 2020-June 2021. [10]80% prediction interval. The observed treatment prescriptions were expected to fall with a probability of 80% within the 80% prediction interval (PI), acknowledging that prescriptions vary slightly from month to month even without major events, e.g. a pandemic. If the number of observed treatment prescriptions fell within the 80% PI, a difference of ±0 was assigned. If the number of observed treatment prescriptions was outside of the 80% PI, the difference to both interval borders was calculated as a number and percentage.

hepatitis C morbidity and mortality related to treatment delay during the COVID-19 pandemic (42) In 2021, the German statutory health insurances included a new screening program for HCV infections for adults with the age of 35 years or older in the general health check-up which is free of charge (43). This may help to catch up the delay in diagnosing HCV infection during the pandemic to reduce the occurrence of long-term consequences of undiagnosed hepatitis C among patients engaged in medical care. Still, Germany should also implement measures to increase the diagnosis and treatment with DAA, especially for key populations.

4.3. Limitations

This study only represents data from patients with statutory health insurance, and patients with private insurance; those treated as refugees or asylum seekers or patients treated in prison settings were not represented. However, 88% of the German population is covered by statutory health insurance. Moreover, the aim of our study was to assess prescriptions over time and the influence of the pandemic hereof and we believe that the proportion of those treated but not covered in our data has remained constant during the study period. Further, some specialized high-selling pharmacies have

refused to share their data with InsightHealth^{TM} which resulted in an estimated loss of ∼5% of DAA data as of 2019. Overall, selection bias should not have impaired the validity of the findings.

The data set that we used for this analysis was anonymized and represented data of redeemed prescriptions at pharmacies. Information on the clinical setting was derived from information on unique doctor numbers, however they did not always allow to differentiate hospital-based outpatient settings from private practices. Therefore, we might have underestimated prescriptions from hospital-based outpatient clinics, and overestimated those from private practices to a small extent. The actual proportion of patients receiving an eight-week regimen of Glecaprevir + Pibrentasvir may slightly differ from our assumption, resulting in a few more or less HCV patients treated per year, which we assumed to be negligible. The data set lacks information on patient data, such as age, gender, reinfection with HCV, number of previous treatment courses with DAA, migration status or HIV status, thus impeding the assessment of trends among different age groups or the analysis of how the COVID-19 pandemic affected different sub-populations.

We restricted the pre-pandemic period to January 2018 to February 2020. This limited the number of data point, especially because the prescription data was only available at a monthly level. Therefore, we refrained from doing a time-series analysis to avoid overfitting of the pre-pandemic data. We did not include more months in the pre-pandemic period as we saw unsystematic changes in prescriptions prior to January 2018, likely due to the approval of new drugs or drug combinations, which would have impaired the validity of our regression models.

4.4. Recommendations

We need further studies to understand the underlying reasons for the regional differences in DAA prescriptions. Comparing the time between HCV diagnosis and treatment initiation before and during the COVID-19 pandemic can shed further light on how the pandemic impacted the continuity-of-care cascade for HCV in Germany, in particular for key populations. Considering the HCV elimination goals, it is important to understand if missed patients from the first pandemic wave were treated later or if they were lost to follow-up. Furthermore, we need to learn from service providers and patients what interventions could increase the number of treated patients.

Hepatitis services need to reach more patients with hepatitis C to minimize the impacts of the pandemic on the reduced number of treated patients. The declining pre-pandemic trend suggests that easy-to-reach patients have been treated. The pandemic disproportionately reduced HCV testing and care points, like low threshold drug services, for key populations, who are simultaneously at high-risk for HCV infections, such as PWID, homeless people or people in prisons (29). Therefore, targeted interventions are necessary to improve access to testing and treatment for key populations to achieve the HCV elimination goals by 2030. This includes improving the collaboration between harm reduction services, opioid substitution facilities and infectious diseases services. Based on the current data, the number of DAA prescriptions need to increase if Germany is to reach the HCV elimination goals. Furthermore, the health-care system should implement screening services to detect patients with HCV-related complications early to minimize the additional treatment costs that the treatment delay caused during the COVID-19 pandemic.

5. Conclusion

Overall, the total number of DAA prescriptions was within the lower realms of predicted counts. However, during the first pandemic wave the number of prescriptions was lower than predicted and monthly trends plummeted. Monthly trends fell also below the pre-pandemic trend during the second and third wave. Practices of the primary care sector managed better to uphold services than hospital-based outpatient clinics. The decline in the number of DAA prescriptions during the pandemic was a global phenomenon. DAA treatment declined less in Germany than compared to most countries represented in the EASL survey. However, the additional decrease in the number of DAA prescriptions due to the pandemic could reduce the possibility of Germany reaching the HCV elimination goal with 90% of infected diagnosed, and 80% of diagnosed eligible patients treated by 2030. To achieve these targets, more HCV-infected patients, including key populations, need to be reached, diagnosed and treated. The delay in treating hepatitis C during the pandemic will cause additional patients with HCV-related complications. Screening programs should aim to diagnose these patients early and link them to specific care to reduce additional costs.

Data availability statement

The data analyzed in this study is subject to the following licenses/restrictions: The data analyzed in this study was obtained from InsightHealth^{TM}, the following restrictions apply: purchasers commercial license restrictions. Requests to access these datasets should be directed to CK, KollanC@rki.de.

Author contributions

Study conception, protocol development, and manuscript preparation: EM, SD, CK, DS, and RZ. Data collection: CK. Data analysis: EM and CK. All authors contributed to the article and approved the submitted version.

Funding

EM was a fellow of the German Postgraduate Training for Applied Epidemiology (PAE), when she conducted this research project.

Acknowledgments

We would like to thank Achim Dörre for his statistical inputs. EM would like to thank Sybille Rehmet for her continuous support.

Conflict of interest

CK: small shareholder in ABBV and MSD manufacturing Glecaprevir/Pibrentasvir and Elbasvir/Grazoprevir SM: speaker bureau: AbbVie, Gilead. HW: serves as clinical trials principal investigator for Abbvie, Altimmune, BMS, Gilead, Janssen, Merck/MSD, MYR GmbH, Novartis, and Vir Biotechnology, has research grants from Abbvie, Biotest, Gilead, Merck/MSD, and Roche, and advises or is on the speakers' bureau for AbbVie, Aligos, Altimmune, Biotest, BMS, BTG, Dicerna, Enanta, Gilead, Janssen, Merck/MSD, MYR GmbH, Roche, and Vir Biotechnology.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpubh.2023.1149694/full#supplementary-material

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frontiers | Frontiers in Public Health

TYPE Brief Research Report
PUBLISHED 02 June 2023
DOI 10.3389/fpubh.2023.1152193

OPEN ACCESS

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RECEIVED 27 January 2023
ACCEPTED 08 May 2023
PUBLISHED 02 June 2023

CITATION
Ndunguru B, Wilfred D, Kapesa A, Kilonzo SD, Mirambo M, Hyera F and Massaga F (2023) Low uptake of hepatitis B vaccination among healthcare workers in primary health facilities in Mwanza region, North-Western Tanzania. *Front. Public Health* 11:1152193. doi: 10.3389/fpubh.2023.1152193

Low uptake of hepatitis B vaccination among healthcare workers in primary health facilities in Mwanza region, North-Western Tanzania

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Background: Despite the availability of hepatitis B vaccines (HBV) in Tanzania, their uptake among healthcare workers (HCWs) in high-level facilities, such as tertiary hospitals where the vaccines are available, is low. However, their uptake among HCWs in primary health facilities remains understudied. The lack of this information limits the scaling up of HBV vaccination programs.

Methodology: A cross-sectional analytical study was conducted between June and July 2022 among HCWs in the Misungwi and Ilemela districts, which were purposefully selected. The sample size was calculated using the Taro Yamane formula, and data were collected using a self-administered questionnaire and analyzed using IBM SPSS® version 25.

Results: A total of 402 HCWs were recruited, their mean age was 34.9±7.77 years, and only 18% (76/402) reported being fully vaccinated. HCWs in Ilemela showed higher uptake (χ^2=23.64, df=1, p=0.00) of the vaccine than HCWs in Misungwi. Being male (aOR=2.38, 95% CI 1.28–4.45, p=0.006), working in an urban setting (aOR=5.75, 95% CI 2.91–11.35, p=0.00), and having an employment duration of more than 2years (aOR=3.58, 95%CI 1.19–10.74, p=0.023) were significantly associated with higher odds of vaccination. Moreover, high perceived susceptibility to HBV infection (aOR=2.20, 95% CI1.02–4.75, p=0.044) and history of needle prick injuries (aOR=6.87, 95%CI 3.55–13.26, p=0.00) were significantly associated with higher odds of HBV vaccination.

Conclusion: Low uptake of HBV vaccine among HCWs in primary health facilities was observed with a noteworthy difference between rural and urban settings. Therefore, advocacy campaigns and resource mobilization toward the promotion of HBV vaccination in primary health facilities are pivotal.

KEYWORDS

coverage of hepatitis B vaccination, hepatitis B virus, healthcare workers, primary health facilities, uptake

1. Introduction

Hepatitis B viral infection (HBV) is a deadly blood-borne vaccine-preventable liver disease affecting more than 2 billion people worldwide (1). It is the top cause of liver cirrhosis and hepatocellular carcinoma (2, 3). Globally, the prevalence of HBV infections is estimated to be around 1.3%, with rates ranging from as low as 0.2% in the United States of America to as high as 8% in Africa (4). In Tanzania, the prevalence of HBV was reported to range from 5.5 to 20% in the general population (3).

Healthcare workers (HCWs) are four times more likely to acquire HBV infection than the general population. This is due to the fact that they encounter many occupational risk exposures such as needle stick injuries and body fluid splashes, among others (5, 6). According to a global policy report on the prevention and control of viral hepatitis among WHO member states, approximately 2 million HCWs are at risk of being infected with HBV each year in a midlist of low vaccination coverage (2). A recent meta-analysis study conducted in Africa and Asia reported the prevalence was reported to be 4.0% and 5.0% in Asia and Africa respectively (7). In Tanzania, the prevalence of HBV infection among HCWs is even higher, ranging from 5.7 to 7% (8, 9).

To prevent HBV infection among HCWs, various interventions have been recommended. These include but are not limited to observing infection control strategies, blood safety through screening before transfusion, and vaccination. Of these, the latter is the most cost-effective intervention, as it conveys a protective efficacy of more than 90 percent (10).

Occupational HBV infections have been reported to affect about 37% of HCWs worldwide (11). In the United States of America, a study conducted between 2002 and 2003 by Somard et al. showed a good response toward HBV vaccination, where 75% of the HCWs had received three doses of the HBV vaccine, meaning that they were fully vaccinated against HBV (12). The coverage of HBV vaccination showed to be higher among nurses and doctors as compared to other HCWs. Another study conducted in Italy in 2019 showed that almost all HCWs had received the HBV vaccination (13). On the other hand, there are limited data in low-middle-income countries (LMICs) on the vaccination coverage among HCWs; however, the available data are useful enough to understand the situation of HBV vaccination status. In LMICs, it is reported that 50% of HBV infections are occupationally related, and there is still low vaccination coverage among HCWs. For instance, a study conducted in Egypt in 2003 found that only 15.8% of HCWs had received three doses of the HBV vaccine. In Tanzania, despite the availability of the HBV vaccine, only one out of five HCWs in tertiary referral hospitals had protective immunity resulting from active vaccination (8).

Factors that have been found to influence vaccination against HBV can be grouped as individual or health system factors. The individual factors that have been found to affect the uptake of the HBV vaccine among HCWs include knowledge and awareness, perceptions, distrust in vaccines, and cost. Health system factors found to affect HBV vaccine uptake include the vaccine availability at a facility followed by a lack of information on where to get the vaccine (14).

Despite the availability of hepatitis B vaccines (HBV) in Tanzania, their uptake among healthcare workers (HCWs) in tertiary hospitals where the vaccines are available is still low, as uncovered in the studies (8, 9). On the other hand, their uptake among HCWs in primary health facilities remains understudied. The lack of this information limits the implementation and scaling up of HBV vaccination programs in these facilities. Therefore, this study aimed to explore the magnitude of HBV vaccination coverage among HCWs in primary health facilities and the factors that influence vaccination among them.

2. Materials and methods

2.1. Study area, design, and participants

This was a cross-sectional analytical study conducted in the Misungwi and Ilemela districts, both located in the Mwanza Region. A total of 339 and 473 HCWs were providing health services in primary health facilities in Ilemela and Misungwi, respectively, during the time of the study. The former district is located in an urban area, whereas the latter is in a rural setting. There are 14 dispensaries in the Ilemela district and 4 public health centers, whereas the Misungwi district has a total of 43 dispensaries and 4 health centers.

2.2. Sample size estimation and sampling techniques

The sample size was calculated by using the Taro Yamane formula (15) to give the minimum sample size required. The obtained sample size was multiplied by 1.5 to cover for the design effect, and the sample size of 402 participants was subsequently reached. The two study areas were purposefully selected to represent rural and urban settings. To ensure representativeness, two-thirds of all primary health facilities from each district were randomly selected. A total of 185 and 217 participants were selected to take part in Misungwi and Ilemela districts, respectively. HCWs from nine dispensaries and two health centers were selected in the Ilemela district, whereas HCWs from two health centers and 14 dispensaries from the Misungwi district were randomly selected from both medical and non-medical personnel.

2.3. Eligibility criteria

2.3.1. Inclusion criteria

The study participants were HCWs working at the selected primary health facilities and had served in that particular facility for more than 1 year.

2.3.2. Exclusion criteria

HCWs born after 2002 were excluded from the study, as they are more likely to have been vaccinated by the national expanded program for immunization.

2.4. Data collection, tools, and procedures

A pre-tested, semi-structured, self-administered questionnaire was used for data collection. The questionnaire had a total of seven

sections. The sections included socio-demographic information of the participants, exposure status to HBV and its risk factors, awareness of HBV, knowledge of the participants on HBV, HBV vaccination status, and their perception toward HBV and vaccines. Awareness was measured using a single question that required a participant to answer whether they had heard of HBV before or not. Knowledge assessment was performed by adapting a work from Abdul Hakeem et al. (16). In this section, participants were asked a total of 10 questions on HBV transmission, prevention, and treatment to obtain an impression of their understanding of the disease. HBV vaccine uptake was determined by a section in the questionnaire where the participants were asked whether they had been vaccinated or not. Then, they were asked about the number of HBV vaccine shots they had received. Participants who had received a total of three shots were regarded as vaccinated, and those who had received less than three shots or none were considered not vaccinated. Perception of HBV vaccine and infection was measured using the six constructs of the health belief model, namely, perceived severity, perceived susceptibility, perceived benefits, perceived self-efficacy, perceived barriers, and cues to action, where each of the constructs had several statements (17). The statements were in the form of questions, with a minimum of two statements on the construct.

