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Diagnosis, treatment and prognosis of viral hepatitis, volume II

Edited by
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Diagnosis, treatment and prognosis of viral hepatitis, volume II

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Editorial: Diagnosis, treatment and prognosis of viral hepatitis, volume II

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Editorial on the Research Topic
Diagnosis, treatment and prognosis of viral hepatitis, volume II

Currently, the global burden of viral hepatitis is mainly caused by five biologically unrelated hepatitis viruses: hepatitis A virus (HAV), hepatitis B virus (HBV), hepatitis C virus (HCV), hepatitis D virus (HDV) and hepatitis E virus (HEV) [(Xiang et al.), (1)]. They have different transmission routes and susceptible population (2, 3). Viral hepatitis is an important global public health problem with high morbidity and mortality rates that seriously endanger the health and quality of life of hundreds of millions of people (4). According to the statistics, 1.1 million died due to viral hepatitis in 2019. Hepatitis B virus and hepatitis C virus infections are responsible for the majority of viral hepatitis deaths. It was estimated that there were approximately 296 million people infected with hepatitis B and 58 million people infected with hepatitis C (5). In 2015, the United Nations adopted a resolution to combat viral hepatitis as part of the agenda to achieve the 2030 sustainable development goals. This was followed in 2016 by the development of the first global strategy to eliminate viral hepatitis (6). In light of this, it is necessary to call on governments, parties and the population to strengthen the prevention, diagnose, and treatment of viral hepatitis in the control of viral hepatitis.

The early diagnosis and prognosis evaluation of viral hepatitis are of great significance for effective treatment. Nevertheless, globally, <5% of population with chronic viral hepatitis are aware of their condition (7). On account of the lack of awareness of viral hepatitis infection, antiviral treatment is often overlooked, which leads to the progression of the disease. Hence, it is of great significance to enhance patients' awareness of the harm of viral hepatitis, to diagnose viral hepatitis infection timely and accurately, to better estimate the prognosis of patients, and to find out new anti-viral treatment. So the appearance of this special issue is very opportune. The purpose of this

Research Topic is to gather articles or reviews that contribute to a better understanding of viral hepatitis and provide new perspective and scientific theoretical grounding for the diagnosis and treatment of viral hepatitis.

To explore the markers or factors related to the occurrence, development and prognosis of viral hepatitis is conducive to the early prevention and treatment of the disease. In this special issue, Chen et al. reported that serum sTim-3 level was increased in patients with HBV, HCV, or HEV infection and gradually elevated in patients with either hepatitis or hepatitis with hepatic fibrosis. The roles of immune checkpoint molecules expressed on CD4 + T cells in chronic asymptomatic HBV carriers (ASCs) with HBeAg-negative were confirmed by Cui et al.. Yu et al. also revealed the associations between sustained low-level expression of HBsAg and mutated S gene sequence characteristics, protein function changes, and HBsAg immune complex formation in ASCs. Yang et al. identified the associations of Fc receptor-like 5 gene polymorphisms and mRNA expression levels with liver fibrosis in CHB patients. Wang et al. revealed that serum pregenomic RNA and hepatitis B core-related antigen were conductive to the prediction of the risk of virological recurrence in CHB patients after discontinuation of nucleos(t)ide analogs. Through the prospective multicenter observational cohorts, Zhu et al. reported the prevalence and adverse consequence of HBV reactivation in CHB patients with acute exacerbations. In additions, Xu et al. suggested the differentiated impact of the different types of acute decompensation events on the subsequent risk of nosocomial infections.

At present, the treatment of viral hepatitis is also one of the key concerns of researchers. Chuang et al. designed a protease-activatable retention probe for tracking HCV NS3/4A protease activity and distribution *via* positron emission topography imaging, which could optimize the protease-based therapies. Through the real-world experience, Peng et al. observed that PEG-IFNα2b add-on therapy was related to elevated eGFR in patients with CHB who received entecavir therapy. Salpini et al. conducted a review that showed that hepatitis B virus DNA integration is a new biomarker of HBV-mediated pathogenetic properties and an obstacle to current HBV therapeutic strategies. Meanwhile, Zhu et al. reviewed current treatment of chronic hepatitis B from clinical aspects.

It is also of great significance to state the relationship between hepatitis and other diseases and its occurrence in special populations, which can deepen people's understanding of viral hepatitis. Chen et al. reported the relationship between hepatitis C and kidney stone in US females. Chen et al. first reported the case of a man with very severe aplastic anemia who developed chronic hepatitis E after hematopoietic stem cell transplantation. Through a meta-analysis, Liu et al. indicated the prevalence of HBV in Chinese pregnant women. Finally, Zhao et al. explained the feasibility of eliminating hepatitis C in China from the perspectives of epidemiology, natural history and intervention.

The purpose of this Research Topic is to provide new perspective and scientific theoretical basis for the diagnosis, treatment and prognosis of various viral hepatitis.

Author contributions

JW and YW drafted and critically revised the work. All authors had the idea for the article.

Conflict of interest

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SYSTEMATIC REVIEW
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Hepatitis B Infection Among Pregnant Women in China: A Systematic Review and Meta-Analysis

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Background: A study of the current situation and characteristics of hepatitis B virus (HBV) infection among Chinese pregnant women is meaningful to provide baseline information for future research and policy making, with an aim to eliminate HBV in China.

Objectives: To provide the epidemiological status of HBV infection among pregnant women in China.

Methods: PubMed, EMbase, The Cochrane Library, and three Chinese databases were searched. Cohort studies and cross-sectional studies on HBV prevalence in Chinese pregnant women, published after 2016, were retrieved. In addition, combined HBV prevalence and 95% confidence interval (CI) were calculated. This research was registered in PROSPERO (CRD42021289123).

Main Results: A total of 42 studies were included in the study, with a sample size of 4,007,518 cases, and 20 provinces in China. The prevalence of HBV in Chinese pregnant women was 6.64% (95% CI: 5.72–7.57%) during the period between 2016 and 2021. Among HBsAg positive pregnant women, the HBeAg positive rate was 25.80% (95% CI: 22.26–29.69%). Moreover, geographic regions with HBV prevalence ranking from high to low were in western China, eastern China, and central China, successively.

Conclusion: The prevalence of HBV in Chinese pregnant women is intermediate endemic, although disparities exist between different regions. Among pregnant women with HBV infection, a high proportion of the patients have strong infectivity. Factors affecting HBV prevalence remain controversial, which demands further studies.

Systematic Review Registration: https://www.crd.york.ac.uk/PROSPERO/, identifier: CRD42021289123.

Keywords: hepatitis B - infectious disease transmission, pregnant women, prevalence, China, systematic review, meta-analysis

BACKGROUND

Hepatitis B infection is caused by the hepatitis B virus (HBV), a leading cause of cirrhosis and hepatocellular carcinoma worldwide (1, 2). In 2016, World Health Organization (WHO) proposed the global health sector strategy on viral hepatitis control, which set the goal to eliminate viral hepatitis as a major public health threat by 2030 (3). One of the targets is to decrease the HBV prevalence in children under the age of 5 years to 0.1%. As mother-to-child transmission is the predominant mode of HBV transmission, blocking this pathway is an essential step to eliminate HBV (4, 5). One effective method is to standardize the health management of pregnant women with chronic HBV infection. China has the largest disease burden of HBV infection in the world (6). However, the current situation and characteristics of HBV infection among Chinese pregnant women remain unclear. Further studies are needed to support the formation of a health policy and future research to eliminate HBV. An observational study with a large sample size was recently conducted to evaluate the national and regional HBV prevalence among Chinese pregnant women (7). The results revealed that the HBV prevalence among pregnant women in China was intermediate endemic between 2015 and 2020, although disparities existed between different regions. Some limitations of that study include lack of demographic information of pregnant women included (such as age, occupation, times of gestation and parity), and information of other HBV serological markers (such as HBeAg, anti-HBs, anti-HBc, anti-HBe, and HBV DNA), which limited the possibility to explore the relationship between these factors and HBV prevalence. A systematic review and meta-analysis was conducted based on literature published after 2016 to provide a better understanding of HBV epidemiology in Chinese pregnant women.

METHODS

This systematic review and meta-analysis was conducted following the criteria of the PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-Analyses) statement guidelines (8), and it was registered in PROSPERO (CRD42021289123).

Search Strategy

Databases including PubMed, EMbase, The Cochrane Library, CNKI (Chinese National Knowledge Infrastructure), Wan-Fang Data, and VIP were searched for cohort studies and cross-sectional studies. In addition, the references of the retrieved studies were reviewed. Literature published between January 1, 2016 and June 1, 2021, reporting HBV prevalence in Chinese pregnant women, were included. A combination of subject words and free text search was used during the searching process. The example search terms are demonstrated in **Table 1**.

TABLE 1 | The example of search terms (PubMed).

#1	(Pregnant Women[Title/Abstract]) OR (Pregnancy[Title/Abstract]) OR ("Pregnant Women"[Mesh]) OR ("Pregnancy"[Mesh])
#2	(Chinese[Title/Abstract]) OR (China[Title/Abstract]) OR ("China"[Mesh])
#3	(HBsAg[Title/Abstract]) OR (hepatitis B[Title/Abstract]) OR (Hepatitis B virus[Title/Abstract]) OR (HBV[Title/Abstract])) OR (Hepatitis B Surface Antigens[Title/Abstract]) OR ("Hepatitis B Surface Antigens"[Mesh])) OR ("Hepatitis B virus"[Mesh]) OR ("Hepatitis B"[Mesh])
#4	#1 AND #2 AND #3

Inclusion and Exclusion Criteria

Inclusion criteria were as follows: (1) participants: pregnant women in China; (2) outcomes: HBV prevalence (HBsAg positive rate); (3) study designs: cohort studies and cross-sectional studies; (4) languages: English and Chinese. All articles were published between 2016 and 2021. The exclusion criteria included HBV prevalence in populations with specific diseases, such as women with intrahepatic cholestasis of pregnancy or preterm birth. If there was overlap in the sample source between different literature (such as participants recruited in the same hospital during the same time period), we included only the most updated report.

Study Selection and Data Extraction

Two independent reviewers (LD and LY) screened articles, extracted data, and cross-checked results. When conflicts occurred, consensus was achieved after further discussion. Titles, abstracts, and full text were reviewed for relevance. Authors of the original studies were contacted by email or telephone for further information when necessary. The following data were extracted from literature: (1) general information of the studies (such as article title, main authors, publication time, study design, and the region where the study was conducted); (2) baseline characteristics of the study populations (such as sample size, age, education level, and occupation); (3) outcome indicators (such as the number of HBsAg positive individuals, HBsAg test methods, HBV serological markers, HBV DNA test results, and mother-to-child transmission rate); (4) key elements for risk of bias assessment.

Risk of Bias Assessment

Two reviewers (LY and NJQ) independently assessed the risk of bias for the studies included and cross-checked the results. Cohort studies were assessed using the Newcastle Ottawa Scale (NOS) (9), and cross-sectional studies were assessed using the Agency for Healthcare Research and Quality (AHRQ) form (10).

Data Analysis

Statistical analysis was conducted with R software. Data were synthesized using a random effect model in consideration of heterogeneity among studies. Moreover, individual proportions and the HBV pooled prevalence were assessed with 95%

Abbreviations: CLIA, chemiluminescence immunoassay; CMIA, chemiluminescence micro particle immunoassay; ECLIA, electrochemiluminescence immunoassay; ELISA, Enzyme-linked immunosorbent assay; HBV, hepatitis B virus; HBsAg, hepatitis B surface antigen; HBeAg, Hepatitis B e Antigens; anti- HBs, Anti-hepatitis B surface antigen antibody; anti-HBc, antibody to hepatitis B core antigen; anti-HBe, Hepatitis B E antibody; HBV DNA, hepatitis B virus deoxyribonucleic acid; PRISMA, Preferred Reporting Items for Systematic Reviews and Meta-Analyses; TRFIA, time resolved fluoroimmunoassay; NR, not reported; WHO, World Health Organization.

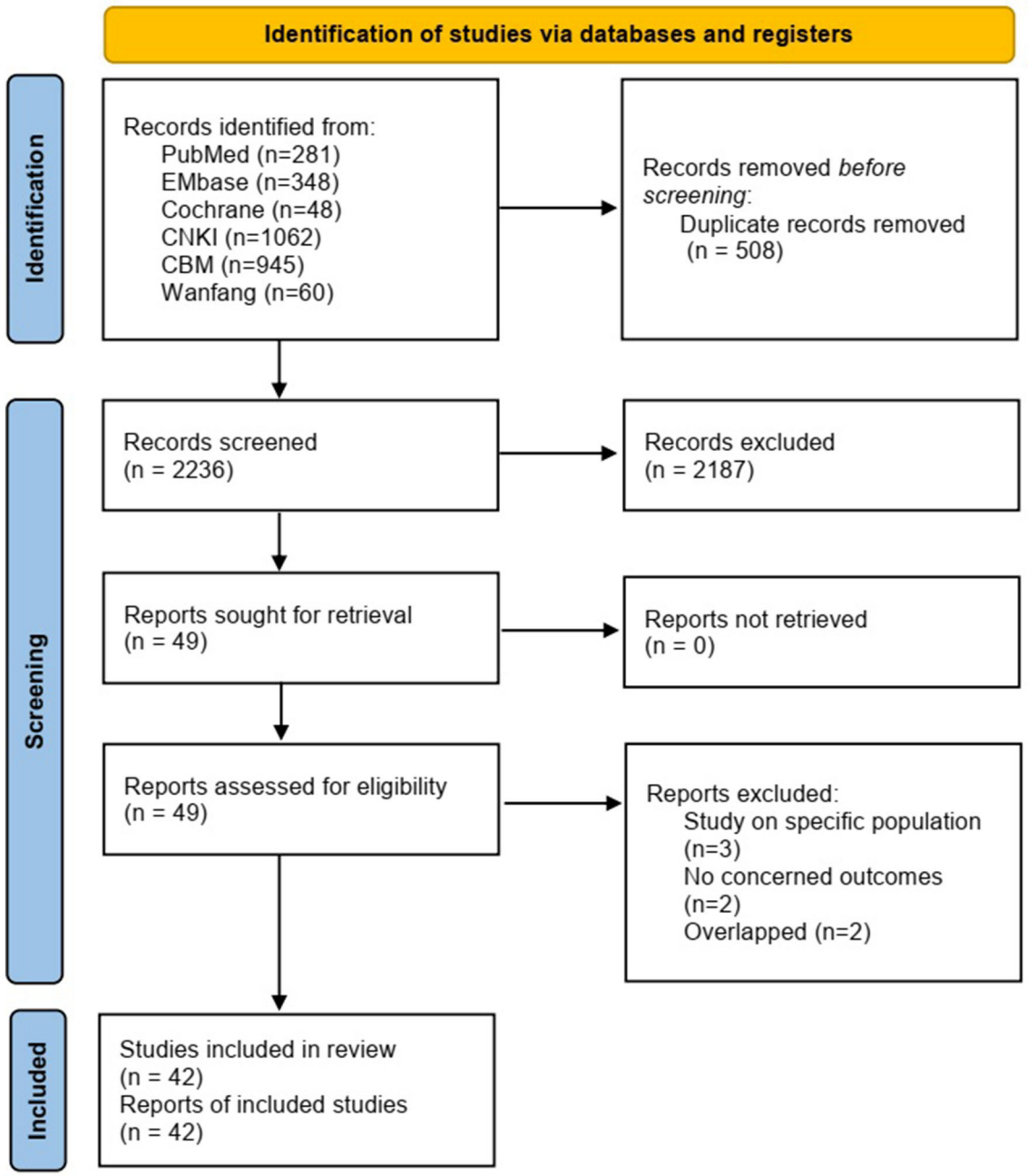

FIGURE 1 | Flow diagram of study selection.

confidence interval (CI). Subgroup analysis and meta-regression were conducted based on the geographical region, economic level of the province, sample source (regions or hospitals), study designs, and study quality to detect the source of heterogeneity. Provinces in China were categorized into three groups based on the geographical regions: eastern China, central China, and western China. In addition, the economic level of the province was categorized as high level (ranks 1–10), medium level (ranks 11–20), or low level (ranks 21–31) based on the rank of per-capita gross domestic product (GDP) among provinces in China in 2020 (11). A sensitivity analysis was conducted by sequential removal of each study, in order to evaluate the individual study's impact on the overall pooled effect. Furthermore, studies with quality score ≤ 3 points were omitted to assess their impact on the overall pooled effect of studies with high risks of bias. Potential publication bias was assessed graphically by funnel plot and examined by Begg's and Egger's tests (significant when $p < 0.05$).

TABLE 2 | Characteristics of included studies.

References	Design	Timespan	Province	Region	Sample source	Sample size	Positive HBsAg	Age (Mean±SD)	Labratory test	Quality score
Sun et al. (32)	Cohort	2005–2017	Yunnan	Western	Medical	49,479	1,624	HBsAg positive: 29.6 ± 4.4 HBsAg negative: 29.7 ± 4.3	NR	7
Wen et al. (41)	Cross-sectional	2015–2019	Guangdong	Eastern	Regional	100,500	9,290	NR	ELISA	2
Du (17)	Cross-sectional	2016–2018	Hubei	Central	Medical	12,861	862	NR	ELISA	3
Liu et al. (28)	Cross-sectional	2013–2016	Yunnan	Western	Medical	15,641	1,234	Median: 28 (26–32)	ELISA	8
Wang et al. (39)	Cross-sectional	2014–2017	Jiangsu	Eastern	Medical	31,457	1,586	28.39 ± 5	CLIA, ECLIA, time-resolved methods	2
Zhang and Li (46)	Cohort	2009–2018	Fujian	Eastern	Medical	85,190	9,699	HBsAg positive: 30.33 ± 4.50	NR	7
Zhang et al. (48)	Cross-sectional	2015–2016	Henan	Central	Medical	1,870	92	27.12 ± 5.31	NR	7
Zhao et al. (49)	Cohort	2011–2018	Fujian	Eastern	Regional	33,437	3,789	HBsAg positive: 29.3 ± 4.3	NR	7
Zou et al. (53)	Cross-sectional	2016–2018	Jiangsu	Eastern	Regional	2,077	78	Range:19–42	Latex immunochromatography	3
Huang (23)	Cross-sectional	2014–2018	Guangxi	Western	Medical	2,861	277	27.5 ± 4.5	NR	3
Zhang et al. (47)	Cross-sectional	2017–2019	Tibet	Western	Regional	496	65	28.62	LAT	4
Cai et al. (13)	Cohort	2014–2015	Anhui	Central	Medical	3,329	346	HBsAg positive: 27 HBsAg negative: 24.3	ELISA, ECLIA	8
Guo et al. (19)	Cross-sectional	2016–2018	Hubei	Central	Medical	7,000	450	Range: 16–45	ELISA	3
He et al. (20)	Cross-sectional	2017	Jiangsu	Eastern	Regional	22,595	873	28.36 ± 4.46	ELISA	4
Kang (24)	Cross-sectional	2014–2018	Tianjin	Eastern	Medical	3466	133	28.76 ± 4.87	TRFIA	3
Tang et al. (35)	Cross-sectional	2014–2015	Guizhou	Western	Medical	2,118	119	26.4 ± 5.3	ELISA	6
Wang et al. (39)	Cross-sectional	2015–2017	Zhejiang	Eastern	Regional	43,628	2,168	NR	ELISA	3
Ying (43)	Cross-sectional	2015–2017	Zhejiang	Eastern	Regional	42,815	2,292	NR	LAT	4
Zhou (51)	Cross-sectional	2017–2018	Tibet	Western	Medical	2,136	192	28.96 ± 5.02	NR	7
Liu et al. (27)	Cross-sectional	2015–2017	Fujian	Western	Medical	14,760	1,221	NR	NR	3
Wang et al. (37)	Cross-sectional	2014–2016	Guangdong	Eastern	Medical	17,722	967	28.7 ± 3.4	ELISA	1
Wei et al. (40)	Cross-sectional	2012–2017	Sichuan	Western	Medical	131,507	9,722	NR	ELISA	5
Zeng et al. (44)	Cross-sectional	2017–2018	Jiangxi	Central	Medical	45,413	953	29.17 ± 5.04	ELISA	3
Ma et al. (29)	Cross-sectional	2016–2017	Qinghai	Western	Medical	60,027	1,912	NR	ELISA	2
Chen et al. (15)	Cross-sectional	2010–2015	Shaanxi Province	Western	Medical	13,451	951	Median:27(19–42)	CMIA	5
Sheng et al. (30)	Cross-sectional	2016	Liaoning	Eastern	Medical	14,314	441	31.1 ± 4.5	CMIA	6
Zhong (50)	Cross-sectional	2015–2017	Chongqing	Western	Medical	1,000	80	24.86 ± 2.55	ELISA	3
Wang et al. (36)	Cross-sectional	2015–2016	Guangdong	Eastern	Medical	13,093	1,424	29.38 ± 4.77	NR	5
Hu (21)	Cross-sectional	2014–2016	Chongqing	Western	Regional	359,570	19,237	NR	NR	6
Bao (12)	Cross-sectional	2011–2015	Guangdong	Eastern	Regional	732,783	63,495	27 ± 4	ELISA	6
Gong et al. (18)	Cross-sectional	2014–2016	Guizhou	Western	Medical	2,648	243	<35: 2,202 ≧35: 446	TRFIA	4
Zhuang et al. (52)	Cohort	2012–2016	Jiangsu	Eastern	Medical	37,264	1,115	Median: 26 (25–29)	ELISA	8
Liang et al. (26)	Cross-sectional	2014-2015	Guangdong	Eastern	Medical	6930	661	NR	CLIA	3
Sun et al. (31)	Cross-sectional	2013–2016	Shanghai	Eastern	Medical	63,109	2,924	20–50	CLIA	4

(Continued)

TABLE 2 | Continued

References	Design	Timespan	Province	Region	Sample source	Sample size	Positive HBsAg	Age (Mean±SD)	Labratory test	Quality score
Huang et al. (22)	Cross-sectional	2013–2014	Fujian	Western	Medical	6,225	720	NR	NR	3
Tang et al. (34)	Cross-sectional	2014	Guangdong	Eastern	Regional	180,9893	182,782	NR	ELISA	3
Cui et al. (16)	Cohort	2012–2015	Jiangsu	Eastern	Medical	21,331	519	HBsAg positive: 27.59 ± 4.02 HBsAg negative: 27.03 ± 4.19	NR	7
Tan et al. (33)	Cohort	2009–2010	Sichuan	Western	Medical	22,374	948	Median: 27 (23–31)	ELISA	7
Wu (42)	Cross-sectional	2013–2015	Shanxi	Central	Medical	16,770	160	NR	ELISA	4
Li et al. (25)	Cross-sectional	2008–2014	Guangdong	Eastern	Medical	22,906	2,164	NR	NR	5
Zhang et al. (45)	Cross-sectional	2014–2015	Guizhou	Western	Medical	18,007	517	25.7	ELISA	3
Chen et al. (14)	Cross-sectional	2015	Guangxi	Western	Regional	115,484	8,751	NR	ELISA	4

ELISA, Enzyme-linked immunosorbent assay; NR, not report; HBsAg, hepatitis B surface antigen; ECLIA, electrochemiluminescence immunoassay; TRFIA, time resolved fluoroimmunoassay; CMIA, chemiluminescence micro particle immunoassay; CLIA, chemiluminescence immunoassay; LAT, latex agglutination test.

RESULTS

Study Characteristics

A total of 2,744 articles were retrieved initially. Following the screening of titles, abstracts, and full text, a total sample size of 4,007,518 pregnant women and 42 studies (12–53) were included in the meta-analysis (**Figure 1**). Seven cohort studies and 35 cross-sectional studies were included (**Table 2**). Generally, the studies included in the analysis were conducted in 20 provinces. The regional distributions included eastern China (19 studies), central China (6 studies), and western China (17 studies). The economic level was ranked as high level (24 studies), medium level (7 studies), and low level (11 studies). The sample size varied from 496 to 1,809,893 across studies.

In terms of quality, all cohort studies included had a total quality score $\geq$ 7 points, with 5 studies achieving 7 points and 2 studies achieving 8 points (**Supplementary Table S1**). For cross-sectional studies, 17 studies had a quality score $\leq$ 3 points, 15 studies had 4–6 points, and 3 studies had $\geq$ 7 points (**Supplementary Table S2**). Most cross-sectional studies did not "explain any patient exclusions from analysis," "describe how confounding was assessed and/or controlled," or "explain how missing data were handled in the analysis."

Prevalence of HBV Infection in Pregnant Women

The HBV prevalence in pregnant women in China was 6.64% (95% CI: 5.72–7.57%) during the period between 2016 and 2021 (**Figure 2**). The HBV prevalence varied from 0.95 to 13.10% across studies (**Figure 3**). Jinzhong City of Shanxi Province reported the lowest prevalence, and Ali City in Tibet reported the highest prevalence. The results revealed that one region was categorized as low endemic area (Shanxi Province), 14 regions were intermediate endemic areas (Jiangxi Province, Liaoning Province, Qinghai Province, Jiangsu Province, Tianjin City, Shanghai City, Henan Province, Yunnan Province, Zhejiang Province, Guizhou Province, Sichuan Province, Hubei Province, Chongqing City, and Shaanxi Province), and 5 regions were high endemic areas (Guangxi Province, Guangdong Province, Anhui Province, Tibet, and Fujian Province). When omitting any study or studies with a risk score $\leq$3 points, the sensitive analysis showed consistent results. The range of the pooled proportion from 6.51% (95% CI: 5.61–7.42%) to 6.78% (95% CI: 5.88–7.69%) was narrow. The funnel graph (**Figure 4**) and the results of Begg's test ($p = 0.0131$) and Egg's test ($p = 0.0388$) revealed potential publication bias.

Subgroup analyses were conducted based on geographical region, economic level, sample source, study design, and the results from risk of bias assessment (**Table 3**, **Supplementary Figures S1–S4**). None of these factors influenced HBV prevalence, which is consistent with the results of the meta-regression. However, the following trends were noticed across subgroups: (1) HBV prevalence during pregnancy was the highest in western China, followed by eastern China, and the lowest in central China; (2) regions with better economic levels were associated with higher HBV prevalence; (3) prevalence tended to be higher if the samples were collected from regions, compared with those from a single or multiple medical institutions.

Seroepidemiology Among HBsAg Positive Pregnant Women

Hepatitis B virus is more contagious when both HBsAg and HBeAg are positive, and when HBsAg is positive with high maternal concentrations of HBV DNA ($>2 \times 10^5$-1×10^6 IU/ml). Twenty studies reported the testing results of HBeAg in pregnant women, and pooled data revealed that the HBeAg positive rate was 25.80% (95% CI: 22.26–29.69%, $I^2 = 98\%$) among HBsAg positive women. Moreover, the number varied from 12.14 to 43.71% across studies. The lowest HBeAg positive rate was found in Anqing City of Anhui Province, and the highest was in Guizhou Province. Subgroup analyses among different regions demonstrated that the HBeAg positive rate was

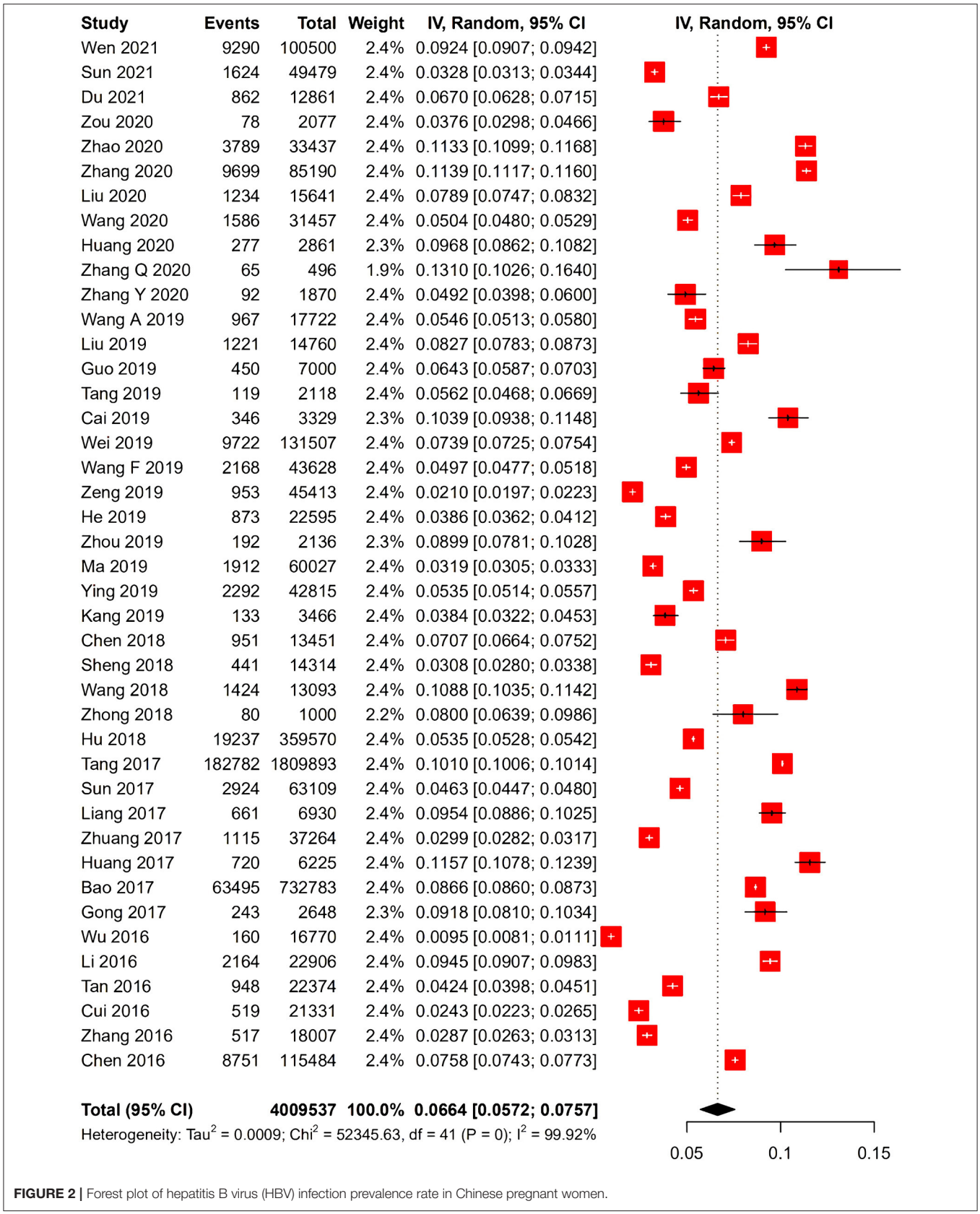

FIGURE 2 | Forest plot of hepatitis B virus (HBV) infection prevalence rate in Chinese pregnant women.

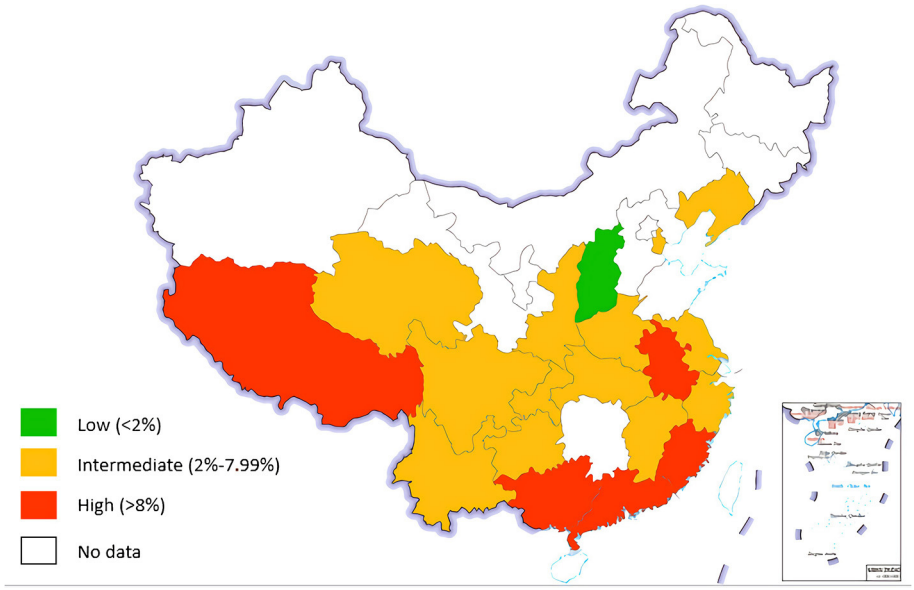

FIGURE 3 | The prevalence of HBV infection among pregnant women by province in China, 2016–2021.

24.92% (95% CI: 19.76–30.92%, I^2 = 98%), 20.79% (95% CI: 15.13–27.88%, I^2 = 88%), and 30.07% (95% CI: 24.90–32.14%, I^2 = 97%), respectively, in eastern, central, and western China.

Five studies reported the HBV DNA level in pregnant women. The pooled data indicated that among HBsAg positive pregnant women, the rate of high HBV DNA level was 26.73% (95% CI: 22.45–31.50%, I^2 = 83%), and the number varied from 16.13 to 36.13% across studies. The rate of high HBV DNA level was the lowest in Shaanxi Province, and the highest in Qiandongnan Prefecture of Guizhou Province.

HBV Prevalence and Demographic Information of Pregnant Women

Twenty-six studies reported the relationship between HBV prevalence and demographic information of pregnant women, which included age, residential address, education level, and occupation. However, whether these factors are related to HBV prevalence remains controversial. Due to the great variations in grouping methods and study designs across studies, a descriptive analysis was performed in this review.

Twenty-three studies focused on the association between age and HBsAg positive rate in pregnant women. Sixteen studies (sample size 1,138,852) suggested that HBsAg positive rate increased with age (12, 13, 17, 19, 20, 23, 24, 27, 28, 30, 31, 33, 36, 39, 40, 42). Two studies (sample size 3,947) revealed that HBsAg positive rate was not statistically different among various age groups (46, 53). Three studies (sample size 94,525) indicated that compared to those with HBsAg negative, HBsAg positive women were older (16, 49, 52). Two studies (sample size 132,254) demonstrated that the age was not statistically different between HBsAg positive and negative groups (32, 48).

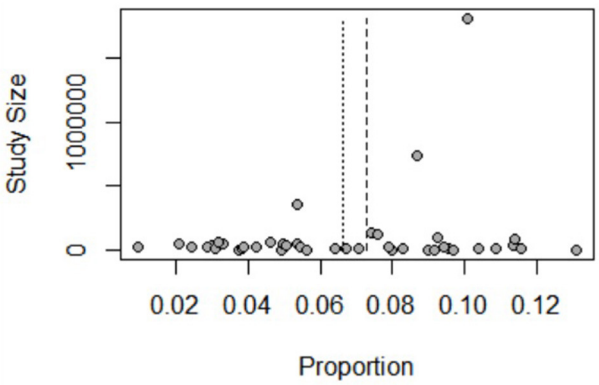

FIGURE 4 | Bias assessment funnel plot of studies reporting HBV infection prevalence rate in Chinese pregnant women, 2016–2021.

Ten studies analyzed the relationship between the times of gestation and parity and HBsAg positive rate. Seven studies (sample size 954,199) suggested that HBV prevalence in primigravida and primiparas was lower (12, 13, 28, 32, 48, 49, 52), while the other three studies (sample size 45,248) showed no relevance (16, 33, 46).

Four studies presented the relationship between the education level and HBsAg positive rate. Two studies (sample size 782,262) showed that HBV positive rate decreased when education level improved (12, 32), and the other two studies (sample size 24,333) demonstrated no relevance (13, 16).

Five studies reported the HBV prevalence among pregnant women residing in rural or urban areas. Three studies (sample size 76,657) demonstrated that the HBsAg positive rate was higher in rural areas when compared to that in urban areas

TABLE 3 | Subgroup analysis of included studies.

		Numbers of Study	Proportion (95% CI)	I^2	*P*
Regional disparities	Western China	16	7.15% (95%CI 5.82–8.48%)	99.6%	0.4592
	Eastern China	20	6.63% (95% CI 5.20–8.06%)	99.9%	
	Central China	6	5.23% (95%CI 2.50–7.96%)	99.6%	
Economic level	1–10	24	6.78% (95% CI 5.66–7.90%)	99.9%	0.4968
	21–31	11	6.46% (95% CI 4.35–8.58%)	99.8%	
	11–20	7	6.64% (95% CI 3.87–8.58%)	99.9%	
Source of data	Regional investigation	11	7.48% (95% CI 5.69–9.27%)	99.9%	0.2906
	Medical institution	31	6.35% (95% CI 5.27–7.43%)	99.8%	
Study design	Cross-sectional study	35	6.65% (95% CI 5.71–7.59%)	99.9%	0.9606
	Cohort study	7	6.57% (95% CI 3.45–9.69%)	99.9%	

(27, 29, 46). However, two other studies (sample size 40,381) presented no statistical differences between rural and urban groups (33, 45). Moreover, two studies (sample size 755,378) showed that the HBsAg positive rate in local population was lower than that in migrant population (12, 20).

Two studies dug into the HBV prevalence among pregnant women with different occupations. All of them revealed differences. One of the studies (sample size 732,783) demonstrated the occupations with HBsAg positive rates from low to high as follows: staff members (8.27%), workers (8.52%), farmers (8.72%), home-based workers (9.17%), and businessmen (10.32%), successively (12). The other study (sample size 15,641) exhibited occupations with HBsAg positive rates from low to high as follows: company administrators (4.65%), farmers (5.41%), workers (7.87%), self-employed workers (7.94%), and unemployed population (9.38%), successively (28).

DISCUSSION

This study systematically searched literature published after 2016, which provided the baseline information of HBV infection among pregnant women in China. Liu et al. (7) revealed that the national HBV prevalence in Chinese pregnant women was 6.17% (95% CI: 6.16–6.18%). The number revealed in our study was 6.64% (95% CI: 5.72–7.57%), which was slightly higher than that from Liu's study. Similar differences were observed regarding regional HBV prevalence. Some possible reasons for the differences are as follows: (1) During 2016 and 2021, only 20 provinces had relevant studies reported, while other provinces such as Inner Mongolia, Beijing, Hebei, Shandong, Jilin, Xinjiang, Ningxia, Gansu, and Heilongjiang had no relevant studies conducted. Most of these provinces without relevant studies were intermediate endemic (2.00–4.99%). Missing of these data led to a high HBV prevalence in our study. (2) Since the establishment and implementation of health policies by the Chinese government in 1992, there has been significant progress in the decline of HBV prevalence (54). The prevalence data included in our study was published between 2016 and 2021, while the data in Liu's (7) study was collected in 2020, which is a later time period when compared with our study. The differences between the two study results were consistent with the decline in HBV prevalence. The same trend was observed in another systematic review published in 2013 (55), which showed a higher HBsAg positive rate (7.6%) among pregnant women when compared with our study.

Our study showed that the regions with HBV prevalence ranking from high to low were in western China, eastern China, and central China, successively, which was consistent with the findings from a systematic review of Chinese population in 2019 (56) and a national HBV serological study conducted in 2014 (57). However, Liu's study (7) showed that the highest prevalence was in eastern China. A possible reason for this difference was that almost no relevant studies were published in some western regions such as Inner Mongolia, Xinjiang, Gansu, and Ningxia. These provinces have low HBV prevalence. Missing of these data led to a high HBV prevalence in our study. The difference between regions might be caused by social factors. Relatively prosperous economy in the eastern region might bring more economic and social exchanges compared with other regions, which increased the risk of HBV transmission (58).

Our study showed that the positive rate of HBeAg (25.80%) is similar to the rate of high HBV DNA ($\geq 2 \times 10^5$-1×10^6 IU/ml) (26.73%) in HBsAg positive pregnant women, which confirmed that the use of HBeAg test in place of HBV DNA test is reasonable. An observational study demonstrated that HBeAg positive rate among HBsAg positive couples was 27.84% in rural China, which decreased significantly with age (59). Both Liu's study and (59) our study demonstrated similar HBeAg positive rates among HBsAg positive pregnant women. Our study indicated that a great number of pregnant women with HBV infection carry high risks of transmitting infection, and special attention should be paid to block mother-to-child transmission in this population. Although the mother-to-child transmission rate declined from 50 to 6% in China since the implementation of HBV vaccination program, the rate remains high (11%) among pregnant women with positive HBeAg (4). In addition, pregnant women with positive HBsAg and HBeAg carry actively replicating virus, are more contagious, and prone to HBV mother-to-child transmission. Therefore, WHO recommends that pregnant women with a high HBV DNA ($\geq 2 \times 10^5$ IU/ml) should receive antiviral therapy during the last trimester of

pregnancy in order to prevent mother-to-child transmission, and HBeAg test can be used as an alternative testing method in settings when antenatal HBV DNA test is not available (60).

The age, times of gestation and parity, residential address, and educational level of pregnant women may affect HBV prevalence, although no consensus was reached across studies. Further studies on these factors were demanded. HBsAg positive rate in pregnant women increased with age, which may be related to the weakening or subsiding of the protective effect from hepatitis B vaccine (12). It was suggested that women of childbearing age should be the prior recipients of hepatitis B vaccine. Additionally, the antibody level of the population should be monitored in real time and a booster dose should be given in time. The HBsAg positive rate of pregnant women living in urban areas or with higher education level is relatively low, which may be related to better access to health care and knowledge about hepatitis B transmission (21). It is important for public education on hepatitis B including its prevention and control methods, especially for populations with high risks for the disease.

Some limitations of our study were as follows. First, our study only included the HBV prevalence data of 20 provinces, and the studies were not distributed equally across regions. Due to the large differences in HBV prevalence among pregnant women in different regions, the situation in regions where no relevant studies were published remains unknown. Besides, there was potential publication bias as revealed by the funnel plot and Begg's and Egger's tests. However, these methods for detecting publication bias are based on the assumptions that when compared with studies with positive and/or significant results, small studies reporting negative results and/or small effects are less likely to be published (61). The studies included in our meta-analysis did not calculate significant levels for their results, accordingly, statistical non-significance is unlikely to be an issue that may have biased publications (62). Hence, the conclusion regarding the presence of publication bias should be drawn with caution. Lastly, due to the great variations in grouping methods and study designs across studies, meta-analysis for factors affecting HBV prevalence was not conducted, a descriptive analysis was performed instead.

CONCLUSION

The prevalence of HBV in Chinese pregnant women is intermediate endemic (6.64%), although disparities exist between different regions. Among pregnant women with HBV infection, a high proportion of patients (25.80%) have strong infectivity, thus, effective mother-to-child blocking methods should be implemented, especially for populations with high risks for the disease. Moreover, factors affecting HBV prevalence among pregnant women remain controversial, and further research is needed to dig into the relationship between different factors and HBV prevalence.

DATA AVAILABILITY STATEMENT

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

AUTHOR CONTRIBUTIONS

DL, YL, and JN: study conduct. YL: data collection. HL and LinaZ: data analysis. JN, CZ, QY, and LiZ: data interpretation. DL and LingZ: drafting article. All authors contributed to the article and approved the submitted version.

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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.2022.879289/full#supplementary-material

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Immune Checkpoint Molecules Expressed on CD4$^+$ T Cell Subsets in Chronic Asymptomatic Hepatitis B Virus Carriers With Hepatitis B e Antigen-Negative

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Background: Chronic hepatitis B virus (HBV) infection remains a major public health problem worldwide. Immune checkpoint molecules expressed on CD4$^+$ T cells play critical roles in chronic HBV infection. However, their roles in chronic asymptomatic HBV carriers (ASCs) with hepatitis B e antigen (HBeAg)-negative remain unclear. In this study, we explored the role of immune checkpoint molecules expressed on CD4$^+$ T cell subsets in chronic ASCs with HBeAg-negative.

Methods: Human peripheral blood mononuclear cells (PBMCs) from the ASCs with HBeAg-negative and healthy controls (HC) were isolated, and immune checkpoint molecules expressed on CD4$^+$ T cell subsets and serum cytokines were detected by flow cytometry. Moreover, the mRNA expressions of immune checkpoint molecules were analyzed by a real-time quantitative PCR assay.

Results: In comparison with HC, CD4$^+$ T cells highly expressed LAG-3, TIM-3, and PD-1 in PBMCs from chronic ASCs with HBeAg-negative. Interestingly, the expressions of TIM-3 and PD-1 on circulating follicular helper T (Tfh) cells in ASCs were significantly high. Moreover, high expressions of LAG-3, TIM-3, and PD-1 were different among Treg, Th1, Th2, and Th17 cells. In addition, the expressions of TIM-3 and CTLA-4 mRNA in PBMCs from ASCs were significantly elevated. However, the frequency of CTLA-4$^+$CD4$^+$ T cell subsets in PBMCs from ASCs was not different from HC. The levels of six cytokines in serum from ASCs were not clearly different from HC.

Conclusion: Immune checkpoint molecules highly expressed on CD4$^+$ T cell subsets indicated an important role in chronic ASCs with HBeAg-negative, which provided potential therapeutic targets in the pathogenesis of chronic HBV infection.

Keywords: hepatitis B virus, asymptomatic HBV carriers, CD4$^+$ T cells, immune checkpoint molecules, chronic hepatitis B virus

INTRODUCTION

Hepatitis B virus (HBV) infection remains a major health problem in the world, which accounts for roughly one-third of the global population (Yuen et al., 2018; Revill et al., 2019; Iannacone and Guidotti, 2022). Although only fewer than 5% of adults with HBV infection can develop chronic hepatitis B (CHB), over 250 million individuals with about one million deaths annually are affected with persistent infection, liver cirrhosis, and hepatocellular carcinoma (HCC) worldwide (Trepo et al., 2014; Yuen et al., 2018; Revill et al., 2019). Moreover, the population with hepatitis B e antigen (HBeAg)-negative is dominant with over 85% in the hepatitis B surface antigen (HBsAg)-positive subjects, and more than 350 million are chronic HBV carriers. The chronic HBV carriers with HBsAg-positive and HBeAg-negative can also develop HCC and liver-associated mortality that are related to baseline age, sex, serum HBV-DNA copies, and alanine aminotransferase (ALT) level (Kumar et al., 2009; Trepo et al., 2014; Koffas et al., 2021). Chronic asymptomatic HBV carriers (ASCs) are major individuals (67–80%) among chronic HBV cases, who have normal levels of ALT and low HBV-DNA copies; although they are not treated commonly by drugs, a long-term follow-up for them is necessary (Kumar et al., 2009; Koffas et al., 2021). Although this "inactive" disease phase of the chronic ASCs with HBeAg-negative is closely associated with a favorable prognosis, it is not a truly "inactive carrier" with no obvious liver disease, and the reactivation and the potential risk of disease progression, including HCC and cirrhosis, are not negligible (Kumar et al., 2009; Puoti, 2013; Tang et al., 2018; Koffas et al., 2021). Therefore, these patients can still potentially develop liver disease for persistent HBV infection.

Chronic and persistent HBV infection is a critical causative agent for T-cell dysfunction, including $CD4^+$ T and $CD8^+$T cells that attenuate or lose the clearance of the virus. The dysfunction of T cells is closely associated with the upregulated immune checkpoint molecules, such as programmed cell death protein-1 (PD-1), lymphocyte activation gene-3 (LAG-3), T-cell immunoglobulin and mucin domain-3 (TIM-3), and cytotoxic T-lymphocyte antigen-4 (CTLA-4) (Bengsch et al., 2014; Park et al., 2016; Salimzadeh et al., 2018; Chen and Tian, 2019; Fisicaro et al., 2020; Ferrando-Martinez et al., 2021). Accumulating evidence indicates that the blockade of immune checkpoint molecules contributes to the restoration of dysfunctional T-cell-mediated adaptive immune responses, which partially promote the clearance of chronic viruses, including HBV, and the remission of the disease (Lang et al., 2019; Jin et al., 2021; Sears et al., 2021). The function of $CD8^+$T cells can be impaired without the help of $CD4^+$ T cells, and $CD8^+$T cells contribute to controlling and clearing viruses (Zuniga et al., 2015; Saeidi et al., 2018; Sears et al., 2021). Unfortunately, the function of $CD4^+$/$CD8^+$T cells is dysregulated in CHB infection, which is closely correlated with high expressions of immune checkpoint molecules on them (Bertoletti and Ferrari, 2012; Dong et al., 2019; Buschow and Jansen, 2021). Therefore, the immune checkpoint molecules on specific $CD4^+$ T cell subsets in chronic HBV infection should be further elucidated in the ASCs with HBeAg-negative, which will contribute to a better understanding of $CD8^+$T cell dysfunction during CHB infection.

In this study, the immune checkpoint molecules (PD-1, TIM-3, LAG-3, and CTLA-4) expressed on specific $CD4^+$ T cell subsets were investigated in peripheral blood mononuclear cells (PBMCs) from chronic ASCs with HBeAg-negative and normal ALT level, and the HBV-specific $CD4^+$ T cell subset-associated cytokines were also detected, which will provide potential therapeutic targets for the development of chronic HBV infection.

MATERIALS AND METHODS

Demographic Characteristics

A total of 22 chronic ASCs with HBeAg-negative and normal ALT levels were enrolled according to the Guideline of Prevention and Treatment for Chronic Hepatitis B (2019 version), and gender- and age-matched 22 healthy controls (HC) were recruited at the First Affiliated Hospital, Zhejiang University School of Medicine (Hangzhou, China) in this study. The individuals were excluded from the study if they have diabetes, autoimmune diseases, hematological system diseases, and other hepatotropic diseases, and the HC were also excluded if they have a vaccine history within 6 months. The ASCs with

TABLE 1 | Demographic characteristics of chronic ASCs with HBeAg-negative and healthy controls.

Variables	ASCs (*n* = 22)	HC (*n* = 22)	Range of reference
Age (years)	47.64 ± 11.12	43.82 ± 7.24	
Gender (M/F)	14/8	14/8	
HBsAg (IU/mL)	1891.83 ± 430.27***	0.01 ± 0.01	0.00 - 0.05
HBeAg (S/CO)	0.41 ± 0.01	0.34 ± 0.01	≥1.00
Anti-HBc (S/CO)	7.89 ± 0.08***	2.11 ± 0.53	≥1.00
Anti-HBe (S/CO)	0.03 ± 0.01***	1.50 ± 0.11	<1.00
Anti-HBs (IU/L)	1.45 ± 0.73**	194.43 ± 61.57	≥10.00
ALT (U/L)	20.14 ± 1.82	21.36 ± 2.04	9 - 50
AST (U/L)	20.77 ± 1.36	18.23 ± 1.18	15 - 40
HBV-DNA (IU/mL)	<30	NA	LOD = 30

*ASCs, symptomatic HBV carriers; HC, healthy controls; M/F, male/female; HBsAg, hepatitis B virus surface antigen; HBeAg, hepatitis B virus e antigen; ALT, alanine aminotransferase; AST, aspartate transferase; LOD, limitation of detection; NA, not applicable. ***P < 0.001.*

Abbreviations: HBV, hepatitis B virus; ASC, asymptomatic hepatitis B virus carriers; HC, healthy controls; PBMCs, peripheral blood mononuclear cells; Tfh, follicular helper T cells; Treg, regulatory T cells; PD-1, programmed cell death protein-1; TIM-3, T-cell immunoglobulin and mucin domain-3; CTLA-4, cytotoxic T-lymphocyte antigen-4; LAG-3, lymphocyte activation gene-3; IFN-γ, interferon-gamma; TNF-α, tumor necrosis factor-α; CXCR5, C-X-C motif chemokine receptor type 5; Th1, T helper cell type 1; Th2, T helper cell type 2; Th17, T helper cell type 17; HBeAg, hepatitis B e antigen; HCC, hepatocellular carcinoma; ALT, alanine aminotransferase; AST, aspartate aminotransferase; HBsAg, hepatitis B surface antigen; CCR6, C–C motif chemokine receptor 6; CXCR3, C–X–C motif chemokine receptor 3.

chronic HBeAg-negative and normal ALT levels who received HBV treatment within 6 months before blood sampling were also excluded. The clinical and laboratory characteristics of ASCs and HC are presented in **Table 1**. Written informed consent of all individuals was obtained according to the Declaration of Helsinki (1964) and was approved by the Medical Ethical Committee of the First Affiliated Hospital, Zhejiang University School of Medicine in this study (approval no. 2021-523).

Serological Analysis of Hepatitis B Virus Markers and Liver Function

Serum ALT and AST levels were detected by an automated biochemical analyzer (Roche cobas p 671, Switzerland). The serum HBsAg, anti-HBs, HBeAg, anti-HBe, and anti-HBc levels were tested by a chemiluminescent microparticle immuno Assay (Abbott Alinity i, United States). The serum HBV-DNA titers were determined by an ABI 7500 fluorescent quantitative PCR system (Applied BioSystems, United States).

Isolation of Human Peripheral Blood Mononuclear Cells

Human fresh PBMCs were isolated from fresh whole blood samples of the individuals by density gradient centrifugation using a Ficoll–Hypaque solution (CL5020, Netherlands) according to the manufacturer's protocol. The isolated PBMCs were washed two times using sterile phosphate-buffered saline (PBS) and transferred into tubes for use in subsequent experiments.

Flow Cytometry Analysis

Human PBMCs were resuspended with 200 μL PBS buffer and incubated with fluorochrome-conjugated antibodies, anti-human Krome Orange-CD4, Alexa Fluor 750-CD45RA, ECD-CD25 (Beckman Coulter Life Science, United States), Pacific blue^{TM}-CXCR5, PerCP/Cy5.5-PD-1, APC-TIM-3, Brilliant violet 650^{TM}-LAG-3, PE/Cy7-CTLA-4, FITC-CCR6, and PE-CXCR3 (BioLegend, United States), for 20 min at room temperature, and matched isotype controls were performed in this study according to the manufacturer's instructions. The samples were detected by the CytoFLEX Flow Cytometer, and the data were analyzed by CyExpert software (Beckman Coulter, United States).

Quantitative Real-Time Polymerase Chain Reaction

Total RNA was extracted from fresh PBMCs by the RNeasy Mini Kit (Qiagen, Germany), which was used to synthesize cDNA by a reverse transcription reagent kit (Takara, China). Real-time quantitative PCR was used to detect the expression levels of target genes in triplicate by Takara SYBR Supermix (Takara, China) by ABI QuantStudio 5 (Applied Biosystems, United States) as previously described (Shen et al., 2020). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal control in this study. The sequences of the specific primers are listed as follows: PD-1 forward 5′-AAGGCGCAGATCAAAGAGAGCC-3′ and reverse 5′-CAACCACCAGGGTTTGGAACTG-3′; TIM-3 forward 5′-GACTCTAGCAGACAGTGGGATC-3′ and reverse 5′-GGTGGTAAGCATCCTTGGAAAGG-3′; LAG-3 forward 5′-GCAGTGTACTTCACAGAGCTGTC-3′ and reverse 5′-AAGCCAAAGGCTCCAGTCACCA-3′; CTLA-4 forward 5′-ACGGGACTCTACATCTGCAAGG-3′ and reverse 5′-GGAGGAAGTCAGAATCTGGGCA-3′; and GAPDH forward 5′-GTCTCCTCTGACTTCAAC AGCG-3′ and reverse 5′-ACCACCCTGTTGCTGTAG CCAA-3′.

Serological Detection of Cytokines

The serum levels of IFN-γ, IL-2, IL-4, IL-6, IL-10, and TNF-α were tested by the flow fluorescence immune microbeads assay (Celgene, China) according to the manufacturer's protocol. In brief, 25 μL fluorescence detection reagent and 25 μL immune microbeads were added to 25 μL serum samples, and the mixed solutions were darkly incubated for 2.5 h at room temperature. Then, they were washed and resuspended with 100 μL PBS buffer, and cytokines were detected by BD FACSCanto II (BD, United States).

Statistical Analysis

Statistical analysis of the experimental data was performed using the Mann–Whitney *U* test or a one-way ANOVA using GraphPad Prism 7 software (GraphPad Software, Inc., CA). The differences were determined between two unpaired groups by Student's *t*-test, and significant differences were determined by paired *t*-tests between the two paired groups. Spearman's correlation coefficients were used to analyze correlations of different variables between the two groups. All P-values < 0.05 were considered to be statistically different.

RESULTS

Expanded Immune Checkpoint Molecules Expressed on CD4+ T Cells in Chronic Asymptomatic Hepatitis B Virus Carriers With Hepatitis B e Antigen-Negative

In this study, there was a significant difference in baseline characteristics, including HBsAg, anti-HBc, anti-HBe, and anti-HBs levels from the patients' chronic ASCs with HBeAg-negative; however, no significant difference was found in ALT and AST levels between 22 chronic ASCs with HBeAg-negative and 22 healthy controls (HC) (**Table 1**). Previous reports indicated that circulating CD4$^+$ T cells with immune checkpoint molecules played a critical role in chronic HBV infection, but the related studies about chronic ASCs were few (Ye et al., 2015; Dong et al., 2019). The frequencies of circulating CD4$^+$ T cells with four immune checkpoint molecules, including TIM-3, LAG-3, CTLA-4, and PD-1, were detected between chronic ASCs with HBeAg-negative and HC by flow cytometry (**Figure 1A**). Compared with HC, the frequency of circulating TIM-3$^+$CD4$^+$ T cells, LAG-3$^+$CD4$^+$ T

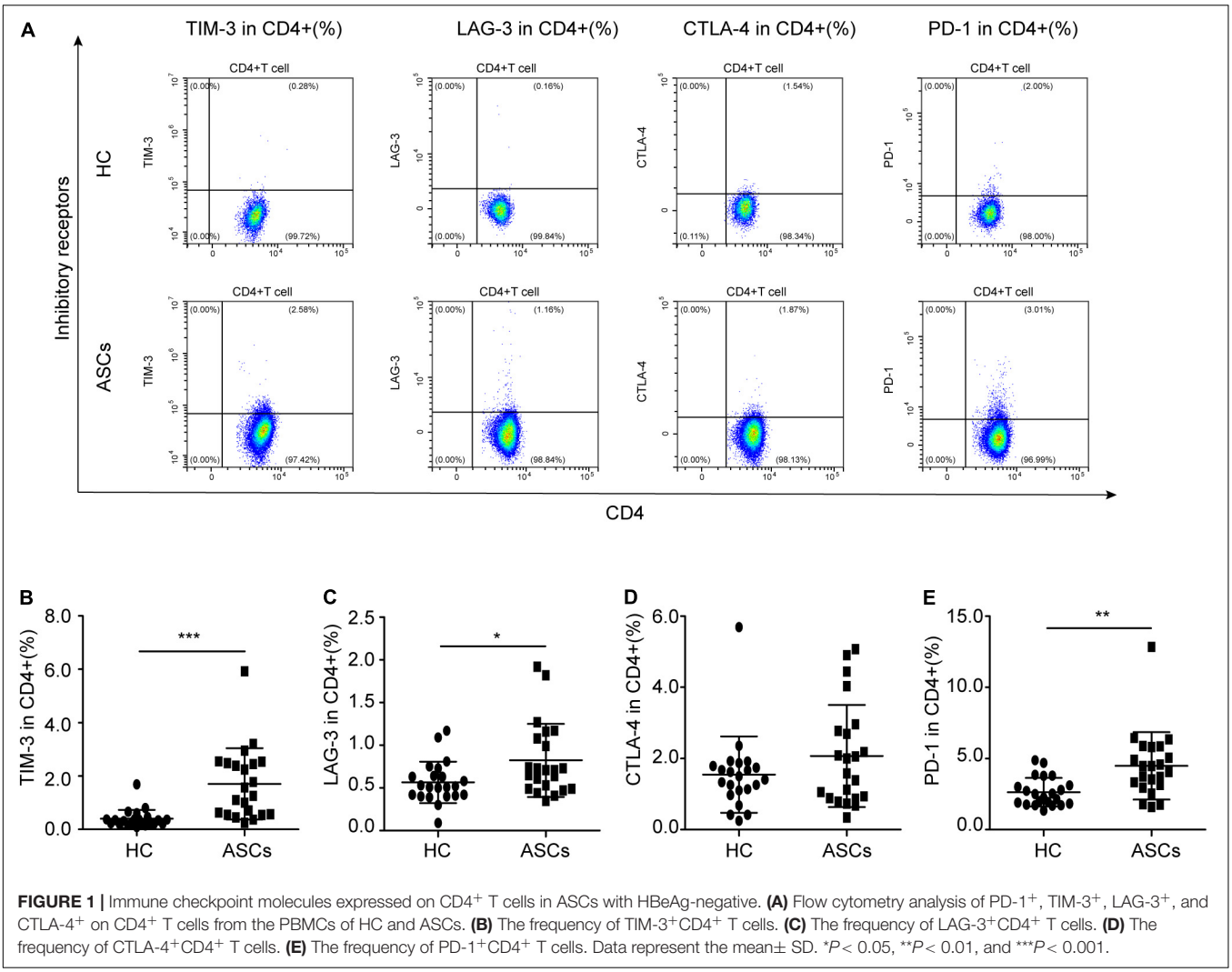

FIGURE 1 | Immune checkpoint molecules expressed on CD4$^+$ T cells in ASCs with HBeAg-negative. **(A)** Flow cytometry analysis of PD-1$^+$, TIM-3$^+$, LAG-3$^+$, and CTLA-4$^+$ on CD4$^+$ T cells from the PBMCs of HC and ASCs. **(B)** The frequency of TIM-3$^+$CD4$^+$ T cells. **(C)** The frequency of LAG-3$^+$CD4$^+$ T cells. **(D)** The frequency of CTLA-4$^+$CD4$^+$ T cells. **(E)** The frequency of PD-1$^+$CD4$^+$ T cells. Data represent the mean± SD. *P< 0.05, **P< 0.01, and ***P< 0.001.

TABLE 2 | The correlation of immune checkpoint molecules on CD4$^+$ T cell subsets and serum HBsAg levels from 22 chronic ASCs with HBeAg-negative.

CD4$^+$ T cell subsets	Spearman r	*P*-value	CD4$^+$ T cell subsets	Spearman r	*P*-value
PD-1$^+$CD4$^+$ T	−0.08978	0.6911	PD-1$^+$Th1	−0.3796	0.0815
LAG-3$^+$CD4$^+$ T	−0.1413	0.5306	LAG-3$^+$Th1	−0.2243	0.3156
TIM-3$^+$CD4$^+$ T	−0.3134	0.1556	TIM-3$^+$Th1	−0.2309	0.3011
CTLA-4$^+$CD4$^+$ T	−0.1293	0.5663	CTLA-4$^+$Th1	−0.1870	0.4048
PD-1$^+$Tfh	−0.1034	0.6471	PD-1$^+$Th2	0.02654	0.9076
LAG-3$^+$Tfh	−0.02598	0.9086	LAG-3$^+$Th2	−0.1169	0.6042
TIM-3$^+$Tfh	−0.2417	0.2784	TIM-3$^+$Th2	−0.1982	0.3766
CTLA-4$^+$Tfh	−0.1593	0.4789	CTLA-4$^+$Th2	−0.07284	0.7473
PD-1$^+$Treg	−0.2465	0.2687	PD-1$^+$Th17	−0.1688	0.4526
LAG-3$^+$Treg	−0.1914	0.3935	LAG-3$^+$Th17	−0.07623	0.7360
TIM-3$^+$Treg	−0.3800	0.0811	TIM-3$^+$Th17	−0.2840	0.2002
CTLA-4$^+$Treg	−0.09317	0.6801	CTLA-4$^+$Th17	−0.07510	0.7398

cells, and PD-1$^+$CD4$^+$ T cells was significantly elevated in ASCs (**Figures 1B,C,E**, respectively). However, the frequency of CTLA-4$^+$CD4$^+$ T cells was not significantly different between chronic ASCs with HBeAg-negative and HC (**Figure 1D**). In addition, there was no significant correlation between immune checkpoint molecules expressed on CD4$^+$ T cells

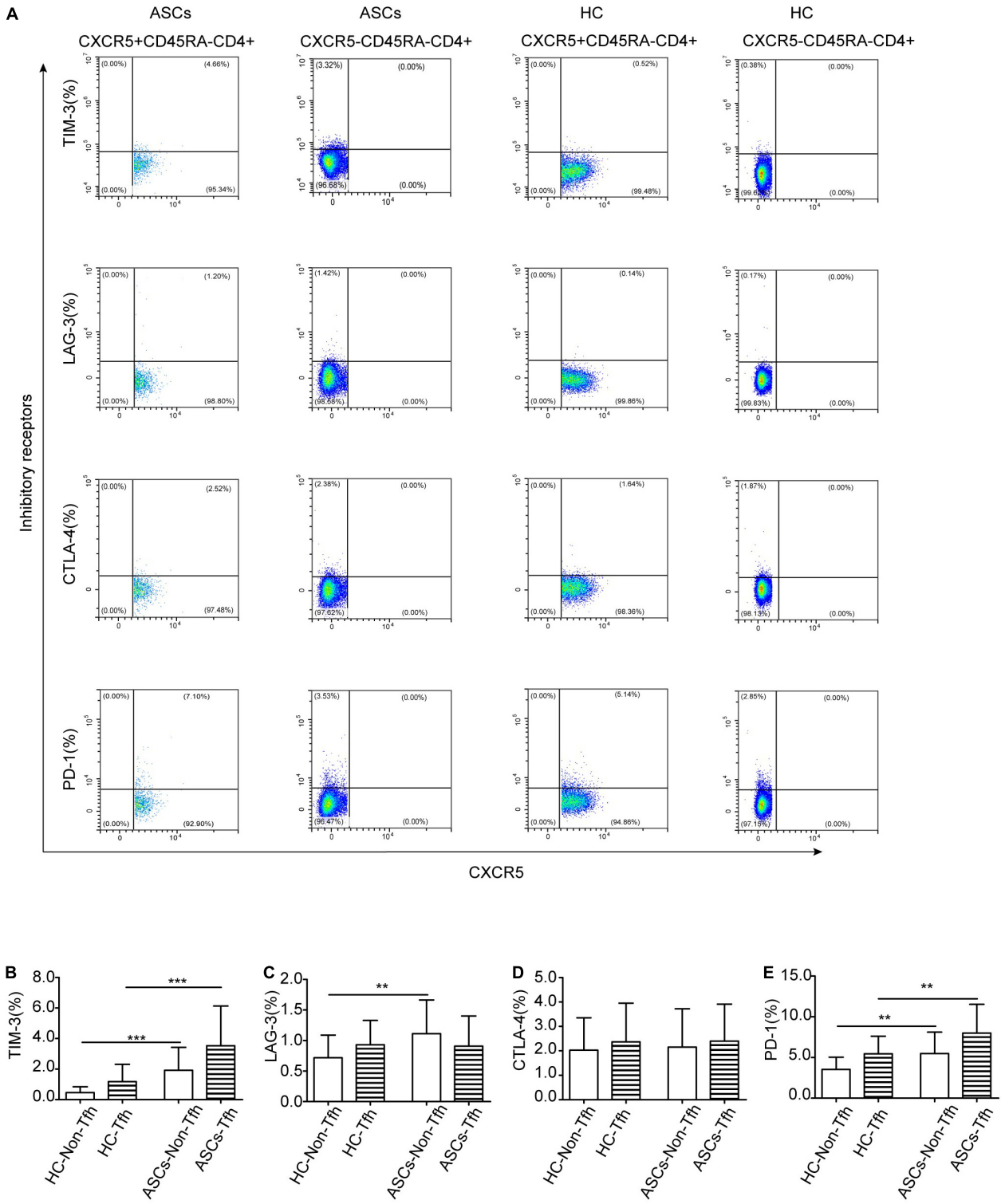

FIGURE 2 | Immune checkpoint molecules expressed on CXCR5$^+$CD45RA$^-$CD4$^+$ Tfh cells. **(A)** Flow cytometry detection of PD-1$^+$, TIM-3$^+$, LAG-3$^+$, and CTLA-4$^+$ on CXCR5$^+$CD45RA$^-$CD4$^+$ Tfh cells and CXCR5$^-$CD45RA$^-$CD4$^+$ Th cells in ASCs and HC. **(B–E)** Analysis of the expression of PD-1$^+$, TIM-3$^+$, LAG-3$^+$, and CTLA-4$^+$ on CXCR5$^+$CD45RA$^-$CD4$^+$ Tfh cells and CXCR5$^-$CD45RA$^-$CD4$^+$ Th cells in ASCs and HC. Data represent the mean± SD. $^{**}P < 0.01$, $^{***}P < 0.001$.

and serum HBsAg levels from chronic ASCs with HBeAg-negative (**Table 2**).