2.5. Data analysis

Data were entered into an Excel® sheet and then exported to IBM SPSS® version 25 for analysis. The continuous variables were summarized as the mean with standard deviation or median with the interquartile range depending on the distribution. The categorical variables were summarized using frequencies and proportions. For the identification of factors associated with the uptake of HBV vaccination, bivariate logistic regression was performed for each independent variable. Variables with value of ps lower than 0.05 were considered statistically significant. Multivariate analysis was performed using a multivariate logistic regression model for all variables with value of ps ≤0.2 during the bivariate analysis.

Knowledge assessment was measured using the following questions in the knowledge section of the questionnaire: How is HBV transmitted? Is HBV a preventable disease? What can be done to prevent HBV infection? If a person is found to be HBV positive, what will be done in Tanzania? Is HBV DNA recombinant vaccine capable of protecting a vaccinated person against HBV infection? Who is at risk of being infected with HBV? Is the HBV vaccine available in Tanzania? What is the minimum number of HBV shots needed to protect against HBV? Is it important to conduct an immune response test after the HBV vaccine? What is to be done after being accidentally in contact with an HBV-infected person's blood or body fluid products? What is the route of the HBV recombinant vaccine? Each correct response scored one mark while the wrong response and I do not know response scored zero. The total scores for each participant were obtained and converted into percentages, and they were categorized as follows: poor knowledge <50%, fair knowledge 50–74%, and good knowledge ≥75% (16).

Perception of HBV vaccination was categorized as good perception and poor perception. The variables were recoded into different variables, and for each of the constructs of the health belief model, a set of questions was asked with the response options of agree, not sure, and disagree. These were subsequently given a score of 3, 2, and 1 point, respectively. Participants with a score above the median on each construct of the health belief model were considered to have a good perception of HBV vaccination, and those with a score below or equal to the median were considered to have a poor perception.

3. Results

3.1. Socio-demographic characteristics of study participants

This study sampled 402 HCWs from 27 health facilities in the Misungwi and Ilemela districts. The mean age of the participants was 34.9 ± 7.7 years (95% CI, 34.1–35.1), and the majority were female 56.5% (227/402). A total of 217 (54%) HCWs were recruited from Misungwi and the rest from the Ilemela district. Nurses were the majority 36.8% (148/402) of all medical cadres who participated in the study. Further details on the above descriptions are tabulated below (Table 1).

3.2. Risk exposures of HBV infection among HCWs in Misungwi and Ilemela districts

Nearly half 46.3% (186/402) of the participants reported having tested for HBV infection, and they all reported a negative status. Additionally, a total of 188 (46.8%) HCWs were reported to have been exposed to needle prick injury in the past year before the study. On the other hand, 90.3% of all the study participants used protective gear when attending to their clients. Table 2 shows hepatitis B risk exposures and protective behavior assessment among the study participants.

3.3. Uptake of hepatitis B vaccine among HCWs

Among all the interviewed HCWs, only 18.9% (76 /402) were fully vaccinated. Most of those who had not received the HBV vaccine 55% (100/182) claimed that the availability of the vaccine at their working facilities was the main hindrance. Table 3 shows the vaccination status and the identified barriers.

3.4. Comparison of vaccination status among HCWs in Ilemela and Misungwi districts

HCWs in the Ilemela district were more likely to be fully vaccinated as compared to their counterparts in Misungwi (29.2% vs. 10.1%; aOR 5.75, 95% CI 2.91–11.35, $p = 0.01$). Generally, healthcare workers working in dispensaries showed higher hepatitis B vaccine completion compared to those working in health centers in both study areas, at 21.35% versus 17.2% (aOR 0.77, 95% CI 0.46–1.27, $p > 0.05$). Table 4 and Figure 1 show the details of the above description.

TABLE 1 Socio-demographic characteristics of study participants (*N*=402).

Variable	Frequency (*n*)	Percentages (%)
Sex		
Male	175	43.5
Female	227	56.5
Age groups		
21–24 years	7	1.7
25-44 years	342	85.1
45-64 years	53	13.2
Districts		
Ilemela	185	46.0
Misungwi	217	54.0
Level of health facility		
Dispensary	164	40.8
Health center	238	59.2
Religion		
Christian	336	83.6
Muslim	68	16.4
Marital status		
Widowed	15	3.7
Divorced	3	7.0
Cohabiting	35	8.7
Single	90	22.4
Married	259	54.4
Education level		
Secondary education	10	2.5
Bachelor's degree	43	10.7
Diploma	154	38.3
Certificate	195	48.5
Medical cadre		
Pharmacist	12	3.0
Medical doctor	33	8.2
Laboratory personnel	44	10.9
Medical attendant	79	19.7
Clinical officer	86	21.4
Nurses	148	36.8
Duration of employment		
1 year	14	3.5
2 years	37	9.2
3 years	39	9.7
4 years	27	6.7
More than 4 years	285	70.9
Departments served		
In patients	219	54.5
Outpatients clinic	92	22.9
Laboratory	59	14.7
Reproductive and child health	155	38.6
Theater	63	15.7
Labor ward	169	42.0
Mortuary	11	2.7

3.5. Factors associated with HBV vaccine uptake among HCWs

During the multivariate logistic regression analysis, a number of the factors showed significant association with full vaccination, including the participants' sex, district, duration of work, history of needle prick injury, and high perceived susceptibility to HBV infection. Male participants had 2.38 higher odds of being vaccinated (aOR = 2.38, 95% CI 1.28–0.45, $p = 0.006$) than to female participants. HCWs in the Ilemela district were five times more likely to be vaccinated (aOR = 5.75, 95% CI 2.91–11.35, $p < 0.01$) than HCWs in Misungwi, and HCWs who had worked for more than 2 years had 3.58 higher odds of being vaccinated (aOR-3.58 95% CI 1.19–10.74, $p = 0.023$) than those who had worked for less than 2 years. On top of that, the participants who had a high perception of susceptibility to HBV infection had 2.20 higher odds of being vaccinated (aOR = 2.20, 95% CI1.02–4.75, $p = 0.044$) than those who had low perception. In addition, HCWs with a history of needle prick injuries were six times more likely to be vaccinated (aOR = 6.87, 95%CI 3.55–13.26, $p < 0.01$) than those who had never encountered accidental needle stick injuries. Details of the above description are tabulated below (Table 5).

4. Discussion

Hospital-acquired infections contribute to a significant loss of human resources for health and affect the quality of services. HBV infection is prominent among healthcare workers across the globe despite being a vaccine-preventable disease. In Tanzania, studies of the uptake of the HBV vaccine have focused on healthcare workers in tertiary hospitals, and little is known about those working in primary facilities. The aim of this study was therefore to determine the extent to which HCWs in primary health facilities are vaccinated against HBV and the associated factors.

This study revealed that less than 20% of HCWs received full vaccination, leaving the rest with no or partial vaccination. These findings are different from studies at tertiary hospitals in Tanzania, where the completion rate has been demonstrated to be higher, ranging from 33 to 70% (7, 18, 19). The difference in uptake could be attributed to vaccine availability, because in high-level facilities the HBV vaccine is usually available and sometimes free of charge (19). On the other hand, the HBV vaccine is not readily available at most primary health facilities in Tanzania, as evidenced by this study. Therefore, the Ministry of Health must consider the HBV vaccine supply throughout lower and higher levels of facilities to increase uptake in these disadvantaged facilities. However, these findings are quite similar to those provided by a study conducted in

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TABLE 2 Exposure to the risk of HBV infection among HCWs in Misungwi and Ilemela districts (*N*=402).

Variable	Frequency (*n*)	Percent (%)
Ever tested for HBV		
Yes	186	46.3
No	216	53.7
HBV status		
Positive	0	0
Negative	186	100
Attended HBV-positive patient		
Yes	170	42.3
No	232	57.7
Needle prick injury in the past year		
Yes	188	46.8
No	214	53.2
How many needle picks in 1 year		
One	105	26.1
Two	48	11.9
Three	22	5.5
Four	11	2.7
More than four	2	0.5
Body fluid splashes from patients		
Yes	334	83.1
No	68	16.9
History of blood transfusion		
Yes	28	7.0
No	374	93.0
History of surgeries		
Yes	99	24.6
No	303	75.4
History of dialysis		
Yes	15	3.7
No	387	96.3
History of invasive procedures such as IV, IM, and endoscopy		
Yes	277	68.9
No	125	31.1
Number of sexual partners		
One	340	84.6
Two	50	12.4
Three	4	1.0
More than three	8	2.0
Condom use during sexual intercourse		
Yes	205	51.0
No	197	49.0
Use of protective gears		
Yes	363	90.3
No	39	9.7
Frequency of protective gear use		
Daily, so long as being at work	298	74.1
Once in a while	65	16.2
Depends on availability	39	9.7

Somalia, where it was found that only 56% of HCWs had received the HBV vaccine, with a very low completion rate of 16.6% (20). This shows that HCWs need to be sensitized more on vaccine completion, because most of them showed higher non-completion status.

In this study, only 43.5% of HCWs had good knowledge of HBV infection. It is therefore important that HCWs are educated about occupational diseases, in particular HBV, to increase their hepatitis B vaccine uptake, as studies have shown a significant association between good knowledge and HBV vaccine uptake (19, 21).

The occupational risk of acquiring HBV infection was also explored in this study, and it was found that splashes of body fluids from patients were the most common occupational accidents at an occurrence of 83.1%, followed by needle stick injuries at 46.8%. The incidences of body fluid splashes are higher than that found in a study in Cameroon, where only 56% of participants reported similar accidents (22). This shows that HCWs are at risk of acquiring infections, given the risk of needle stick injuries and body fluid splashes, which might be infected and expose them to infection acquisition.

The factors that were found to be significantly associated with HBV vaccine uptake include the sex of the participant, area of residence, duration of employment, history of needle prick injury, and high perception of susceptibility to HBV infection.

Residents of the Ilemela district showed higher rates of vaccine completion compared to HCWs in Misungwi, which represents the rural setting of the study. The findings exemplify studies in other regions of Africa, where urban residents showed higher HBV vaccine uptake compared to rural residents (23, 24). This is because there is limited research and supplies in rural settings compared to urban settings, which could be a cause of this compromise. Therefore, a focus should also be centered on rural health facilities to protect this vulnerable group.

Another factor that showed a significant association with vaccination was a history of needle stick injuries. Needle stick injuries are very common among HCWs; most studies report a prevalence range between 40 and 65% (5, 6, 25). This predisposes them to the risk of acquiring HBV infection and therefore the need for vaccination to prevent infection acquisition. Therefore, this finding emphasizes the need for sensitization and resource mobilization campaigns among HCWs on infection prevention control strategies to reduce the risk of acquiring HBV and other blood born infections. This will help increase their vaccination rates and reduce their risk of acquiring HBV infection through occupational accidents.

Male respondents were more likely to uptake full vaccination than female participants in this study. This finding is incongruent with other studies conducted elsewhere in Africa, most of which reported higher vaccination coverage among female participants (26–28). This was related to the fact that female people are usually more conscious

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TABLE 3 Vaccination status of HCWs and barriers to vaccination (*N*=402).

Variable	Frequency (*n*)	Percentage (%)
HBV vaccination status		
Vaccinated with at least one shot	220	54.7
Never been vaccinated	182	45.3
Number of shots received		
Never vaccinated	182	45.3
One	45	11.2
Two	99	24.6
Three	76	18.9
Reasons for not vaccinating		
Unavailability of vaccine at my facility	100	54.9
I have never thought of getting the HBV vaccine	33	18.1
I do not know where to find the HBV vaccine	4	2.2
I have not heard of the HBV vaccine	6	3.3
No enough motivation	18	9.9
No money to cover the cost	7	3.8
Not ready to be vaccinated	5	2.7
Other reasons	9	4.9

TABLE 4 Comparison of vaccination status among HCWs in Ilemela and Misungwi districts (*N*=402).

District	Fully Vaccinated *n* (%)	Not vaccinated and partial vaccination n (%)	aOR value	value of *p*
Ilemela	54 (29.2%)	131(70.8%)		
Misungwi	22 (10.1%)	195 (89.9%)	5.75 (2.91–11.35)	<0.01

when it comes to disease prevention and control (26). The implication of this finding in our study is that male people have now become more responsive to their health and are willing to take part in the whole process of disease prevention.

Duration of employment was significantly associated with full vaccination status among the participants. The participants who had worked for more than 2 years were more likely to have complete vaccination than those who had less than 2 years of employment in this study. The findings are in line with the findings in the Muhimbili national hospital, where employees who had a long duration of employment were likely to have been vaccinated fully (7). Moreover, studies conducted in Ghana and Uganda among medical students showed vaccination to be associated with advanced years of study among participants (28, 29). The cause of this may be an increased understanding of HBV disease with years of work and study that drive HCWs and students to receive vaccines.

Moreover, HCWs who perceived higher susceptibility to HBV infection showed a significant HBV vaccination rate. This is probably a good sign, as this shows that with a higher perception of infection the prevention strategies are likely to be taken into account, as evidenced by the study participants; however, the vaccination rate is not yet satisfactory. The findings are in line with a study conducted among HCWs in Ethiopia, which showed a significant vaccination rate with the perception of higher susceptibility to HBV infection (6, 26). However, this finding is different to most other studies that showed that participants who perceived higher HBV infection susceptibility still had low vaccination rates (5, 6, 25, 26). Therefore, national programs should emphasize vaccination, as it is possible to vaccinate with a higher perception of infection acquisition.

Most of the studies only focus on only disease status and screening, but not on the linkage to HBV vaccination centers, of which this study is an example. Therefore, it is important to have more studies that screen, provide free vaccines to, and link the diseased HCWs to treatment clinics for follow-up. The studies and campaigns should be conducted at least twice a year to capture HCWs who are newly employed, as this study showed that those who had worked for less than 2 years were unlikely to have received the HBV vaccine. This will help to increase coverage of HBV vaccination and hence reduce HCW–patient transmission of HBV. Moreover, it is important to have HBV vaccination as a compulsory requirement before employment to increase vaccine uptake among newly employed HCWs.

5. Conclusion

This study showed low coverage of the hepatitis B vaccine among healthcare workers despite the existence of risky exposures. Therefore, advocacy campaigns and HBV vaccine mobilization in primary health facilities are pivotal to saving this important population group. Moreover, there is a need for continuous medical training on infection prevention and control. The HBV vaccine should be forwarded as one of the prerequisites for employment among HCWs to protect them from the risk of acquiring HBV infection during their practice. Further studies on seroprevalence are recommended among HCWs in primary levels of facilities to attain reliable data on the prevalence of HBV infection among HCWs in these facilities and HBV vaccine uptake.

5.1. Limitations of the study and recommendations

This study used only questionnaires to assess vaccine uptake and HBV status among HCWs. It did not go further to assess HBsAg or HBsAb status among HCWs, which are measures of HBV infection status and exposure status to HBV. Furthermore, vaccination status was self-reported among respondents, and there was no available documentation to verify that they were vaccinated. It is therefore important that as vaccination programs continue operating documentation should be given to the clients to have proof of

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TABLE 5 Factors associated with full HBV vaccine uptake among HCWs in Misungwi and Ilemela districts (n=402).