Immune Checkpoint Molecules Expressed on Tfh Cells in Chronic Asymptomatic Hepatitis B Virus Carriers With Hepatitis B e Antigen-Negative

Accumulated evidence showed the critical role of follicular helper T (Tfh) cells with high expressions of immune checkpoint molecules including TIM-3 and PD-1 in viral infection, including chronic HBV infection (Ye et al., 2015; Vella et al., 2017; Zhuo et al., 2017; Crotty, 2019; Yan et al., 2020; Cui et al., 2021; Liu et al., 2022). In this study, effector $CD45RA^{-}CXCR5^{+}CD4^{+}$ Tfh cells with immune checkpoint molecules were detected by flow cytometry (**Figure 2A**). The frequency of circulating $CD4^{+}$ T cells with immune checkpoint molecules was detected between chronic ASCs with HBeAg-negative and HC by flow cytometry (**Figure 2A**). Compared with HC, the frequency of circulating $TIM\text{-}3^{+}CD45RA^{-}CXCR5^{+}CD4^{+}$ Tfh cells and $PD\text{-}1^{+}CD45RA^{-}CXCR5^{+}CD4^{+}$ Tfh cells was notably increased, which also was higher than that of $TIM\text{-}3^{+}CD45RA^{-}CXCR5^{-}CD4^{+}$ T cells and $PD\text{-}1^{+}CD45RA^{-}CXCR5^{-}CD4^{+}$ T cells in ASCs (**Figures 2B,E**). However, there was no significant difference in the frequency of $LAG\text{-}3^{+}CD45RA^{-}CXCR5^{+}CD4^{+}$ Tfh cells $CTLA\text{-}4^{+}CD45RA^{-}CXCR5^{+}CD4^{+}$ Tfh cells, $TIM\text{-}3^{+}CD45RA^{-}CXCR5^{-}CD4^{+}$ T cells, and $PD\text{-}1^{+}CD45RA^{-}CXCR5^{-}CD4^{+}$ T cells between chronic ASCs with HBeAg-negative and HC (**Figures 2B–E**). In addition, there was no significant correlation between immune checkpoint molecules expressed on Tfh cells and serum HBsAg levels from chronic ASCs with HBeAg-negative (**Table 2**).

Immune Checkpoint Molecules Expressed on Treg Cells

Previous reports indicated that Treg cells with high expressions of immune checkpoint molecules played an essential role in cancer types, autoimmune diseases, and viral infections (Andrews et al., 2019; Schnell et al., 2020; Granito et al., 2021). In this study, $CD45RA^{-}CD25^{+}CXCR5^{-}CD4^{+}$ Treg cells with immune checkpoint molecules were detected by flow cytometry (**Figure 3A**). In comparison with that of HC, the frequency of $CD45RA^{-}CD25^{+}CXCR5^{-}CD4^{+}$ Treg cells with TIM-3 or PD-1 and the frequency of $LAG\text{-}3^{+}CD45RA^{-}CD25^{+}CXCR5^{-}CD4^{+}$ Treg cells in chronic ASCs with HBeAg-negative were significantly high (**Figures 3B,C,E**). Moreover, similar significant results were found for the frequency of $CD45RA^{-}CD25^{-}CXCR5^{-}CD4^{+}$ T cells with TIM-3 or PD-1 (**Figures 3B,E**). However, there was no significant difference in the frequency of $CTLA\text{-}4^{+}CD45RA^{-}CD25^{+}CXCR5^{-}CD4^{+}$ Treg cells between chronic ASCs with HBeAg-negative and HC (**Figure 3D**). In addition, there was no significant correlation between immune checkpoint molecules expressed on Treg cells and serum HBsAg levels from chronic ASCs with HBeAg-negative (**Table 2**).

Immune Checkpoint Molecules Expressed on Other $CD4^{+}$ T Cell Subsets in Chronic Asymptomatic Hepatitis B Virus Carriers With Hepatitis B e Antigen-Negative

Previous studies showed that human Th1 ($CXCR3^{+}CCR6^{-}$), Th2 ($CXCR3^{-}CCR6^{-}$), and Th17 ($CXCR3^{-}CCR6^{+}$) cells from PBMCs were distinguished based on the expressions of chemokine receptors CXCR3 and CCR6 on $CD4^{+}$ T cells (Maecker et al., 2012; Kubo et al., 2017). In this study, to further investigate the frequency of immune checkpoint molecules expressed on other $CD4^{+}$ T cell subsets in chronic ASCs with HBeAg-negative and HC, flow cytometry was used to detect these immune checkpoint molecules, and human Th1 ($CXCR3^{+}CCR6^{-}$), Th2 ($CXCR3^{-}CCR6^{-}$), and Th17 ($CXCR3^{-}CCR6^{+}$) cells were also defined by gating $CD45RA^{-}CXCR5^{-}CD4^{+}$ T cells from PBMCs of ASCs and HC (**Figure 4A**). The results showed that the frequency of $TIM\text{-}3^{+}$Th1 cells, $TIM\text{-}3^{+}$Th2 cells, $LAG\text{-}3^{+}$Th17 cells, $PD\text{-}1^{+}$Th2 cells, and $PD\text{-}1^{+}$Th17 cells was significantly increased; However, the frequency of $TIM\text{-}3^{+}$Th17 cells, $LAG\text{-}3^{+}$Th1 cells, $LAG\text{-}3^{+}$Th2 cells, $CTLA\text{-}4^{+}$Th1/2/17 cells, and $PD\text{-}1^{+}$Th1 cells was not varied considerably in chronic ASCs with HBeAg-negative in comparison with HC (**Figures 4B–E**). Interestingly, Th1 cells highly expressed TIM-3, CTLA-4, and PD-1 compared with Th2 and Th17 cells (**Figures 4B,D,E**), Th1 and Th17 cells were highly expressed LAG-3 compared with Th2 cells in chronic ASCs with HBeAg-negative (**Figures 4B,C**). In addition, there was no significant correlation between immune checkpoint molecules expressed on Th1, Th2, and Th17 cells and serum HBsAg levels from chronic ASCs with HBeAg-negative (**Table 2**).

Immune Checkpoint Molecules mRNA Expression in Chronic Asymptomatic Hepatitis B Virus Carriers With Hepatitis B e Antigen-Negative

To further explore the mRNA levels of PD-1, TIM-3, LAG-3, and CTLA-4, the total RNA in PBMCs isolated from ASCs and HC was used to assess the expression levels of the four molecules by PCR method. The results displayed that the relative mRNA levels of TIM-3 and CTLA-4 genes were notably increased in the PBMCs from the ASCs in comparison with those from the HC (**Figures 5A,D**), but PD-1 and LAG-3 mRNA levels were not notably different between the ASCs and HC (**Figures 5B,C**).

Serum Levels of Cytokines in Chronic Asymptomatic Hepatitis B Virus Carriers With Hepatitis B e Antigen-Negative

To further investigate the immune status of ASCs with negative HBeAg, serum levels of some important cytokines were tested by flow cytometry. The results showed that decreased levels of serum IL-2, IL-4, and TNF-α were not notably different in chronic ASCs with negative HBeAg in comparison with HC (**Figures 6A,B,E**). Serum IL-6, IL-10, and IFN-γ levels were not significantly

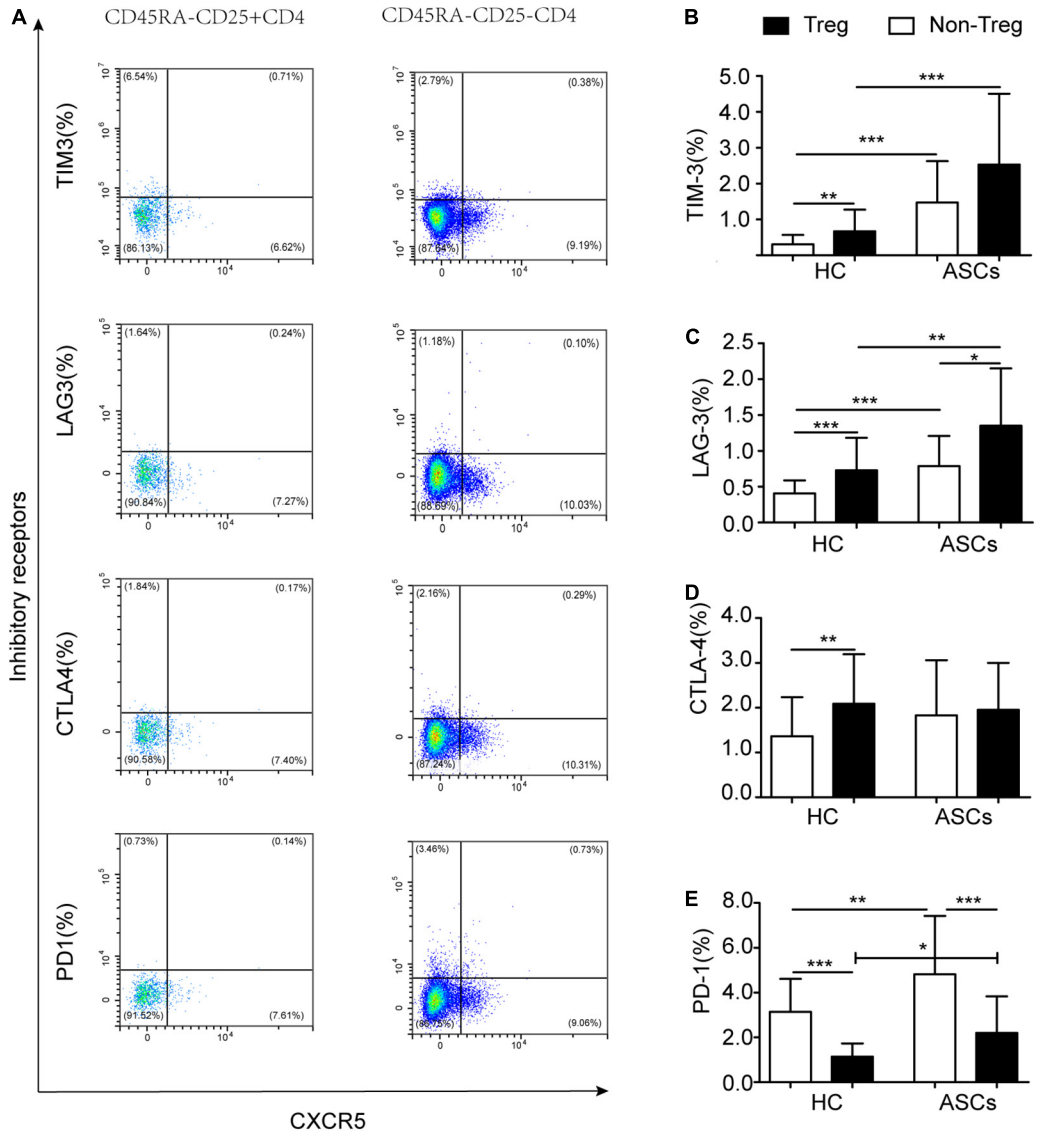

FIGURE 3 | Immune checkpoint molecules expressed on CD45RA$^-$CD25$^+$CD4$^+$ Treg cells in HBV carriers. **(A)** Flow cytometry detection of PD-1$^+$, TIM-3$^+$, LAG-3$^+$, and CTLA-4$^+$ on Treg cells. **(B–E)** Different expressions of PD-1$^+$, TIM-3$^+$, LAG-3$^+$, and CTLA-4$^+$ on Treg cells in ASCs and HC. Data represent the mean ± SD. *P< 0.05, **P< 0.01, and ***P< 0.001.

changed in chronic ASCs with HBeAg-negative in comparison with HC (**Figures 6C,D,F**). However, there was no correlation between these cytokine levels and HBsAg concentrations in chronic ASCs with HBeAg-negative (data not shown).

DISCUSSION

In this study, we provided an insight into the immune checkpoint molecules expressed on circulating human CD4$^+$ T cell subsets from chronic ASCs with HBeAg-negative. The results showed that immune checkpoint molecules (TIM-3, LAG-3, and PD-1) expressed on CD4$^+$ T cells were significantly upregulated in PBMCs from chronic ASCs with HBeAg-negative. Interestingly, a comprehensive analysis displayed a high frequency of circulating TIM-3$^+$or PD-1$^+$ Tfh cells in chronic ASCs with HBeAg-negative. Moreover, increased expressions of TIM-3, LAG-3, and PD-1 molecules on Th1, Th2, Th17, and Treg cells and TIM-3 and CTLA-4 mRNA levels in PBMCs from chronic ASCs with HBeAg-negative in comparison with HC were notably different. The chronic ASCs with HBeAg-negative can still develop into the stage of CHB with HBeAg-positive and end-stage liver disease including HCC and liver fibrosis (Kumar et al., 2009; Puoti, 2013; Tang et al., 2018; Koffas et al., 2021). Previous reports have shown that the co-inhibitory molecules on T cells contributed to the development of pathogenesis in patients with chronic HBV infection (Salimzadeh et al., 2018; Lang et al., 2019; Jin et al., 2021; Sears et al., 2021). Taken

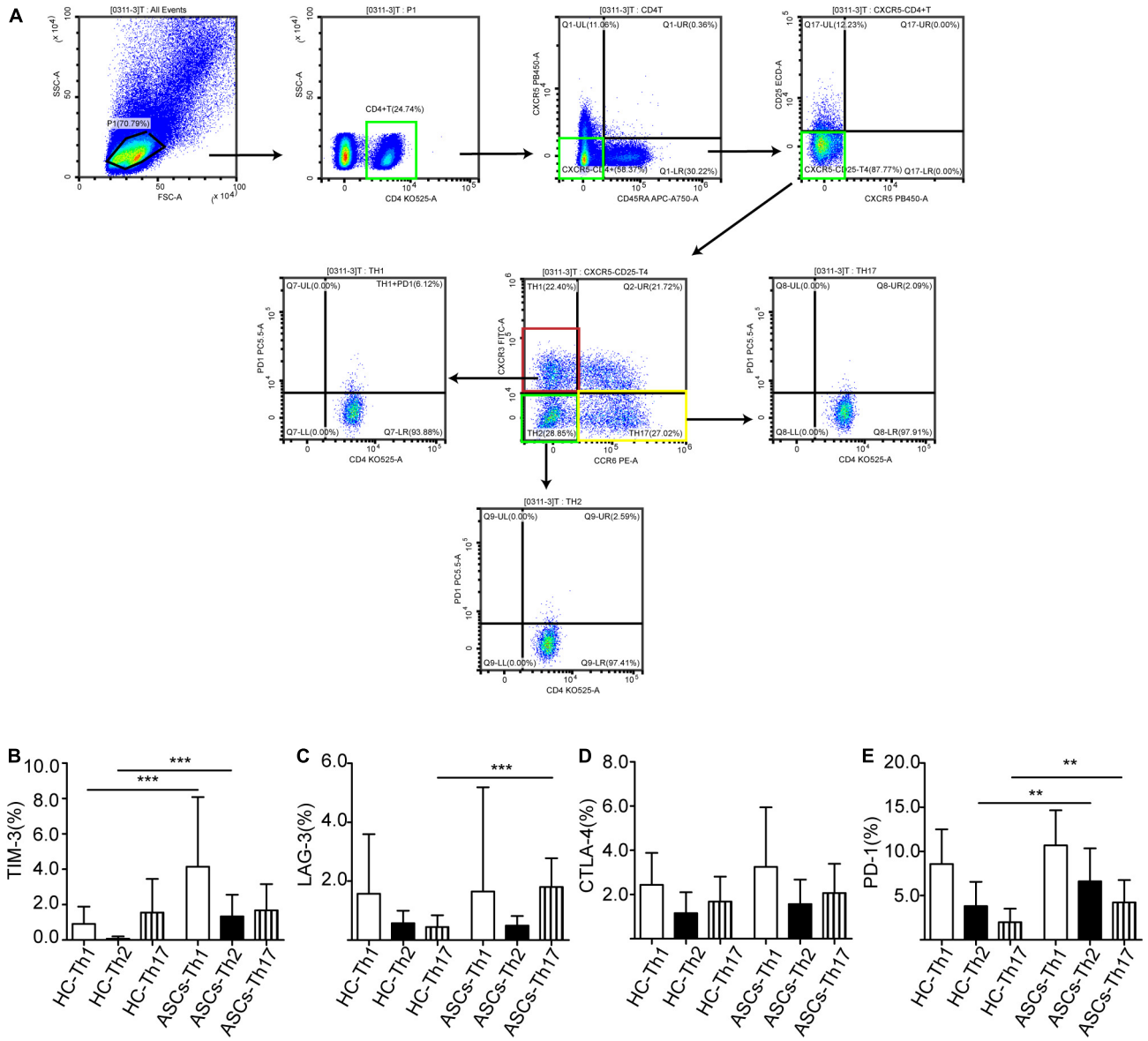

FIGURE 4 | CD4$^+$ T cell subsets showed different levels of immune checkpoint molecules. **(A)** Analysis of CD4$^+$ T cell subsets. **(B)** TIM-3$^+$ expression on Th1, Th2, and Th17 cells. **(C)** LAG-3$^+$ expression on Th1, Th2, and Th17 cells. **(D)** CTLA-4$^+$ cells expressed on Th1, Th2, and Th17 cells. **(E)** PD-1$^+$ expression on Th1, Th2, and Th17 cells. Data represent the mean ± SD. $^{**}P < 0.01$; $^{***}P < 0.001$.

together, these findings indicated that elevated expressions of immune checkpoint molecules (TIM-3, LAG-3, and PD-1) on CD4$^+$ T cell subsets might play a critical role in chronic ASCs with HBeAg-negative.

CD4$^+$ T cell dysfunction in chronic HBV infection affected the function of other immune cells, including HBV-specific CD8$^+$T cells and B cells, and resulted in disruption of the host's immune responses and persistent HBV infection (Trepo et al., 2014; Yuen et al., 2018; Revill et al., 2019; Iannacone and Guidotti, 2022). Dysfunctional CD4$^+$ T cells are closely correlated with the upregulation of immune co-inhibitory molecules expressed on CD4$^+$ T cells that usually played an immunosuppressive function, such as TIM-3, PD-1, LAG-3, and CTLA-4 (Park et al., 2016; Salimzadeh et al., 2018; Dong et al., 2019; Fisicaro et al., 2020; Ferrando-Martinez et al., 2021). Previous reports showed an increased frequency of PD-1$^+$CD4$^+$ T cells, TIM-3$^+$CD4$^+$ T cells, LAG-3$^+$CD4$^+$ T cells, and CTLA-4$^+$CD4$^+$ T cells in chronic HBV infection, and the blockade of the co-inhibitory receptors contributed to the restoration of CD4$^+$ T cell proliferation and function and promoted cytotoxic effector of CD8$^+$T cells that played critical roles in viral clearance and disease pathogenesis of sustained HBV infection (Peng et al., 2008; Dong et al., 2017, 2019; Buschow and Jansen, 2021; Xiong et al., 2021). However, these studies did not display detailed data on ASCs of patients with chronic HBV. Our results showed an expanded frequency of PD-1$^+$CD4$^+$ T cells, TIM-3$^+$CD4$^+$ T

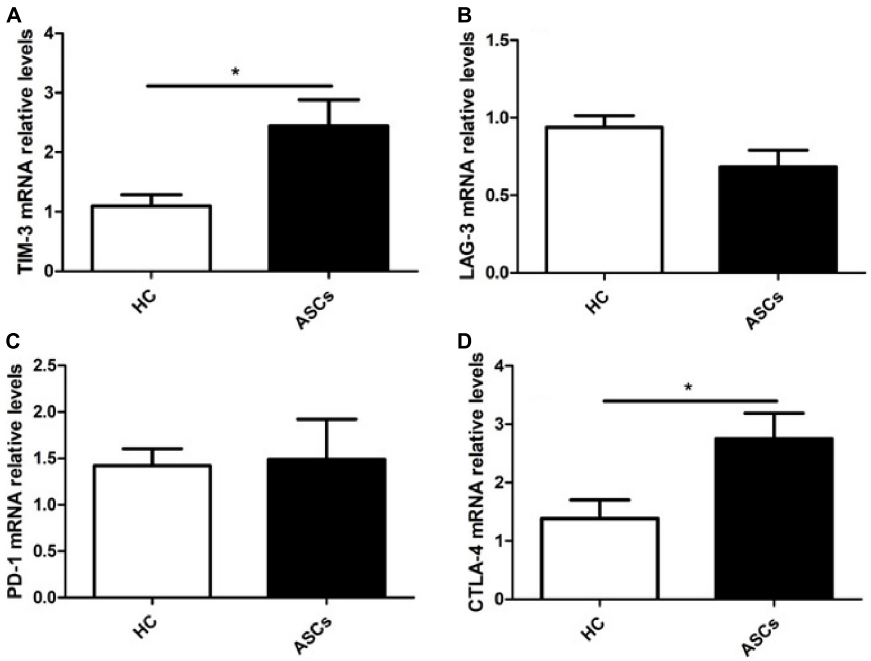

FIGURE 5 | Expression levels of immune checkpoint molecules mRNA in PBMCs from chronic ASCs. **(A)** The relative levels of TIM-3 mRNA. **(B)** The relative levels of LAG-3 mRNA. **(C)** The relative levels of PD-1 mRNA. **(D)** The relative levels of CTLA-4. HC: $n = 6$, ASCs: $n = 9$. Data represent the mean ± SEM. $^*P < 0.05$.

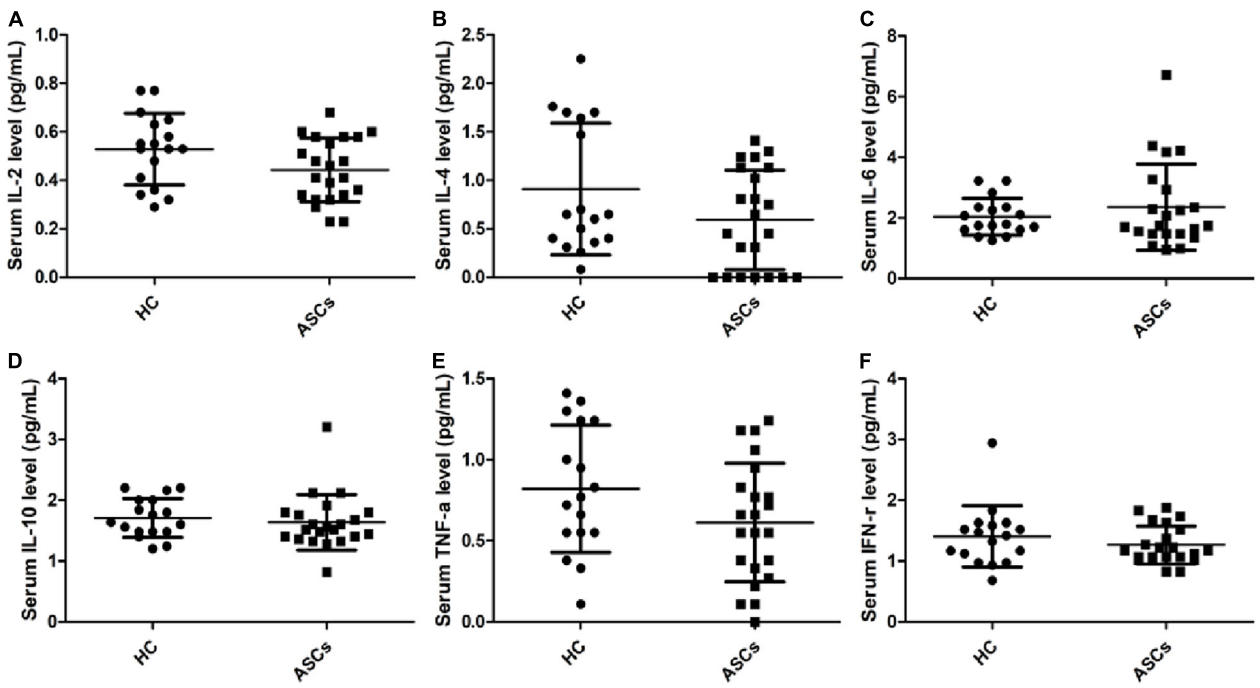

FIGURE 6 | Expression levels of six cytokines in serum from chronic ASCs. **(A)** The relative levels of IL-2. **(B)** The levels of IL-4. **(C)** The levels of IL-6. **(D)** The relative levels of IL-10. **(E)** The relative levels of TNF-α. **(F)** The relative levels of IFN-γ. Data represent the mean ± SD.

cells, and LAG-3$^+$CD4$^+$ T cells, but not CTLA-4$^+$CD4$^+$ T cells in chronic ASCs with HBeAg-negative in comparison with HC. The number and status of the patients possibly affected the statistical analysis of CTLA-4$^+$CD4$^+$ T cell frequencies in this study. In addition, the mRNA levels of TIM-3 and CTLA-4 expressions, but not PD-1 and LAG-3 mRNA, were notably

increased in chronic ASCs with HBeAg-negative in comparison with HC, which were not completely consistent with previous reports (Yu et al., 2009; Zhang et al., 2014; Cheng et al., 2021; Liu et al., 2021). The discrepancy may be associated with the number and the status of the special population who are the "inactive carrier" phase of chronic HBV infection, and these co-inhibitory receptors are also expressed in other immune cells, including CD8$^+$T cells, innate lymphoid cells (ILCs), and NK cells (Kumar et al., 2009; Puoti, 2013; Riva and Chokshi, 2018; Tang et al., 2018; Mariotti et al., 2019; Fisicaro et al., 2020; Koffas et al., 2021; Mak et al., 2021).

Accumulating evidence indicates that high expressions of immune checkpoint molecules (TIM-3, PD-1, CTLA-4, and LAG-3) on CD4$^+$ T cells are related to decreased secretion of cytokines and proliferation capacity of T cells, such as IFN-γ^+Th1, IL-4$^+$Th2, IL-17A$^+$Th17, and IL-21$^+$Tfh cells, except for PD-1^{high} Tfh cells that have the capacity of inducing B-cell response and the generation of specific antibodies against antigens or viruses (Gibney et al., 2016; Park et al., 2016; Saeidi et al., 2018; Buschow and Jansen, 2021). However, Treg cells with increased expressions of immune checkpoint molecules displayed a high immunosuppressive function on HBV-specific Th1 and Tfh responses and promoted IL-10 and TGF-ß1 secretion (Stoop et al., 2005; Buschow and Jansen, 2021). This study showed that the frequencies of circulating TIM-3$^+$or PD-1$^+$ Tfh cells and TIM-3, LAG-3, and PD-1 molecules on Th1, Th2, Th17, and Treg cells were significantly expanded in chronic ASCs with HBeAg-negative in comparison with HC, which were partially accordant with previous reports (Stoop et al., 2005; Wang R. et al., 2018; Wang X. et al., 2018). These findings implied that chronic HBV infection resulted in the host's immune disorder and dysregulation by upregulating immune checkpoint molecules, including TIM-3, PD-1, CTLA-4, and LAG-3 on T cells, that induced T-cell dysfunction in chronic HBV infection. In addition, our results showed that serum cytokines (IL-2, IL-4, and TNF-a) had a low trend in chronic ASCs with HBeAg-negative in comparison with HC, which were partially consistent with previous reports (Ayada et al., 2006; Wang X. et al., 2018; Zhong et al., 2021). These cytokines play a critical role in regulating CD4/CD8$^+$T cell proliferation and differentiation and the production of various specific antibodies to control persistent HBV infection (Buschow and Jansen, 2021; Mak et al., 2021; Zhong et al., 2021).

Our study showed some interesting results, including four important immune checkpoint molecules expressed on CD4$^+$ T cells and their subsets in chronic ASCs with HBeAg-negative. However, several limitations are listed in the following. First, the sample size was relatively small. Second, the function of these CD4$^+$ T cells and their subsets with high expressions of the immune checkpoint molecules should be explored by a large number of samples in further study. The function of the co-inhibitory molecules on CD4$^+$ T cells includes the cellular metabolism, cytokines secretion, differentiation, and helper capacity for CD8$^+$T cells and B cells, which will be precisely explored by multi-omics methods.

CONCLUSION

In summary, during chronic ASCs with HBeAg-negative infection, CD4$^+$ T cells and their subsets, including Th1, Th2, Th17, Tfh, and Treg cells, showed high expressions of immune checkpoint molecules (TIM-3, PD-1, CTLA-4, and LAG-3) and decreased the secretion of cytokines. Currently, immune checkpoint-based therapies were used in many diseases, including cancer types, autoimmune diseases, and viral infections, which showed satisfactory curative effects for patients and animals. Taken together, immune checkpoint-based therapies have potential values for chronic ASCs with HBeAg-negative. These findings could provide potential therapeutic implications for chronic HBV infection by regulating CD4$^+$ T cells' functional therapy.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Medical Ethical Committee of the First Affiliated Hospital, Zhejiang University School of Medicine (Approval No. 2021-523). 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

DC wrote the manuscript. DC and DJ performed the experiments and analyzed the data. DC, DJ, CY, and YL collected the clinical data and samples. DC, DJ, and XL revised the manuscript. DC, YC, and JX conceived the topic and revised the manuscript. All authors approved the submitted version.

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REVIEW
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Human Adenovirus Associated Hepatic Injury

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Human adenovirus (HAdV) is a common virus, but the infections it causes are relatively uncommon. At the same time, the methods for the detection of HAdV are varied, among which viral culture is still the gold standard. HAdV infection is usually self-limited but can also cause clinically symptomatic in lots of organs and tissues, of which human adenovirus pneumonia is the most common. In contrast, human adenovirus hepatitis is rarely reported. However, HAdV hepatitis has a high fatality rate once it occurs, especially in immunocompromised patients. Although human adenovirus hepatitis has some pathological and imaging features, its clinical symptoms are not typical. Therefore, HAdV hepatitis is not easy to be found in the clinic. There are kinds of treatments to treat this disease, but few are absolutely effective. In view of the above reasons, HAdV hepatitis is a disease that is difficult to be found in time. We reviewed and summarized the previously reported cases, hoping to bring some relatively common characteristics to clinicians, so as to facilitate early detection, early diagnosis, and early treatment of patients.

Keywords: human adenovirus, hepatitis, pathogenesis, pathology, therapy

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INTRODUCTION

Human adenoviruses (HAdVs), the widespread pathogens of the Adenoviridae family, are non-enveloped, double-stranded deoxyribonucleic acid (DNA) viruses. About 60 years earlier, HAdV had been independently discovered by Rowe et al. and written by Hilleman et al. (1, 2).

Human adenovirus possesses a similar icosahedral capsid with a diameter of approximately 90 nm and contains the trimeric fibrin with variable length (3, 4). The capsid is wrapped in a tightly packed viral protein and DNA genome (between 34 and 37 kbp), known as core (5, 6). HAdV is divided into serotypes (1–104) together with single species (A-G) (**Table 1**; http://hadvwg.gmu.edu/) through conventional serotype, viral DNA restriction enzyme digestion, sequencing, and the serotype-specific PCR.

In immunocompetent individuals, HAdV infection usually causes upper respiratory, gastrointestinal (GI), or conjunctival involvement. The vast majority of cases are self-restricting, while in the immunocompetent hosts, fatal and disseminated infections are rare. HAdV can sometimes result in serious infections in the immunocompromised individuals, for example, hemorrhagic cystitis, gastroenteritis, pneumonia, encephalitis, nephritis, enterocolitis, hepatitis, or the disseminated diseases, leading to the evident incidence rate and mortality. Some serotypes can lead to more seeking infections, even to the death of immunodeficient infants and patients. For instance, HAdV7, HAdV5, and HAdV3 are the causes of acute respiratory infections, and the recently, the outbreak of HAdV7 at the rehabilitation center of New Jersey caused 11

TABLE 1 | Current spectrum of known HAdVs.

A	12, 18, 31, 61
B	3, 7, 11, 14, 16, 21, 34, 35, 50, 55, 66, 68, 76–79
C	1, 2, 5, 6, 57, 89, 104
D	8–10, 13, 15, 17, 19, 20, 22–30, 32, 33, 36–39, 42–49, 51, 53, 54, 56, 58, 59, 60, 63, 64, 65, 67, 69–75, 80–88, 90–103
E	4
F	40, 41
G	52

The HAdV species (A-G) and types (104) belonging to individual species are indicated.

deaths (https://www.nj.gov/health/cd/topics/adenovirus.shtml), whereas HAdV41 and HAdV40 are known to result in persistent and acute gastroenteritis in children (7).

On account of their genetic heterogeneity, HAdV species have very diverse tropism, leading to the infection of various tissues and organs. Some of HAdV species E and D (HAdV-D) mainly result in keratoconjunctivitis (EKC) (8), the infections of HAdV-C, B, and A can cause GI, respiratory, and urinary diseases, and the GI disease is also resulted from HAdV-E and F (9, 10). Subgroup D adenovirus serotype is known for its tendency to the eye, leading to epidemic EKC or conjunctivitis (11). Hepatitis is mainly correlated with subgroup C serotypes (HAdV 5, 2, and 1), especially serotype 5 infection is related to the occurrence of hepatitis in liver transplant recipients (12). In the following, we will review HAdV and HAdV hepatitis.

EPIDEMIOLOGY OF HADV

Adenovirus infection can be resulted from latent virus reactivation or contact with the infected individuals. Adenovirus can be transmitted by the inhalation of infected aerosols, person-to-person contact, fecal-oral route, direct conjunctival inoculation, or contact with infected blood or tissue. Infections occur at any time of the year without apparent seasonal (12), despite the great majority of epidemics occurs in early spring or winter (13). In the meanwhile, HAdV has a worldwide distribution. The regional or local epidemics are described, but many infections occur as sporadic events. HAdV is resistant to numerous disinfectants; however, ethanol solution (95%) is a useful disinfectant (14).

Adenovirus infections are most common in the healthy children (especially children under 4 years) (15), adults in the closed environment (such as military and college), and immunocompromised patients. In immunocompetent people, HAdV make up exceed 50% of pneumonia cases and febrile respiratory illness (FRI) among the unvaccinated recruits, in the United States, simultaneously globally (16).

In immunocompromised patients, adenovirus infections are almost described in the recipients of solid organ transplantation (SOT) together with hematopoietic stem cell transplant (HSCT). In patients with human immunodeficiency virus (HIV) infection, the incidence of HAdV infection is between 12 and 28%. Among the recipients of HSCT, for the HAdV infection, its incidence is between 3 and 47%. Available data indicate that the incidence rate of allogeneic (from 5 to 47%) HSCT receptor is much greater than that of the autologous HSCT receptor (between 2.5 and 14%) (17, 18). Furthermore, the studies have shown that the adenoviral infections are more prevalent in the patients containing acute graft-versus-host disease (GVHD), severe T-cell depletion, cord-blood donors, human leukocyte antigen (HLA) mismatch, and patients who use of alemtuzumab (10). In addition, a wide variety of SOT populations have been reported to be infected with adenovirus, including those receiving liver, renal, heart, intestinal, and lung transplants. The HAdV infection incidence in the recipients of SOT is between 5 and 22%, generally within the first 6 months after the transplantation. For the adenovirus infection, its risk factors in the recipients of SOT contain liver transplant and small bowel recipients, patients receiving anti-lymphocyte antibodies, child transplant recipients, and patients with recipient negative or donor positive adenovirus status (19).

The incubation period of HAdV infection is determined by the transmission mechanism and virus serotype, between a few days and more than a week. In patients with normal immune function, the viral shedding from throat of patients with common cold will fall off for about 1–3 days; the virus shedding from throat, nose, eyes, or stool of patients with pharyngeal conjunctival fever were for 3–5 days; the virus shedding from corneal conjunctivitis eyes were for 2 weeks; the virus shedding from stool or throat of children with systemic or respiratory diseases were for 3–6 weeks. Despite the details of virus shedding in the immunocompromised patients are restricted, the shedding time is generally longer. The proposed positions of viral latency contain the intestine, pharynx (adenoids and tonsils), lymphocytes, and urinary tract, despite some of them are still controversial (20).

HIGH-RISK FACTORS OF HADV HEPATITIS

Hepatitis can be caused by a variety of causes. If not treated, it can lead to liver transplantation, death, or acute liver failure (ALF). Possible causes of hepatitis contain drugs, autoimmune hepatitis, toxins, venous obstruction, ischemia, and metabolic together with infectious causes, containing cytomegalovirus (CMV), varicella zoster virus (VZV), hepatitis A to E, Epstein–Barr virus (EBV), herpes simplex virus (HSV), and human adenovirus. Although human adenovirus (HAdV) hepatitis was rarely been reported, HAdV hepatitis can occur in immunocompromised patients. First of all, we will mainly introduce the high-risk factors of HAdV hepatitis.

In a recent study, 66% of the patients were patients with pediatrics. This observation is very consistent with the outcomes of a recent meta-analysis, which found that 64% of cases of HAdV hepatitis occurred in children (21, 22). In the pediatric setting, the most prevalent predisposing condition may be liver transplantation. In the meantime, children may also develop HAdV hepatitis in the process of immunosuppression after the transplantation of hematopoietic stem cell, chemotherapy for solid malignancies or lymphoblastic leukemia, and severe combined immunodeficiency (SCID) (23).

However, some studies have shown that the proportion of HAdV hepatitis in adult patients who are immunosuppressed after the transplantation of hematopoietic stem cell is higher than that in adult patients after liver transplantation (24, 25). Adults are probably developed HAdV hepatitis when SOT other than the liver or chemotherapy leads to immunosuppression (26). All in all, in SOT recipients, most reported cases of HAdV hepatitis appeared in the recipients of liver transplant. What is more, it was reported that one patient was just given chemotherapy about a year prior to the HAdV infection. Despite he was not immunosuppressed actively, his residual lymphoplasmacytic lymphoma was found to involve kidney, spleen, and bone marrow at the autopsy, and HAdV infection was discovered in his liver. It is well known that lymphoma may cause a certain degree of immunosuppression (27). The study suggests that the immunosuppression degree resulted from lymphoma is probably enough to cause patients to develop the fatal HAdV hepatitis (22).

In conclusion, most risk factors for HAdV hepatitis include SOT, human immunodeficiency virus infection, chemotherapy, the transplantation of hematopoietic stem cell, SCID, and lymphoma (21, 28).

PATHOGENESIS OF HADV HEPATITIS

Despite the human adenoviral hepatitis has been reported before, it mainly appears in the manner of small series and individual case reports and does not always emphasize the pathogenesis. The infection of human adenovirus rarely leads to serious pathological changes; however, the intravenous injection of high-dose replication-deficient HAdV vector into the body of patients will lead to rapid innate immune system activation, resulting in disseminated intravascular coagulation, cytokine storm syndrome, hepatotoxicity, and thrombocytopenia. Meanwhile, HAdV-C5 vectors have high hepatic tropism, and the intravascular administration of HAdV-C5 vectors can result in acute liver toxicity. So, the pathogenesis of hepatitis caused by HAdV-C5 vectors can be partly represented the hepatitis caused by HAdV.

In the article of Alemany et al., after intravenous virus administration, virus particles can be isolated in endothelial cells of splenic sinuses and liver, tissue macrophages, and platelets *via* directly binding to the receptors of cell surface (29, 30). In addition, tissue resident macrophages in the liver, for instance, Kupffer cells, can also isolate a large number of HAdV particles (31–33). Coagulation factors that contain Gla domain can mediate the entry of virus into hepatocytes through binding to the main viral capsid protein hexon (34). These coagulation factors contain FX, FIX, and FVI, and FX has the highest affinity with the HAdV-C5 hexon (35). It had been proven that protein associated with LDL receptor and heparan sulfate proteoglycan (HSPG) can mediate the cell entry of the coagulation factor HAdV-C5 complexes existed *in vitro* (36). Nevertheless, macrophages residing in the tissues are only the first line of defense against the invading pathogens, and the activation of innate immune system sensors was triggered by these cells. Then, 10 min after the HAdV-C5 intravenous injection, in the liver, the Kupffer cells activated the transcription of proinflammatory cytokine IL-1a (33). Subsequently, IL-1a is synthesized continuously, which triggers the generation of pro-inflammatory cytokines depended on IL-1RI, containing TNF-a and IL-6, as well as chemokines CXCL2, CXCL1, and CCL2 (33). Then, IL-1a is constantly being synthesized and triggers the generation of pro-inflammatory cytokines depended on IL-1RI, containing TNF-a and IL-6, as well as chemokines CXCL2, CXCL1, and CCL2 (33). In the meantime, after the isolation of HAdV from blood, Kupffer cells undergo necrotic death within 60 min after intravenous injection of HAdV. For the death of necrotic Kupffer cell, the result is the activation of systemic inflammatory response, resulting in the release of pre-synthesized cytosolic IL-1a together with other proinflammatory mediators, containing HMGB1, in circulation (37). When necrotic cells are released, systemic and local IL-1RI signals are initiated by IL-1a (38). Thus, IL-1a activation can also cause severe immunopathology of the infected liver.

The cells of natural killer (NK) existed in a liver-directed gene transfer mouse model were found to play a major role in eliminating hepatocytes infected with the HAdV-C5-based vector (39). Within 3 days after the intravenous injection of vector, NK cells existed in the spleen were activated highly and expressed IFN-c, glutamate B, and perforin. Besides, in the cells of NK, the expression of the cytotoxic effectors is completely determined by the functional IFN-I signal. The research has suggested that on account of the lack of IFN-I signal, there is a cell population dysfunction of NK and the prolonged duration of transgenic hepatocytes infected by virus. Therefore, the removal of virus infected cells from liver is principally mediated *via* the activated cells of NK. The same research exhibits that viral infection cell lysis mediated by NK cell requires the main NK cell activation receptor NKG2D (40). NK cells mediate antiviral innate immune responses by producing cytokines and dissolving infected cells when activated (41–43). Activated NK cells help to control the early stage of infection and promote the in-depth initiation for the virus-specific immunity of CD8+ T-cell. In one study, the aggravation of hepatocellular damage observed during human adenovirus infection associated with a raising expression of IFN-γ and the CD8+ T-cell absolute numbers in liver (44). This is in accordance with other liver infection models, where the emergence of hepatocyte injury is related to the obtainment of the effector function *via* CD8+ T-cells (45).

Combined with the previous studies, the indirect effect related to immune damage is more important than direct effect of viral infection. As a recent study shows that disseminated infections caused by HAdV-C in immunocompromised hosts are known to trigger severe acute inflammatory responses, which are manifest in part by high blood levels of inflammatory cytokines and chemokines, such as IL-1, IL-6, TNF-α, IFN-a/b, IFN-c, and IL-8 (46). While promoting virus-infected cells to be cleared from the body, these inflammatory cytokines and chemokines aggravate virus-induced inflammation, severe immune pathology, and damage to healthy tissues, leading to multiple organ dysfunction and even death. In addition, Fejer et al. have shown that intravenous or intraperitoneal administration of HAdV-C2 or

HAdV-C5 leads to the development of hypersensitivity to secondary challenge of mice with lipopolysaccharide (LPS) (47).

PATHOLOGICAL FEATURES OF HADV HEPATITIS

Histopathological assessment is the gold standard for diagnosing the HAdV hepatitis. When tissues are infected with human adenovirus, routine examination of eosin-stained and hematoxylin slides can exhibit different degrees of regeneration, reactivity, and inflammation. By *in situ* and immunoperoxidase hybridization, we can confirm the presence of the virus in tissues (such as liver) (19). Human adenovirus-infected cells (which known as "smudge cells") are a typical type of the intranuclear inclusions containing thin cytoplasmic margins and large nuclei, basophilic inclusions.

Recently, the research exhibited twelve human adenoviral hepatitis cases, containing autopsy specimens and biopsy (22). All of the above cases possessed histological evidence of liver injury and laboratory or immunohistochemical (IHC) evidence of the infection of active human adenovirus (e.g., electron microscopy, polymerase chain reaction (PCR), virus culture, and other additional approaches). The two pathologists review all of the cases (KBS and JPTH), and all the cases were discussed and agreed. They examined various morphological findings, containing granulomatous inflammation, varying degrees of necrosis, and the existence of inclusion bodies, lymphocytes, and neutrophils.

In this paper, tissue sections of all the cases revealed non-banded coagulative hepatocyte necrosis, and necrosis was divided into spotty hepatocyte necrosis, submassive necrosis, and massive necrosis, with a corresponding percentage of necrosis ranging from <5 to 95%. Punctate hepatocyte necrosis was employed for expressing the apoptosis or necrosis of isolated hepatocytes in lobules, whereas massive necrosis referred to extensive necrosis of nearly all or all normal liver lobules. The submassive necrosis was applied for expressing a moderate number of necrosis, generally manifested as confluent necrotic region. Despite this necrosis is generally completely non-banded, half of the cases confirmed raised involvement in the periportal area on the biopsy specimens. On the contrary, autopsy revealed complete non-banded necrosis. The change from extreme punctate, focal necrosis to massive, and wide necrosis seems to be determined by the progression and timing of HAdV infection as well as sampling. Notably, the percentage of necrosis in this small biopsy sample is probably underlying misleading on account of the restrictions of sampling and the liver heterogeneous involvement. Histological variations revealed that there exists some anatomical heterogeneity in liver. In a case, despite peripheral liver biopsy revealed only rare granuloma and focal portal vein lymphocyte infiltration, the biopsy of discrete liver lesions guided by simultaneous image observed *via* abdominal MRI and CT exhibited a large number of intranuclear inclusions and necrosis. A total of 33% of the patients with imaging reports provided for review suggested heterogeneous or focal imaging outcomes. Necrosis will also progress as the progresses of disease. The comparison of autopsy specimen and biopsy specimen patients indicated that the percentage of necrosis raised from 20 to 60% from the initial biopsy time.

In all cases in this paper, characteristic stains in hepatocytes to inclusion bodies in vitreous nuclei were reflected in tissue sections. These inclusions are most prevalent at the edge of the necrotic region, however, sometimes scattered in lobules. Despite these inclusions are nearly basophilic, a few cases suggest that the inclusions have glassy eosinophilic regions. Well-developed and particularly large inclusion bodies usually possess a central eosinophilic glassy appearance, and basophilic chromatin is marginalized. Besides, in a case (8%; 1/12), inclusion bodies were also found in the bile duct epithelium. Adenoviral ascending biliary hepatitis has formerly been reported to be associated with the GI infection existed in the immunosuppressed children, which may be the outcome of GI ascending infection entering biliary tract (48). Nonetheless, there was few related inflammations in most cases (58%, 7/12). If exists, inflammation is lymphoid and focal, while no neutrophilic inflammation was identified.

IMAGING CHARACTERISTIC OF HADV HEPATITIS

Although the imaging characteristics of HAdV hepatitis are often non-specific, radiology plays a more essential role in the suspected patients with HAdV hepatitis, it is conducive to exclude other causes. Non-specific images of magnetic resonance imaging and computed tomography revealed periportal edema and liver enlargement. The clear low attenuation regions and uneven enhancement existed on the CT, whereas periportal edema possessed high signal intensity regions on MRI T2-weighted images (49). Among some patients with HAdV hepatitis, we can also find multiple hypodense lesions or single hypodense lesion in CT.

In one case, a biopsy guided by simultaneous image of a liver lesion revealed massive necrosis, while the lesion was thought to represent an abscess when it was first found on a CT scan of the abdomen because of the lesion with an intrahepatic rim-enhancing (22). In the same study, a patient's lesion was mistaken for cirrhosis by ultrasound because of the heterogeneously necrotic appearance of the liver. Another patient was explicated *via* CT scan as a lymphoma involving the liver. In fact, these two patients were diagnosed with HAdV hepatitis. Similar heterogeneous enhancement and clear low attenuation areas have been described in the previous reports and were radiologically mistaken for metastases, other infections, or hepatolenticular degeneration (HLD) (24–26, 50). Therefore, these different imaging findings and interpretations confirm the significance of histological evaluation of liver biopsy. Simultaneously, it is significant to be familiar with the most related key radiological features and then combined with the key clinical information, which is probably afforded sufficient information to describe the characteristics of the lesion. In the end, we can also successfully differentiate HAdV hepatitis from other diseases.

DIAGNOSIS OF HADV

Human adenovirus can be detected through indirect or direct immunofluorescence, routine or shell bottle culture, together with the PCR of the infected sites (e.g., urine, stool, blood, swabs, nasopharyngeal aspirates, bronchoalveolar lavage, and washings) (51). The available diagnostic approaches for the infections of adenovirus [e.g., electron microscopy, culture, histopathology, detection of antibodies, direct antigen detection, PCR, immunohistochemistry, and *in situ* hybridization (ISH)] are determined by the collected samples and infection location.

Traditional or shell bottle culture is a gold standard, but it may not be sensitive to some samples (e.g., blood) and is probably take several days to induce the cytopathic effect (CPE). Adenovirus-related CPE is typically expressed as the grapevine, with irregular aggregates composed of enlarged circular cells revealing characteristic refractable intranuclear inclusions. CPE slowly develops and variations can be found within 28 days after the inoculation, most of which occur in the first culture week (12). The time of detection is determined by various factors, containing the serotypes or adenovirus subsets, viral load inoculated, culture condition, or cell lines utilized.

The electron microscopy can be employed for identifying the HAdV, particularly in stool. What is more, immunoelectron microscopy can improve the detection sensitivity. Generally speaking, although the number of enteric adenoviruses in stool is much larger than the number of isolates of respiratory adenovirus, in the stool, the existence of adenovirus cannot diagnose GI diseases. In the past, these approaches for the identification of adenovirus in the clinical specimens (especially GI specimens) were a main method, but their convention use has decreased in the past several years.

Histopathological evaluation is still a gold standard for diagnosing the diseases of invasive adenovirus. This technique for the detection of adenovirus has been described above.

Fluorescent antibody (FA) detection can be employed for the direct detection (generally from the respiratory samples), simultaneously for identifying virus isolates cultured by shell bottle method and traditional methods. Despite FA is extensively utilized, its sensitivity is restricted, especially in detecting the double infection (52).

Adenovirus nucleic acid can be determined through a variety of molecular approaches. Since the N-terminal area of hexagonal gene and trans-activated area of E1A are very conservative between serotypes, primers for these areas are generally utilized for PCR detection (53).

Polymerase chain reaction is an extensively utilized approach to detect the adenovirus through amplifying and determining virus genome, which greatly replaces the virus culture together with other old diagnostic approaches (10). Through polymerase chain reaction, we can test stool, blood, respiratory secretions, and tissue specimens. Quantitative and qualitative PCR approaches can be utilized. Simultaneously, PCR possesses high rapidity and sensitivity. A novel molecular detection approach known as immune-PCR is expected to improve the sensitivity of fecal detection owing to it eliminates the requirement for extracting the nucleic acid (54).

Immunohistochemical staining can determine the HAdV hexagonal antigen existed in the tissue (55). In ISH together with IHC technologies for detecting the adenovirus is conducive to identify the infection of HAdV in tissue sections and enhance the sensitivity as an auxiliary means of morphological examination. In the meantime, they may localize disease and provide evidence for causality (56).

Although the sensitivity of instance electron microscopy is low, the specificity is high. The sensitivity of direct antigen detection is lower than the specificity. On the contrary, the sensitivity of detection of antibodies is higher than the specificity. Combining ISH with immunohistochemistry techniques for the detection of adenoviruses can increase the sensitivity. PCR's both sensitivity and specificity are high. The sensitivity and specificity of culture and histopathology are also high, but they are more time-consuming and laborious. In conclusion, despite HAdV-positive detection may be regarded as the diagnosis of the adenovirus infection in a proper clinical environment, PCR of infection site or pathological evidence of the invasive adenovirus disease is still required to determine causality.

CLINICAL FEATURES OF HADV INFECTION

Respiratory Tract Involvement

Human adenovirus makes up at least 5–10% of childhood infections and 1–7% of the adult respiratory infections (RTIs) (12). For the HAdV RTI, its characteristic symptoms consist of fever, cough, tonsillitis, and pharyngitis. Among children, GI symptoms may be present concomitantly (57). Symptoms are usually self-limiting (within 2 weeks) in immunocompetent patients, and infection can lead to the type-specific immunity. Nonetheless, pneumonia appears in 20% of infants and newborns, and death from HAdV pneumonia has been expresses in formerly healthy adults or children (58). In 10–30% of cases, immunocompromised persons may develop into severe respiratory failure and/or transmission, and mortality rate of severe HAdV pneumonia may be as high as 50% (59).

Ocular Involvement

Manifestations of ocular HAdV infection consist of non-specific conjunctivitis, pharyngeal conjunctival fever, and EKC (60). The most prevalent species related to the ocular mucosa acute infection is HAdV-D, which causes the clinical ocular pathogenesis, whereas HAdV-D is reported to be related to EKC outbreak, which may induce vision loss, recurrent, or chronic keratitis. David Dyer, Xiaohong Zhou, Ashrafali Mohamed Ismail, James Chodosh, Jaya Rajaiya, and Donald Seto expressed the clinical pathology for this disease and the virus molecular phenotype inducing the epidemic EKC (8). What is more, conjunctivitis occurs predominantly with HAdV-B (61).

GI Infection

Acute HAdV infections of the GI tract occur predominantly with HAdV-F (notably HAdV-40 and−41) and can cause a lot of symptoms, containing hemorrhagic colitis, diarrhea, gastroenteritis, pancreatitis, cholecystitis, and hepatitis (62). Even if the major position of the infection is respiratory tract, HAdV

infections can also cause GI symptoms, particularly in young children (15). The researches have indicated that most invasive infections seem to be induced *via* the reactivation of virus. Adenovirus persists in children's GI tract. Intestinal epithelium is the major position of virus replication, which precedes the invasion event. As a result, regular monitoring of the existence and load of adenovirus in the fecal samples has become the main diagnostic approach of patient monitoring. However, the site of reactivation in the adult setting is unclear. Hence, peripheral blood sample monitoring is a conversion method for the adult patients.

Urinary Tract Infection

In patients undergoing HSCT and SOT, HAdV can induce urinary tract infections (UTIs). Its classical manifestations contain hematuria, dysuria, the dysfunction of renal transplantation and hemorrhagic cystitis (HC) (63). Despite there have been reports of dialysis-dependent or fatal renal failure, obstructive uropathy, necrotizing tubulointerstitial nephritis, and fatal dissemination, HAdV UTIs usually abate spontaneously. Most common species associated with HC is HAdV-B, including HAdV-11, -34, -35, -3, -7, and -21 (64). Diagnosis can be confirmed through culture or PCR in serology or urine, and the viral infection of the renal tubular epithelial cells containing intranuclear inclusions and stained cells was demonstrated by renal biopsy.

Liver Infection

We almost reviewed all case reports in the recent 10 years and summarized the liver function index of these patients (22, 23, 26, 65–75). The average of ALT was 1,607 U/L, with a range from 87 to 9,150 U/L. The average of AST was 4,671 U/L, with a range from 76 to 16,050 U/L. The average of TBIL was 6.2 mg/dL, with a range from 0.4 to 31.3 mg/dL. The average of ALP was 820 U/L, with a range from 123 to 2,802 U/L. The average of γ-glutamyltransferase was 790 U/L, with a range from 98 to 1,563 U/L. In a case report written by Kawashima et al., their observation raises the possibility that elevated γ-glutamyltransferase could be a sentinel marker for HAdV hepatitis. In subsequent studies by Onda et al., they also suggest that an increase in γGTP (100 IU/L or more) appears more than 2 weeks before the onset of hepatitis may be useful for the early diagnosis of HAdV hepatitis. All in all, adenovirus hepatitis is more common in children, the elderly, and adults with compromised immune systems than other types of hepatitis, and its onset is more acute. In other viral hepatitis, except viral hepatitis A and E often cause acute hepatitis, viral hepatitis B, C, and D usually cause chronic hepatitis. On the laboratory examination, adenovirus hepatitis shows markedly elevated liver enzymes, which should be differentiated from drug-induced liver injury. In addition, the other clinic features of HAdV hepatitis will be described below.

Other Disease

Despite 10–30% of HSCT recipients develop the disseminated disease with the infection of HAdV, in the immunocompetent hosts, disseminated infection of HAdV is rare (76). At the same time, rare manifestations for the infection of HAdV contain cardiomyopathy and myocarditis, meningitis, encephalitis, pulmonary dysplasia, mononucleosis-like syndrome, sudden infant death, and intussusception in children (77).

THERAPY OF HADV HEPATITIS

The first and most significant treatment is still the reduced immunosuppression and supportive care. Nevertheless, there do not exist consensus on which immunosuppressive drugs to stop or decrease and the time to restart immunosuppression. Remission of viremia after reduction of immunosuppression shows the significance of the immune recovery during infection (78, 79). On the other hand, it is difficult to identify the primary cause that is on account of an increase in antiviral therapy and a decrease in immunosuppression or the combination of these treatments in the resolution of the disease (80).

Timely initiation of antiviral therapy is necessary to prevent or successfully control human adenovirus hepatitis. The currently available immunotherapy and agents against HAdV based on the nucleoside analogs are not supported through the randomized prospective clinical experiment of brincidofovir and cidofovir (cidofovir's lipid-conjugated prodrug) that seem to be useful for treating progressive, disseminated and severe human adenovirus diseases, which include HAdV hepatitis.

Despite cidofovir is a cytosine nucleotide analog, which suppresses the viral DNA polymerase, has been reported that its efficacy depends partly on patients' partial immune recovery, the most commonly used antiviral medicant is still cidofovir. Meanwhile, cidofovir has main side effects, including severe nephrotoxicity and neutropenia. So, the side effects of cidofovir limit its wide application in clinic.

In comparison with cidofovir, brincidofovir has less renal toxicity and has better oral biological availability and enables administration once or two times a week. Although brincidofovir also has some toxic side effects, such as GI symptoms, it is the most effective anti-adenoviral medicant. The recent studies used the lipid conjugate of cidofovir to give a preemptive treatment in patients with HAdV and reveal promising outcomes, namely, the drug allows a considerable proportion of patients to clear HAdV, even without the reconstitution of immune (81, 82). A few reports have emphasized the life-saving potentiality of this drug for the immunocompromised patients (83, 84).

Ribavirin is a type of nucleoside analog that appears to possess an antiviral activity only against HAdV-c (serotypes 6, 5, 2, and 1) (85). However, the significant anti-adenovirus activity of ribavirin in patients is uncertain, and its main side effect is anemia. All in all, ribavirin is not recommended to treat serious HAdV infections. Ganciclovir is a common antiviral drug, but it needs the phosphorylation to reach the active condition. Nevertheless, human adenovirus is unreasonable to treat adenovirus infection owing to the lack of thymidine kinase (86). The efficacy of other commonly used drugs, which include foscarnet and vidarabine, in treating HAdV hepatitis is highly questionable.

Besides using antiviral medicant, intravenous immunoglobulin may be beneficial, especially in patients with hypogammaglobulinemia. In addition to the treatments described above, immunotherapy (for instance the adoptive transfer for the treatment of HAdV-specific T cells) has been employed for exceeding 10 years to combat invasive HAdV infections that do not respond to virostatic drugs (87). This is an adoptive immunotherapy that could result in a polyclonal T-cell graft, containing enriched numbers of HAdV-specific CD4+ and CD8+ T cells. Additionally, it would be better that making a transfer of specific T cells before the onset of symptoms and increasing viral load. The potential side effect of adoptive T-cell immunotherapy is GVHD. Because the isolation procedure is an enrichment of virus-specific T cells and it may result in a mixed population with a potential contamination of alloreactive T cells, which carry the risk of GVHD induction and aggravation of viral infections. Several studies have suggested that the existence of HAdV-specific T cells and immune reconstitution are very significant to clear the infection of HAdV, which gives a foundation for the treatment of human adenovirus infection (88–90). The initial studies had shown that timely initiation for the treatment is especially significant for controlling the infection of HAdV successfully. Proper immune cells from various sources have been investigated (87). Except for a variety of methods of separating HAdV-specific T cells from their respective SCT donors, other sources of virus-specific T cells were employed, containing T-cell line libraries constructed from healthy donors revealing prevalent HLA polymorphisms or partial HLA-matched third-party donors (87, 91–95). The clear link between successful virus control and timely immunotherapy initiation supports the concept of affording an off-the-shelf supply of HAdV-specific T cells (96, 97). Simultaneously, in the recipients of child SCT, the average time span between the first onset of adenoviremia and HAdV amplification exceeding critical threshold in continuous stool samples was as long as 11 days (98). During this time, separation and amplification of Don-induced HAdV-specific T cells can also be adoptive transferred after or before viremia (92). Specifically, in high-risk groups, it can be imagined to produce the virus-specific T cells prior to allogeneic SCT to guarantee access to the targeted immunotherapy timely. In conclusion, immunotherapy is an important part of combating HAdV infection in immunocompromised patients because of the current limitations of antiviral drugs.

SUMMARY OF HADV HEPATITIS CASE REPORT

In 2014, Ronan et al. published a review on the adenoviral hepatitis clinical characteristics, containing the follow-up reports as of July 2021. A number of 107 adenoviral hepatitis cases have been reported in the literature (21–23, 26, 65–74). Next, we will summarize these cases briefly.

Of the 107 confirmed HAdV hepatitis cases, the great majority of the patients were children under the age of 18, and the proportion was 61% (**Table 2**). In contrast, 42 were adults. There were slightly more male patients than female patients. In our study, the recipients of liver transplant and bone marrow transplant are 48 and 27 cases, respectively, 15 cases received chemotherapy recently, and five cases were patients with serious combined immunodeficiency. Compared with the Ronan's article, the number of patients receiving bone marrow transplant has increased, probably because of hematology science is now better developed. HAdV hepatitis caused by other diseases does not change significantly from the past, except for the addition of one patient who was underlying chronic lymphocytic leukemia (CLL). Diagnosis of HAdV hepatitis was made by patients' liver biopsy or on autopsy. The method used to identify adenovirus has been described above. Among 107 patients, the most common method was immunohistochemistry in the liver tissue. In the meantime, viral culture was also commonly used. The application ratio of these two methods was 62 and 61%, respectively, while the application proportion of electron microscopy was 45%. Comparatively speaking, PCR and ISH were relatively less used. Liver necrosis is the most prevalent histopathological manifestation. The stain cells and viral inclusion bodies could also be found in liver histopathology. Additionally, the initial symptom was fever in 84 patients. Other prevalent symptoms when present contained discomfort or drowsiness, jaundice, and diarrhea. In some situations, alkaline phosphatase, alanine aminotransferase (ALT), and aspartate aminotransferase (AST) also rapidly increased. Abdominal CT scan revealed that 15 of the 19 patients had multiple low-density lesions, while a single hypodense lesion was found in 3 of these patients. However, few positive findings were in CT imaging, suggesting the concealed of HAdV hepatitis.

In 107 cases, only 59 patients' treatments were accurately described, while the most useful treatment was reduced immunosuppression (**Table 2**). Of the 27 patients, the only improvement of treatment was the decrease of immunosuppression, of which 15 survived. A number of eight other patients with antiviral drugs (ribavirin or cidofovir) and immunosuppressants also survived. Among the 12 patients, 6 were successful in liver transplantation. Intravenous immunoglobulin therapy alone or cidofovir therapy alone was unsuccessful in all 6 patients of HAdV hepatitis, while 2 of 3 patients survived using IVIG and cidofovir together. It was unfortunate that the overall prognosis for patients with hepatitis HAdV is poor, with only 30 of 107 published cases (28%) surviving. In conclusion, the great majority of HAdV hepatitis cases are fatal. The hepatitis of HAdV has few characteristic signs and symptoms, but it is generally hard to diagnose and delayed treatment is not uncommon.

CONCLUSIONS

Among viral infections, human adenovirus infection is relatively rare, but HAdV infection is also of great clinical significance. Among immunocompetent patients, HAdV infection usually causes upper respiratory, GI, or conjunctival involvement, and the most cases are self-limited. In contrast, disseminated and severe infections (e.g., pneumonitis, gastroenteritis, hemorrhagic

TABLE 2 | Characteristics of 107 patients with HAdV hepatitis.

Basic conditions	Number of patients	Proportion of patients
Age		
Pediatric (0–17)	65	61%
Adult (≥18)	42	39%
Gender		
Male	42	54%
Female	36	46%
Underlying condition		
Liver transplant	48	45%
Bone marrow transplant	27	25%
Chemotherapy	15	14%
SCID	5	4%
HIV infection	4	4%
Renal transplant	2	2%
Heart transplant	2	2%
Neonates (no known comorbidity)	2	2%
CLL	2	2%
Method of adenovirus detection in the liver (*N* = 107)		
Immunohistochemistry	66	62%
Culture	65	61%
Electron microscopy	48	45%
Polymerase chain reaction	16	15%
In situ hybridization	4	4%
Liver histopathology (*N* = 82)		
Necrosis	72	88%
Intranuclear inclusions	55	67%
Smudge cells	21	26%
Presenting symptoms (*N* = 92)		
Fever	84	91%
Lethargy/malaise	22	24%
Diarrhea	13	14%
Jaundice	8	9%
CT imaging findings (*N* = 19)		
Multiple hypodense lesions	15	79%
Single hypodense lesion	3	16%
Normal	1	5%
Outcome (*N* = 107)		
Survival	30	28%
Death	77	72%

Treatment (*N* = 59)	Number of patients	Survival
Reduced immunosuppression	27	15
Reduced immunosuppression + antiviral	9 cidofovir	7
	2 ribavirin	1
Liver re-transplantation	12	6
Cidofovir + IVIG	3	2
IVIG alone	4	0
Cidofovir alone	2	0

TABLE 3 | Characteristics of HAdV hepatitis.

The route of infection	Inhalation of infected aerosols Person-to-person contact Fecal-oral route Direct conjunctival inoculation Contact with infected blood or tissue
Methods of detection	Instance electron microscopy Culture Histopathology Detection of antibodies Direct antigen detection Polymerase chain reaction Immunohistochemistry *In situ* hybridization
Treatment	Reduced immunosuppression Reduced immunosuppression + antiviral drug (cidofovir/ribavirin) Liver re-transplantation Cidofovir + IVIG IVIG alone Cidofovir alone Brincidofovir Adoptive transfer of HAdV-specific T cell therapy

cystitis, nephritis, encephalitis, and enterocolitis) are more common among immunocompetent patients. However, HAdV hepatitis is uncommon, the common causes of hepatitis contain drugs, venous obstruction, ischemia, autoimmune hepatitis, toxins, and metabolic cause together with infectious causes, involving CMV, VZV, hepatitis A-E, EBV, and HSV. In our review, we mainly introduced human adenovirus hepatitis. And we summarize some characteristics of HAdV hepatitis (**Table 3**). The high-risk factors of HAdV hepatitis include SOT, the virus infection of human immunodeficiency, chemotherapy, the transplantation of hematopoietic stem cell, SCID, and lymphoma. The review of reported cases with HAdV hepatitis shows that the most common risk factor is liver transplantation. With the development of hematology science, the number of patients receiving bone marrow transplant has increased, and the proportion of this factor is also increasing. There are few studies on the pathogenesis of human adenovirus hepatitis, but they mainly focus on the study of adenovirus vector-induced liver disease. According to the current reports, we found that NK cells and CD8+ T cells play a major effect in the elimination of hepatocytes that infected with vector based on HAdV-C5. We expect that there are more innate and adaptive immunity studies related to HAdV hepatitis in the future. Now, the available diagnostic methods of HAdV include culture, electron microscopy, histopathology, detection of antibodies, direct antigen detection, PCR, immunohistochemistry, and ISH. Surprisingly, there is an even newer detection method, next-generation sequencing, which can help to type adenoviruses. In an appropriate clinical setting, a positive next-generation sequencing (NGS) test can be considered a diagnosis of adenovirus infection. So far, few cases that used NGS to detect HAdV have been reported, and the effectiveness of this method remains to be observed. Histopathologic assessment is a gold standard for diagnosing the HAdV hepatitis, and the histopathologic features include hepatic necrosis, viral inclusions, and smudge cells. For the HAdV hepatitis, its imaging characteristics are generally non-specific, but radiology exerts a

more significant effect in suspected patients with HAdV hepatitis and is conducive to eliminate other causes. In our review, we can find multiple hypodense lesions or single hypodense lesion in CT among some patients with HAdV hepatitis. However, the positive findings are still relatively rare and are still not supported by large sample data. In terms of treatment, despite the presently available anti-HAdV drugs based on the nucleoside analogs are not supported through the prospective randomized clinical trials, antiviral medicant is partly useful. Besides, intravenous immunoglobulin and immunotherapy (e.g., adoptive transfer of HAdV-specific T-cell therapy) may be beneficial. More importantly, reducing immunosuppression is necessary. In the end, 107 human adenovirus hepatitis cases reported in the literature were gathered. We find that the clinical symptoms of HAdV hepatitis are not typical, and the most common symptom is fever. Other symptoms include lethargy or malaise, diarrhea, and jaundice. In some situations, alkaline phosphatase, ALT, and AST also rapidly increased. Generally speaking, HAdV hepatitis possesses few characteristic signs and symptoms, but it is often hard to diagnose and delayed treatment is not uncommon. So, HAdV hepatitis is usually fatal. We expect to collect further information on large samples to find clinical features of human adenovirus hepatitis in the next study.

AUTHOR CONTRIBUTIONS

NZ and XH designed the concept of the study. NZ, XH, and YW wrote the first draft of the manuscript. HR and KW edited the subsequent versions of the manuscript. All authors contributed to the article and approved the submitted version.