Variable	Fully vaccination	Partial or no vaccination	Crude OR (95% CI)	value of p (95%CI)	Adjusted OR	value of p (95% CI)
Age						
15–44 years	69 (19.8%)	280 (80.2%)	Reference			
45 years and above	7 (13.2%)	46 (86.8%)	0.62 (0.27–1.43)	0.26		
Sex						
Female	38 (16.7%)	189 (83.3%)	Reference			
Male	38 (21.7%)	137 (78.3%)	1.38 (0.84–2.28)	0.20	2.38 (1.28–4.45)	0.006
District						
Misungwi	22 (10.1%)	195 (89.9%)	Reference			
Ilemela	54 (29.2%)	131 (70.8%)	3.65 (2.12–6.29)	<0.01	5.75(2.91–11.35)	<0.01
Facility level						
Dispensary	35 (21.3%)	129 (78.7%)	Reference			
Health center	41 (17.2%)	197 (82.8%)	0.77 (0.46–1.27)	0.30		
Years of employment						
Less than 2 years	5 (9.8%)	46 (90.2%)	Reference			
More than 2 years	71 (202%)	280 (79.8%)	2.33 (0.89–6.09)	0.076	3.58(1.19–10.74)	0.023
Medical cadre						
Medical attendant	10 (12.7%)	69 (87.3%)	Reference			
Nurse	24 (16.2%)	124 (83.8%)	0.49 (2.36–10.62)	<0.01	0.09 (0.36–0.25)	<0.01
Laboratory personnel	14 (31.8%)	30 (68.2%)	0.42 (2.07–7.94)	<0.01	0.16 (0.05–0.29)	<0.01
pharmacist	1 (8.3%)	11 (91.7%)	0.48 (1.19–3.30)	0.013	0.30 (0.12–0.78)	0.013
Clinical officer	7 (8.1%)	79 (91.9%)	1.10 (2.83–16.92)	<0.01	0.06 (0.02–0.51)	0.01
Medical doctor	20 (60.6%)	13 (39.4%)	0.53 (2.85–17.36)	0.01	0.06 (0.02–0.16)	<0.01
Needle prick injuries						
No	19 (8.9%)	195 (91.1%)	Reference			
Yes	57 (30.3%)	131 (69.7%)	4.47 (2.54–7.85)	<0.01	6.87(3.55–13.26)	<0.01
Perceived susceptibility						
Low susceptibility	15 (9.3%)	146 (90.7%)	Reference			
High susceptibility	61 (25.3%)	180 (74.7%)	3.30 (1.80–6.04)	<0.01	2.20 (1.02–4.75)	0.044
Perceived severity						
Low severity	73 (19.0%)	311 (81.0%)	Reference			
High severity	3 (16.7%)	15 (83.3%)	0.85 (0.24–3.02)	0.80		
Perceived benefits						
Low benefits	74 (21.0%)	279 (79.0%)	Reference			
High benefits	2 (4.1%)	47 (95.5%)	0.16 (0.04–0.68)	0.005	0.34 (0.07–1.53)	0.159
Perceived efficacy						
Low efficacy	28 (13.2%)	184 (86.8%)	Reference			
High efficacy	48 (25.3%)	142 (74.7%)	2.22 (1.33–3.12)	0.002	0.90 (0.44–1.84)	0.774
Barriers to action						
high barriers	35 (15.0%)	199 (85.0%)	Reference			
Low barriers	41 (24.4%)	127 (75.6%)	1.84 (1.11–3.04)	0.017	1.08 (0.58–2.03)	0.80
Cues to action						
Less motivated to vaccinate	32 (12.5%)	225 (87.5%)	Reference			
Highly motivated to vaccinate	44 (30.3%)	101 (69.7%)	3.06 (1.84–5.11)	<0.00	1.81 (0.92–3.58)	0.087
Knowledge						
Poor and average knowledge	41 (18.1%)	186 (81.9%)	Reference			
Good knowledge	35 (20.0%)	140 (80.0%)	1.13 (0.69–1.87)	0.62		

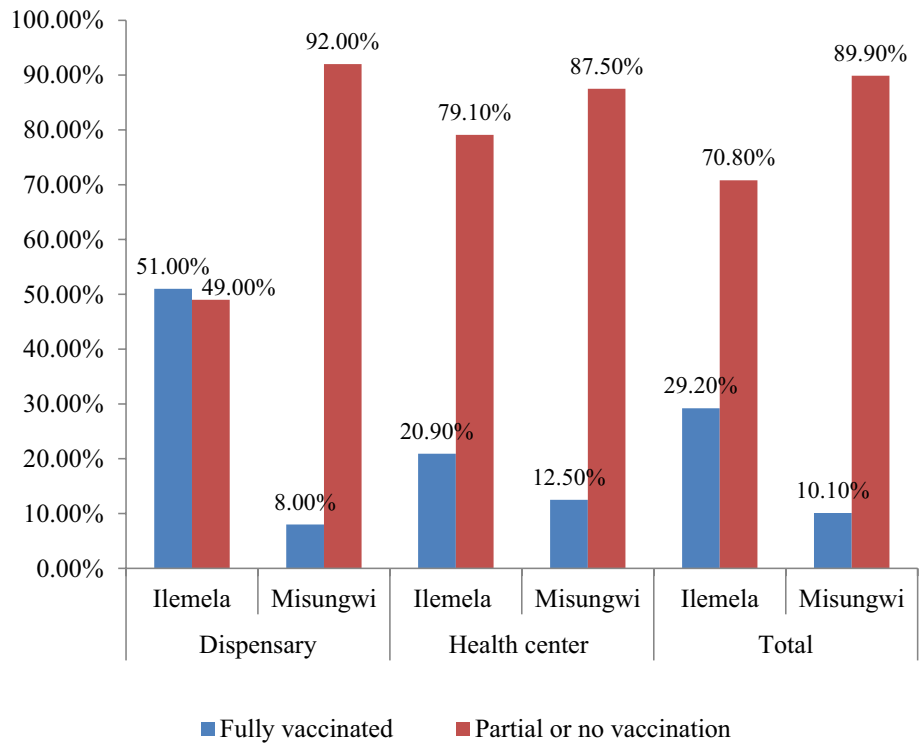

FIGURE 1
Comparison of vaccination status among HCWs in Ilemela and Misungwi districts by facility levels.

vaccination against HBV. Moreover, further studies should be conducted that assess the vaccination status among these HCWs through the measurement of HBV antibodies acquired through vaccination to attain more reliable data on seroprevalence.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The studies involving human participants were reviewed and approved by the Joint Catholic University of Health and Allied Sciences–Bugando Research and Ethics Committee (CREC/573/2022). The participants provided their written informed consent to participate in this study.

Author contributions

BN, FM, and AK designed the study and conception. BN, FH, and DW conducted and supervised data collection, data analysis, and interpretation of the findings. BN and DW wrote the first draft of the manuscript. SK, FM, MM, and AK critically reviewed the manuscript and approved its submission. All authors approved the submission of the manuscript.

Acknowledgments

The assistance offered by the local authorities in the Misungwi and Ilemela districts, healthcare workers, and all study participants is highly appreciated. The authors are thankful to the Catholic University of Health and Allied Sciences (CUHAS)–Bugando joint ethics and review committee for allowing this study to be conducted.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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frontiers | Frontiers in Cellular and Infection Microbiology

TYPE Original Research
PUBLISHED 16 June 2023
DOI 10.3389/fcimb.2023.1151899

OPEN ACCESS

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RECEIVED 26 January 2023
ACCEPTED 12 May 2023
PUBLISHED 16 June 2023

CITATION
Li J, Dong X-Q, Cao L-H, Zhang Z-Q, Zhao W-F, Shang Q-H, Zhang D-Z, Ma A-L, Xie Q, Gui H-L, Zhang G, Liu Y-X, Shang J, Xie S-B, Liu Y-Q, Zhang C, Wang G-Q, Zhao H and China HepB Related Fibrosis Assessment Research Group (2023) Factors associated with persistent positive in HBV DNA level in patients with chronic Hepatitis B receiving entecavir treatment. *Front. Cell. Infect. Microbiol.* 13:1151899. doi: 10.3389/fcimb.2023.1151899

Factors associated with persistent positive in HBV DNA level in patients with chronic Hepatitis B receiving entecavir treatment

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Introduction: The clinical significance of persistent positive in Hepatitis B Virus (HBV) DNA level in patients receiving antiviral therapy is not well known. We investigated factors associated with persistent viremia (PV) in patients with chronic hepatitis B (CHB) given 78-week entecavir.

Methods: A total of 394 treatment-naïve CHB patients who had undergone liver biopsy at baseline and week 78 of treatment were analyzed in this prospective multicentre study. We identified patients with PV (above the lower limit of quantification, 20 IU/ml) after 78 weeks of entecavir therapy. Stepwise, forward, multivariate regression analyses of specified baseline parameters were apllied to identify factors associated with PV. Futhermore, we assessed the incidence of hepatocellular carcinoma (HCC) in all patients using models of the risk of HCC development.

Results: Of the 394 patients, 90 (22.8%) still with PV after 78-week antiviral treatment. Factors associated significantly with PV (vs complete virological response, CVR) were HBV DNA level ≥8 log10 IU/mL (OR, 3.727; 95% CI, 1.851-

7.505; P < 0.001), Anti-HBc level < 3 log10 IU/mL (OR, 2.384; 95% CI, 1.223-4.645; P=0.011), and HBeAg seropositivity (OR, 2.871; 95% CI, 1.563-5.272; P < 0.001). Patients with PV were less likely to have fibrosis progression and HCC development than those with the CVR. Of the 11 HBeAg-positive patients with HBV DNA level ≥8 log10 IU/mL and Anti-HBc level < 3 log10 IU/mL at baseline, 9 (81.8%) had persistent positivity in HBV DNA level and 0 had fibrosis progression at week 78 of treatment.

Discussion: In conclusion, HBV DNA level ≥8 log10 IU/mL, Anti-HBc level < 3 log10 IU/mL and HBeAg seropositivity at baseline contribute to PV in patients with CHB receiving 78-week antiviral treatment. In addition, the rate of fibrosis progression and the risk of HCC development in patients with PV were kept low. The complete protocol for the clinical trial has been registered at clinicaltrials.gov (NCT01962155 and NCT03568578).

KEYWORDS

chronic hepatitis B, persistant viremia, HBV DNA, anti-hepatitis B virus core antibody, fibrosis, carcinoma

Introduction

Chronic hepatitis B (CHB) affects more than 250 million people worldwide and causes annual mortality of nearly 1 million from cirrhosis, hepatocellular carcinoma and other diseases associated with hepatitis B virus (HBV) infection (World Health Organization, 2016; Polaris Observatory Collaborators, 2018). Nucleos(t)ide analogues (NAs) approved for CHB treatment have been demonstrated to reduce HBV disease progression, reverse liver fibrosis and decrease the risk of hepatocellular carcinoma (HCC) development. Entecavir (ETV) and tenofovir disoproxil fumarate (TDF) are clinically used as first-line nucleos(t)ide analogue (NA) antivirals for the treatment of patients with CHB, and both have reasonably improved the rates of virological suppression, biochemical and serological response. Both drugs display high genetic barriers with very minimal resistance and high rates of viral suppression (Tenney et al., 2009; Chang et al., 2010; Yang et al., 2013).

In controlled clinical trials, most but not all patients with CHB experience undetectable serum HBV DNA levels on oral antiviral therapy. Reported rates of undetectable serum HBV DNA (<300 copies/mL) range from 67% to 97% among hepatitis B e antigen–positive (HBeAg+) patients (Chang et al., 2006; Gish et al., 2007; Pan et al., 2012; Marcellin et al., 2013) and 90% to 97% among HBeAg-negative (HBeAg-) patients (Lai et al., 2006; Marcellin et al., 2013). Precisely why a proportion of patients do not achieve an undetectable serum HBV DNA level despite apparently effective antiviral treatment has not been fully explored. Both the stability of the HBV covalently closed circular DNA (cccDNA) in the hepatocyte nucleus (Allweiss and Dandri, 2017), and the resistance of HBV to NAs (Sinn et al., 2011) are believed to be some mechanisms.

Some reports suggested that persistent positive in HBV DNA level after NA therapy was associated with a higher risk of hepatocellular carcinoma (HCC) occurrence and fibrosis progression (Kim et al., 2017; Sun et al., 2020). However, it had also been reported that low-level viremia (LLV, <2,000 IU/mL) during treatment was not a predictive factor for HCC and cirrhotic complications in patients with treatment-naïve CHB and good adherence to ETV treatment (Lee et al., 2020). And Lee et al. suggested that episodic LLV among untreated patients with compensated cirrhosis did not increase the risk of disease progression compared with maintained virological response status (Lee et al., 2022). As a result, it remains unclear whether it is more beneficial to continue original NAs or to switch/add another NA in order to prevent liver-related events including fibrosis progression and HCC development in persistent viremia (PV, above the lower limit of quantification, 20 IU/ml) patients. The AASLD 2018 hepatitis B guidance suggested that persons with persistent LLV on ETV or TDF monotherapy should continue monotherapy, but the quality and certainty of this evidence was very low (Terrault et al., 2018).

Therefore, the objectives of this study were to identify factors associated with PV and investigate whether patients with PV were associated with HCC development and fibrosis progression, so as to further explore the next treatment options for patients with PV, by analyzing data collected from a well-characterized cohort of CHB patients that have been treated with ETV for 78 weeks.

Materials and methods

Patients

This multi-center, prospective, longitudinal study included 780 Chinese treatment-naïve patients with CHB who were consecutively admitted from 24 teaching hospitals located in Mainland China between October 2013 and May 2021. Patients recruited in the

cohort study were those with hepatitis B surface antigen (HBsAg) positive for at least 6 months and negative of other forms of chronic liver diseases (CLD), decompensate liver cirrhosis or HCC. Patients who had received treatments with either bicyclol or antiviral drugs within 26 weeks before the recruitment were excluded. The specific inclusion and exclusion criteria had been described previously (Deng et al., 2015). Demographic data were collected at baseline. Clinical data, including blood test results and liver stiffness measurement (LSM) were recorded at the time of liver biopsy at baseline and every 26 weeks of follow-up in local hospitals. Paired liver biopsies were performed at baseline and week 78. The study was approved by the Ethics Committee of Peking University First Hospital and the other 23 teaching hospitals. All patients gave informed consent for research use of their clinical data and liver biopsy specimens. All authors had access to the study data and reviewed and approved the final manuscript.

Laboratory assessments

Blood specimens were routinely obtained on the same day of liver biopsy in local hospitals. Serum HBV DNA quantitation was detected at central laboratory using Roche COBAS TaqMan platform (lower limit of detection 20 IU/mL) according to manufacturer's instructions. Levels of HBV serological markers (HBsAg/anti-HBs, HBeAg/anti-HBe) were measured by relevant Roche Elecsys® assays (Roche Diagnostics, Penzberg, Germany). Quantitative detection of anti-hepatitis B virus core antibody (Anti-HBc) was performed using Sandwich enzyme-linked immunosorbent assays (Jia et al., 2014). The level of HBV DNA, HBsAg, and Anti-HBc were expressed as $\log_{10}$ IU/mL.