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Increase in Serum Soluble Tim-3 Level Is Related to the Progression of Diseases After Hepatitis Virus Infection

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Background: Viral hepatitis is a widespread and serious infectious disease, and most patients with liver cirrhosis and hepatocellular carcinoma are prone to viral infections. T cell immunoglobulin-and mucin-domain-containing molecule-3 (Tim-3) is an immune checkpoint molecule that negatively regulates T cell responses, playing an extremely important role in controlling infectious diseases. However, reports about the role of serum soluble Tim-3 (sTim-3) in hepatitis virus infection are limited. Therefore, this study explored changes in sTim-3 levels in patients infected with hepatitis B virus (HBV), hepatitis C virus (HCV), and hepatitis E virus (HEV).

Methods: This study applied high-sensitivity time-resolved fluorescence immunoassay for the detection of sTim-3 levels. A total of 205 cases of viral hepatitis infection (68 cases of HBV infection, 60 cases of HCV infection, and 77 cases of HEV virus infection) and 88 healthy controls were quantitatively determined. The changes in serum sTim-3 level and its clinical value in hepatitis virus infection were analyzed.

Results: Patients with HBV infection (14.00, 10.78–20.45 ng/mL), HCV infection (15.99, 11.83–27.00 ng/mL), or HEV infection (19.09, 10.85–33.93 ng/mL) had significantly higher sTim-3 levels than that in the healthy control group (7.69, 6.14–10.22 ng/mL, $P < 0.0001$). Patients with hepatitis and fibrosis infected with HBV (22.76, 12.82–37.53 ng/mL), HCV (33.06, 16.36–39.30 ng/mL), and HEV (28.90, 17.95–35.94 ng/mL) had significantly higher sTim-3 levels than patients with hepatitis without fibrosis (13.29, 7.75–17.28; 13.86, 11.48–18.64; 14.77, 9.79–29.79 ng/mL; $P < 0.05$).

Conclusion: sTim-3 level was elevated in patients infected with HBV, HCV, or HEV and gradually increased in patients with either hepatitis or hepatitis with hepatic fibrosis. It has a certain role in the evaluation of the course of a disease after hepatitis virus infection.

Keywords: viral hepatitis, liver fibrosis, soluble Tim-3, time-resolved fluorescence immunoassay, diagnostic value

INTRODUCTION

Hepatitis virus infection is one of the major public health concerns globally with high mortality and morbidity, affecting hundreds of millions of people (1). In addition to hepatitis A virus (HAV), hepatitis B virus (HBV), hepatitis C virus (HCV), hepatitis D virus (HDV), and hepatitis E virus (HEV) cause chronic infections. HBV and HCV are the main causes of chronic diseases and liver cancer (2). Viral hepatitis is caused by the complex interaction between the replication of the hepatitis virus and the host immune response (3). CD8 + T cells play an important role in immune response to viral infections. In acute hepatitis virus infection, CD8 + T cells mainly eliminate the virus by producing tumor necrosis factor-α (TNF-α) and interferon-γ (IFN-γ) (4). In chronic hepatitis infection, as infection progresses, the effector functions of T cells gradually decrease (including the reduction of proliferation and cytotoxicity), and their ability to produce IL-2, TNF-α, and IFN-γ decreases (5, 6). That is, CD8 + T cells show a depleted phenotype. The degree of depletion of hepatitis virus-specific CD8 + T cells showcase varying clinical results (7–10).

T cell immunoglobulin-and mucin-domain-containing molecule-3 (Tim-3) is a type I transmembrane protein that negatively regulates immune response and is involved in the dysfunction and failure of CD8 + T cells in HBV and HCV infections (11). Tim-3 expression increases in HBV/HCV-specific CD8 + T cells, and this increase is related to the failure of CD8 + T cells in patients with HBV/HCV infection (12–15). In addition, the combination of Tim-3 and its ligand galectin-9 can increase the expression level of the Tim-3 protein during the activation of regulatory T (Treg) cells, and the increase in Tim-3 level can induce helper T lymphocyte 1 (Th1) cell apoptosis and inhibit Th1-type immune response, thereby regulating Th1/Th2 balance in patients with hepatitis B (13, 16). The immune function of membrane-bound Tim-3 during chronic HBV and chronic HCV infection and its role in diseases has been explored, but the clinical importance of changes in serum soluble Tim3 (sTim-3) levels during viral hepatitis, especially HEV infection, remains unclear. Therefore, this study detected the level of sTim-3 in the serum of patients with different viral hepatitis infections (HBV, HCV, and HEV), and analyzed the diagnostic value of serum sTim-3 in these patients.

PATIENTS AND METHODS

Serum Samples

A total of 205 patients with hepatitis virus infection from Zhejiang Xiaoshan Hospital and Wuxi NO.5 People's Hospital were included in the study. Patients comprising 68 patients infected with HBV (46 patients with hepatitis and 22 patients with hepatitis and fibrosis), 60 patients infected with HCV (47 patients with hepatitis and 13 patients with hepatitis and fibrosis), and 77 patients infected with HCV (50 patients with hepatitis and 27 patients with hepatitis and fibrosis). In addition, 88 healthy individuals were also included in the study. The samples were included in two batches successively. The first batch included 50 healthy people, 90 hepatitis patients, and 32 patients with hepatitis and fibrosis. The first batch was included from 2020.1 to 2020.6. The second batch was included 38 healthy people, 53 hepatitis patients, and 30 patients with hepatitis and fibrosis. The second batch was included from 2020.7 to 2020.12.The healthy control group was composed of adults who came to the hospital for physical examination and had no history of liver disease and were negative for HBV, HCV, and HEV infections. A total of 68 patients tested positive for hepatitis B surface antigen positivity, 60 patients tested positive for anti-HCV IgG positivity, and 77 patients tested positive for anti-HEV IgG positivity. Patients with hepatitis and fibrosis had undergone liver biopsy with significant fibrosis (METAVIR status F3-F4; F3, numerous septa without cirrhosis; and F4, cirrhosis). A complete medical history was taken and physical examination carried out in all patients and controls. Exclusion criteria were HIV infection, HAV infection, auto-immune hepatitis, non-alcoholic fatty liver diseases (excluded based on histopathological findings), patients with uncontrolled psychiatric disorders, cardiac diseases (cardiomyopathy, arrhythmias, ischemia, myocarditis and significant valvular disease), severe comorbid diseases (renal failure, hypertension), congenital liver disease, a history of alcohol intake and pregnancy.

Serum collection and storage: venous blood (5 mL) was collected from each participant and centrifuged at 4,000 rpm for 10 min. The supernatant (serum) was stored at −80°C for subsequent use. Serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), albumin (ALB), hyaluronic acid (HA), direct bilirubin (Dbil), hepatitis B surface antigen (HBsAg), and total bilirubin (Tbil) were provided by the hospital.

The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki. Written informed consent was obtained from all patients and the study was approved by the hospital's ethics committee (approval no. 2020-023-1).

Detection Methods

Reagents and instruments: two monoclonal antibodies against different Tim-3 epitopes (capture and detection antibody), and Tim-3 antigen were purchased from Sino Biological Inc. (Beijing, China). The enhancement solution was provided by Zhejiang Boshi Biotechnology Co., Ltd., ProClin-300, Tris, Sephadex-G50, bovine serum albumin (BSA), and other reagents were purchased from Shanghai Xibao Biotechnology Company (Beijing, China).

A time-resolved immunofluorescence analyzer was purchased from Foshan Daan Medical Equipment Co., Ltd., and 96-well plates were purchased from Xiamen Yunpeng Technology Development Co., Ltd.

Buffer compositions: coating buffer (50 mmol/L Na_2CO_3-$NaHCO_3$; pH 9.6); elution buffer (50 mmol/L Tris-HCl, 0.2% BSA, and 0.05% ProClin; pH 7.8); washing buffer (50 mmol/L Tris-HCl, 0.9% NaCl, 0.02% Tween-20, and 0.01% Proclin300; pH 7.8); blocking solution (50 mmol/L Tris-HCl, 0.9% NaCl, 1% BSA, and 0.05% NaN_3; pH 7.8); labeling buffer (50 mmol/L

Na_2CO_3-$NaHCO_3$; pH 9.0); analysis buffer (50 mmol/L Tris-HCl, 0.9% NaCl, 0.5% BSA, 0.0008% DTPA, 0.0005% PHloxine B, 0.01% Tween-40, and 0.05% Proclin300; pH 7.8).

TRFIA's double antibody sandwich method for sTim-3 detection. The experimental steps were as follows (17):

- (1) Antibody coating: the capture antibody was diluted to 2 μg/mL with coating buffer, and 100 μL of the diluted capture antibody solution was added to each well of the 96-well plate and overnight at 4°C. The plate was washed once with washing buffer, and 150 μL of blocking solution was added to each well. After blocking at room temperature for 2 h, the blocking solution was discarded. After drying, stored at −20°C until further use.
- (2) Labeling antibody: 300 μg of the detection antibody was added to an ultrafiltration tube. Through ultrafiltration, the buffer of the antibody to be detected was converted into a labeling buffer with pH 9.0. The collected antibody was mixed with 30 μL of 2 mg/mL diethylenetriaminetriacetic acid (DTTA)-Eu^{3+}, and the mixture was incubated at 30°C overnight. The next day, the labeled antibody was purified with Sephadex G50 and elution buffer. Finally, the Eu^{3+}-labeled antibody (Eu^{3+}-McAb) was collected and stored at −20°C.
- (3) The sTim-3 antigen was diluted with analysis buffer to different concentrations (6.25, 12.5, 25, 50, and 100 ng/mL), and a concentration of 0 indicated the standard buffer.
- (4) Determination of sTim-3 concentration in serum: 100 μL of standard solution or serum sample were added to a 96-well microtiter plate coated with anti-Tim-3 capture antibody. The plate was incubated at 37°C with shaking for 1 h and washed twice with washing buffer. Then, 100 μL of Eu^{3+}-McAb (diluted 1:1000 with assay buffer) was added to each well, incubated at 37°C for 1 h, and washed six times with washing buffer. Approximately 100 μL of enhancement solution was added to each well, and the plate was incubated with shaking for 3 min. Finally,

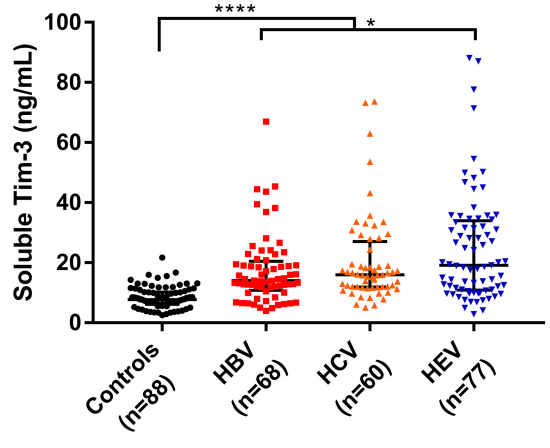

FIGURE 1 | sTim-3 content in uninfected v/s in patients infected with HBV, HCV, and HEV. *$P < 0.05$; ****$P < 0.0001$.

TABLE 1 | Logistic analysis of factors influencing hepatitis or liver fibrosis after HBV, HCV and HEV infection.

	Controls vs. HBV					Controls vs. HCV					Controls vs. HEV				
	β	*S.E*	*Wald*	*P*	*OR*	*95%CI*	*β*	*S.E*	*Wald*	*P*	*OR*	*95%CI*	*β*	*S.E*	*Wald*	*P*	*OR*	*95%CI*
Gender	−2.092	0.429	23.813	<0.0001	0.123	0.053–0.286	−0.695	0.347	4.003	0.045	0.499	0.253–0.986	−0.018	0.391	0.216	0.642	0.834	0.387–1.795
Age	−0.032	0.011	8.064	0.005	0.948	0.948–0.990	0.001	0.012	0.012	0.914	1.001	0.978–1.025	0.024	0.014	2.926	0.087	1.024	0.997–1.052
ALT	0.058	0.012	21.794	<0.0001	1.06	1.034–1.086	0.046	0.011	16.818	0.001	1.047	1.024–1.071	0.051	0.012	17.867	<0.0001	1.052	1.028–1.077
AST	0.142	0.028	25.041	<0.0001	1.153	1.090–1.219	0.129	0.027	22.124	<0.0001	1.138	1.078–1.201	0.133	0.031	18.421	<0.0001	1.142	1.075–1.214
ALB	0.384	0.075	26.359	<0.0001	1.468	1.268–1.700	0.337	0.065	26.957	<0.0001	1.4	1.233–1.590	0.334	0.063	28.176	<0.0001	1.397	1.235–1.580
Dbil	1.453	0.258	31.6	<0.0001	4.274	2.576–7.093	1.078	0.191	31.747	<0.0001	2.939	2.020–4.277	0.928	0.212	19.132	<0.0001	2.528	1.668–3.831
sTim-3	0.287	0.051	31.274	<0.0001	1.332	1.205–1.473	0.348	0.062	31.627	<0.0001	1.416	1.254–1.598	0.326	0.069	22.449	<0.0001	1.386	1.211–1.586

HBV, hepatitis B virus; HCV, hepatitis C virus; HEV, hepatitis E virus.

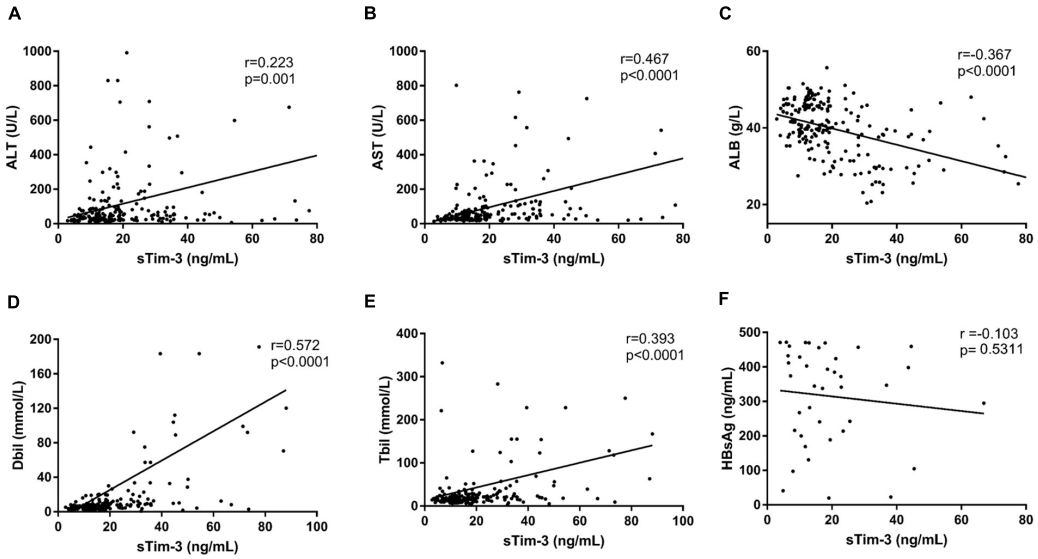

FIGURE 2 | Relationship between sTim-3 levels and liver function indexes in patients with hepatitis virus infection. **(A)** Correlation analysis of sTim-3 and ALT levels showed a significant positive correlation. **(B)** Correlation analysis of sTim-3 and AST levels showed a significant positive correlation. **(C)** Correlation analysis of sTim-3 and ALB levels showed a significant negative correlation. **(D)** Correlation analysis of sTim-3 and Dbil levels showed a significant positive correlation. **(E)** Correlation analysis of sTim-3 and Tbil levels showed a significant positive correlation. **(F)** Correlation analysis of sTim-3 and HbsAg levels was not significant.

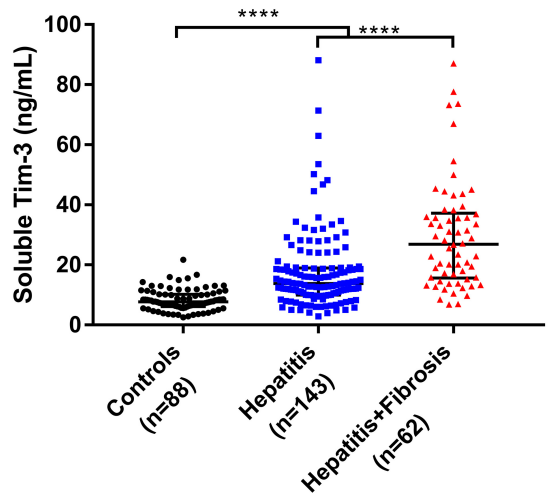

FIGURE 3 | Levels of sTim-3 in normal individuals, patients with hepatitis, and patients with hepatitis and fibrosis. ****$P < 0.0001$.

a time-resolved immunofluorescence analyzer was used to measure the fluorescence counts.

Statistical Analysis

Data were expressed as median or quartile. Statistical analysis was performed using SPSS software version 21.0 (IBM Corporation, Armonk, NY, United States). Mann–Whitney test was performed for the comparison of serum indicator levels in the patients v/s controls. Binary regression was used to analyze influencing factors. The correlations among the values were determined by calculating Spearman's correlation coefficient. GraphPad Prism (GraphPad Software Company) was used in drawing the receiver operating characteristic (ROC) curve for the determination of the best cutoff value of sTim-3 level and evaluation of the sTim-3 performance in distinguishing between the hepatitis and hepatitis with fibrosis groups.

RESULTS

Association of Serum Soluble Tim-3 Levels in Hepatitis B Virus, Hepatitis C Virus, and Hepatitis E Virus Infection-Associated Disease Conditions

Figure 1 shows that the sTim-3 concentrations in patients infected with HBV (14.00, 10.78–20.45 ng/mL), HCV (15.99, 11.83–27.00 ng/mL), or HEV (19.09, 10.85–33.93 ng/mL) were significantly higher than those in the healthy controls (7.69, 6.14–10.22 ng/mL, $P < 0.0001$). The sTim-3 concentrations in the HEV-infected patients were significantly higher than those in the HBV-infected patients ($P < 0.01$).

Logistic Analysis of Factors Influencing Hepatitis or Liver Fibrosis After Hepatitis B Virus, Hepatitis C Virus, and Hepatitis E Virus Infection

Table 1 showing occurrence of hepatitis with or without fibrosis as the outcome variable, with gender, age, ALT, AST, ALB, Dbil, and sTim-3 levels as independent variables for logistic regression analysis. ALT (OR = 1.060, $P < 0.0001$; 1.047, $P = 0.001$; 1.052, $P < 0.0001$), AST (OR = 1.153, $P < 0.0001$; 1.138,

TABLE 2 | Laboratory parameters of patients with hepatitis and hepatitis with liver fibrosis caused by HBV, HCV, and HEV infections.

Variables	Controls	HBV		HCV		HEV	
		Hepatitis	Hepatitis + fibrosis	Hepatitis	Hepatitis + Fibrosis	Hepatitis	Hepatitis + fibrosis
Gender M/F	41/47	31/15	7/15	28/19	8/5	31/19	9/18
Age	56.00 (38.50–66.75)	41.50 (32.75–51.50)	55.00 (40.00–69.00)	53.00 (46.00–58.00)	55.00 (46.00–71.00)	53.00 (48.75–67.00)	59.00 (51.00–68.00)
ALT (U/L)	19.95 (16.85–25.5)	45.50 (25.00–86.00)	42.00 (28.00–241.50)	38.00 (21.00–68.00)	39.00 (21.00–81.00)	46.00 (21.00–75.00)	77.00 (32.25–233.50)
AST (U/L)	21.00 (18.80–25.97)	33.50 (25.75–57.75)	51.00 (32.00–244.50)*	36.00 (25.00–55.00)	37.00 (24.00–97.50)	55. 50 (28.00–131.25)	55.00 (33.00–108.00)
ALB (g/L)	15.20 (11.90–19.30)	46.55 (39.85–48.67)	36.10 (31.55–40.75)*	45.50 (41.70–46.40)	31.30 (29.50–39.00)*	38.90 (36.85–42.20)	31.40 (28.10–37.60)
Dbil (mmol/L)	2.70 (2.23–3.37)	5.25 (4.07–7.40)	11.50 (5.05–106.75)*	6.00 (3.90–7.37)	14.20 (8.55–33.15)*	6.20 (3.40–12.20)	14.70 (8.20–57.30)
Tbil (mmol/L)	15.20 (11.90–19.20)	15.00 (12.33–23.50)	19.00 (12.50–24.50)	17.00 (12.00–22.00)	34.00 (18.50–63.00)*	16.00 (10.87–27.75)	34.00 (19.00–63.00)
HA (ng/mL)	57 (45.00–84.00)	68.46 (37.46–102.25)	87.00 (67.00–231.79)*	81.50 (40.85–122.23)	109.00 (99.00–727.27)*	79.00 (71.00–98.50)	120.17 (92.00–503.65)*
sTim-3 (ng/mL)	7.69 (6.74–10.22)	13.29 (7.75–17.28)	22.76 (12.82–37.53)*	13.86 (11.48–18.64)	33.06 (16.36–39.30)*	14.77 (9.79–29.79)	28.90 (17.95–35.94)*

*ALT, alanine aminotransferase; AST, aspartate aminotransferase; ALB, albumin; Dbil, direct bilirubin; Tbil, total bilirubin; HA, hyaluronic acid; HBV, hepatitis B virus; HCV, hepatitis C virus; HEV, hepatitis E virus. *Significant differences in hepatitis and liver fibrosis in each parameter in the same virus infection ($P < 0.05$).*

$P < 0.0001$; 1.142, $P < 0.0001$), ALB (OR = 1.468, $P < 0.0001$; 1.400, $P < 0.0001$; 1.397, $P < 0.0001$), Dbil (OR = 4.274, $P < 0.0001$; 2.939, $P < 0.0001$; 2.528, $P < 0.0001$) and sTim-3 (OR = 1.332, $P < 0.0001$; 1.416, $P < 0.0001$; 1.386, $P < 0.0001$) were independent risk factors for hepatitis or liver fibrosis after HBV, HCV, and HEV infection, respectively.

Correlations of Serum Soluble Tim-3 Levels With Other Parameters

As shown in **Figure 2**, the sTim-3 levels in patients with HBV, HCV, or HEV infection were highly positively correlated to serum ALT ($r = 0.223$, $P = 0.001$), AST ($r = 0.467$, $P < 0.0001$), Dbli ($r = 0.572$, $P < 0.0001$), and Tbil ($r = 0.393$, $P < 0.0001$) and significantly negatively correlated with serum ALB ($r = -0.367$, $P < 0.0001$). Next, we checked for any possible correlation between HBsAg and sTim-3 levels in HBV infection. As shown in **Figure 2F**, we analyzed the correlation between the levels of hepatitis B virus surface antigen and sTim-3 in patients with hepatitis B virus infection; the result showed that the correlation between HBsAg and sTim-3 was not significant (r = -0.103, $P = 0.5311$).

Correlation Between Serum Soluble Tim-3 Level and Hepatitis or Liver Fibrosis Caused by Hepatitis B Virus, Hepatitis C Virus, and Hepatitis E Virus Infection

We further divided the patients infected with HBV, HCV, or HEV into hepatitis group and hepatitis with fibrosis group. **Figure 3** shows that the sTim-3 concentrations in the control, hepatitis, and hepatitis with fibrosis groups gradually increased, and the differences were significant ($P < 0.0001$).

As shown in **Table 2**, the sTim-3 concentrations (22.76, 12.82–37.53; 33.06, 16.36–39.30; 28.90, 17.95–35.94 ng/mL) in patients infected with HBV, HCV, or HEV with liver fibrosis were significantly higher than those in patients with hepatitis (13.29, 7.75–17.28; 13.86, 11.48–18.64; 14.77, 9.79–29.79 ng/mL). In addition, in the HBV-infected group, the serum AST, Dbil, HA, and ALB concentrations in patients with hepatitis and fibrosis were significantly different compared to those in patients with hepatitis ($P < 0.05$). In the HCV-infected group, the serum levels of ALB, Dbil, HA and Tbil in patients with liver fibrosis were significantly different from those in patients with hepatitis ($P < 0.05$). However, in the HEV-infected group, except for sTim-3 and HA, no significant differences were observed in these indicators between hepatitis and hepatitis with fibrosis groups.

Diagnostic Value of Serum Soluble Tim-3 in Distinguishing Hepatitis From Hepatitis With Fibrosis After Hepatitis Virus Infection

We divided the samples into two batches according to the time of inclusion of samples. The first batch was included from 2020.1 to 2020.6 and the second batch was included from 2020.7 to 2020.12.The second batch is used as the validation set. The ROC

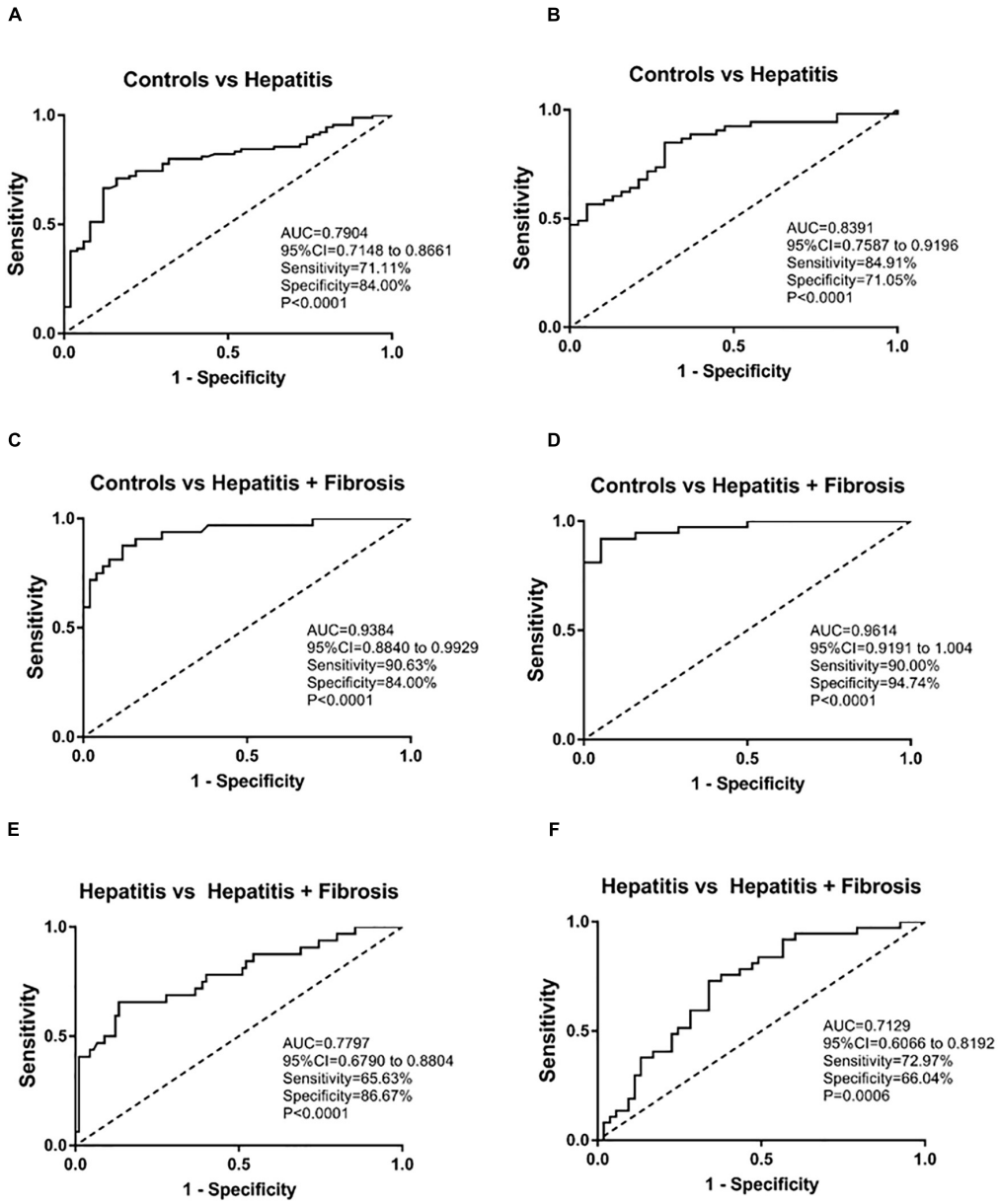

FIGURE 4 | ROC analysis of sTim-3 to determine its diagnostic value for patients with hepatitis and hepatitis with fibrosis. **(A)** ROC curve of sTim-3 in healthy controls and patients with hepatitis (the first batch of included samples). **(B)** ROC curve of sTim-3 in healthy controls and patients with hepatitis (the second batch of included samples). **(C)** ROC curve of sTim-3 in healthy controls and patients with hepatitis and fibrosis (the first batch of included samples). **(D)** ROC curve of sTim-3 in healthy controls and patients with hepatitis and fibrosis (the second batch of included samples). **(E)** ROC curve of sTim-3 in patients with hepatitis and hepatitis with fibrosis (the first batch of included samples). **(F)** ROC curve of sTim-3 in patients with hepatitis and hepatitis with fibrosis (the second batch of included samples).

curve was used in evaluating the role of sTim-3 in distinguishing hepatitis from hepatitis with fibrosis. As shown in **Figures 4A,C,E** sTim-3 can be effectively used in distinguishing healthy controls and patients with hepatitis (AUC (area under the curve), 0.7904; sensitivity, 71.11%; specificity, 84.00%), controls and patients with hepatitis and fibrosis (AUC, 0.9384; sensitivity, 90.63%; specificity, 84.00%), and hepatitis and hepatitis with fibrosis as well (AUC, 0.7797; sensitivity, 65.63%; specificity, 86.67%). In the validation set, as shown in **Figures 4B,D,F**, sTim-3 can also be effectively used in distinguishing healthy controls and patients with hepatitis (AUC, 0.8391; sensitivity, 84.91%; specificity, 71.05%), controls and patients with hepatitis and fibrosis (AUC, 0.9614; sensitivity, 90.00%; specificity, 94.74%), and hepatitis and hepatitis with fibrosis as well (AUC, 0.7129; sensitivity, 72.97%; specificity, 66.04%).

As shown in **Table 3**, we compared the positive detection rates of sTim-3 and HA in patients with hepatitis and hepatitis with fibrosis. The cut off for sTim-3 was determined by the

Youden index in **Figure 4E**, where the cut off value was 19.45 ng/mL. At this cut off value, and positivity rates of 21.68% and 66.13%, respectively, sTim-3 can effectively distinguishing between hepatitis and hepatitis with fibrosis. In addition, combined detection of HA and sTim-3 can improve the positive detection rate of hepatitis with fibrosis up to 77.43%.

DISCUSSION

We studied the role of sTim-3 in HBV, HCV, and HEV infections. Our results suggest that the sTim-3 concentrations in patients with hepatitis were significantly higher than those in the control group and were closely related to ALT and AST levels. sTim-3 is known to be related to the progression of infectious diseases, such as tuberculosis (18), HBV (10), and HIV (19). Serum sTim-3 may also be related to HCV and HEV infections and disease progression. Our study provides new information on the clinical diagnostic and surveillance value of sTim-3 in infectious diseases.

It is well known that Th cells play a key role in the regulation of the dynamic balance of the body's immune response and an important regulatory role in HBV infection. Th cells secrete a variety of cytokines, increase the activity of CD8 + T cells, and promote cellular immune responses (20) improving the body's ability to clear viruses. However, they also aggravate liver cell damage. ALT and AST are liver function parameters, and their levels increase in a patient's serum upon cellular damage (10, 21). Increase in bilirubin level during viral hepatitis is related to liver cell damage and the degree of hepatic necrotizing inflammation (22), while reduction in ALB level is also related to the severity of liver synthetic function damage (10). The present study showed that sTim-3 levels in patients infected with HBV, HCV, or HEV were positively correlated with serum ALT, AST, Dbil, and Tbil levels, and sTim-3 levels were negatively correlated with ALB. The results indicate that sTim-3 can reflect the degree of liver cell damage in patients infected with HBV, HCV, or HEV to a certain extent and help in monitoring and evaluation of disease development.

This study showed that the sTim-3 concentrations in patients infected with HBV, HCV, and HEV were significantly higher than those in the healthy controls. The possible reason could be the accumulation of tissue-related memory Treg cells expressing high levels of Tim-3 in liver during the inflammatory stage of viral hepatitis infection (23). In addition, Tim-3 is mainly present on the surface of Th1 cells. When the body is invaded by viruses, Th1 levels increase during the activation of T cell immunity. Tim-3 expression on Th1 cells increases, which inhibits their excessive proliferation, and regulates the balance of Th1/Th2 (24), and exerts a negative immunomodulatory effect. Tim-3 expression also increases in depleted T cells and natural killer cells in patients infected with HBV/HCV (12, 13, 25, 26). Tim-3 mainly exists in three forms: full-length, spliced, and detached. Membrane bound Tim-3 can be cleaved from cell surfaces by metalloproteinases ADAM10 and ADAM17 to produce sTim-3 (27). sTim-3 can be a result of shedding from the cell surfaces after metalloprotease activity, and sTim-3 level may sequentially reflect membrane Tim-3 level. Therefore, sTim-3 levels in patients with three types of viral hepatitis infection were higher than those in the normal control group. The level of sTim-3 in patients infected with HEV was higher than that in patients infected with HBV, possibly because hepatitis caused by HEV is a systemic disease. Recently, studies have systematically explained the many extrahepatic manifestations of HEV infection, which are mainly related to diseases such as the kidney system, autoimmune system, and pancreatitis (28). Moreover, studies have shown that sTim-3 plays an important immunoregulatory role in kidney disease (17, 29), pancreatitis (30), and autoimmune diseases (31), resulting in its high serum levels. Similarly, sTim-3 levels were higher in patients infected with HEV as well.

sTim-3 concentration is elevated in patients infected with HBV and gradually increases in the serum of patients with liver cirrhosis and liver cancer (10). These findings are consistent with our research. In addition to HBV infection, our study reported for the first time that sTim-3 increased in patients infected with HCV and HEV as well and further increased in patients with hepatitis along with liver fibrosis. sTim-3 likely participates in pathogenesis by regulating the balance between Th1 and Th2. Liver fibrosis is a manifestation of tissue damage and inflammation, and viral hepatitis is one of its causes (32). Especially after hepatitis virus infection, without intervention treatment, liver fibrosis develops easily. Tim-3 may promote the progression of liver fibrosis by regulating the secretion of cytokines (TNF-α, IL-10, and IFN-γ) by immune cells, thereby regulating the progression of liver disease (33). Our data showed that Dbil and ALB in the serum of patients infected with HBV or HCV and hepatitis or hepatitis with fibrosis showed significant differences, but there was no difference among patients infected with HEV. Hence, sTim-3 has the advantage of distinguishing HEV-infected hepatitis and hepatitis with fibrosis. The dysfunction of monocytes is an important reason for the development of liver cirrhosis, and studies have confirmed that Tim-3 plays an important role in the expression of monocytes (26). High expression of Tim-3 may limit the activation of monocytes, thereby inhibiting cytokine production, which further promotes liver fibrosis progression. In addition, studies have shown that in patients with liver cirrhosis the CD^+ T cell phenotype is altered, and high expression of Tim-3 in the cell membrane is one of the characteristics (34). This may be the

TABLE 3 | Comparison of the positive rate of sTim-3 and HA to distinguish hepatitis from hepatitis with liver fibrosis.