Liver stiffness measurement

LSM was performed on fasting patients at baseline and week 78 using 1-dimensional ultrasound TE (FibroScan®, Echosens, Paris, France) in local hospitals. All operators who had no knowledge of the patients' clinical data were trained according to the manufacturer's recommendations. LSM values are expressed in units of kilopascals (kPa). Only a procedure with at least ten valid measurements, an interquartile range (IQR)/median value (IQR/M) <30% and a success rate >60% was considered reliable (Sandrin et al., 2003).

Fibrosis-4 and AST to platelet ratio index scores

The two noninvasive indexes for fibrosis were calculated at baseline and week 78 based on the following formulas:

$$\text{FIB}-4 = (\text{age} \times \text{AST})/(\text{platelet count})/[\times 10^9/\text{L}] \times \text{ALT}^{1/2})$$

(Sterling et al., 2006);

$$\text{APRI} = ([\text{AST}/\text{ULN}]/\text{platelet count}[\times 10^9/\text{L}]) \times 100$$

(Wai et al., 2003).

Liver histological assessment

Ultrasonographic-guided liver biopsies were performed at baseline (on day before starting antiviral therapy) and week 78 in each institute. A minimum of 20 mm of liver biopsy with at least 11 portal tracts was considered adequate for diagnosis. All liver biopsies were blindly and independently reviewed by 2 hepatopathologists from Beijing You An Hospital affiliated to Capital Medical University. When discrepancies occurred, final decision was made by the third experienced hepatopathologist who was also responsible for reassessment of 10% samples by random drawing. Necro-inflammation and fibrosis were assessed with the Ishak scoring system (Ishak et al., 1995). Fibrosis was scored as follows: F0-1, no/mild fibrosis; F≥2, moderate fibrosis; F≥3, significant fibrosis; F≥4, advanced fibrosis; and F≥5, cirrhosis. Histological inflammation grading was performed using the modified histology activity index (HAI), and the scored as follows: HAI 0-4, zero to mild inflammation; HAI 5-18, moderate to severe inflammation. Histological improvement was defined as ≥2-point decrease in the HAI score and without concurrent worsening of the fibrosis score 78 weeks after the therapy initiation. Fibrosis improvement was defined as ≥1-point decrease in the Ishak fibrosis score, whereas ≥1-point increase was considered as fibrosis progression. Inflammation improvement was defined as ≥2-point decrease in the HAI score.

Statistical analyses

Statistical analysis was performed using SPSS 20.0 (SPSS Inc., Chicago, IL, USA). Patients' characteristics were summarized as median (range), or numbers of cases and percentages, as appropriate. Continuous variables were compared using Student t test or Mann-Whitney test, categorical variables were compared using Chi-squared test or Fisher's exact test. Univariate and multivariate logistic regression analysis were used to identify independent predictors associated with persistent positivity in HBV DNA level after 78-week antiviral therapy. Factors significant in univariate analyses were included in the multivariate model using the forward selection procedure; predictors were retained in the model if the P value was less than 0.10. All statistical tests were two-sided, and $P<0.05$ was considered statistical significance.The original data was shown in https://github.com/Xiaoqind/Factors-associated-with-PV.

Results

Study population

Of 780 Chinese treatment-naïve CHB patients who met the eligible criteria and with qualified liver biopsy at baseline, 504 (64.6%) patients received entecavir-based therapy and were prospectively followed to 78 weeks for second liver biopsy. Ishak fibrosis scores was available at all two time-points (baseline and

week 78) for 394 patients (Supplementary Figure 1). In total, 90 patients (22.8%) had PV at week 78.

Predictors of persistent positive in HBV DNA level after 78-week antiviral therapy

Baseline demographic and disease characteristics of patients with PV and patients with complete virological responses (CVR, below the lower limit of quantification, 20 IU/ml) at week 78 are summarized in Table 1. Compared with the group that achieved CVR, patients with PV were younger. A higher proportion of patients with PV were HBeAg positive and this group had higher baseline HBV DNA and HBsAg levels, and lower Anti-HBc level compared with the CVR group. Baseline liver biopsy analysis showed that patients with PV had less fibrosis than patients with CVR (Ishak fibrosis score>3: 43.3% vs 56.6%; *P*=0.027), but the prevalence of necroinflammation was similar (HAI ≥5: 77.8% vs 70.7%; *P*=0.189). There was no difference between the two groups in the terms of sex, BMI, PLT, ALT, ALB, TBIL, AFP, CR, FPG, FIB-4, APRI, LSM, patients with family history of HCC or hepatitis B.

Furthermore, according to different baseline HBV DNA levels, we analyzed the virological responses in all patients after 78-week antiviral therapy (Figure 1). Among patients with baseline HBV DNA level <2 $\log_{10}$ IU/mL, none has showed PV. As baseline HBV DNA levels increased, the proportion of patients with PV also increased after 78 weeks of treatment (when the HBV DNA levels were between 2-3, 3-4, 4-5, 5-6, 6-7,7-8, and ≥8, there were 4.3%, 10.6%, 10.3%, 17.9%, 27.1%, 29.0% and 58.1% patients occurred PV, respectively). Similarly, in patients with PV, the higher the baseline HBV DNA level was, the greater the proportion of patients has developed PV (when the HBV DNA levels were between 2-3, 3-4, 4-

TABLE 1 Baseline demographics and disease characteristics of 394 patients with and without complete virological response at week 78 (univariate analysis).

Variables	Total n=394	Persistent viremia n=90	Complete virological response n=304	*P*-value*
Age (years) ≤30y, n (%)	77 (19.5)	27 (30.0)	50 (16.4)	**0.004**
Male gender, n (%)	294 (74.6)	69 (76.7)	225 (74.0)	0.611
BMI ≥25 kg/m^2, n (%)	134 (34.0)	32 (35.6)	102 (33.6)	0.725
PLT (×10^9/L)	155.0 (121.0-194.0)	155.0(127.3-199.0)	155.0 (118.3-193.8)	0.609
ALT ≤ 40 IU/ml, n (%)	123 (31.2)	24 (26.7)	99 (32.6)	0.289
ALT ≤ 30 IU/ml for male and ≤ 19 IU/ml for female, n (%)	47 (11.9)	8 (8.9)	39 (12.8)	0.311
Albumin ≥35 g/L, n (%)	373 (94.7)	88 (97.8)	285 (93.8)	0.135
TBIL ≤ 17.1 μmol/L, n (%)	245 (62.2)	53 (58.9)	192 (63.2)	0.463
TC ≤ 5.18 mmol/L, n (%)	332 (84.3)	71 (78.9)	261 (85.9)	0.111
TG ≤ 1.69 mmol/L, n (%)	351 (89.1)	81 (90.0)	270 (88.8)	0.752
Cr ≤ 106 IU/ml for male and ≤ 97 IU/ml for female, n (%)	387 (98.2)	88 (97.8)	299 (98.4)	0.661
FPG ≥7.0 mmol/L, n(%)	11 (2.8)	1 (1.1)	10 (3.3)	0.461
HBV DNA ≥8 log^{10}IU/mL, n(%)	43 (10.9)	25 (27.8)	18 (5.9)	**<0.0001**
HBsAg ≥4 log10 IU/mL, n(%)	53 (13.5%)	27 (30.0)	26 (8.6)	**<0.0001**
HBeAg positive, n (%)	226 (57.4%)	73 (81.1)	153(50.3)	**<0.0001**
qAnti-HBc<3log^{10}IU/mL), n(%)	49 (12.4%)	22 (24.4)	27 (8.9)	**<0.0001**
FIB-4	1.5 (1.0-2.3)	1.4 (0.9-2.3)	1.6 (1.0-2.4)	0.329
<1.45, n(%)	166 (42.1)	44 (48.9)	122 (40.1)	
1.45-3.25, n(%)	191 (48.5)	39 (43.3)	152 (50.0)	
>3.25, n(%)	37 (9.4)	7 (7.8)	30 (9.9)	
APRI	0.7 (0.4-1.3)	0.9 (0.4-1.4)	0.7 (0.4-1.3)	0.636
≤0.5, n(%)	105 (26.6)	23 (25.6)	82 (27.0)	
0.5-1.5, n(%)	186 (47.2)	40 (44.4)	146 (48.0)	
>1.5, n(%)	103 (26.1)	27 (30.0)	76 (25.0)	

(Continued)

TABLE 1 Continued

Variables	Total n=394	Persistent viremia n=90	Complete virological response n=304	*P*-value*
LSM (kPa)	11.7 (8.1-17.6)	12.2 (8.6-19.0)	11.5 (7.8-17.2)	0.774
<7.4, n(%)	76 (19.3)	15 (17.4)	61 (20.1)	
7.4-9, n(%)	57 (14.5)	12 (13.0)	45 (14.8)	
9-12, n(%)	78 (19.8)	17 (17.8)	61 (20.1)	
≥12, n(%)	183 (46.4)	46 (41.8)	137 (45.1)	
HAI ≥5, n(%)	285 (72.3)	70 (77.8)	215 (70.7)	0.189
Fibrosis stages >3, n(%)	211 (53.6)	39 (43.3)	172 (56.6)	**0.027**
HAI ≥5 or F≥3, n(%)	365 (92.6)	82 (91.1)	283 (93.1)	0.527
Patients with family history of HCC, n(%)	48 (12.2)	14 (15.6)	34 (11.2)	0.270
Patients with family history of hepatitis B, n(%)	188 (47.7)	46 (51.1)	142 (47.0)	0.495

P value: comparision between patients with with and without complete virological response at week 78. %, percentage, range from 0-100%。
BMI, body mass index; ALT, Alanine transaminase; TBil, total bilirubin; CR, creatinine; FPG, fasting plasma glucose; PLT, platelet counts; TC total cholesterol; TG triglyceride; AFP, alpha fetoprotein; LSM, liver stiffness measurement; HBsAg, hepatitis B surface antigen; HBeAg, hepatitis B e antigen; HBV, hepatitis B virus; Anti-HBc, hepatitis B core antibody; HAI, histology activity index; ULN upper limit of normal; FIB-4, fibrosis-4; APRI, AST to platelet ratio index. The meaning of the bold values is to highlight statistically significant indicators.

5, 5-6, 6-7, 7-8, and ≥8, the proportions were 1.1%, 5.6%, 6.7%, 15.6%, 21.1%, 22.2% and 27.8%, respectively.) (Figure 2).

In a multivariate analysis (Table 2), 3 baseline variables were identified to be associated independently with PV at week 78: baseline HBV DNA level ≥8 $\log_{10}$ IU/mL (odds ratio [OR], 3.727; 95% confidence interval [CI], 1.851-7.505; *P*<0.001), baseline Anti-HBc level<3 $\log_{10}$ IU/mL (OR, 2.384; 95% CI, 1.223-4.645; *P*=0.011), and baseline HBeAg seropositivity (OR, 2.871; 95% CI, 1.563-5.272; *P*<0.001). As shown in Table 2, among patients with baseline HBV DNA level ≥8 $\log_{10}$ IU/mL, 58.1% of patients had PV at week 78 compared with 18.5% of those with CVR. Similarly, 44.8% of patients with Anti-HBc level<3 $\log_{10}$ IU/mL had PV at week 78 compared with 19.7% of those with CVR. In HBeAg+ patient, 32.3% had persistence viremia and 10.1% achieved CVR at week 78 (both *P*<0.001). Among HBeAg+ patients with HBV DNA level ≥8 $\log_{10}$ IU/mL and Anti-HBc level<3 $\log_{10}$ IU/mL at baseline, 81.8% had PV at week 78 compared with 10.1% of HBeAg- patients with HBV DNA level<8 $\log_{10}$ IU/mL and Anti-HBc level ≥3 $\log_{10}$ IU/mL (*P*<0.001) (Figure 3). Of the 11 HBeAg-positive patients with baseline HBV DNA level ≥8 $\log_{10}$ IU/mL and Anti-HBc level<3 $\log_{10}$ IU/mL, none had shown fibrosis progression at week 78 of treatment (*P*<0.001).

On-treatment characteristics associated with persistent positive in HBV DNA level

On-treatment serum immunological, biochemical, and histologic responses associated with PV patients are summarized in Table 3.

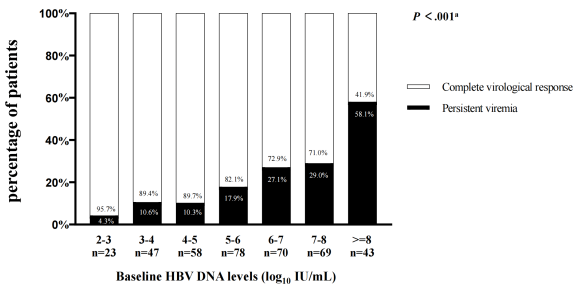

FIGURE 1
The virological responses in patients after 78-week antiviral therapy according to different HBV DNA levels at baseline. [a]Comparisons by the Chi-squared test (between 2-3, 3-4, 4-5, 5-6, 6-7 ,7-8, and ≥8 groups).

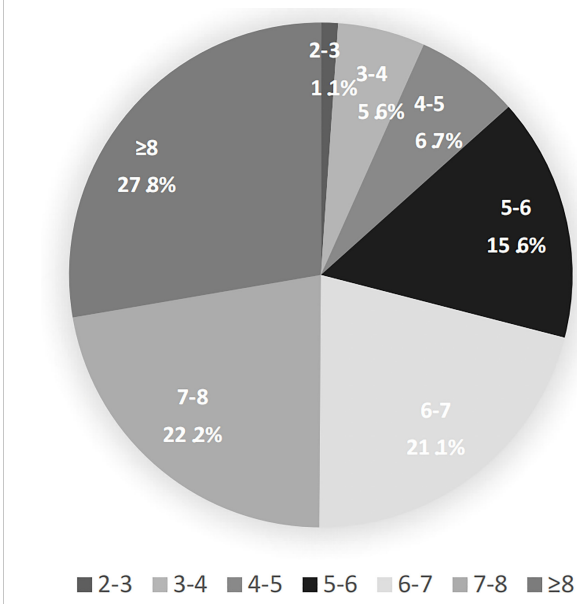

FIGURE 2
The proportion of patients in persistent viremia group according to different HBV DNA levels at baseline.

TABLE 2 Baseline characteristics associated with persistent viremia (multivariate analysis).

Parameters	OR	95% CI	P value
HBV DNA level ≥8 log10 IU/mL	3.727	1.851-7.505	**<0.0001**
Anti-HBc level<3 log10 IU/mL	2.384	1.223-4.645	0.011
HBeAg positive vs negative	2.871	1.563-5.272	0.001
Age (years) ≤30y			0.241
HBsAg ≥4 log10 IU/mL			0.402
Fibrosis stages >3			0.106

OR, odds ratio; CI, confidence interval; HBV, hepatitis B virus; Anti-HBc, hepatitis B core antibody; HBeAg, hepatitis B e antigen. The meaning of the bold values is to highlight statistically significant indicators.