		Positive rate		
	Cut off	**Controls**	**Hepatitis**	**Hepatitis + fibrosis**
sTim-3	19.45 ng/mL	0.00%	21.68%	66.13%
HA	110 ng/mL	0.00%	4.90%	35.48%
sTim-3 or HA		0.00%	25.87%	77.42%

*The cut off for sTim-3 was determined by the Youden index of **Figure 4E**, and HA concentration greater than 110 ng/mL was considered positive (commonly used in clinical practice).*

reason for higher sTim-3 level in patients with liver fibrosis than in patients with hepatitis. These results indicate that sTim-3 level is related to the progression of the disease (the degree of liver damage) after hepatitis virus infection and is a potential marker for monitoring the progression of the disease upon hepatitis virus infection.

The results of this study indicate that sTim-3 has a certain diagnostic value to detect the degree of liver fibrosis in patients with hepatitis. HA is a commonly used clinical indicator for diagnosing liver fibrosis. When we combined sTim-3 and HA to diagnose liver fibrosis, the positivity rate significantly increased. Faster and more accurate diagnosis tools combined with effective treatment of viral hepatitis will help prevent proliferation of the disease. HEV infection is usually asymptomatic but can cause acute liver damage and even liver failure (35). Therefore, understanding the pathogenesis of hepatitis virus is essential for the treatment of the disease. In animal models, Tim-3 pathway blockade combined with PD-1 and CTLA-4 blockade can increase the production of T cells, reduce the expression of Treg, and restore T cell mediated immune response (36). Blocking the Tim-3/galectin-9 pathway might restore the secretion of some cytokines (IL-2, TNF-α, and IFN-γ) and T cell proliferation (37). These finding provides novel insights into the treatment of viral infectious diseases.

This study has several limitations including the small number of patients in the subgroups of different clinical diseases, and the lack of functional studies concerning the role of sTim-3 in the CD8 + T -cell exhaustion in viral hepatitis infection. We found that the combined detection of sTim-3 and HA can improve the positive detection rate of patients with liver fibrosis, but it only demonstrated moderate performance for discriminating hepatitis from liver fibrosis. Therefore, further studies are required to verify and extend our findings.

CONCLUSION

sTim-3 levels were significantly increased in patients infected with HBV, HCV, or HEV. The quantitative detection of sTim-3 levels can be a tool to distinguish viral hepatitis from hepatitis with fibrosis. The level of sTim-3 is related to disease progression upon hepatitis virus infection.

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/s.

ETHICS STATEMENT

This manuscript is part of a study which was approved by the Wuxi No.5 People's Hospital (approval no. 2020-023-1) and our study obtained informed consent from all participants. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

LC, BH, YD, and HP contributed to conception and design of the study. LC organized the database, performed the statistical analysis, and wrote the first draft of the manuscript. XZ, YQ, LC, SZ, YW, XY, and CL wrote sections of the manuscript. All contributed to manuscript revision, read, and approved the submitted authors version.

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METHODS
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Highly Efficient Methods to Culture Mouse Cholangiocytes and Small Intestine Organoids

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Background: Organoids, which enable disease modeling and drug screening closer to an *in vivo* environment, can be isolated and grown from organs such as the brain, small intestine, kidney, lungs, and liver. To facilitate the establishment of liver and small intestinal organoids, we developed efficient protocols for cholangiocytes and intestine crypts collecting and organoid culturing.

Methods: Cholangiocytes were collected from intrahepatic bile ducts, the gallbladder, and small intestine crypts by gravity settling and multistep centrifugation methods. The cells isolated were embedded with Matrigel and grew in three-dimensional spheroids in a suitable culture medium. The stability of organoid cells was assessed by subculture, cryopreservation, and thawing. RNA and DNA extraction of organoids, as well as immunostaining procedure, were also optimized. Hand-picking procedures were developed and performed to ensure similar growth characteristics of organoids.

Results: A large number of cholangiocytes and small intestine crypts were collected under these protocols. Cholangiocytes developed into cyst-like structures after 3–4 days in Matrigel. After 1–2 weeks of cultivation, small intestinal organoids (in-orgs) developed buds and formed a mature structure. Compared to organoids derived from the gallbladder, cholangiocyte organoids (Cho-orgs) from intrahepatic the bile ducts grew more slowly but had a longer culture term, expressed the cholangiocytes markers Krt19 and Krt7, and recapitulated *in vivo* tissue organization.

Conclusions: Our protocols simplified the cell collection procedure and avoided the possibility of exposing tissue-derived stem cells to mechanical damage or chemical injury by gravity settling and multistep centrifugation. In addition, our approach allowed similar growth characteristics of organoids from different mammalian tissue sources. The protocol requires 2–4 weeks to establish a stable organoid growth system. Organoids could be stably passaged, cryopreserved, and recovered under protocol guidance. Besides, the organoids of cholangiocytes and small intestines retained their original tissue characteristics, such as tissue-specific marker expression, which prepares them for further experiments such as preclinical *in vitro* trials and mechanism research studies.

Keywords: organoids, cholangiocytes, small intestine, 3D culture, intestinal crypt, liver

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INTRODUCTION

Organoids are *in vitro* three-dimensional (3D) cultures grown from primarily isolated cells or stem cells (Dutta et al., 2017). Three-dimensional culture enables organoid growth from healthy (Huch and Koo, 2015) or diseased (van de Wetering et al., 2015) human or animal primary tissues. Organoids have potential as preclinical models (Lancaster and Knoblich, 2014; Dutta et al., 2017; Zhang et al., 2021) for drug screening (Kondo and Inoue, 2019) and in mechanistic research (Lancaster and Knoblich, 2014; Vives and Batlle-Morera, 2020). Standardized good manufacturing practice (GMP)-compliant scalable organoids may enable the replacement of damaged human organs (Dossena et al., 2020). What's more, in the widespread spread of world pandemics such as SARS-CoV-2, organoids were also applied to evaluate the intestinal function changes (Zhou et al., 2020), which disclosed a deeper insight into unique gene mutations in gastrointestinal disease (Li, 2021).

As for the cellular composition of organs, the liver mainly comprises hepatocytes, cholangiocytes, and nonepithelial cells (Duncan et al., 2009). The small intestine is almost formed of the intestinal epithelium, which contains intestinal crypt-villus units and $Lgr5^+$ stem cells (Sato et al., 2009), which potentially own stem cell properties. For organoid growth, Matrigel is needed to provide an *in vitro* 3D environment for stem cell growth. Besides, the proliferation of hepatic and small intestinal stem cells requires epidermal growth factor (EFG), the R-spondin 1 (R-SPO1) protein (notch signaling components) (Sato et al., 2009), growth factors (e.g., N2, B27, and Noggin), hepatocyte growth factor (HGF), and fibroblast growth factor (FGF), as mentioned before.

Based on the above research studies, we developed and improved an efficient method for collection of cholangiocytes and intestinal crypts by gravity settling and multistep centrifugation, which not only simplified the steps of cell separation but also improved the rate of cell acquisition. However, during the experiment, we found a phenomenon that stem cells derived from different mice present different growth characteristics, with some developing into mature organoids in 7–10 days and the others taking more than 10 to develop into mature forms. Therefore, we established an organoid hand-picking procedure (**Figure 1**) to ensure that the organoids had similar growth characteristics and avoid excessive differences in organoid growth, which is convenient for disease modeling and mechanism research.

Under our protocol guidance, the organoids can be passaged, cryopreserved, and thawed, and they are suitable for genetic analysis and immunochemical staining. Compare with other stem cell isolation methods, our procedure does not require additional mechanical equipment (e.g., flow cell sorter) and chemical experiments, like antibody combination, which avoids the possibility of exposing stem cells to mechanical damage or chemical injury. Besides, our protocol makes it is easy for beginners to get started and does not require physical exertion. Most importantly, the cell growth rates of stem cells gained from different mammals are unified before further experiments.

Organoids generated from the bile ducts and small intestine have the characteristics of the original tissue, which makes them suitable for disease modeling, drug screening, mechanism research, etc. As recent studies show, organoids may also have a therapeutic investigation potential for diseases such as primary sclerosing cholangitis (PSC) (Loarca et al., 2017), intestinal cancer (Aberle et al., 2018), and inflammatory bowel diseases (IBD) (Meir et al., 2020). Our protocol may provide huge support for the early-stage preparation of such research studies.

Abbreviations: 3D, three dimensional; PSC, primary sclerosing cholangitis; IBD, inflammatory bowel diseases; EGF, epidermal growth factor; FGH, fibroblast growth factor; HGF, hepatocyte growth factor; ROCK, Rhokinase; IF, immunofluorescence; IHC, immunohistochemistry.

EXPERIMENTAL DESIGN

Cell Isolation

To isolate bile ducts (cholangiocytes) from mouse liver, the ducts and gallbladder can be digested. In brief, the liver was perfused with PBS 1× through the inferior vena cava before removing the liver tissue. If a small number of cholangiocytes is needed, or if cho-orgs must be cultured rapidly, cholangiocytes can be obtained from mouse gallbladder. After digestion, ducts and crypts from the small intestine can be collected by gravity settling followed by multistep centrifugation. If cholangiocytes need to be counted, bile ducts can be further digested using a single-cell TrypLE Express medium.

Organoid Culture and Establishment of Stable Organoid Lines

To establish a long-term organoid culture, we picked and collected larger organoids to passage and to unify the whole growth speed of organoids in the same culture well. As reported by Huch, when culturing organoids for further experiment, the elements of organoid size, proliferation rate, physical morphology, formation rate, and expression of markers/genes are all needed to be considered (Huch et al., 2013). Therefore, we described an easy hand-picking procedure to achieve this goal. We also provided two methods of cho-orgs culture, one from intrahepatic bile ducts and the other from gallbladder cholangiocytes. These two types of organoids both expressed cholangiocyte specific markers like Krt7 and Krt19, and displayed different transcriptional profiles and differentiation capacity as demonstrated by Casey A. Rimland (Rimland et al., 2021), which provided a basis for selection according to purpose, such as disease simulation or *in vitro* modeling. Besides, the methods of home-made Wnat3a and R-SPO1 were described by Broutier (Broutier et al., 2016), which were indispensable in organoid culture. When a stable culture line is established, organoids can be maintained for a long time.

Organoid Analysis

To research the gene changes of the organoids, after the removal of Matrigel from the organoid cultures, the procedure of DNA or RNA extraction from the organoids was almost similar with normal cells. Matrigel was removed first using a pre-cold basal medium. It was suggested to use a cell recovery

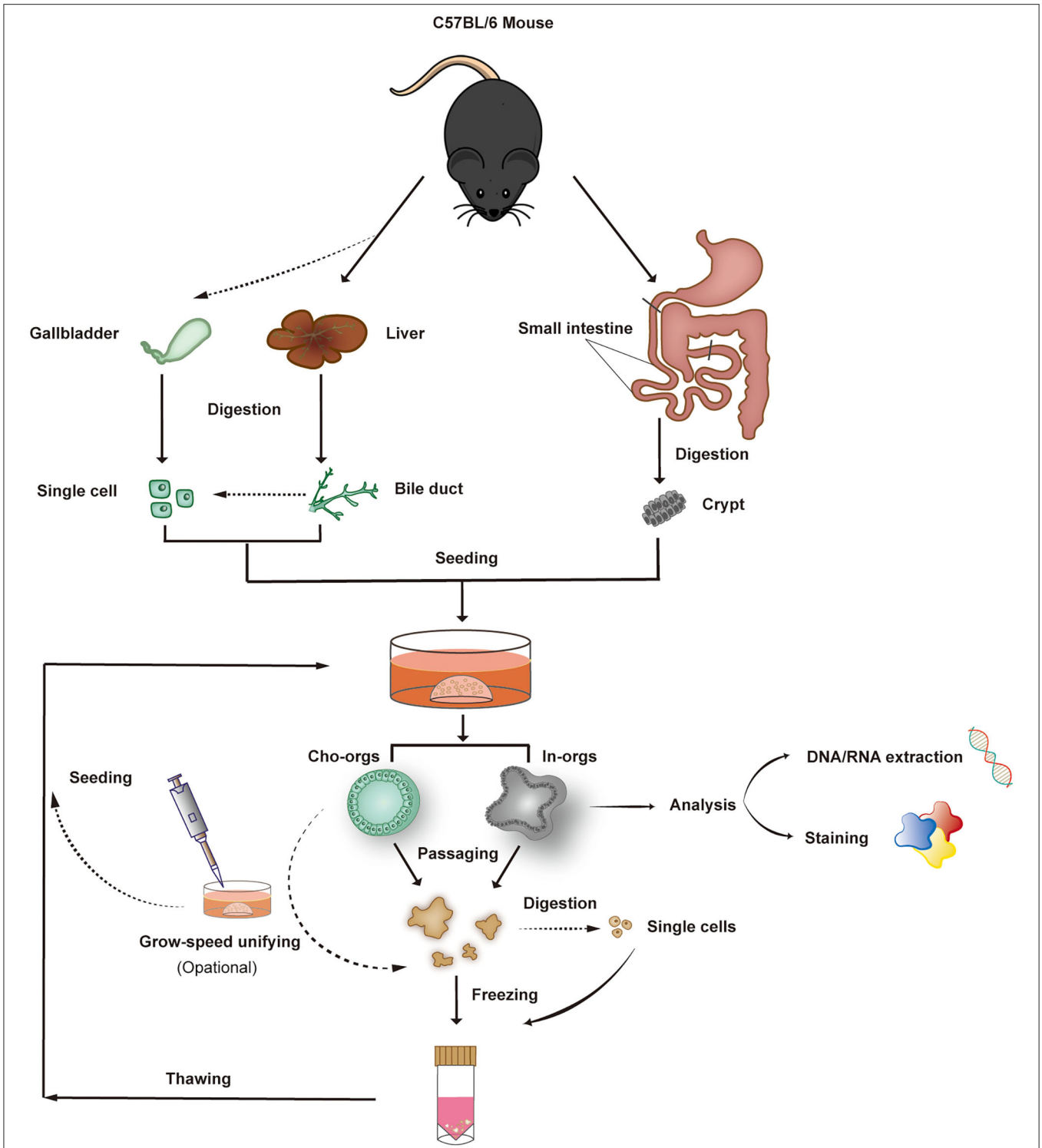

FIGURE 1 | Organoid isolation, culture, growth-rate harmonization, passaging, and freezing. Bile ducts, single bile duct (or gallbladder) cells, and crypts from the small intestine derived from C57BL/6 mice form 3D structures after 7–14 days of culture (5–7 days for gallbladder-derived organoids) in Matrigel. After expansion, the organoids can be characterized or frozen in liquid nitrogen.

solution to remove the Matrigel without disrupting organoid structures for immunofluorescence and immunohistochemical staining analysis. Whole-mount immunofluorescence staining enabled 3D structure observation and characterization, such as detecting aimed antibody signal distribution. For observation of cellular morphology and storage of organoids for further staining

analysis, paraffin sectioning and immunohistochemical staining are preferred.

MATERIALS

Animals

Male or female mice of any genetic background and ranging in age from 6 weeks to 1.5 years (weight 20–28 g) can be used for cho-org culture; for in-org culture, ≤ 6-week-old C57BL/6 mice should be used.

Reagents

- Collagenase D (Roche, cat. no. 1108866001).
- Dispase II (Life Technologies, cat. no. 17105-041).
- DNase I (Sigma-Aldrich, cat. no. DN25).
- TrypLE Express (Life Technologies, cat. no. 12605-028).
- EDTA (Sangon Biotech Inc., Shanghai, CAS: 60-00-4).
- Matrigel matrix, phenol-red-free (BD, cat. no. 356231).
- Advanced DMEM/F-12 (Life Technologies, cat. no. 12634-010).
- DMEM, high glucose, GlutaMAX, pyruvate (Life Technologies, cat. no. 11995-065).
- GlutaMAX (100×; Life Technologies, cat. no. 35050-068).
- HEPES (Life Science Products & Services, cat.no. HB0264).
- Penicillin/streptomycin (Corning, cat.no.30002297).
- B27 Supplement 50×, minus vitamin A (Life Technologies, cat. no. 12587-010).
- N2 Supplement 100× (Gibco, Life Technologies, cat. no. 17502-048).
- L-glutamine (Gibco, Life Technologies, cat. no.25-005-CI).
- N-acetylcysteine (Sigma-Aldrich, cat. no. A0737-5MG).
- Nicotinamide (Sigma-Aldrich, cat. no. N0636).
- Recombinant mouse (Rm) FGF10 (Peprotech, cat. no. 100-26).
- Rm EGF (Life Technologies, cat. no. PMG8043).
- Rm HGF (Peprotech, cat. no. 100-39).
- [Leu15] - Gastrin I human (PL Laboratories, cat. no. P2000646).
- Rho kinase inhibitor Y-27632 dihydrochloride (Sigma-Aldrich, cat. no. Y0503).
- Recombinant human (Rh) Noggin (Peprotech, cat. no. 120-10C).
- Wnt3a-conditioned medium (home-made).
- Rspo1-conditioned medium (home-made).
- Freezing solution (Life Technologies, cat. no. 12648-010).
- Cell recovery solution (Corning, cat. no. 354253).

Additional reagents (**Supplementary Table S1**), instruments (**Supplementary Table S2**), and antibodies (**Supplementary Table S3**) are listed in **Supplementary Materials**.

REAGENT SETUP

Mouse Liver Digestion Medium

The digestive medium should be prepared fresh and used immediately. Collagenases D and II were dissolved in a sterile washing medium (see below) at a concentration of 0.125 mg/ml and supplemented with 0.1 mg /ml DNase I (dissolved in sterile H_2O).

Mouse Liver Basal Medium

Advanced DMEM/F-12 was added with 1% penicillin/streptomycin, 1% GlutaMAX, and 10 mM HEPES. It can be stored at 4°C for 1 month.

Mouse Liver Isolation Medium

The mouse liver isolation medium was a mouse liver expansion medium supplemented with 25 ng/ml recombinant human Noggin, 30% (vol/vol) Wnt3a-conditioned medium, and 10 μM Rho kinase (ROCK) inhibitor (Y-27632 if single cells were cultured). The medium was stored at 4°C for up to 2 weeks.

Mouse Liver Wash Medium

The DMEM (high glucose, GlutaMAX, and pyruvate) supplemented with 1% FBS and 1% penicillin/streptomycin. The medium was stored at 4°C for up to 1 month.

Mouse Liver Expansion Medium

Mouse liver basal medium supplemented with B27 50×, 1 mM N-acetylcysteine, 5% (vol/vol) Rspo1, 10 mM nicotinamide,10 nM Gastrin I, 50 ng/mL EGF, 100 ng/ml FGF10, and 50 ng/ml HGF. Store the medium at 4°C for up to 2 weeks.

Mouse Intestine Expansion Medium

Advanced DMEM/F-12 supplemented with 10 mM HEPES, 2 mM L-glutamine, N2 50×, B27×, 50 ng/ml EGF, 100 ng/ml Noggin, and 10% (vol/vol) R-spondin 1. Store it at 4°C for up to 1 month.

Mouse Intestine Digestion Medium

The PBS 1× supplemented with 10 mM HEPES, 1%(vol/vol) L-glutamine, 1 mM EDTA, 1% penicillin/ streptomycin, and 5% FBS.

Regent Preparation for Immunofluorescence (IF)

PBS-BSA 1% (vol /vol): 1 g BSA per 100 ml PBS 1×. Store at 4°C for 2 weeks.

PBT 0.1% (vol /vol): 1 ml Tween 20 per 1,000 ml PBS 1×. Store at 4°C for 4 weeks.

Washing buffer: 1 ml Triton X-100 and 2 g BSA per 1 L PBS 1×. Store at 4°C for 2 weeks.

F-G clearing solution: 2.5 M fructose and 60% glycerin. Store at 4°C in the dark for up to 1 month.

Regent Preparation for Immunohistochemistry (IHC)

Alcohol 96% (vol/vol): dilute 100% alcohol with purified water.

Eosin solution 0.5% (wt/vol): Dissolve 0.5 g eosin in 100-ml 96% alcohol.

PROCEDURE

I. Culture of mouse cholangiocytes and small intestinal organoids: cell isolation

Mouse liver duct cells may be collected from the liver or gallbladder.

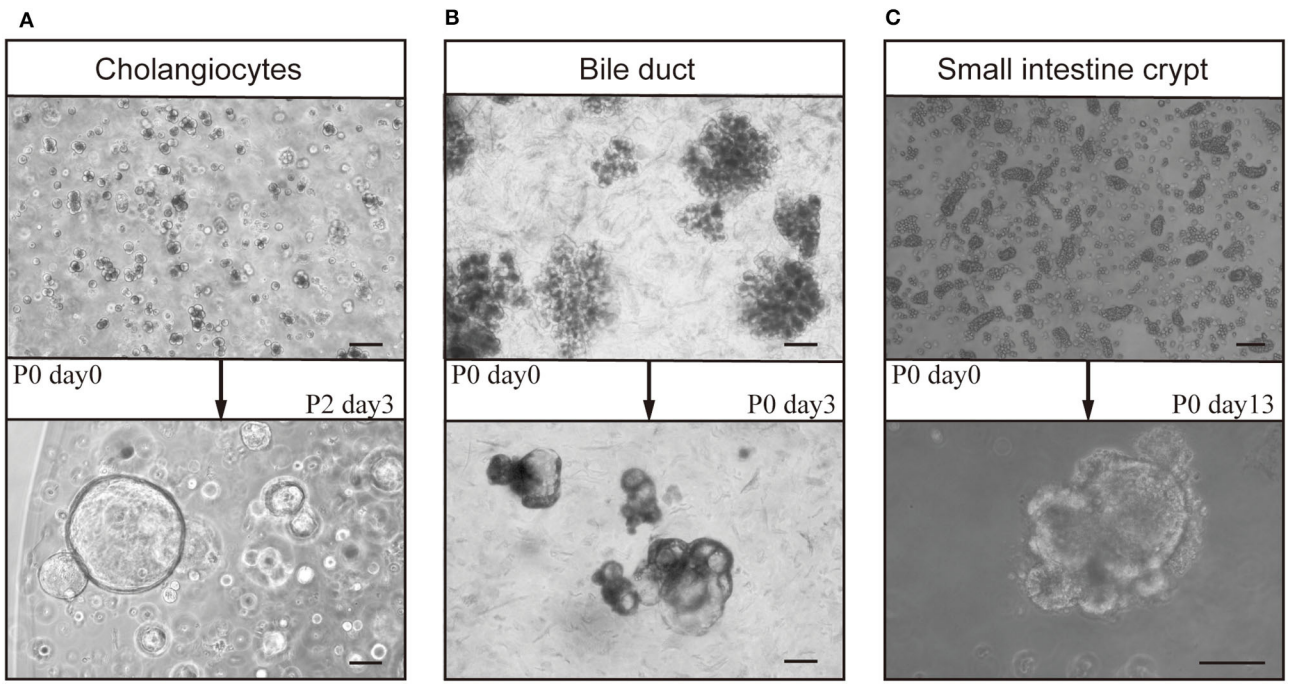

FIGURE 2 | Culture of organoids from bile ducts and small intestines of mice. After gravity settling and multi-step centrifugation, cholangiocytes (derived from bile ducts), bile ducts, and crypts from the small intestine formed organoids after ∼14 days in culture. Cholangiocytes (day 0) from **(A)** bile ducts formed cholangiocyte organoids (cho-orgs) (day 3, passage 2) (bar, 100 μm), and those from **(B)** intrahepatic bile ducts (day 0) formed cho-orgs (day 3) (bar, 100 μm). **(C)** Crypts from the small intestine (day 0) (bar, 200 μm) formed small intestinal organoids (in-orgs) (day 13) (bar, 100 μm).

I.i. Collection of bile ducts from mouse liver

1) To collect liver tissue, anesthetize the mouse and expose the liver. Find the inferior vena cava using a sterile swab, place a sterile cotton ball beside the liver, inject PBS 1× using a 10-mL injector, and cut off the portal vein immediately when the liver swells. By standard surgical procedures, remove the liver as an entire organ and transfer it to a 10-cm Petri dish.
2) Place the Petri dish on ice and transfer it to a biological safety cabinet. Preheat the digestion solution to 37°C.
3) Cut the liver tissue into small pieces (<1 mm^3) using fine scissors. Transfer the small pieces to a 50-mL sterile centrifuge tube, add up to 5 ml precooled washing medium, and pipette it up and down several times using a 10-ml pipette to wash the minced tissue. Repeat the washing procedure.
4) Transfer the tissue to a new 50-ml sterile centrifuge tube. Add 5 ml pre-warmed digestion medium. Shake the tube at 120–160 rpm and 37°C for ∼2 h.
5) During incubation, check the appearance of bile ducts using a light microscope. Pipette up and down the supernatant in a biological safety cabinet using a 1-ml pipette, and transfer ∼200 μl of the solution to a glass slide for observation (**Figure 2B**, upper row). If no bile ducts are present, return the solution to the shaker. Perform the checking at 20–30 min intervals. Ducts usually appear after 60 min.
6) When bile ducts appear, transfer the digestion supernatant to a fresh 50-ml centrifuge tube, add the same volume of precooled washing medium, and centrifuge at 80 g for 4 min at 4°C. Discard the supernatant and add 15 mL precooled washing medium; repeat the centrifugation to remove the remaining digestion solution.
7) Add the precooled washing medium to the pellet and pipette it up and down to mix. Place the 50-mL centrifuge tube upright on ice for 30 min.
8) Remove the supernatant without disrupting the pellet. Add 5 ml precooled washing medium, transfer the mixture to a fresh 15-ml centrifuge tube, and centrifuge at 60 g for 2 min at 4°C.
9) Remove the supernatant carefully. Add 1 ml precooled basal medium, transfer the mixture to a 1.5-ml microcentrifuge tube, and centrifuge at 500 rpm for 2 min at 4°C.
10) The pellet can be directly cultured. If there is a need to collect single bile cells, follow the next procedure.

I.ii. Enrichment of single cholangiocytes

1) Resuspend bile ducts in 5 ml prewarmed TrypLE solution supplemented with 5 μl DNase I (10 mg/ml). Using narrow 1,000 μl tips, pipette the solution up and down to mix and incubate at 37°C for 2–10 min.
2) Check the solution every 2 min using a bright-field microscope. Stop the digestion when majority (85–95%) of

the mixture consists of single cells by adding the precooled wash medium (**Figure 2A**, upper row).

3) Add 10 ml cold washing medium to stop the digestion. Transfer the mixture to a 50-ml centrifuge tube through a 70-μm filter and centrifuge at 300–350 g for 5 min at 4°C. Remove the supernatant and repeat the centrifugation to wash out any remaining TrypLE. Add 1–5 ml washing medium to resuspend the pellet.
4) Enumerate the cells using a standard cell-counting chamber.

I.iii. Collection of cholangiocytes from gallbladder

Prewarm the mouse liver digestion medium and TrypLE solution (supplemented with 0.1% [vol/vol) DNase I [10 mg/ml]) to 37°C.

1) By standard surgical procedures, anesthetize the mouse and expose the liver. Strip the gallbladder using two ophthalmic forceps and transfer it to a 6 mm dish containing precooled mouse liver washing medium.
2) Cut the gallbladder into small pieces using ophthalmic scissors, transfer the pieces to a 15-ml sterile centrifuge tube using a 1,000 μl Eppendorf pipette, add ∼5 ml precooled washing medium, gently pipette the solution up and down, and centrifuge it at 4°C, 200–250 g, for 4 min to remove bile.
3) Discard the supernatant and add 5 ml mouse liver digestion medium. Shake the tube at 120–180 rpm and 37°C for 1 h.
4) Add 5 ml precooled washing medium to stop the digestion, centrifuge at 4°C and 200–250 g for 4 min and discard the supernatant.
5) Resuspend the gallbladder tissue in 1 ml prewarmed TrypLE solution and pipette it up and down 30 times to isolate single cholangiocytes.
6) Incubate the tube for 2–4 min in a 37°C culture bath and pipette it up and down 30 times.
7) Add t∼5 ml precooled wash medium and filter the mixture through a 70 μm mesh into a 50-ml centrifuge tube using a 1,000 μl Eppendorf pipette. Pipette the fluid into a 15-ml centrifuge tube, and centrifuge at 4°C and 300–350 g for 4 min.
8) Discard the supernatant and enumerate the cells. The plates are ready for Matrigel embedding and 3D organoid culture.

I.iv. Collection of crypts from mouse small intestine

1) Anesthetize the mouse; by standard surgical procedures, expose and remove the intestine using ophthalmic forceps. Cut the optional intestine segment 1 cm above the end of the ileum and 2–3 cm below the stomach, and transferred them to a 10 cm dishes containing PBS 1×.
2) Put the 10-cm dish on ice. Squeeze out the intestinal contents using forceps and cut the tissue longitudinally. Use a blunt instrument (e.g., the curved part of curved dissecting forceps) to scrape the intestinal villi, wash twice or thrice and cut the tissue into 1–2 cm pieces.
3) Transfer the pieces to a 50 ml centrifuge tube and add 20 ml mouse intestine digestion medium. Put the tube on a shaker at 120–160 rpm and 4°C and incubate for about 30 min.
4) Carefully remove the supernatant and resuspend the tissue in a new 50 ml centrifuge tube, add ∼25 mL PBS 1×, and vigorously pipette the solution up and down 30–50 times to isolate crypts from the tissue.
5) Filter the supernatant through 100- and 70-μm meshes into a new 50 ml centrifuge tube.
6) Allow the tube to stand for 8 min and discard the supernatant or transfer them to a new tube for another 8 min-standing circulation. Gather the plates together and check the proportion of the crypts. Add 1–5 ml PBS 1× to the white sediment and pipette it up and down gently. Observe the mixture under a light microscope.
7) If the proportion of single cells is too high, add ∼25ml precooled PBS 1×, and repeat the 8-min standing circulation procedure in step (D-6) to collect a higher proportion of crypts.
8) Count the crypts using a counting board.

II. Seeding and culture: cholangiocytes/bile ducts and crypts

1) Prewarm 24-well (or 48-well) culture plates at 37°C for at least 30 min. Pre-dissolve Matrigel and keep it on ice.
2) Resuspend an appropriate number of cholangiocytes, duct structures, or crypts (e.g., 5,000 cells or 250 duct/crypt structures per well of a 24-well plate) in Matrigel for seeding. For example, use a volume of 40 μl per 24-well plate or 20 μl per 48-well plate.
3) Put Matrigel in the plates and mix gently. Add a droplet of the mixture (basal matrix and cultures) to the center of each well to prevent the droplet from touching the edges. Incubate for 15–20 min at 37°C until Matrigel solidifies.
4) Add the appropriate medium to each well (500 μl per well for a 24-well plate or 250 μl per well for a 48-well plate) (liver isolation medium for cho-org culture, intestine expansion medium for in-org culture).
5) Incubate the plates at 37°C in a 5% CO_2 atmosphere. For cho-orgs, after 3 days, change the isolation medium with the expansion medium and incubate for ∼14 days. For in-orgs, retain the intestine expansion medium. Change the medium every 3–4 days. Organoids will start to develop on days 3–5.

III. Organoid passage

1) Prewarm culture plates for 1 h–overnight. Place Matrigel on ice and thaw before use. Prewarm the TrypLE Express solution (∼2 ml per tube) in a water bath at 37°C.
2) To disrupt the basal matrix, add 500–1,000 μl precooled basal medium (for cho-orgs) or PBS 1× (in-orgs), and pipette the mixture up and down using a 1,000 μL pipette. Transfer the organoid suspension (three wells for 24-well culture plates or six wells for 48-well culture plates) to a 15 ml centrifuge tube, add ∼10 ml precooled basal medium (cho-orgs) or PBS 1× (in-orgs) to the top, and mix by pipetting vigorously 5–10 times to wash away Matrigel.

3) Centrifuge the tube at 200–250 g for 5 min at 4°C.
4) Discard the supernatant, leaving 100 μl mixture.
5) Add the prewarmed TrypLE Express solution and mix by vigorously pipetting up and down using a 1,000 μl pipette. Transfer the tube to a water bath at 37°C for 1–4 min, checking every 2 min under a light microscope. When majority (85–95%) of the material is single cells, add ∼10 ml precooled basal medium to stop digestion.
6) Centrifuge the tube at 300–350 g for 4 min at 4°C, and carefully aspirate the supernatant.
7) Organoids can be mechanically dissociated and split at a 1:3–1:6 ratio. Resuspend the cells in Matrigel (40–50 μl per well for 24-well plates or 20–25 μl per well for 48-well plates). Pipette the mixture up and down gently to resuspend the cells. Add a droplet of the mixture to the center of each well. Incubate for 15–20 min to allow for Matrigel polymerization.
8) Overlay the cultures with the expansion medium (500 μl per well for 24-well plates or 250 μl per well for 48-well plates).
9) Replace the medium every 2–3 days.

IV. Harmonizing the growth rates of organoids

Organoids developed from different primary cells grew at different rates, for some experimental needs, organoids in different growing state are hard to perform experiments. To resolve this problem, we purified cho-orgs with similar growth rates using the procedure below, which can be repeated once to thrice according to growth situation. In addition, intestinal organoids can also use the following method to uniform growth rate.

1) When cho-orgs have budded for 2–3 days (5–7 days after seeding), check their growth state daily under a light microscope.
2) Before cells aggregate in the larger cho-orgs, prepare to selectively passage then, as mentioned in III-1, prewarm the culture plates and TrypLE Express solution, and thaw the Matrigel before use.
3) Move the plates to a sterile environment. Under a light microscope, transfer the larger cho-orgs to the precooled basal medium using a 10-μl pipette.
4) Subsequent steps are the same as for organoids passage; please follow steps III-3–8.

V. Cryopreservation and thawing of organoids

V.i. Freezing organoids

1) At least one confluent well (24-well plate) or two confluent wells (48-well plates) of organoids are needed per cryovial tube.
2) Proceed as in steps (III-1–5) to digest the organoids into single cells and resuspend the cells in 500 μl precooled freezing medium per well (24-well plates) or two wells (48-well plates). Transfer the mixture to cryovials (500 μl each), and immediately place them on ice. Transfer the cryovials at −80°C and then to liquid nitrogen after 24 h. The organoids can be stored for years.