Compared with patients with CVR, patients with PV were less likely to achieve HBeAg loss (21.9% vs 35.9%; *P*=0.034); however, no differences in the reduction of HBsAg level were shown. Significant differences in histologic responses were observed, where patients with PV were less likely to have a worse LSM value (0% vs 5.9%, *P*=0.038) and Ishak fibrosis score (11.1% vs 21.4%, *P*=0.029) than patients with CVR at week 78. In addition, there was no significant difference between the two groups in obtaining normal ALT levels (92.2% vs 91.4%, *P*=0.816) and histological improvement (58.9% vs 50.3%, *P*=0.153) after 78-week antiviral therapy.

Furthermore, we divided the patients with fibrosis progression into 3 groups based on baseline Ishak fibrosis score: group 1 with Ishak fibrosis score between 0-2 (33 patients); group 2 with Ishak fibrosis score between 3-4 (39 patients); group 3 with Ishak fibrosis score between 5-6 (3 patients). There were 5 (15.2%), 5 (12.8%) and 0 (0%) patients in groups 1, 2 and 3, respectively, with PV (Supplementary Figure 2).

Estimated risk of HCC occurrence using different HCC risk scores

Considering that some models evaluating the risk of HCC development which suitable for the untreated patients with chronic HBV infection include the serum HBV DNA level as a constituent variable, we selected the models which suitable for the treated patients to avoid overestimating the incidence of HCC. According to the CAMD score (Hsu et al., 2018), more patients with PV were observed in the low-risk group (CAMD score, <8; 73.3% vs 56.9%), whilst less patients with PV were in the high-risk group (CAMD score, >13; 4.4% vs 8.9%), compared to the patients with CVR (*P* =0.018). Although there was no significant difference according to the AMAP score (Fan et al., 2020), AASL-HCC score (Yu et al., 2019), HCC-ESCAVT score (Lim et al., 2020), CAMPA score (Lee et al., 2020), HCC-RESCUE score (Sohn et al., 2017), and mPAGE-B HCC score (Kim et al., 2018), more patients with PV were in the low-risk group than patients with CVR (Table 4).

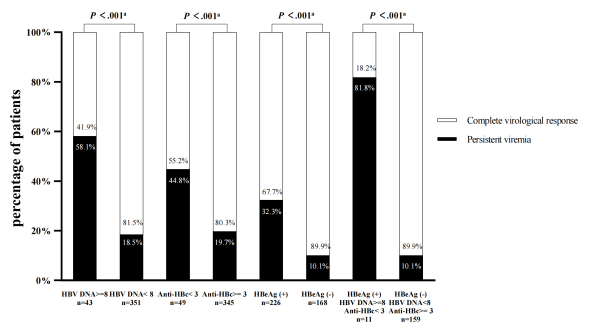

FIGURE 3
Effects of baseline HBV DNA level ≥8 log10 IU/mL, Anti-HBc level<3 log10 IU/mL and HBeAg status on persistent viremia at week 78. [a]Comparisons between the related two groups (HBV DNA level ≥8 log10 IU/mL vs <8 log10 IU/mL; Anti-HBc level<3 log10 IU/mL vs ≥3log10 IU/mL; HBeAg+, HBV DNA level ≥8 log10 IU/mL and Anti-HBc level<3 log10 IU/mL vs HBeAg- , HBV DNA level<8 log10 IU/mL and Anti-HBc level ≥3 log10 IU/mL).

Discussion

In the era of NAs therapy, most patients with CHB achieve CVR and the majority experience fibrosis regression including the reversal of cirrhosis (Chang et al., 2006; Lai et al., 2006; Chang et al., 2010; Xu et al., 2015). However, despite these highly beneficial outcomes, a small number of patients still fail to achieve CVR, and the clinical significance of a PV with respect to the outcome of CHB is not clear. In this analysis of predictive factors for PV after 78 weeks of ETV treatment in a well-characterized cohort of CHB patients, we found that HBV DNA level ≥8 $\log_{10}$ IU/mL, Anti-HBc level<3 $\log_{10}$ IU/mL, and HBeAg seropositivity are independently predictive of failure to achieve CVR. Furthermore, of the 11 HBeAg-positive patients with HBV DNA level ≥8 $\log_{10}$ IU/mL and Anti-HBc level<3 $\log_{10}$ IU/mL at baseline, 9 (81.8%) had persistent positive in HBV DNA level and 0 had fibrosis progression at week 78 of treatment. Of the 90 patients with PV, only 10 (11.1%) had fibrosis progression and 0-7 (0-7.8%) were with high risk of HCC occurrence according to different HCC risk scores.

Higher levels of HBV DNA are associated with an increased risk for HCC and cirrhosis (Chen et al., 2011), but its impact on virological response is debatable. Yuen et al. reported that 100% of treatment-naïve HBV patients who received entecavir for 3 years with baseline HBV DNA <8 $\log_{10}$ copies/mL had undetectable HBV DNA (<12 IU/mL), whereas only 75% of patients with baseline HBV DNA ≥8 $\log_{10}$ copies/mL did (Yuen et al., 2011). Gordon et al. reported that equal virologic responsiveness between CHB patients

TABLE 3 Different treatment responses between persistent viremia group and complete virological response group (univariate analysis).

	Total n=394	Serum HBV DNA level at week 78		P-value*
		Persistent viremia (n=90)	Complete virological response (n=304)	
Serum immunological responses				
HBeAg loss at week 78, n/N (%)	71/226 (31.4)	16/73 (21.9)	55/153 (35.9)	**0.034**
HBsAg level decrease at week 78, n/N (%)	155/394 (68.6)	28/90 (31.1)	107/304 (35.2)	0.473
>1 log decline in qHBsAg, n/N (%)	38/394 (9.6)	13/90 (14.4)	25/304 (8.2)	0.079
Biochemical response				
ALT normal at week 78, n/N (%)	361/394 (91.6)	83/90 (92.2)	278/304(91.4)	0.816
>1 log decline in qAnti-HBc, n/N (%)	194/394 (49.2)	52/90 (57.8)	142/304 (46.7)	*0.065*
Histologic responses				
APRI score progression at weei 78, n/N (%)	4/394 (1.0)	0/90 (0)	4/304 (1.3)	0.620
FIB-4 score progression at weei 78, n/N (%)	34/394 (8.6)	9/90 (10)	25/304 (8.2)	0.598
LSM value progression at week 78, n/N (%)	18/394 (4.6)	0/90 (0)	18/304 (5.9)	**0.038**
Fibrosis progression at week 78, n/N (%)	75/394 (19.0)	10/90 (11.1)	65/304 (21.4)	**0.029**
Inflammation improvement at week 78, n/N (%)	247/394 (62.7)	58/90 (64.4)	189/304 (62.2)	0.695
Histological improvement at week 78, n/N (%)	206/394 (52.3)	53/90 (58.9)	153/304 (50.3)	0.153

ALT, Alanine transaminase; HBV, hepatitis B virus; Anti-HBc, hepatitis B core antibody; HBeAg, hepatitis B e antigen; HBsAg, hepatitis B surface antigen; LSM, liver stiffness measurement. The meaning of the bold values is to highlight statistically significant indicators.
*P value provided in Tables 3, 4: comparison between patients with and without complete virological response at week 78.

with high viral load (HVL) (HBV DNA ≥9 $\log_{10}$ copies/mL) and with non-HVL, 98.3% of HVL and 99.2% of non-HVL patients achieving HBV DNA <400 copies/mL by week 240 (Gordon et al., 2013). Chan et al. reported that 55% of HBeAg-positive patients with high levels of HBV DNA (mean baseline level of HBV DNA of 8.41 $\log_{10}$ IU/mL) and normal levels of ALT treated with TDF had levels of HBV DNA <69 IU/mL at week 192 (Chan et al., 2014). In our analysis, only 77.2% of treatment-naïve CHB patients who received entecavir for 78 weeks achieve CVR, 81.5% of patients with baseline HBV DNA <8 $\log_{10}$ IU/mL achieved CVR (HBV DNA <20 IU/mL), whilst the CVR rates were only 41.9% in patients with baseline HBV DNA ≥8 $\log_{10}$ IU/mL. The poor efficacy of ETV may be related to previous using of lamivudine (LAM) and telbivudine (LdT), but the proportion of such patients was similar between the two groups. This finding may be attributed to the short time courses of antiviral therapy or some patients may be in immune-tolerant phase. Larger longitudinal studies are warranted to explore this factor further and its potential effect on the virological response to antiviral treatment in CHB patients.

Previous studies have shown that rates of CVR in clinical trials of CHB patients were different between HBeAg+ and HBeAg- patients, and the former tended to have lower rates of CVR compared to the latter (Chang et al., 2006; Lai et al., 2006; Gish et al., 2007; Pan et al., 2012; Marcellin et al., 2013). Consequently, the observed associations between HBeAg-positive and a PV state is not entirely surprising. Among 875 treatment-naive chronic hepatitis B virus (HBV) mono-infected patients, 377 patients with low-level viremia (LLV; <2,000 IU/mL), Kim et al. reported that HBeAg status was the only significant factor associated with LLV (Kim et al., 2017), which coincided with our results.

Our finding that Anti-HBc level at baseline is associated independently with failure to achieve CVR on NAs therapy is novel and may relate to the intensity of liver inflammation. It remains controversial of the association between ALT levels and liver inflammation. Some studies have suggested that substantial CHB patients with normal ALT levels exhibit severe liver damage. Chao et al. reported that approximately one fifth (20.7%) of CHB patients with ALT ≤ 40 IU/L may have significant hepatic fibrosis. The corresponding proportion was 27.8% even when the newer ULN of 30 IU/L (males) and 19 IU/L (females) was applied (Chao et al., 2014).

Recent studies proposed that serum anti-HBc levels could serve as a promising marker for predicting the severity of liver inflammation and exhibited a high diagnostic accuracy in CHB patients with normal ALT (Song et al., 2015; Zhou et al., 2017). In that studies, Anti-HBc can accurately reflect the inflammation of the liver. In our analysis, the levels of ALT at baseline were comparable between the two groups, but the level of Anti-HBc in patients with PV was lower than patients in CVR (3.6 vs 3.9 $\log_{10}$ IU/mL, P<0.001), which indicated that Anti-HBc is a more responsive indicator of liver inflammation than ALT. Of 49 patients with Anti-HBc level<3 $\log_{10}$ IU/mL, 22 (44.9%) failed to

10.3389/fcimb.2023.1151899

TABLE 4 Risk of HCC development in 394 patients with and without complete virological response at different HCC risk prediction models.

Variables	Total n=394	Persistent viremia n=90	Complete virological response n=304	P-value*
CAMD score				**0.018**
low-risk group, n(%)	239 (60.7)	66 (73.3)	173 (56.9)	
intermediate-risk group, n(%)	124 (31.5)	20 (22.2)	104 (34.2)	
high-risk group, n(%)	31 (7.9)	**4 (4.4)**	**27 (8.9)**	
AMAP score				0.204
low-risk group, n(%)	162 (41.1)	43 (47.8)	119 (39.1)	
intermediate-risk group, n(%)	184 (46.7)	40 (44.4)	144 (47.4)	
high-risk group, n(%)	48 (12.2)	**7 (7.8)**	**41 (13.5)**	
AASL-HCC score				0.091
low-risk group, n(%)	177 (44.9)	48 (53.3)	129 (42.4)	
intermediate-risk group, n(%)	195 (49.5)	40 (44.4)	155 (51.0)	
high-risk group, n(%)	22 (5.6)	**2 (2.2)**	**20 (6.6)**	
HCC-ESC$_{AVT}$ score				0.424
low-risk group, n(%)	281 (71.3)	**69 (76.7)**	**212 (69.7)**	
intermediate-risk group, n(%)	100 (25.4)	19 (21.1)	81 (26.6)	
high-risk group, n(%)	13 (3.3)	2 (2.2)	11 (3.6)	
CAMPAS score				0.538
low-risk group, n(%)	110 (27.9)	**29 (32.2)**	**81 (26.6)**	
intermediate-risk group, n(%)	151 (38.3)	31 (34.4)	120 (39.5)	
high-risk group, n(%)	133 (33.8)	30 (33.3)	103 (33.9)	
HCC-RESCUE score				0.111
low-risk group, n(%)	264 (67.0)	68 (75.6)	196 (64.5)	
intermediate-risk group, n(%)	104 (26.4)	19 (21.1)	85 (28.0)	
high-risk group, n(%)	26 (6.6)	**3 (3.3)**	**23 (7.6)**	
mPAGE-B HCC score				0.679
low-risk group, n(%)	254 (64.5)	**60 (66.7)**	**194 (63.8)**	
intermediate-risk group, n(%)	138 (35.0)	30 (33.3)	108 (35.5)	
high-risk group, n(%)	2 (0.5)	0 (0)	2 (0.7)	

HCC, hepatocellular carcinoma; CAMD, cirrhosis, patient age, male sex, and diabetes; AMAP, age, male, albumin-bilirubin, platelets; AASL, age, albumin, sex, liver cirrhosis; ESC, e antigen seroclearance; AVT, antiviral therapy; HCC-RESCUE, HCC-Risk Estimating Score in CHB patients Under Entecavir; mPAGE-B, modified PAGE-B; CAMPAS, cirrhosis on ultrasonography, age, male gender, platelet count, albumin and liver stiffness. The meaning of the bold values is to highlight statistically significant indicators.
*P value provided in Tables 3 and 4: comparison between patients with and without complete virological response at week 78.

achieve CVR, which suggested that some of patients with low levels of Anti-HBc may be in the immune tolerance phase.

Evidence has emerged that incomplete virologic suppression, particularly intrahepatic viral transcriptional activity, was associated with abnormal liver histopathology in a cross-sectional study (Wang et al., 2017). Sun et al. suggested that detectable low-level HBV DNA was associated with fibrosis progression in patients with chronic HBV infection during 78 weeks of entecavir therapy (Sun et al., 2020). But in our study, patients with PV were less likely to have a worse LSM value (0% vs 5.9%, *P*=0.038) and fibrosis progression (11.1% vs 21.4%, *P*=0.029) than patients with CVR during 78 weeks of entecavir therapy. Furthermore, Patients with PV were more likely to have a low-risk of HCC development than those with CVR. High viral load (HVL) (HBV DNA≥8 $\log_{10}$ copies/mL), positive HBeAg, slightly liver inflammation (Anti-HBc <3 $\log_{10}$ IU/mL), low-risk of fibrosis progression and HCC development, all these suggested that these particular PV patients may be in the immune tolerance phase (Sarin et al., 2016; European Association for the Study of the Liver, 2017; Terrault et al., 2018). Even with potent drugs like entecavir, their virological response was unsatisfactory. Hence, the initiation of antiviral therapy could be delayed or waiting for stronger and more effective drugs may be

alternative choice. Further prospective studies with larger sample size and longer follow-up are needed to confirm this finding and may provide some hint to optimization of histological and longterm clinical outcomes of antiviral therapy.