V.ii. Thawing of organoids

1) Prewarm a 15 ml tube with 10 ml basal medium (for cho-orgs or in-orgs) at 37°C. Prewarm a 24- or 48-well plate according to need.
2) Incubate the cryovial in a 37°C water bath and remove when the frozen cell mass is almost completely thawed. Transfer the thawed cell aggregates to the prewarmed basal medium and pipette it up and down gently.
3) Centrifuge the tube at 250–300 g for 4 min at 4°C.
4) Remove the supernatant without disturbing the pellet.
5) Proceed as in steps (II-1–3) to seed the cells in Matrigel and add the appropriate expansion medium (500 μl per well for 24-well plates or 250 μl per well for 48-well plates) to each well.
6) Replace the medium every 2–3 days.

VI. Analysis of organoids

To characterize the organoids, use option (VI.i) for immunofluorescence analysis, (VI.ii) for immunohistochemical staining, and (VI.iii–iv) for isolation of DNA (iii) or RNA (iv),

VI.i. Immunofluorescence staining

1) To collect the organoids completely, remove the expansion medium, and add 500–1,000 μl precooled Cell Recovery Solution to each well.
2) Gently shake the plate horizontally at 4°C for 30–60 min to disrupt Matrigel.
3) Cut off the top of 1,000 μl tips. Blow the tips twice in precooled 1% (v/v) PBS-BSA, and wash 15 ml centrifuge tubes using 1% (v/v) PBS-BSA to precoat the tips and tubes.
4) Transfer the mixture to the precoated tubes using the precoated tips, add PBS 1× to ∼10 ml and mix gently. Centrifuge at 70 g and 4°C for 4 min, and carefully remove the supernatant.
5) Repeat the PBS 1× wash steps once or twice to wash out Matrigel completely.
6) Fix the organoids by resuspending in 4% (w/v) paraformaldehyde at 4°C for 45 min and mix once or twice.
7) Add ∼10 ml precooled 0.1% (vol /vol) PBT, mix, and centrifuge at 70 g and 4°C for 4 min.
8) Remove the supernatant and resuspend the organoids in the precooled washing buffer. Transfer the mixture to a 24-well plate (>200 μl per well) and incubate at 4°C for 15 min.
9) When the organoids sink to the bottom, tilt the culture plate at 45° and remove the washing buffer to leave about a 200-μl liquid.
10) Add a two-fold concentration of primary antibody (diluted in 0.5% PBS-BSA) (200 μL) to each well. Incubate overnight at 60 rpm and 4°C.
11) Add 1 ml washing buffer to each well and pipette gently.

12) When the organoids sink to the bottom (3 min), remove 1 ml washing buffer, add 1 mL washing buffer, and incubate for 30 min.
13) Repeat step (11) at least twice.
14) When the organoids sink to the bottom, remove the washing buffer to leave a 200 μl liquid.
15) Add a two-fold concentration of secondary antibody (diluted in 0.5% PBS-BSA) (200 μl) to each well. Incubate overnight at 60 rpm and 4°C.
16) Repeat 10–13. Transfer the organoids to 1.5 ml EP tubes and centrifuge at 70 g and 4°C for 4 min. The organoids can be stored in the washing buffer at 4°C for 2 days.
17) Remove the washing buffer without touching the plates.
18) Add the F-G clearing solution (>50 μl, RT) to the EP tube using top-cut 200-μl tips.
19) Add an appropriate volume of DAPI and incubate for 20 min at room temperature. The organoids can be stored in the F-G clearing solution for 1 week at 4°C or 6 months at −20°C.
20) Transfer the organoids to a 24-well plate using 200-μl top-cut tips.
21) The organoids can be subjected to immunofluorescence imaging.

VI.ii. Immunohistochemical staining of organoids

1) To obtain the organoids completely, perform steps VI-C 1–6.
2) Remove 4% (w/v) paraformaldehyde, add ∼10 ml precooled PBS 1×, and centrifuge at 70 g and 4°C for 4 min.
3) Repeat the washing step once or twice.
4) Remove the supernatant and add ∼10 ml 70% alcohol. The organoids can be stored at 4°C in 70% alcohol for 1 week.
5) Adjust the water bath to 65°C and prewarm Histowax (∼3 ml per sample), plastic straws, soft EP tubes, and a metal mold. Be careful to keep the materials dry.
6) Place a centrifuge tube on ice upright until the organoids sink to the bottom. Remove the supernatant, resuspend the organoids in 0.5% (w/v) eosin solution to dehydrate, and stain for >30 min.
7) When the organoids sink to the bottom, discard the supernatant and resuspend in 100% alcohol to wash for at least 30 min. Repeat these wash steps thrice.
8) Resuspend the organoids in dimethylbenzene and wash thrice for ∼30 min each.
9) Remove the dimethylbenzene.
10) Place the centrifuge tube containing the organoids mentioned above in a 65°C water bath, absorb appropriate volume of prewarmed liquid Histowax (≤500 μL) to the organoids using the prewarmed plastic straws, quickly pipette twice or thrice, and transfer the mixture to the prewarmed soft EP tubes.
11) Incubate the soft EP tubes at 65°C overnight.
12) Transfer the soft EP tubes to RT or a cold area to solidify Histowax.
13) Peel off the tube carefully and cut the Histowax to a smaller size suitable for embedding. This step concentrates the organoids, facilitating their staining and visualization.
14) Re-embed the small wax block to a new Histowaxin metal mold.
15) Cool the mold and take out Histowax, which can be stored at RT for years.
16) Cut Histowax into pieces for further staining.

VI.iii. Isolation of DNA

1) Perform the procedure in main text III 2–3 to remove Matrigel.
2) Discard the supernatant, add ∼1 ml precooled PBS 1×, and pipette the mixture up and down thrice. Transfer the mixture to 1.5 ml EP, and centrifuge at 250 g and 4°C for 4 min.
3) Resuspend the organoids in PBS 1×, transfer them to a 1.5 ml EP tube, and centrifuge at 300 g and 4°C for 4 min.
4) Discard the supernatant and add 30 μl Direct-PCR solution and 0.5 μl proteinase K per sample.
5) Incubate at 60°C overnight.
6) Centrifuge at ≥8,000 g at RT for 10 min.
7) DNA can be stored at 4°C.
8) If necessary, purify the DNA using a DNA purification kit.

VI.iv. Isolation of RNA

1) To collect the organoids, follow steps VI.iii 1–3 to collect cell mass.
2) Remove the supernatant, add 350 μl RLT to each 1.5 ml microcentrifuge tube, and vortex-mix for 30 s.
3) To promote cell mass lysis, draw the mixtures at least 5 times without RNase using a sterile syringe fitted with a no. 20 needle.
4) The mixtures can be stored at −80°C for 1 month.
5) RNA can be extracted following the RNeasy Mini Kit (Qiagen, Hilden, Germany) protocol guides.

Troubleshooting

Troubleshooting advice can be found in **Table 1**.

Step Times

Step I. i–ii: collection of mouse liver bile-duct cells: 4 h.
Step I. iii: collection of mouse gallbladder cholangiocytes: 2 h.
Step I. iv: isolation of mouse intestinal crypts: 2 h.
Step II: seeding of mouse cholangiocytes or crypts: 30 min.
Step III: passaging of mouse organoids: 30–45 min.
Step IV: harmonizing of organoid growth rate: 40–60 min.
Step V: cryopreservation and thawing of mouse organoids: 30 min each.
Step VI: analysis of organoids: 2 days for isolation of DNA, 40–60 min for isolation of RNA, 3 days for immunofluorescence staining, and 2 days for immunohistochemical staining.

RESULTS

Establishment of Organoid Cultures

The bile ducts, cholangiocytes, and intestine crypts embedded in Matrigel developed into 3D structures (**Figure 2**). The cho-orgs from cholangiocytes were single cystic spheres, while the primary cho-orgs from bile ducts comprised multi-cystic sphere-like organoids but can be separated after passaging and grow into

TABLE 1 | Troubleshooting.

Step	Problem	Possible reason	Solution
I.i-5	Low yield after digestion	Over- or under-digestion of liver tissue	Check the duct cells every 20 min during digestion, (**Figure 2A** upper row)
I.i-7 I.ii-2	Large amount of cell debris	Over-digestion of the liver tissue or the gravity sinking time was too long	When large flakes of ducts appear, stop the digestion; shorten the gravity sinking time
II-2	Air bubbles in the Matrigel	The Matrigel was pipetted too quickly	Pipetted slowly when seeding; if bubbles are formed, centrifuged tube at 4°C to push the bubbles to the top of the mixture
II-3	Matrigel solidified in the tube or the tip	Over-temperature of Matrigel when seeding	Keep the Matrigel mixture on ice and pre-cold the tips; perform the seeding procedure as soon as possible
III-5	Low formation rate of passaged organoids	Over-digestion of organoids or mechanical separation too fierce.	Check the single cells under a microscope every 2 min; slow down the pipetting speed.
III-7	More cell clumps after digestion	Not enough digestion time or too little mechanical blowing	Increase the digestion time; increase the number of mechanical blowing (10-30 times) before neutralizing the digestion
VI.ii-10	Wax blocks touch softly	Not enough incubation of wax blocks in cold area or too much vapor mixed in the wax.	Prolong the incubation time of wax in cold area and keep care of the vapor
VI.ii-13	Wax blocks crack easily	The wax block has not set completely; peel off the soft EP tube too fiercely.	Prolong the incubation time of wax in cold area; Cut the soft EP tube by fine scissor before peeling off the tube
VI.iv-1	Cell clumps tend to stick to the wall of the tube or on the tips	The Matrigel is not completely removed	Before washing with PBS 1×, washing the Matrigel-cell mixture by medium 1-2 more times; and then avoid the 1000 μL tips going too deep under the liquid surface when washing with PBS 1×

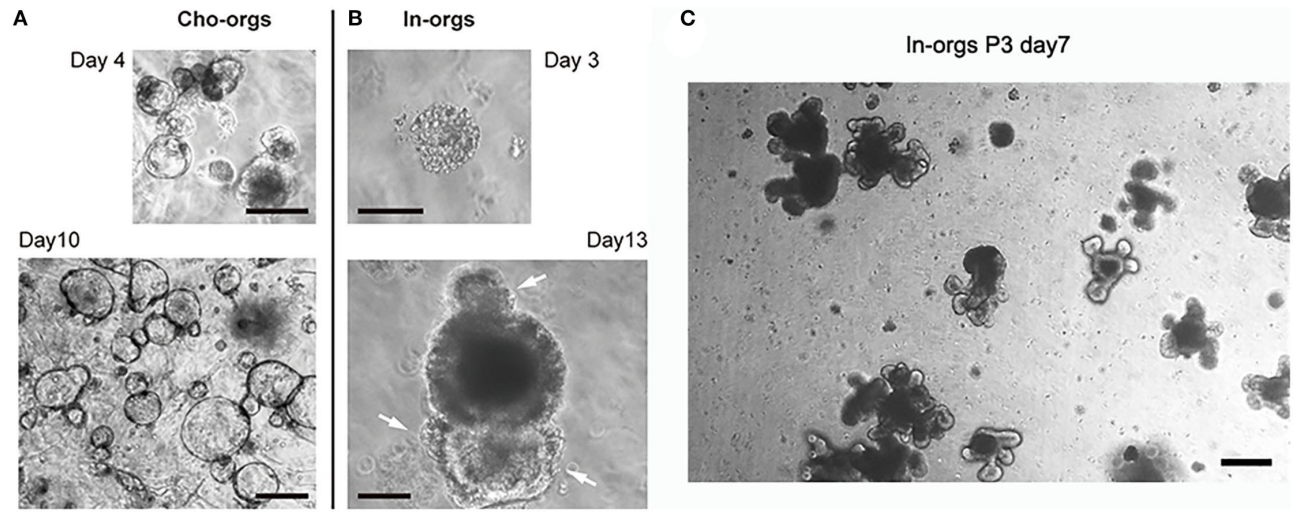

FIGURE 3 | Development of cho-orgs and in-orgs. Cholangiocytes (bile ducts) and crypts from the small intestine embedded in Matrigel developed into cyst-like structures. **(A,B)** Cells congregated in the cysts of in-orgs (day 3) and were outside the cho-orgs (day 4); after culturing for 10–13 days, the cho-orgs (day 10) and in-orgs (day 13) increased in size, and the in-orgs developed three buds (white arrow) to form a mature structure (bar, 100 μm). **(C)** Mature in-orgs after culturing for 7 days after passage, they were bud-shaped and had aggregated cell clumps in the organoids (bar, 100 μm).

single cystic spheres, as in the case of cho-orgs derived from single cholangiocytes. Besides, the In-orgs were bud-shaped and had aggregated cell clumps in the organoids.

Growth of Organoids

After seeding for 2–4 days, primary stem cells from the cholangiocytes or small intestine started to develop into cyst-like structures (**Figure 3A**). After 10–14 days, the organoids increased in size and matured (**Figure 3B**) and could be passaged at a 1:3–1:4 ratio for further expansion (**Figures 2A**, **3C**).

Characteristics of Cho-Orgs and In-orgs

Cho-orgs expressed the cholangiocytes markers Krt19 (**Figure 4A**) and Krt7 (**Figure 4B**), showed high proliferative activity, and consisted of more than one cell layer (**Figure 4C**).

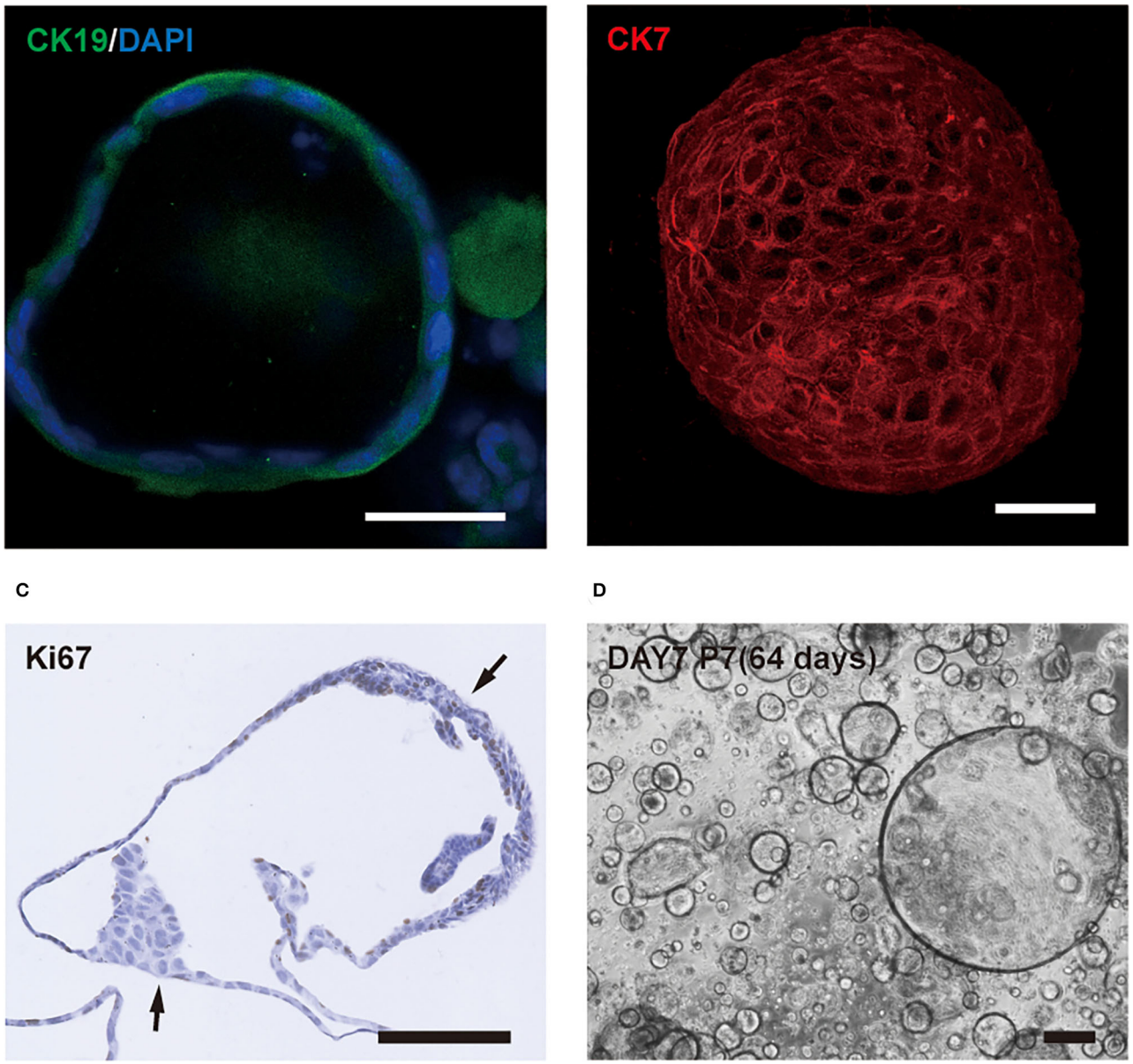

FIGURE 4 | Characteristics of cholangiocytes-organoids (cho-orgs). **(A)** Immunofluorescence staining for the cholangiocyte marker CK19 (green) (bar, 100 μm). **(B)** Immunofluorescence staining for CK7 (red) in a 3D structure (bar, 100 μm). **(C)** The cho-orgs consisted of more than one cell layer and express the proliferation marker Ki67 (bar, 100 μm). **(D)** The cho-orgs from intrahepatic bile ducts were cultured for 7 days after seeding and passaged seven times for a total culture duration of >64 days (bar, 200 μm).

We found a phenomenon that the cho-orgs from the intrahepatic bile ducts grew slower than the cho-orgs from gallbladders. Formation of mature organoids, about 200 nm in size, took only about 5 days for gallbladder-derived bile duct cells compared to the 7-14 days for primary intrahepatic bile duct cells. However, the latter could be passaged for a longer period (**Figure 4D**).

Besides, the in-orgs expressed the enterocyte cell marker CK20 before they matured (**Figure 5A**), and the cell connections were stable as shown in the complete tight junction protein CLAUDIN-1 staining results (**Figure 5B**). In addition, the intestinal permeability of the in-orgs was proved to be normal, and the fluorescent dye did not enter the cavity of the organoid, as identified by FITC-dextran-4KD (FD4) staining analysis (**Figure 5C**).

It is worth mentioning that the digestion time of the organoids in TrypLE Express solution is crucial for the viability of the next generation of organoids during passaging. On

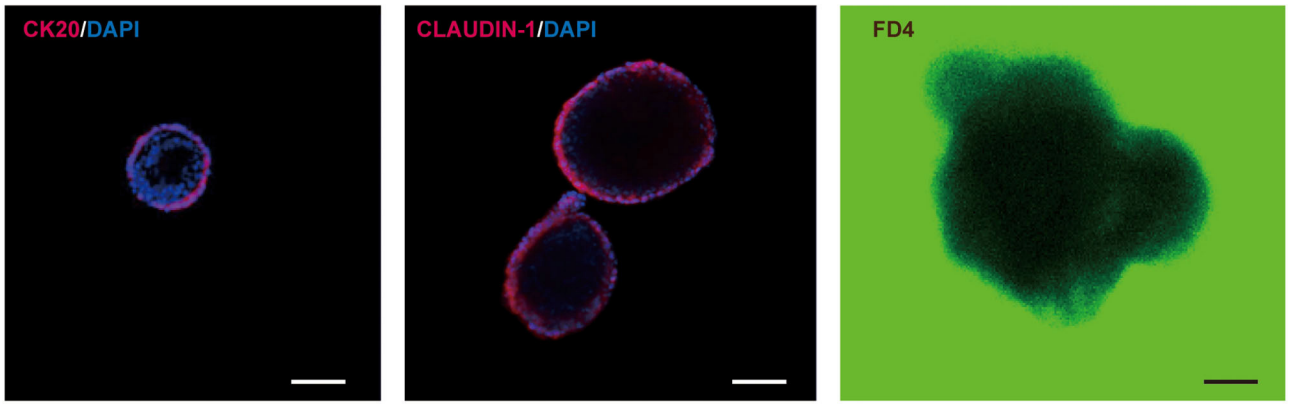

FIGURE 5 | Characteristics of small intestinal organoids (in-orgs). **(A)** Immunofluorescence staining for the enterocyte cell marker CK20 (red) (bar, 100 μm). **(B)** Immunofluorescence staining for the complete tight junction protein CLAUDIN-1 (red) (bar, 100 μm). **(C)** Fluorescent dye FITC-dextran-4KD (FD4) absorbing results, observed with a laser confocal Microscope. The in-orgs are on a green background (bar, 100 μm).

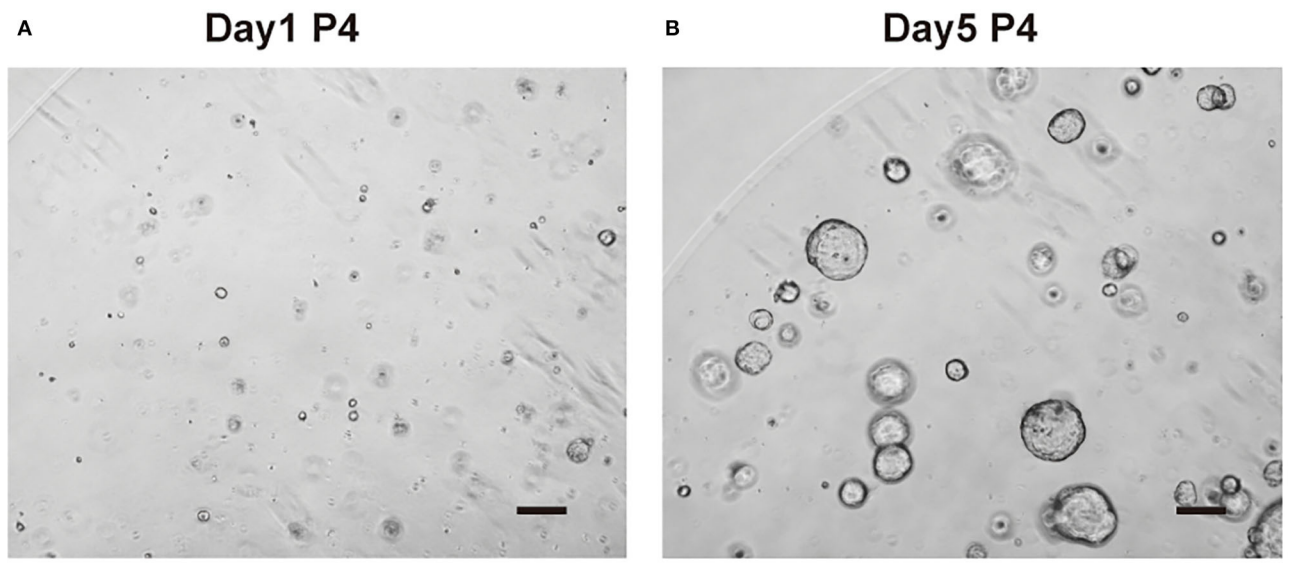

FIGURE 6 | Organoid culture after thawing. **(A)** Cholangiocytes-organoids (cho-orgs) were cultured for 1 day after thawing (bar, 200 μm) (P4, passage 4). **(B)** Cho-orgs are cultured for 5 days after thawing (bar, 200 μm).

the one hand, prolonged incubation time leads to over-digestion and lower cell viability; on the other hand, insufficient digestion can lead to decrease in the number of organoids. Therefore, the digestion situation of organoids should be monitored under a light microscope. The optimum time of digestion can be determined when 80–90% of cells are single cells.

Stable Growth of Cho-Orgs After Cryopreservation and Recovery

The organoids grew stably after thawing, as observed by light microscopy (**Figure 6**). After stable culture, homogenization of growth rate manipulation, the organoids were ready for RNA/DNA isolation, immunochemical staining, cryopreservation, or further experiments.

CONCLUSIONS

Our protocols have high repeatability if tissue dissociation steps are performed correctly, and the composition of medium is precise. Besides, we simplify the cell collection procedure by gravity settling and multistep centrifugation to collect cholangiocytes/bile duct and intestine crypts from mouse liver cholangiocytes or small intestine,

although some unwanted cells may also be collected during the process, which will disappear gradually after 1–2 passages of the organoids. In addition, when experiments are operated properly, the possibility of exposing stem cells to mechanical damage or chemical injury will be avoided. After passaging, cryopreservation, and thawing, the organoids are in a stable growth state and suitable for mechanistic analysis, preclinical research, drug screening or further experiments.

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/s.

ETHICS STATEMENT

The animal study was reviewed and approved by the First Affiliated Hospital, College of Medicine, Zhejiang University (reference number 2020-1088).

AUTHOR CONTRIBUTIONS

WC and HC contributed to the design of the study, method optimization, and writing of the manuscript. WC, QY, and RW contributed to organoid culture and experiments. WC, BF, JC, YX, and JY contributed to method optimization. WC and QY contributed to the production of Figures. LL conducted study supervision. All authors have read and approved the final version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2022.907901/full#supplementary-material

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REVIEW
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Feasibility of Hepatitis C Elimination in China: From Epidemiology, Natural History, and Intervention Perspectives

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Hepatitis C imposes a heavy burden on many countries, including China, where the number of reported cases and the incidence of hepatitis C virus (HCV) increased yearly from 2005 to 2012, with a stable trend after 2012. The geographical distribution of HCV infections varies widely in China, with the northwest and southwest regions and the Henan Province showing a high disease burden. Elderly, men, sexually active people, drug users, migrants, blood transfusion recipients, and renal dialysis patients have become the target populations for hepatitis C prevention and control. It is important to improve the diagnosis rate in high-risk groups and asymptomatic people. Identifying secondary HCV infections, especially in HCV patients co-infected with the human immunodeficiency virus (HIV) is a priority of hepatitis C prevention and control. Enhancing universal access to direct antiviral agents (DAAs) treatment regimens is an effective way to improve the cure rate of HCV infection. For China to contribute to the WHO 2030 global HCV elimination plan, strategic surveillance, management, and treatment program for HCV are needed.

Keywords: hepatitis C, epidemiology, natural history, interventions, elimination

INTRODUCTION

Hepatitis C is a blood-borne disease caused by the hepatitis C virus (HCV), leading to acute or chronic infection. Newly infected individuals are usually asymptomatic. Approximately 30% (15%–45%) of people with acute infection clear the virus by themselves within 6 months, with the remaining 70% (55%–85%) developing chronic hepatitis C (World Health Organization, 2020). HCV infections have led to a serious disease burden. According to the World Health Organization (WHO), around 71 million people worldwide are infected with chronic HCV, and there were 1.75 million new cases of HCV infection worldwide in 2015 (World Health Organization, 2017a). The WHO proposed a hepatitis C elimination plan in 2016 that projects a 90% reduction in new HCV infections and a 65% reduction in deaths globally by 2030 (World Health Organization, 2020). Many countries around the world are working to achieve this goal.

China is actively responding to WHO's plan through the Healthy China Initiative (2019–2030), aiming to increase blood-borne disease testing, high-risk occupational screening, and universal safe sex (The State Council of the People's Republic of China, 2019). However, China still has one of the highest rates of hepatitis C infection in the world (Hajarizadeh et al., 2013; World Health Organization, 2017a). HCV infections showed a slow upward tendency from 2012 to 2017 (Gao et al., 2019). In addition, 9,795,000 HCV infections and 45,300 HCV deaths have been reported in 2016, which were both high prevalence and high mortality rates (World Health Organization, 2017a; CDA Foundation, 2020).

There is no vaccine against HCV and prevention measures rely mostly on screening improvement and health promotion (Gomaa et al., 2017). However, routine medical examinations in China do not include HCV detection, resulting in a very low number of HCV cases detected through active surveillance. Only 18% of people with HCV were diagnosed in China in 2016 (CDA Foundation, 2020). The main measure against HCV is medical therapy. Direct antiviral agents (DAAs) have changed the traditional treatment regimen for HCV infection and have been shown to reduce the disease burden of HCV, thus offering hope for a successful HCV elimination.

Despite their effectiveness, DAAs have not been well implemented in China due to several factors. Therefore, it is critical to further explore effective measures to reduce HCV morbidity and mortality in China and thus contribute to the WHO 2030 HCV Elimination Plan. Previous studies have explored the proportion of asymptomatic and symptomatic individuals with acute and chronic HCV infection. Further studies should elucidate the course of infection in patients to clarify the natural history of HCV infection and to distinguish between acute and chronic stages of HCV infection. This distinction, which can be effective delineation of important populations and segments, can be useful for the elimination of hepatitis C.

FACTORS INFLUENCING THE FEASIBILITY OF HEPATITIS C ELIMINATION IN CHINA

Understanding the epidemiological characteristics of HCV infections, such as population, temporal, and spatial distribution, can help to take targeted prevention and control measures against HCV. In recent years, the incidence of hepatitis C in China has been slowly increasing. In addition, the unbalanced spatial distribution of disease burden and genotypes further increases the challenge of preventing and treating HCV infection. Although the detection of HCV has improved in recent years, the reporting rates in China have not improved significantly (European Association for the Study of the Liver, 2018). A large number (85%–90%) of people with HCV are currently asymptomatic (Ozaras and Tahan, 2009). This phenomenon has led to the majority of HCV-infected patients ignoring them that have hepatitis C, and the detection rate of active HCV is very low. This greatly increases the difficulty of preventing and controlling hepatitis C. Therefore, there is a need to further investigate key areas and populations and propose targeted strategies.

EPIDEMIOLOGICAL CHARACTERISTICS OF HEPATITIS C IN THE MAINLAND OF CHINA

Temporal Distribution

Previous studies found that the number of reported hepatitis C cases in China increased slowly from 1997 to 2003 with an annual increase of 27.89% and rapidly increased from 2004 to 2011 with an average annual increase of 48.79% (Qin et al., 2013). According to the Chinese Center for Disease Control and Prevention, a study found that there was a sharp increase from 52,927 cases in 2005 to 201,622 cases in 2012, with a steady trend from 2012 to 2017 (**Figure 1A**). Previous studies also found consistent with our results (Liu et al., 2018). Furthermore, the number of new cases showed seasonality, with a peak in March every year. The trend in incidence rate was also similar (**Figure 1B**). The number of deaths due to hepatitis C also showed fluctuations with an average of 10 deaths reported per month. According to an epidemiological survey of serum specimen testing in China in 2006, the prevalence of HCV infection in the total population was 0.43%. However, the actual reporting rate for that year was only 5.41 per 100,000 (Chen et al., 2011). Only one in 10 infected individuals may be detected.

Spatial Distribution

The geographical distribution of hepatitis C in China varies widely. The top three provinces with the greatest number of new confirmed cases in 2017 were Guangdong (23,927 cases), Henan (21,446 cases), and Hunan Province (14,241 cases). The three provinces with the lowest new confirmed cases were Ningxia Hui Autonomous Region (803 cases), Tianjin City (784 cases), and Tibet Autonomous Region (58 cases; **Figure 2A**). The geospatial distribution of the incidence and new cases of HCV varies considerably among provinces due to differences in population size. The geographical distribution of incidence rates in China in 2017 showed a trend of high in the northern regions and low in the southern regions. The higher incidence rates concentrated in the northwestern regions of China (including Xinjiang Uyghur Autonomous Region, Qinghai, Inner Mongolia Autonomous Region, Ningxia Hui Autonomous Region, Gansu, and Shaanxi Province). The highest incidence of HCV infection was recorded in the Xinjiang Uyghur Autonomous Region (44.77 cases/100,000 people), while the lowest incidence rate was found in Beijing (3.87 cases/100,000 people; **Figure 2B**). A previous study also found that the highest incidence of hepatitis C was observed in Xinjiang (Moriyama and Rahman, 2018). Sociocultural and economic factors may play a key role in HCV transmission (Zhou and Zhou, 2013; Moriyama and Rahman, 2018); specifically, low socioeconomic conditions, poor public health programs,

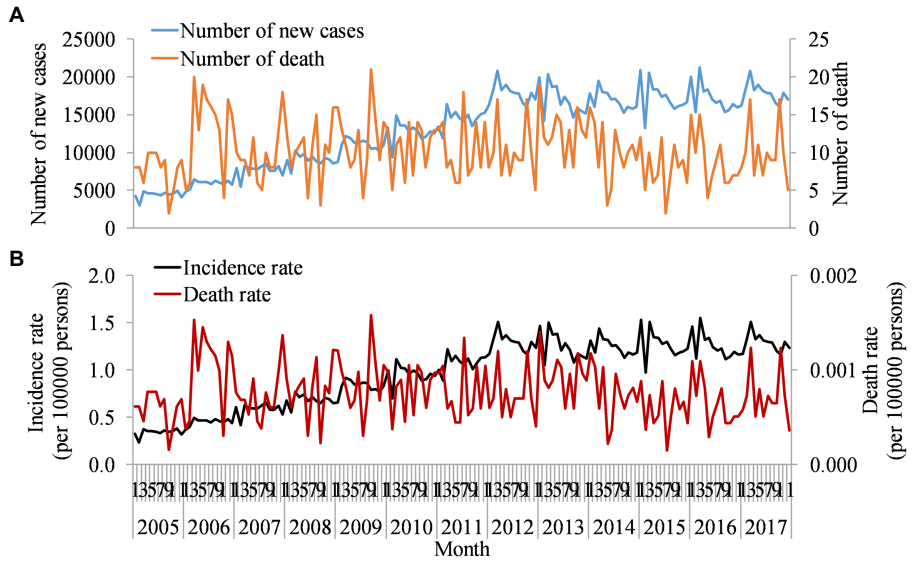

FIGURE 1 | Monthly reported number of new cases, deaths, incidence, and death rates of hepatitis C in China, 2005–2017. Orange line in plot **(A)** used left axis represents new cases, and blue line used left axis represents death cases of hepatitis C virus (HCV) per month reported by Chinese Centre for Disease Control and Prevention (CDC). Plot **(B)** is the rate calculated by new cases and death cases in plot **(A)** after adjusting for population.