Our study has several limitations. First, the definition of PV was based on a single serum HBV DNA measurement at week 78. Although HBV DNA was measured at week 0, 26, 52 and 78 in local hospitals, respectively, we selected week 0 and 78 data detected at central laboratory to ensure the consistency of the results. Second, the follow-up time was relatively short. There was a possibility that PV observed at week 78 may turn to CVR after longer follow-up. Third, the development of drug-associated mutations was not investigated. However, considering the good compliance of patients and the extremely low resistance rate of entecavir, HBV mutations are unlikely to be the main cause of PV during antiviral therapy. Finally, our study used only ETV as a first choice for treatment of naïve CHB patients. Other NAs may have differential results. Therefore, the result of present study applies only to the patients using ETV.

In conclusion, our study suggested that HBV DNA level ≥8 $\log_{10}$ IU/mL, Anti-HBc level<3 $\log_{10}$ IU/mL and HBeAg seropositivity at baseline contributed to persistent positivity in HBV DNA level in patients with CHB receiving 78-week antiviral treatment. The risk of HCC development and the rate of fibrosis progression in these patients were low. Maybe these particular PV patients were in the immune tolerance phase, and the initiation of antiviral therapy could be delayed or waiting for stronger and more effective drugs may be another choice. Further well-designed prospective studies with large-scale and longer follow-up are needed to confirm this finding and may provide some hint to judge true immune tolerance phase.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding authors.

Ethics statement

The study was approved by the Ethics Committee of Peking University First Hospital and other 23 teaching hospitals. The patients/participants provided their written informed consent to participate in this study.

Author contributions

JL, X-QD, HZ and G-QW designed the experiments; HZ and G-QW provided the overall principle and direction of the study; X-QD and JL gathered and analyzed data, and drafted the manuscript; X-QD, Y-QL, and CZ done the laboratory examination. L-HC, Z-QZ, W-FZ, Q-HS, D-ZZ, A-LM, QX, H-LG, GZ, Y-XL, JS, S-BX and China HepB Related Fibrosis Assessment Research Group had participated in acquisition of data, revision of the manuscript for important intellectual content. All authors contributed to the article and approved the submitted version.

Funding

This study was supported by China Mega-Project for Infectious Diseases (grant numbers 2017ZX10203202, 2013ZX10002005) and China Mega-Project for Innovative Drugs (grant numbers 2016ZX09101065).

Acknowledgments

We gratefully acknowledge the members of China HepB-Related Fibrosis Assessment Research Group for assisting patient recruitment and data acquisition. We gratefully acknowledge Dr. Xiaomeng Wang from the University of Manchester for her critical reading of the manuscript and suggestions.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2023.1151899/full#supplementary-material

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10.3389/fcimb.2023.1151899

Glossary

HBV	Hepatitis B Virus
CHB	chronic hepatitis B
HCC	hepatocellular carcinoma
CVR	complete virological response
PV	persistent viremia
NAs	Nucleos(t)ide analogues
TDF	Tenofovir disoproxil fumarate
ETV	entecavir
HBeAg	hepatitis B e antigen
CLD	chronic liver diseases
ALT	alanine aminotransferase
cccDNA	covalently closed circular DNA
INR	international normalized ratio
PLT	platelet counts
TBil	total bilirubin
AFP	alpha fetoprotein
Anti-HBc	anti-hepatitis B virus core antibody
KPa	kilopascals
IQR	interquartile range
FIB-4	Fibrosis-4
APRI	AST to platelet ratio index
HAI	histology activity index
OR	odds ratio
CI	confidence interval
LLV	low-level viremia
HVL	High viral load
BMI	body mass index
CR	creatinine
FPG	fasting plasma glucose
LSM	liver stiffness measurement
HBsAg	hepatitis B surface antigen
HBeAg	hepatitis B e antigen
HBV	hepatitis B virus
ULN	upper limit of normal
CAMD	cirrhosis, patient age, male sex, and diabetes
AMAP	age, male, albumin-bilirubin, platelets
AASL	age, albumin, sex, liver cirrhosis
ESC	e antigen seroclearance
AVT	antiviral therapy
HCC-RESCUE	HCC-Risk Estimating Score in CHB patients Under Entecavir
mPAGE-B	modified PAGE-B
CAMPAS	cirrhosis on ultrasonography, age, male gender, platelet count, albumin and liver stiffness

frontiers | Frontiers in Cellular and Infection Microbiology

TYPE Original Research
PUBLISHED 11 July 2023
DOI 10.3389/fcimb.2023.1092936

OPEN ACCESS

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RECEIVED 08 November 2022
ACCEPTED 13 June 2023
PUBLISHED 11 July 2023

CITATION
Jia Y, Zou X, Yue W, Liu J, Yue M, Liu Y, Liu L, Huang P, Feng Y and Xia X (2023) The distribution of hepatitis C viral genotypes shifted among chronic hepatitis C patients in Yunnan, China, between 2008–2018.
Front. Cell. Infect. Microbiol. 13:1092936.
doi: 10.3389/fcimb.2023.1092936

The distribution of hepatitis C viral genotypes shifted among chronic hepatitis C patients in Yunnan, China, between 2008–2018

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Object: The hepatitis C virus (HCV) is prevalent across China, with a distinctive genotypic distribution that varies by geographical region and mode of transmission. Yunnan is one such geographical region wherein the local population continues to experience a high level of HCV infection, severely straining public health resources. This high prevalence is likely due to the increased incidence of intravenous drug use in that region, as Yunnan is a major point of entry for illegal heroin into China.

Methods: We investigated 510 individuals with chronic HCV infections in Yunnan Province from 2008 through 2018. Using reverse transcription PCR and Sanger sequencing to amplify and sequence samples. Bayesian analyses was performed to estimate the common ancestors and Bayesian skyline plot to estimate the effective viral population size. Molecular network was conducted to explore the characteristics of HCV transmission.

Results: We successfully amplified and sequenced a total of 503 viral samples and genotyped each as either 3b (37.6%), 3a (21.9%), 1b (19.3%), 2a (10.5%), HCV-6 (10.1%), or 1a (0.6%). Over this 11-year period, we observed that the proportion of 3a and 3b subtypes markedly increased and, concomitantly, that the proportion of 1b and 2a subtypes decreased. We also performed Bayesian analyses to estimate the common ancestors of the four major subtypes, 1b, 2a, 3a, and 3b. Finally, we determined that our Bayesian skyline plot and transmission network data correlated well with the changes we observed in the proportions of HCV subtypes over time.

Conclusions: Taken together, our results indicate that the prevalence of HCV 3a and 3b subtypes is rapidly increasing in Yunnan, thus demonstrating a steadily growing public health requirement to implement more stringent preventative and therapeutic measures to curb the spread of the virus.

KEYWORDS

hepatitis C virus, genotype, RT-PCR, Bayesian analysis, network, transmission

1 Introduction

The hepatitis C virus (HCV) is a global pandemic that continues to rise and constitutes a major threat to public health. The World Health Organization (WHO) has reported that approximately 58 million people have been infected with HCV as of 2022, an estimated 1.5 million new infections occur annually (WHO, 2022), and the prevalence varies among countries. HCV infection can lead to chronic hepatitis C (CHC), cirrhosis, hepatocellular carcinoma, and even death (Calvaruso and Craxi, 2020). Presently, China has the highest HCV disease burden of any single country in the world, and its cases alone account for over 14% of global CHC infections (Li et al., 2019), thus indicating a pressing need for more effective mitigation of the effects of the virus upon the public.

HCV is a single-stranded, positive-sense RNA virus, with a genome of ~9.6 kb in length that encodes a single open reading frame (ORF) flanked by 5′ and 3′ untranslated regions (UTRs). Modern genetic diversity and phylogenetic analyses indicate that the virus is classifiable into eight major genotypes (GTs) and 92 distinct subtypes (Jia et al., 2021). Different HCV GTs have distinct geographical distributions and differ in their responses to antiviral therapies. In general, GTs 1, 2, and 3 are distributed globally, whereas the other five tend to be endemic to certain geographic locales. For example, GT4 is primarily restricted to the Middle East and North Africa, GT5 to South Africa, GT6 to South Asia, GT7 to Central Africa (Chen et al., 2017), and GT8 to India (Borgia et al., 2018). GTs 1, 2, 3, and 6 are the predominant HCV strains found in China today, especially subtypes 1b, 2a, 3a, 3b, and HCV-6 (Zhou et al., 2017).

Yunnan sits on the border of southwest China, adjacent to the so-called "Golden Triangle," the region where the borders of Myanmar, Laos, and Vietnam meet, well known for its substantial production of opium. Thus, due to this unique geographical circumstance, Yunnan serves as an important drug trafficking route into the mainland of China (Wan et al., 2016). Indeed, historically, Yunnan has been the main channel of heroin entry into China. Unfortunately, HCV transmission and prevalence are critically high in this region and are associated with the migration of the populace and illicit drug trafficking activities. Previous studies have shown that, unlike in mainland China, GT3 was the major GT in Yunnan Province among injection drug users (IDUs), followed by GT6 and GT1, prior to 2008 (Xia et al., 2008). However, research by another group on the Yunnan area from 2009 to 2013 among IDUs found that the distribution of HCV GT subtypes shifted such that HCV-6 was now the predominant GT, followed by GT3b and GT3a (Zhang et al., 2013).

However, little is known about the distribution of HCV GTs across the Yunnan Province. Therefore, we investigated the dynamics of the HCV GT distribution among CHC individuals from 2008 to 2018. Simultaneously, we sought to more comprehensive understand the changes, origins, and spread of the predominant HCV genotypes across Yunnan over time by employing evolutionary and transmission network analyses.

2 Materials and methods

2.1 Ethical statement

All subjects gave their informed consent for inclusion before they participated in the study. The study was conducted in accordance with the Declaration of Helsinki, and the protocol was approved by the Ethics Committee of Yunnan Provincial Hospital of Infectious Disease (Approval No. YNACC [2015]-12).

2.2 Study population

A total of 510 chronic CHC individuals were enrolled in Yunnan Province of China, from January 2008 to December 2018. All the participants provided written informed consent prior to enrollment. Plasma samples were collected from whole blood samples using EDTA tripotassium salt. After centrifugation at 4000 rpm for 10 minutes, the supernatant serum was carefully collected and stored at -80°C for further HCV RNA analysis.

2.3 HCV RNA extraction and gene amplification

HCV RNA was extracted from 200-µl serum samples using the MiniBest viral RNA/DNA extraction kit, following the manufacturer's protocol. The isolated RNA was amplified by nest PCR of *NS5B* region of strain H77 (nt 8266-9303), which was usually used to determine the HCV genotype and subtypes. PCR primers and conditions were reported in previously described studies (Yue et al., 2020).

2.4 Sequencing, HCV genotyping and phylogenetic analysis

The HCV *NS5B* gene PCR products were detected using 1.0% agarose gel electrophoresis under UV illumination, purified using a DNA purification kit, and sequenced by Tsingke Biological Technology Co. on an ABI 3730XL automated DNA sequencer. Sequencing date were aligned using Clustal version 1.8.1 and then processed by BioEdit version 7.1.5 software. HCV *NS5B* sequences were genotyped after alignment with reference sequences from the GenBank (available at: http://www.ncbi.nlm.nih.gov/genbank/) according to co-analyses with a total of 111 reference sequences of 1a, 1b, 2a, 3a, 3b, 6a, 6b, 6c, 6d, 6e, 6f, 6g, 6h, 6i, 6j, 6k, 6l, 6m, 6n, 6o, 6p, 6q, 6r, 6s, 6t, 6u, 6v and 6w subtypes. Under the GTR+G+I (general time reversible + gamma distribution + invariant sites) model, Maximum-likelihood (ML) tree was constructed using MEGA version 6.0.6, with bootstrap values estimated as 1000 replications.

2.5 Evolutionary analysis

To estimate the epidemic history of the four main HCV subtypes in Yunnan Province, China, four sequence data sets of 1b, 2a, 3a and 3b were assembled. All the data set sequences were from our study, due to few Yunnan sequences had been reported. All the four data sets were aligned and manicured. Then Bayesian coalescent analyses were performed using the Markov chain Monte Carlo (MCMC) algorithm implemented in the BEAST version 1.7.5 under the uncorrelated log-normal relaxed clock model with the GTR+G+I nucleotide substitution model, a coalescent Bayesian skyline plot tree prior, and a relaxed uncorrelated lognormal molecular clock model. For the four subtypes, the evolutionary prior rates were different, and were chosen for their best-fitting by estimating directly from the data sets. For the last, 1.0E-03, 9.0E-04, 9.3E-04 and 2.4E-3 for subtype 1b, 2a, 3a and 3b were used, respectively. Each MCMC analysis was run for 300 million chains, and a tree was output every 10,000 chains. The estimated effective sampling size (ESS) was ≥ 200. Posterior probability densities were determined in Tracer version 1.7.1, and 10% of each chain was discarded as burn-in. The maximum clade credibility (MCC) tree was summarized with Tree Annotator version 1.7 and scanned using FigTree version 1.4.0. In addition, population dynamics were constructed under a coalescent Bayesian skyline plot tree prior and a piecewise linear skyline model with 10 groups using BEAST version 1.7.5. The Bayesian skyline plot was reconstructed using Tracer version 1.7.1.

2.6 Transmission network analysis

Based on the four data sets, all sequences were aligned, and pairwise genetic distance were computed using the Tamura-Nei 93 (TN93) method. We performed a sensitivity analysis on the genetic thresholds of subtype 1b, 2a, 3a and 3b separately (Ge et al., 2021). A more conservative (0.001 substitutions/site) to more liberal thresholds (0.05 substitutions/site) were estimated to selected the optimal genetic distance threshold, which was established by the maximum number of clusters below this threshold). We proceeded, visualized and analyzed the identified potential transmission clusters using the Gephi version 0.9.2. A node represents a sequence or an individual, and links (edges) represent connections between different individuals, reflecting their potential transmission relationships, with more links, the node may have higher transmission risk. Pairs were de-fined as connected components of the network comprising 2 nodes, while clusters were more than 2 nodes. Singletons were defined as those greater than the genetic distances threshold sequences. In addition, I roughly divided the sequences into three time periods, 2008~2010, 2011~2014, and 2015~2018, which nodes were rendered with brown, green, and red, respectively.

3 Results

3.1 Demographic characteristics

This retrospective study included 510 individuals with CHC from Yunnan, China, and spanned 11 years, from 2008 to 2018. The demographic characteristics of the 510 subjects are summarized as follows. The mean age ± standard deviation of participants was 42.5 ± 13.1 years and the majority were male (300, 58.82%). The following clinical characteristics were identified: mean alanine transaminase (ALT), 63.2 ± 58.4 IU/liter; mean aspartate transaminase (AST) 61.8 ± 71.4 IU/liter; and mean HCV RNA, 5.9 ± 2.1 log10 IU/ml.

3.2 HCV subtyping and changes across time

HCV genotyping was successfully performed for 503 (98.63%) of the 510 participants. From the 11 years' worth of sequences, we constructed a circular phylogenetic tree. We found that the most frequent subtypes were 3b (37.6%, 189), then 3a (21.9%, 110), 1b (19.3%, 97), and then 2a (10.5%, 53). Minor identified sequences were mainly GT6 (10.1%, 51), which included 6n (7.2%, 36), 6a (1.6%, 8), other HCV-6 subtypes (1.4%, 7), as well as 1a (0.6% 3) (Figure 1). We observed obvious differences in HCV subtype composition during the study period. However, there was no significant difference in the overall number of sequences annually. Interestingly, rather than finding that GT1b was most prevalent, we determined that GT3b was the predominant HCV GT between 2008 and 2018 (GT1b 28.26% to 8.16%; GT3b 19.57% to 48.98%). Simultaneously, the frequency of HCV genotype 3a gradually increased every year (from 6.52% to 32.65%). In contrast, 2a and 1a prevalence decreased from 21.74% to 0 and from 6.52% to 0, respectively. The proportion of GT6 remained stable over time, however, with a lower prevalence but more complexity in its subtypes (Figure 2).

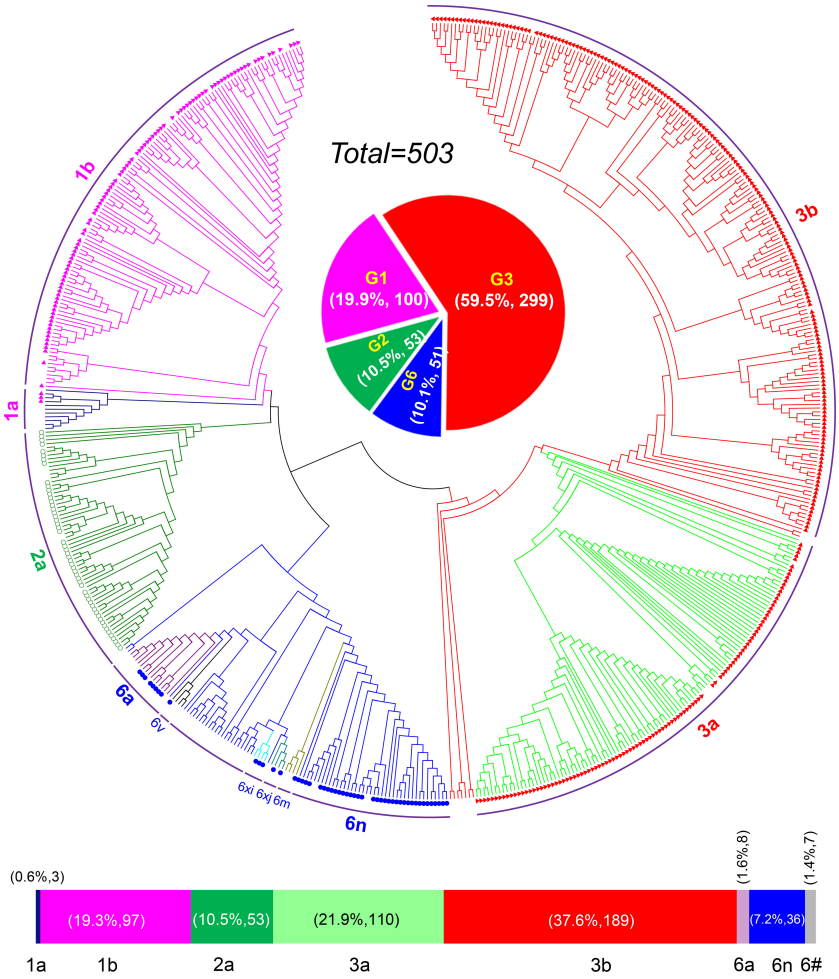

FIGURE 1
HCV genotyping and prevalence in Yunnan Province from the year of 2008 to 2018.

3.3 Evolutionary and demographic histories of four HCV subtypes

MCC trees were constructed for each of our four datasets, each corresponding to a different HCV subtype. From these analyses, we estimated that the common ancestor of the 1b, 2a, 3a, and 3b strains in Yunnan Province date to 1932 (95% highest probability density [HPD], 1912-1959), 1938 (95% HPD:1921-1960), 1961 (95% HPD:1941-1984), and 1976 (95%HPD:1960-2002), respectively (Figure 3).

For subtype 1b, we tentatively identified clades I–IV, with posterior probabilities of 0.99, 0.97, 1.00, and 0.96, respectively. CladeI had the earliest scaled divergence time around 1965, followed by cladeII in 1971, and clades III and IV in 1976. Clade IV was the predominant subgroup, with 48 terminal branches (Figure 3A).

Among the four data sets, subtype 2a comprised only 53 sequences. Based on the highly supported monophyletic clade, we found four clades of I-IV in 2a with 0.96, 1.00, 0.99, and 1.00 posterior probabilities, respectively. The estimated time of the most recent common ancestor (tMRCA) of clade I was 1971. Clades II-IV dated to 1975, 1981, and 1982, respectively. However, clade I contained the majority, with 15 sequences (Figure 3B).

Within the 3a Bayesian tree, three clades (I-III) of closely related sequences with high posterior probabilities were identified. Up to 32 sequences clustered within clade II shared a common ancestor from 1980. Clade II was the second largest with 19 sequences branching out from 1975, and clade III had 5 sequences and branched out from 1995 (Figure 3C).

In contrast to the three HCV subtypes above, the Bayesian tree for 3b appeared to be more divergent, with each clade containing only a few sequences. Within this tree, we defined a total of VII well-supported clades with high posterior probabilities ranging from 0.84–1.00, and all emerging within the past 40 years. Estimated tMRCAs were 1993 for clade I, 1997 for clade II, 2001 for clades III, and 2002 for clade IV. Clades V and VI diverged around 2003. And finally, clade VII appeared to diverge as recently as 2010 (Figure 3D).

3.4 Bayesian Skyline Plot (BSP) analysis

We estimated the number of individuals infected with HCV subtypes 1b, 2a, 3a, and 3b by performing BSP analyses using the Yunnan HCV sequences reported in the present study. The

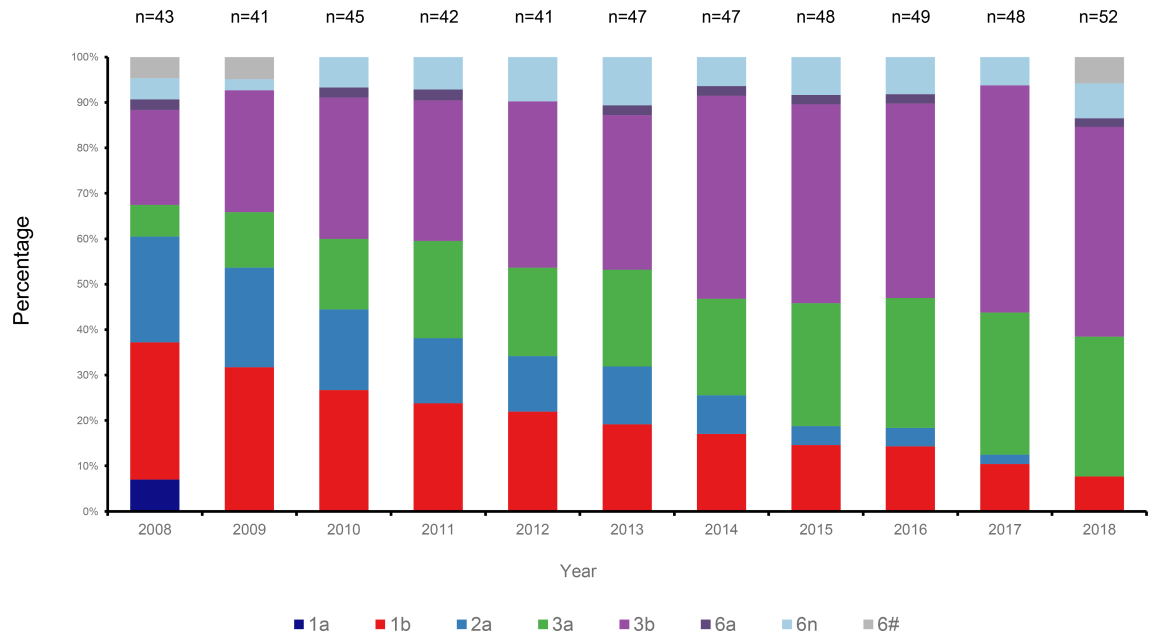

FIGURE 2
Changes of HCV subtypes over time in Yunnan Province from the year of 2008 to 2018.

estimated viral effective population size (Ne) for subtypes 1b and 2a showed a logarithmic-like growth phase from 1980 to 1995, which was followed by a stabilization of the 1b viral population and a steady decline in the size of the 2a population (Figures 4A, B). In contrast, the BSP for the 3a subtype displayed a rapid exponential growth phase from 2009–2012 that leveled off until 2018 (Figure 4C). Finally, the 3b subtype BSP showed a clear exponential growth phase from 1995 to 2007 but then tended toward stability over the next 10 years with only a slight decrease in Ne size (Figure 4D).

3.5 Transmission networks

Next, we constructed transmission networks to model how the HCV strain distribution evolved over time in Yunnan. From this, we identified 48 putative transmission clusters, composed of 234 individuals (52.12%; 234/449). According to the TN93, under the thresholds of 2.3%, 3.3%, 2%, and 1.7% genetic distances of 1b, 2a, 3a, and 3b, respectively, the largest number of clusters was contained in the transmission network. Out of this, 12 clusters were identified among the 54 GT1b sequences, nine among the 36 GT2a sequences, 12 among the 70 GT3a sequences, and 15 among the 74 GT3b sequences (Figure 5A). Across the generated networks, the GT3a and GT3b proportions were 18.57% and 27.03% in 2008–2010, 54.29% and 31.08% in 2011–2014, and 27.14% and 41.89% in 2015–2018, respectively. These results clearly indicated that their relative proportions increased rapidly over time. Both GT3a and GT3b sequences were concentrated into large clusters, implying that the 3a and 3b subtypes, particularly GT3b, have spread quickly across the network over recent years. In contrast, the respective proportions of GT1b and GT2a sequences declined over time: 48.15% and 55.56% in 2008–2010, 31.48% and 36.11% in 2011–2014, and 20.37% and 8.33% in 2015–2018, respectively. Interestingly, most of the recent GT1b and GT2a sequences clustered in pairs, indicating a limited ability to spread (Figure 5B).

4 Discussion

Owing to China bearing the greatest HCV disease burden out of all the countries of the world, a growing number of studies have focused upon characterizing the distribution of the five major HCV subtypes (1b, 2a, 3b, 6a, and 3a) within its borders (Yan et al., 2012; Chen et al., 2017). However, the distribution of HCV GTs and subtypes is not uniform across regions (Zhang et al., 2013). Previous studies have demonstrated that HCV-1b is the predominant HCV GT in most parts of China. However, HCV-2a dominates in northern China. In the south, the genotypic composition tends to be more complex (Chen et al., 2017). GT1b is the major HCV GT in Shanghai, followed by GT3a and finally 3b (Qu et al., 2021). Similar to Shanghai, in Jiangsu the most predominant GTs are 1b, 3a, 3b, and 6a (Qi et al., 2016). Hainan sequences were classified into six GTs: 6a (35%), 1b (31%), 3b (16%), 2a (8%), 3a (6%), and 1a (4%) (Wu et al., 2016). In Guangdong Province, GT1b is the main GT and is followed by GT6a, which replaced 2a as the second most common GT from 2004 onward (Chen et al., 2017). Interestingly, in contrast to the provinces mentioned above, we found the most prevalent GT in Yunnan to be GT3b, followed by GT3a, GT1b, and GT2a, along with various minor GT6 subtypes. Our findings concord well with the results of another recently published study, in which researchers detected 3b isolates in 23 (41.8%) out of 53 donors from Yunnan (Lu et al., 2014) and 24 (30%) from among IDUs (Xia et al., 2008). However, a survey summarizing the changes

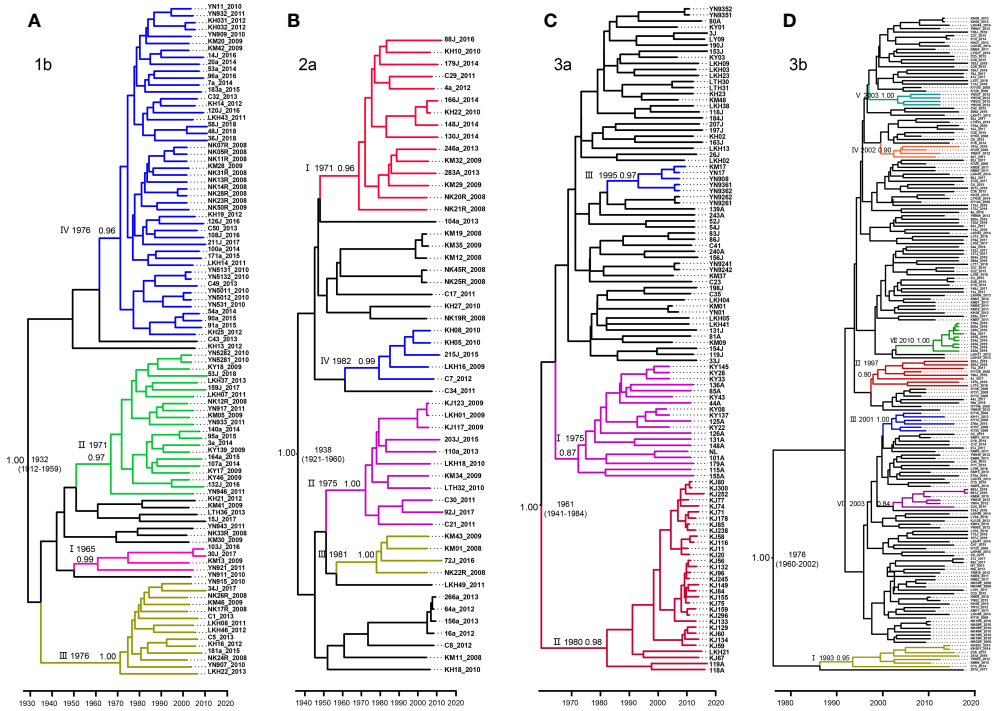

FIGURE 3
Maximum clade credibility (MCC) tree of the four major HCV subtypes estimated based on partial NS5B gene in Yunnan Province. **(A)** 1b, **(B)** 2a, **(C)** 3a and **(D)** 3b.

in the proportions of HCV GTs over time in southwest China before 2017 indicated that GT1b and GT2a decreased while GT3 increased (Zhang et al., 2017). Consistent with the review's findings, we similarly demonstrated an increased prevalence of 3a and 3b that was accompanied by a decrease in genotypes 1b and 2a in Yunnan. HCV GT3 is associated with a higher risk of liver fibrosis, cirrhosis and cancer than other HCV genotypes (Xu et al., 2022). GT3a and GT3b are the most common subtypes of HCV GT3. GT3a is prevalent worldwide, while GT3b is predominantly found in Southeast and East Asian countries. GT3b has become the second most common subtype in southwest and southern China, including the provinces of Sichuan, Yunnan, Chongqing, Guizhou and

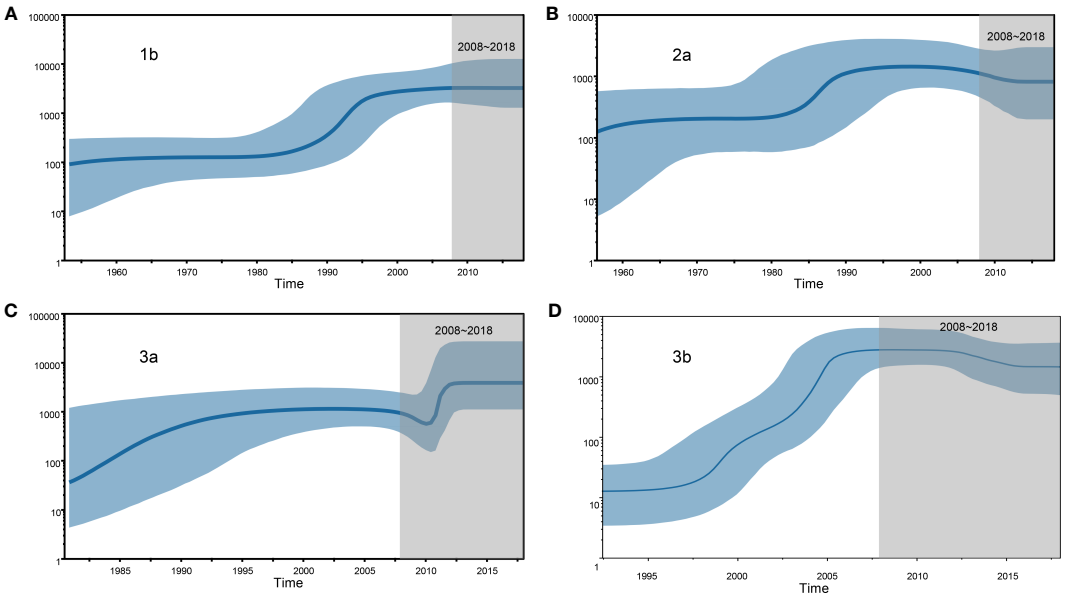

FIGURE 4
Demographic history of the four HCV subtypes is inferred by Bayesian Skyline Plot (BSP). **(A)** 1b, **(B)** 2a, **(C)** 3a and **(D)** 3b.

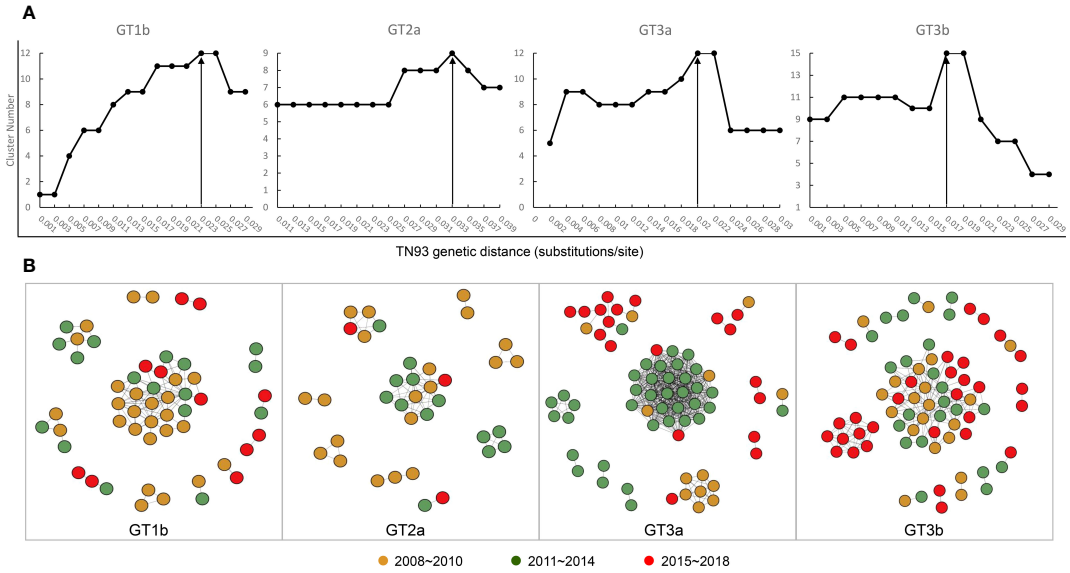

FIGURE 5
Transmission network definition and visualization. **(A)** Determination of transmission clusters genetic distance through Tamura-Nei 93 (TN93) algorithms. **(B)** Transmission network of the four HCV subtypes shifted between 2008 to 2018.

Guangdong (Xu et al., 2022). Although the recent discovery of direct-acting antivirals (DAAs) has revolutionized treatment, most patients achieve a sustained virologic response (SVR) of more than 95% (Abulitifu et al., 2022). However, GT3 has a lower SVR than other genotypes, mainly because advanced cirrhosis combined with the presence of resistance-associated substitutions can influence the response to DAA treatment. In China, the DAAs sofosbuvir/velpatasvir or sofosbuvir/ledipasvir are commonly used as treatment regimens for patients with HCV GT3 infection, to which RBV is added for the treatment of patients with GT3 HCV-related compensated cirrhosis and decompensated cirrhosis (Abulitifu et al., 2022). Some studies have shown a significantly lower SVR12 for the GT-3b subtype compared to GT-3a in China, which may be explained by the fact that significant differences in the prevalence of resistance-associated substitutions (RAS) were observed between HCV GT3a and GT3b. More than 90% of subtype 3b HCV strains have baseline RASs at A30K+L31M in the NS5A region (Liu et al., 2022). Worryingly, our results showed a rapidly increasing epidemiological profile of HCV type 3 in Yunnan from 2008 to 2018. Therefore, it is necessary to strengthen the detection of HCV genotypes, subtypes and drug-resistant mutations in Yunnan. In addition, our results showed that HCV GT2a and GT1a decreased from 21.74% to 0 and from 6.52% to 0, respectively. In addition, our results showed that HCV GT2a and GT1a decreased from 21.74% to 0 and from 6.52% to 0, respectively. This may be explained by the fact that treatment of HCV type 2a infected individuals achieved SVR greater than 95% in both the pegylated-interferon-α plus ribavirin and DAA treatment periods (Ishiguro et al., 2015; Li et al., 2022). However, only three cases of HCV GT1a were identified in this study and the change in prevalence is not representative or convincing.

Distinct evolutionary histories have been found for each HCV subtype. In this study, only HCV subtypes 1b, 2a, 3a and 3b were analyzed for evolutionary history because they are the four most common genotypes in Yunnan, while the remaining subtypes 1a, 6a, 6n, etc. were too few in number to be analyzed for evolutionary purposes. We found that the most recent common ancestors of the 1b, 2a, 3a, and 3b strains, as calculated based upon variations in the *NS5B* gene (371 bp), were estimated to be 1932 (95% HPD, 1912-1959), 1938 (95% HPD:1921-1960), 1961 (95% HPD:1941-1984), and 1976 (95%HPD:1960-2002), respectively. We observed a similar divergence time for subtypes 1b, 2a, and 3a in the *E1* region (Wu et al., 2016). It has been shown that in China, the common ancestor of all 1b strains may date to 1942, 2a to 1932, and 3a to 1959. This modest genetic diversity may account for the different gene regions and sequences identified. Notably, there were some differences in subtype 3b. Our results showed that the estimated tMRCAs for all Yunnan strains was approximately 1976. The divergence at 1976 consisted of clade I, or another large branch of the 3b MCC tree using the *E1* gene reported previously, which speculated that 3b isolates originated from Yunnan Province, with the entire tree branching out earlier in 1942 (Wu et al., 2016; Wang et al., 2019). Because published reference sequences for *NS5B* were limited, it is difficult to conduct a reliable overall evolutionary analysis and explore the geographic origins of the four subtypes from China solely using the *NS5B* sequence. However, the present study provides an effective evolutionary rate by performing BEAST analyses of *NS5B* to estimate the dates of strain divergences and thus, effectively characterizes the evolution of the four major subtypes within Yunnan Province.

The history of HCV dissemination varies by mode of transmission. HCV GTs 1 and 2 infections are most likely to be transmitted by blood transfusion, whereas infections of GTs 3 and 6 are associated with having a history of intravenous drug abuse (Wang et al., 2019). To estimate the history of the predominant

HCV subtypes in Yunnan, BSP analyses was per-formed separately for each subtype. The results revealed that a phase of rapid population expansion of 1b, 2a, and 3a subtypes occurred during 1980–1995, which may coincide with a period of unsafe blood drawing equipment utilized across China, an incident that led to ~500,000 blood donors being infected with HCV (Shi et al., 1999; Xu et al., 2013). The increase could also be a consequence of the Chinese "open door" policy of the late 1970s that resulted in the increased importation of illicit goods (Wang et al., 2019). From 2009 to 2012, 3a showed a rapid exponential phase, which may be associated with the major transmission route of 3a often concentrated on the IDU network via known drug trafficking routes. From 1990 to 2008, transmission via the IDU network and blood transfusions may have accounted for the continued growth in the population size of HCV 3b infections. Alternatively, this increase could also be partially attributable to a growing number of travelers between Yunnan and other Southeast Asian countries. After the rapid growth period of 3b, its BSP curve leveled off before slightly declining. However, several HCV 3b infections have been identified, leading to opportunities to spread into the uninfected population through numerous transmission routes.

Molecular transmission networks have been used to monitor and control emerging outbreaks of HCV. However, it is difficult to identify a unified standard genetic threshold for HCV transmission networks using different genes or even the same gene. Epidemiologically defined outbreaks and epidemiologically unrelated individuals have been re-ported to have a relatedness distance of 3.77% in hypervariable region 1 (HVR1) (Campo et al., 2016). Another publication separately defined *NS5B* (650 bp), *Core-E2* (920 bp), and HVR1 (100 bp) through inter- and intra-person applications of lower and higher cutoffs: 0.018 and 0.020, 0.03 and 0.06, 0.15 and 0.19, respectively, to identify transmission clusters (Olmstead et al., 2015). A recent study used the *Core-E2* (minus HVR1) region of 1221 bp by calculating the TN93 genetic distance to identify the most epidemiologically relevant cut-off, and determined that to be 0.03 substitutions/site for inferring the network (Bartlett et al., 2017). In the present study, we decided to employ the TN93 algorithms, which have been used in HIV infections recently, to perform a sensitivity calculation to measure genetic distances between transmission clusters (Ge et al., 2021). The transmission network results are consistent with our expectations.

Our study does have some limitations. The HCV blood samples collected for this study were provided by the Yunnan Provincial Key Laboratory of Clinical Virology Team, an interferon clinical practice base in Yunnan Province approved by the China Hepatitis Control Foundation, which has 120 sub-centres in Yunnan Province covering 16 prefectures and 120 counties. A random sample of 50 HCV-positive cases was selected each year for this survey, and although the annual random sample was small, the sample in this study is reasonably representative of HCV prevalence in Yunnan Province. To better characterise the epidemiological changes in the distribution of HCV genotypes in Yunnan, it is important to increase the sample size in the future. Another limitation is that sample transmission routes were not obtained, making it difficult to accurately define transmission networks. Finally, the samples we used for BEAST analyses and transmission networks were derived solely from the Yunnan area; therefore, we were unable to analyze the geographic transmission into Yunnan from other regions.

5 Conclusions

In conclusion, we evaluated our recent data from 2008–2018 on HCV genotypic distribution dynamics in the Yunnan Province of southern China, which showed that HCV subtypes 3b and 3a had gradually increased to be the predominant GTs of the region, and that 1b and 2a concomitantly decreased. In 2018, 3b became the most predominant sub-type, followed by 3a and then 1b. We performed Bayesian analyses to construct MCC trees for each subtype, which estimated when the common ancestors of 1b, 2a, 3a and 3b, respectively, existed. Furthermore, our BSP and transmission network analyses supported our findings that the distribution of HCV subtype had markedly shifted over time. Overall, our results are of great significance for the epidemiological investigation and prevention of HCV infection, and are especially useful for tracking individuals within transmission clusters.

Data availability statement

The data presented in the study are deposited in the Genbank repository, accession number OR210427-OR210929. Further inquiries can bedirected to the corresponding authors.

Ethics statement

All subjects gave their informed consent for inclusion before they participated in the study. The study was conducted in accordance with the Declaration of Helsinki, and the protocol was approved by the Ethics Committee of Yunnan Provincial Hospital of Infectious Disease (Approval No. YNACC [2015]-12). The patients/participants provided their written informed consent to participate in this study.

Author contributions

YF and XX conceived and designed the experiments. YJ, XZ, JL, and YL performed the experiments and analyzed the data. YJ wrote the original manuscript. WY and LL reviewed and edited the manuscript. WY, MY, and PH provide technical guidance. YF and XX supervision. All authors contributed to the article and approved the submitted version.

Funding

This research was funded by the Yunnan Major Scientific and Technological Projects (202202AG050013), National Natural Science Foundation of ChinaA (82060612), the Reserve Talents Project for Young and Middle-Aged Academic and Technical Leaders of Yunnan Province (2019HB012), and Youth Talent

Program of Yunnan "Ten-thousand Talents Program" (YNWR-QNBJ-2018-054).

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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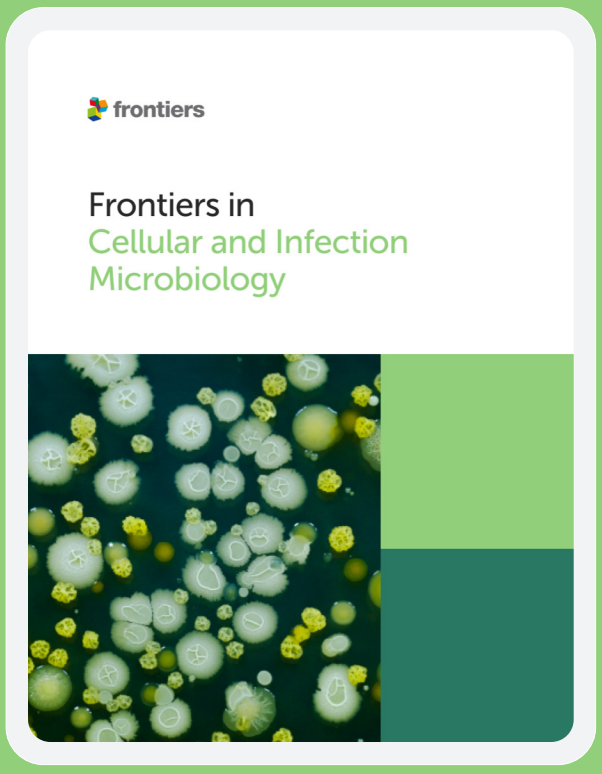

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