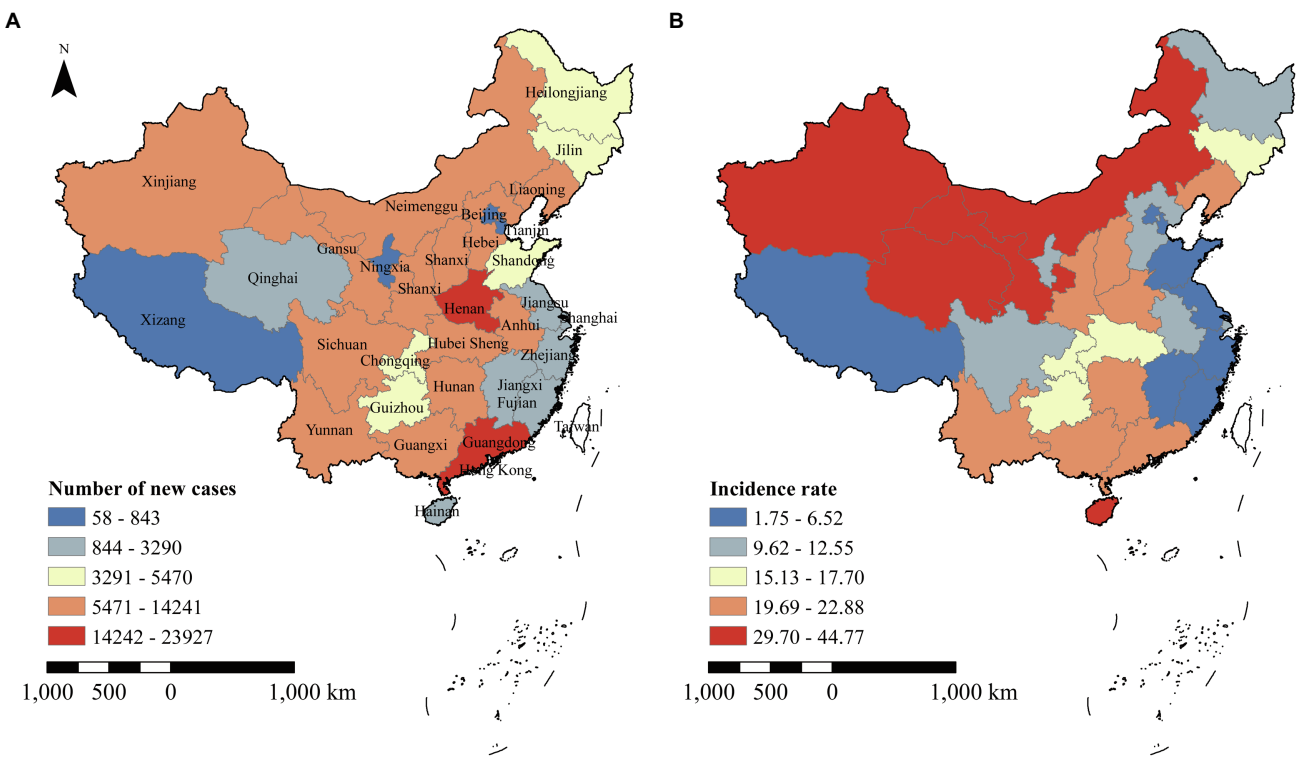

FIGURE 2 | Number of new cases and incidence of HCV infection reported in China in 2017. Plot **(A)** represents the HCV cases reported by China CDC in 2017. Plot **(B)** represents incidence rate calculated by the population of each province in China.

and indigenous customs contribute to an increased burden of HCV (Abdelhakam and Othman, 2018). A previous study found that healthcare access in ethnic minority regions was worse than in the non-minority region (Mali Xia et al., 2014; Wang and Pan, 2016). Some ethnic minorities, located in border areas, may have more drug trafficking and use, potentially contributing

to the high incidence rate of HCV infection (Zhou et al., 2016). Besides, the governments in developed areas may invest more money and resources in healthcare and people's health awareness than in underdeveloped areas (Zhou and Zhou, 2013). There may be urban–rural inequality of opportunity in healthcare in China (Ma et al., 2021). Therefore, the Chinese government should strengthen economically and culturally appropriate, regional-specific interventions to curb the HCV-transmitted infection epidemic, especially in the northwestern and southwestern regions as well as in the Henan Province.

In addition, the regional HCV infection rates among Chinese injecting drug users (IDUs) also varied considerably, with the lowest HCV infection rate among IDUs being 11.43% (Shaanxi) and the highest being 90.77% (Hubei). HCV prevalence is also high in IDUs in the Yunnan Province, Guangxi Zhuang Autonomous Region, Hunan Province, Xinjiang Uygur Autonomous Region, etc. (Xia et al., 2008). The lowest hepatitis C infection rate among non-injecting drug users is 0% (Anhui Province) and the highest is 40.00% (Fujian Province; Bao and Liu, 2009). Another study conducted HCV detection among 2,000 injecting drug users in Yunnan Province and found that 77% of the participants were infected with HCV (Zhou et al., 2012). Furthermore, the prevalence of HCV seropositivity among IDUs in China was the highest in the southwest (77.7%, 95%CI: 69.9%–85.4%), followed by the south (76.2%, 95%CI: 65.9%–86.4%). In terms of specific regions, the Sichuan Province had the highest prevalence of HCV antibodies (91.7%, 86.6%–95.3%), followed by the Guangxi Zhuang Autonomous Region (86.1%, 95%CI: 81.8%–90.4%; Bao et al., 2019).

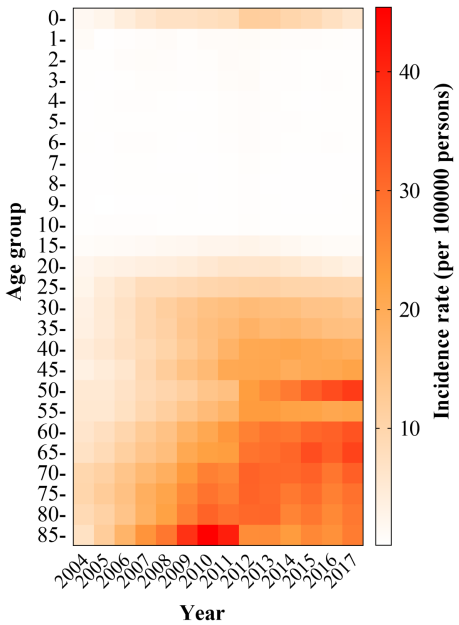

FIGURE 3 | Incidence of reported HCV infection in different age groups in China, 2004–2017. The reported incidence rate by different age groups was obtained from a website (https://www.chinacdc.cn/).

Population Distribution

The age distribution of reported HCV incidence varies. The main characteristic was that HCV incidence tends to affect younger people, while the prevalence remained high in older adults, especially in those aged 60. In 2004, the prevalence was higher in people aged 25–44 and 55 or older, while in 2017 the prevalence was more evenly distributed in all age classes above 25 (**Figure 3**). The incidence of HCV infection in infants (0–1-year-old) rose and then fell, with a peak in 2012 (11.71 cases/100,000) during the 2004–2017 period. Mother-to-infant transmission has become the most common routine of hepatitis C virus infection, with risk factors including titer of HCV RNA, IgM positivity, high viral load, active drug use, and HIV coinfection in the mother (Ohto et al., 1994; Dal Molin et al., 2002; Syriopoulou et al., 2005; Squires and Balistreri, 2017). There was a significant upward trend in prevalence between 2004 and 2012, with a smoothing trend in prevalence among people aged 85 years after 2012. The incidence of hepatitis C was a clear upward trend since 2012 among people aged 50–55, with the highest incidence (37.061 cases/100,000) in 2017. In addition, a previous study has found that the prevalence of hepatitis C generally increases with age (Li et al., 2020). Another study also demonstrated that the seropositivity was correlated with high age (≥60 years; Xu et al., 2019).

A study in the Liaoning Province showed that the seropositivity was significantly higher in men than in women, with detection rates ranging from 0.18 to 2.40% for men and 0.20 to 2.07% for women (Li et al., 2020). One study reported a prevalence ratio of 1.6:1 for men and women (Cornberg et al., 2011). This gender difference is directly related to risk behaviors, such as unprotected sex in men who have sex with men (MSM), sharing syringes, and tattooing (Rao et al., 2019).

Hepatitis C is present in various occupational groups in China, and retirees, farmers, workers, and accounted for the largest proportion (Xin-rong and Qing-long, 2019; Yang et al., 2019; Tian et al., 2020; Wang and Han-bing, 2020). The prevalence among female sex workers ranged from 0.32 to 1.14%, and it was even higher among female sex workers with lower socioeconomic status (Chen et al., 2015). The prevalence was 0.3%–0.5% for male truck drivers and passengers and 0.2% for pregnant women and young students (Wang et al., 2013).

The use of infected syringes is currently one of the main routes of HCV transmission in China. The prevalence was 66.97% among IDUs and 18.30% among non-injecting drug users (Bao and Liu, 2009). A study showed a high disease burden of HCV infection among IDUs in China, with high seropositivity of 71.6% (95%CI: 65.7%–77.6%; Bao et al., 2019). Another study found that there was a 60.1% (95% CI: 52.8%–67.0%) prevalence of hepatitis C among outpatients on methadone maintenance treatment (Zhuang et al., 2012). The prevalence of HCV infection among IDUs in China is higher than that in general drug users. The main reason is certainly the sharing of needles, syringes, or other drug-related paraphernalia, leading to cross-contamination (Midgard et al., 2016; Chinese Society of Hepatology, Chinese Medical Association, and Chinese Society of Infectious Diseases, Chinese Medical Association, 2020). This indicates that effective interventions for the prevention

and control of HCV infection among IDUs should be implemented.

Human immunodeficiency virus and HCV have the same mode of transmission and share common risk factors. Immunocompromised individuals are vulnerable to HCV infection, and high active antiretroviral therapy (HAART) is associated with hepatotoxicity. Therefore, HIV/HCV co-infection is common. Previous studies have found a 24.7% HCV prevalence among HIV-infected individuals (Yu et al., 2020). As of October 2020, a cohort study of HIV patients in Guangxi Zhuang Autonomous Region, China, found that 8.1% co-infected with HCV (Jia et al., 2022). A cross-sectional survey in Yunnan Province reported 6.5% of a total of 5,922 HIV/AIDS cases were infected with HCV (Li et al., 2021). As of October 2021, the number of people living with HIV in China reached 1.14 million and is still increasing (Xian, 2021), implying that there might be 74,100–281,580 cases of HIV/HCV co-infection in China.

Blood transmission is another major route of HCV infection, with a prevalence of 166.56 per 100,000 among first-time blood donors and 15.21 per 100,000 among regular blood donors (Fu et al., 2019). The prevalence of hepatitis C in hemodialysis patients was approximately 10%, which is higher than the general population (Jadoul et al., 2019).

With the transformation of China's economy and the proposal of the National New Urbanization Plan (2014–2020), the orderly growth of the agricultural transfer population has been encouraged. As a result, the scale of domestic migration in China has been constantly expanding. Rural workers began to migrate to urban areas for better employment opportunities. However, a large proportion of them are limited to 3D (i.e., dirty, dangerous, and difficult) occupations. Migrants remain a socially vulnerable group with high health risks. One study indicated that the prevalence of hepatitis C among Chinese internal migrants reached 0.45%, which is 3.8 times higher than in the general population (Zou et al., 2014).

Among other high-risk groups for HCV infection, MSM are represented with a higher prevalence of 0.7%–1.2% than the general population (Chow et al., 2014). HCV prevalence among clandestine sex workers ranged from 0.7 to 0.9% and 0.8 to 0.9% among men attending sexually transmitted disease (STD) clinics (Wang et al., 2013).

Genotypic Distribution

Hepatitis C virus is classified into seven genotypes (HCV 1–7), including 67 established subtypes and 20 provisional subtypes (Messina et al., 2015). Genotype 1 is the most prevalent globally (46.2%) followed by genotype 3 (30.1%). Most Asian countries also display a predominance of HCV-1 and HCV-3 (McOmish et al., 1994). HCV subtypes 1–6 have been identified in China, with 1b, i.e., 62.78% (95% CI: 59.54%–66.02%) and 2a, i.e., 17.39% (95% CI: 15.67%–19.11%) being the two main subtypes (Zhang et al., 2017a). The distribution of genotypes varied greatly across the country (**Figure 4**). The provinces with the highest proportion of 1a subtypes were Tianjin (84%), Shanghai (80%), Sichuan (79%), Henan (79%), and Hubei (74%).

FORMS OF HEPATITIS C

Hepatitis C is divided into two forms, i.e., acute and chronic hepatitis C. However, asymptomatic people remain the main source of contamination whatever the form might be. Therefore, it is essential to analyze the transmission of HCV caused by asymptomatic carriers (**Figure 5**). As already highlighted, HCV is mainly transmitted through used syringes and needles, reuse of medical equipment, blood transfusions, and unprotected sexual contact (World Health Organization, 2020). However, mother-to-child transmission is rare. Acute hepatitis C is defined as the first 6 months after initial infection (Hajarizadeh et al., 2013), when HCV is detectable in peripheral blood in susceptible individuals after 1–3 weeks of exposure (Farci et al., 1991). The viral titer will reach a peak after 1–2.5 months (Liu et al., 2012). Viremia is also high at this stage. About 10%–15% of infected individuals become symptomatic after an incubation period of 2 months (range: 1–3 months). Symptoms last for 2–12 weeks and are usually mild and mainly non-specific such as drowsiness and muscle pain, but jaundice may also occur (Hoofnagle, 1997; Maheshwari et al., 2008). Symptomatic acute patients are easily detected, but the majority (85%–90%) of infections is asymptomatic (Ozaras and Tahan, 2009). Therefore, misdiagnosis is a frequent occurrence. Approximately 10%–15% of asymptomatic patients, as well as 25%–50% of symptomatic patients, will experience spontaneous clearance of HCV within the first 3 months (Maheshwari et al., 2008; Ozaras and Tahan, 2009). Spontaneous clearance of hepatitis C virus is more likely to occur in younger age groups and children (Corey et al., 2006; Micallef et al., 2006). Spontaneous clearance of hepatitis C virus infection is approximately twice as likely in women as in men (Kong et al., 2014; Xiong et al., 2017).

Hepatitis C chronicity is defined as the persistence of the hepatitis C virus in the bloodstream for more than 6 months. About 55%–85% of acute infections will evolve into a chronic stage (Chinese Society of Hepatology, Chinese Medical Association, and Chinese Society of Infectious Diseases, Chinese Medical Association, 2020; World Health Organization, 2020). Both asymptomatic (85%–90%) and symptomatic (50%–70% of infected individuals) may develop chronic hepatitis C after 4 months (range: 3–5 months) (Maheshwari et al., 2008; Ozaras and Tahan, 2009). Most chronic hepatitis C patients are also asymptomatic, making this population significant for the impact of hepatitis C transmission. A very small percentage of chronic hepatitis C patients are self-clearing, but recovery rates of 71%–100% can be achieved after 2–3 months of treatment (Grebely et al., 2011). However, patients who develop severe lesions may die from the disease. Although HCV infection can be spontaneously cleared and recovered with treatment, the risk of secondary infection remains, with the likelihood of secondary infection varying from 0 to 6% (Martinello et al., 2017). In addition, patients coinfected with HIV and HCV are at greater risk of secondary infection (3%–15%; Westbrook and Dusheiko, 2014; Martinello et al., 2017).

The parameters of the natural history of HCV in China are also consistent with those estimated by other countries. In China, approximately 40 million people are infected with

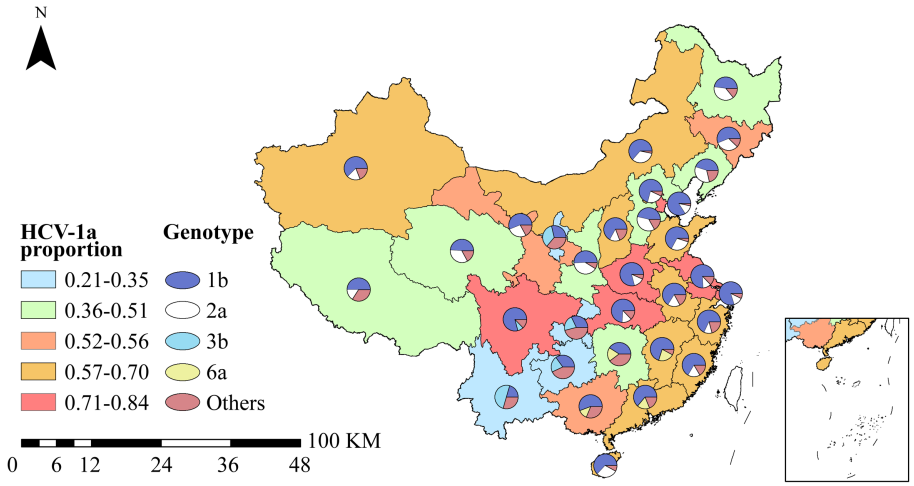

FIGURE 4 | Distribution of HCV genotypes in China. The color of map background adopts a square legend, indicating proportion genotype 1a of HCV to all genotype in each province. Pie charts represent the proportion of five genotypes of HCV in each province.

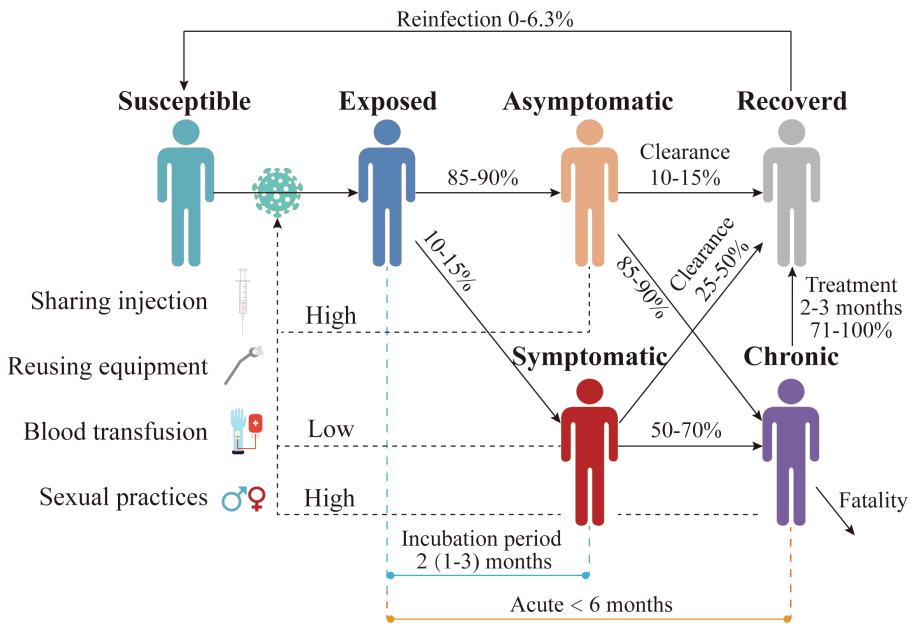

FIGURE 5 | Natural history of HCV infection in humans. People with different colors indicate the infection status of HCV. The solid lines represent the transition of each status, and the numbers above them represent the transition ratio or time. The dotted lines indicate the main transmission routes. "High" means the main transmission route, and "low" means the secondary route.

HCV (Li et al., 2012), and it is estimated that 50%–85% of all individuals infected with HCV develop chronic hepatitis. Another study indicated that the proportion of acute patients who are transformed into chronic patients is about 82.62% (Jia et al., 2019). In Hebei Province of China, a 9-year follow-up of hepatitis C virus infection study found that 12 (8.4%) of 142 cases were negative for both HCV RNA and anti-HCV (Fan et al., 2004). Another study showed that 92 in 416 cases (22.44%) would occur spontaneous clearance after at least 6 months (Kong et al., 2014). Meanwhile, the spontaneous clearance of HCV in 100 HCV-infected Chinese blood donors was 24.0% in China (Liu et al., 2013). A study reported that the mean incubation period of 99 HCV-infected patients was close to 31–45 days in a county of China. The Chinese guidelines for the prevention and treatment of hepatitis C summarized the rate of sustained virologic response (SVR) in hepatitis C patients after 12 weeks of DAAs treatment and found that most DAAs were highly effective (more than 85%; Chinese Medical Association and Chinese Society of Infectious Diseases, 2019). Furthermore, a study showed that an intention-to-treat analysis

of a real-life observational study revealed more than 90% of SVR rates of 192 patients in China (Zeng et al., 2017).

PREVENTION AND CONTROL STRATEGIES IN CHINA

There is no effective vaccine to prevent HCV infection. According to the HCV infection prevention and control guidelines in 2019, the main prevention and control measures for hepatitis C include screening and management, strict screening of blood donors, prevention of medical and broken skin-mucous membrane transmission, prevention of sexual transmission, prevention of mother-to-child transmission, and active treatment and management of infected individuals (Chinese Society of Hepatology, Chinese Medical Association, and Chinese Society of Infectious Diseases, Chinese Medical Association, 2020).

HCV Infection Prevention and Control Policy in China

China has adopted some active strategies to strengthen HCV infection prevention and control. HCV infection elimination has been included in the "Healthy China 2030" plan (The State Council of the People's Republic of China, 2016). China has established HCV Infection Elimination Alliance in 2017, and the established pilots to conduct free screening for HCV in high-incidence counties (People 's Daily Online, 2017). The government also issued guidelines for HCV infection prevention and treatment in 2019. This series of policies emphasizes the determination and efforts to eliminate HCV infection in China.

Early Screening for HCV

Early screening for HCV infection is very important and is currently focused on high-risk groups, such as drug users, blood transfusion recipients, and renal dialysis patients (Alter and Margolis, 1998). The diagnosis rate of HCV in China is low. In 2016, only 18% of HCV-infected people were diagnosed, which is significantly lower than the WHO target of 72% diagnosis rate (Virtual Community of Hepatitis C, 2017). One study advocates that the entire general population should be screened for HCV as screening is still lacking even in high-risk populations (Mak et al., 2018). In addition, the presence of a large proportion of asymptomatic infected individuals further increases the difficulty of efficiently screening for HCV (Ozaras and Tahan, 2009).

Positive Treatment and Management of HCV Infection

Although early screening can greatly reduce the HCV transmission, the prevention and control of HCV should still be based on active treatment. Most studies used SVR to assess the effectiveness of the treatment (Miriam and Harold, 1998). DAAs are considered ideal treatment for people with chronic HCV infection (SVR > 90%; Virtual Community of Hepatitis C, 2017). The WHO recommended that all patients over 18 with chronic hepatitis should be treated with DAAs (World Health Organization, 2016). DAAs therapy cures most people with HCV infection within a short period of time (usually 12–24 weeks; Mak et al., 2018). According to WHO, about 5 million people were treated with DAAs as of 2017, but this was still far from the global goal of achieving 80% treatment by 2030 (Ozaras and Tahan, 2009).

The genotypes of hepatitis C in China are mainly type 1 and type 3. According to the new Chinese guidelines of hepatitis C, the SVR12 rates for genotypes 1a, 1b, 2, 3a, 3b, and 6 were 100, 100, 100, 95, 76, and 99%, respectively, after 12 weeks of treatment with sofosbuvir/velpatasvir (Chinese Medical Association and Chinese Society of Infectious Diseases, 2019). There is no vaccine to prevent HCV, but combination therapy with DAAs can cure more than 95% of chronic HCV (Chinese Hospital Infection Control Branch of Preventive Medicine, Chinese Society Branch of the Medical Association, Chinese Society Prevention and Control Branch of Preventive Medicine, 2021). In general, DDAs are very effective against hepatitis C, with most drugs achieving and SVR rate of more than 80% after 12 weeks of treatment. Most DAAs are already approved in China. China's HCV elimination plan in September 2021 also identifies a cure rate of more than 95% of HCV patients within 10 years (China Liver Health, 2021).

The Management Systems of Hepatitis C Prevention and Treatment in China

To widely promote the screening and management of HCV-infected patients, several platforms have been established in China using information technology, such as the HCV community-self-management system for patients with liver disease, and the HCV Screening and Management Information Platform of Shengjing Hospital of China Medical University (Virtual Community of Hepatitis C, 2016; Wang et al., 2018). The HCV Screening and Management Information Platform integrates screening, alerting, reporting, and management intervention functions. These platforms combined with hospital information systems, help achieve regulatory tracking of HCV-infected patients and improve management efficiency.

Other Measures for HCV Infection Prevention and Treatment

Other prevention and control measures have been implemented to further reduce the incidence of hepatitis C, such as the popularization of condoms, the strict control of nosocomial infections, the enhanced management and health education for HCV-infected patients. It is vital to increase public awareness of the prevention and control of hepatitis C (Chinese Society of Hepatology, Chinese Medical Association, and Chinese Society of Infectious Diseases, Chinese Medical Association, 2020).

SUGGESTIONS FOR THE IMPROVEMENT OF THE PREVENTION AND CONTROL OF HCV IN CHINA

China still has a long way to go to achieve the WHO goal of hepatitis C elimination by 2030. China has proposed a work program for the elimination of hepatitis C from 2021

to 2030, with seven key tasks, including strengthening HCV awareness and education, providing comprehensive interventions for key populations, enhancing standardized treatment and improving treatment rates, strengthening medicine supply, establishing and improving information management systems, and increasing detection capacity and implementing medical insurance policies (Health Commission of Hebei Province, 2021). To further reduce the burden of hepatitis C in China, we make the following recommendations (**Table 1**).

1. Screening for HCV should be conducted in the general population, to increase the diagnosis rate and thus improve the effective control of HCV. For example, the American CDC recommended a one-time HCV screening for all individuals born between 1945 and 1965 (AASLD/IDSA HCV Guidance Panel et al., 2015). Similar action should be implemented. In addition to increasing screen rates based on risk factors, prenatal screening of pregnant women also needs to be reinforced (World Health Organization, 2017b).
2. Hepatitis C virus epidemic is usually exacerbated by the transmission of the virus among those who are vulnerable and at high risk, such as drug users, sex workers, the MSMs, and minorities. The optimal, cost-effective intervention comprises screening of vulnerable populations, ensuring that all individuals newly diagnosed with HCV receive DAA (Heffernan et al., 2021). We recommended strengthening the screening in vulnerable and high-risk populations. Screening of people at high risk for HCV could more effectively identify people with HCV infection and thus provide an entry point for them to access care, treatment, and support. It might be useful to develop mathematical models and AI systems to prioritize more vulnerable and high-risk populations (gender, age class, occupation, comorbidities, genotype, etc.). Further studies could provide reasonable screening protocols, such as the comprehensive screening program recommended in the United States for high-risk age groups.
3. The long-term regular management and follow-up of HCV-infected patients also need to be further strengthened. Health managers may consider improving national HCV surveillance management system to guarantee the attendance and recovery rate of HCV-infected patients.
4. Although efficient therapies for HCV already exist, they are expensive in certain cases (Li et al., 2019). Treatment costs need to be reduced and DAAs should be considered for inclusion in health insurance.
5. Some scholars have noted that integrating HCV healthcare and surveillance systems, strengthening primary care level, and coordinating government initiatives are essential in providing effective HCV preventive measures and treatments (Chow et al., 2014; Sun et al., 2021). Disparities in healthcare resources across the provinces increased following the economic reform, and the socioeconomic status is associated with healthcare resources (Bakkeli, 2021). The eastern regions with better economic status and more healthcare investment have high-quality healthcare resources (Guo et al., 2021). Residents from the affluent eastern regions are more likely to use preventive care than those from the central and northeastern regions (Huang et al., 2016). Rural residents have significantly less access to healthcare services than urbanites (Zhu et al., 2017; Zhang et al., 2017b). Thus, regional-specific strategies should be proposed and conducted to prevent HCV infections and manage HCV patients, based on local HCV prevalence, government healthcare funding, and disparities in healthcare access.
6. To achieve hepatitis C elimination, hepatitis C prevention and control strategies have been proposed and implemented in most regions of China. In accordance with China's HCV prevention and control strategy, some provinces with high incidence rates had further strengthened prevention and control. For example, Guangdong has increased its emphasis on mobile big data to implementation of education and monitoring. Henan emphasized the role of health education and opinion leaders. Several provinces have initiated sentinel surveillance for HIV and hepatitis C. As of November 2020, Qinghai Province has established 21 national sentinel sites, covering basically all provinces for all types of high-risk populations (The People's Republic of China, 2020). Zhejiang Province decided to launch basic medical insurance for hepatitis C (antiviral treatment) outpatient medical expenses to be paid by disease type in 2019 [Notice on Carrying out Basic Medical Insurance Hepatitis C (Antiviral Treatment) Outpatient Zhejiang Provincial Medical Security Bureau, 2019]. Tianjin launched a capitation payment scheme for

TABLE 1 | Recommendations to further reduce the burden of hepatitis C in China.

Screening and management	Strengthen the screening of hepatitis C in high-risk groups to improve the awareness of prevention and treatment of the general population.
	Testing of donated blood.
	(1) High-risk populations. We recommended that health agencies emphasized testing in vulnerable populations, such as drug users, sex workers, and minorities.
	(2) Increase testing rates in the general population.
	Closed-loop management of screen-positive patients through a multi-sectoral collaborative screening and referral pathway for HCV infection.
Treatment	Three DAAs had been covered in national health insurance, including glatavir/elbasvir (genotype 1/4), sofosbuvir/velpatasvir (genotypes 1–6), and sofosbuvir/ledipasvir (genotype 1/4/5/6).
Healthcare setting	Hand hygiene: including surgical hand preparation and hand.
	Safe cleaning of equipment.
	Safe handling and disposal of sharps and waste.
	Improved access to safe blood.
	Reducing the exposure of the newborn to maternal blood to prevent mother-to-child transmission.
Health education and training of health personnel	Prevention of sexual contact transmission. (1) Managed MSM and people with multiple partners. (2) Universal condom use. (3) Provided proper sex education to adolescents.
	Counseling patient education on prevention and control of HCV to improve diagnosis and treatment rates.
	Regular training on the topic of occupational exposure to HCV infection.

outpatient medical institutions for hepatitis C and a per-case payment scheme for outpatient medical institutions (The People's Republic of China, 2018).

CONCLUSION

This review elaborated the feasibility of hepatitis C elimination in China from an epidemiological, natural history, and intervention perspectives. From 2005 to 2012, the incidence of HCV in China showed an increasing trend, with a stable trend after 2012. The disease burden of HCV is usually heavy among those vulnerable and high-risk populations such as drug users, sex workers, the MSMs, minorities, etc. By interpreting the natural history of hepatitis C, we suggest that the focus should be on transmission from asymptomatic populations. Improving the diagnosis rates, treatment, management, and health education in high-risk populations and asymptomatic individuals is essential to slow or even curb the HCV epidemic. The country should pay more attention to establishing sentinel sites for HCV to observe the epidemic and evaluate interventions. The spatial distribution of hepatitis C incident rates indicates regional disparities, with the higher incidence rates concentrated on the northwestern part of China. Several factors such as health care, socio-economy, and customary practices, may contribute to differences in hepatitis C prevalence, screening, and treatment. The eastern regions with better economy may spend more on health care. Therefore, regional-specific surveillance and management system should be established to better prevent and control the transmit of HCV.

AUTHOR CONTRIBUTIONS

TC and RF conceptualized and designed the study. ZZ and MC prepared the manuscript and figures. ZZ, MC, YG, SY, and GA contributed to literature search. TC, RF, YG, SY, and GA contributed to the revision and reviewed the final manuscript. All authors contributed to the article and approved the submitted version.

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Serum Pregenomic RNA Combined With Hepatitis B Core-Related Antigen Helps Predict the Risk of Virological Relapse After Discontinuation of Nucleos(t)ide Analogs in Patients With Chronic Hepatitis B

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Background and Aim: Cessation of nucleos(t)ide analogs (NAs) therapy in patients with chronic hepatitis B (CHB) is uncommon. Although criteria for discontinuation appear in some guidelines, the indicators for assessing discontinuation of NAs are limited, whether NAs can be safely ceased remains a difficult clinical issue. Our study aimed to investigate the role of serum pregenomic RNA (pgRNA) and hepatitis B core-related antigen (HBcrAg) at the end of treatment (EOT) in guiding the safe discontinuation of NAs in CHB patients.

Methods: This is a retrospective study, clinical data of all CHB patients who discontinued NAs treatment at West China Hospital between June 2020 and January 2021 were collected, including EOT pgRNA, HBcrAg, hepatitis B surface antigen (HBsAg), etc. All patients should meet the Asian-Pacific guideline for discontinuation. Observing virological relapse (VR) rates during 1 year of NAs discontinuation and analyzing the relationship between EOT pgRNA, HBcrAg, and VR.

Results: A total of 64 patients were enrolled in this study and 33 (51.5%) patients experienced VR in 1 year. EOT pgRNA positivity (OR = 14.59, $p = 0.026$) and EOT higher HBcrAg levels (OR = 14.14, $p = 0.001$) were independent risk factors for VR. The area under the receiver-operating characteristic (AUROC) value of EOT HBcrAg for VR was 0.817 ($p < 0.001$), optimal cut-off value was 3.3 log10 U/mL. Patients with EOT pgRNA positivity and EOT HBcrAg >3.3 log10 U/mL were more likely to experience VR after discontinuation of NAs (88.9 vs. 45.5%, $p = 0.027$).

Conclusion: According to current guidelines, a higher VR rate occurs after cessation of NAs. EOT pgRNA positivity and higher HBcrAg level carries a higher risk of VR. Combining these novel markers can better help us assess whether patients can safely cease NAs treatment.

Keywords: chronic hepatitis B, discontinuation, hepatitis B core-associated antigen, nucleos(t)ide analogs, pregenomic RNA

INTRODUCTION

HBV-infected patients are at increased risk of cirrhosis, liver failure and hepatocellular carcinoma (HCC), nearly one million people die each year from HBV-related end-stage liver disease and its complications (Cooke et al., 2019). Due to the presence of intrahepatocellular covalently closed circular DNA (cccDNA), a long course of antiviral therapy is required for chronic hepatitis B (CHB) patients. Long-term nucleos(t)ide analogs (NAs) therapy can improve liver histology and reduce the risk of liver-related complications through sustained viral suppression. However, the financial burden, patient compliance, and drug side effects have made the question of whether NAs therapy can be discontinued in CHB patients become a hot clinical issue.

Although criteria for discontinuation of NAs appear in some guidelines, the indicators for assessing discontinuation are limited. The ideal endpoint for NAs therapy in patients with CHB is the loss of hepatitis B surface antigen (HBsAg), but it can only be achieved in a very small number of patients and requires decades of treatment (Chevaliez et al., 2013). To address this issue, the Liver Association guidelines suggest alternative endpoints to NAs therapy (Liaw et al., 2012; Terrault et al., 2016). The Asian-Pacific guideline recommend that in hepatitis B e antigen (HBeAg) positive patients, NAs treatment can be stopped when HBeAg seroconversion with undetectable HBV DNA has been maintained for at least 12 months. In HBeAg-negative patients, the treatment discontinuation can be considered if patients have been treated for at least 2 years with undetectable HBV DNA documented on three separate occasions 6 months. Following Asian-Pacific guideline, studies have demonstrated that very high rates of virological relapse (VR) after discontinuation and recommended continued NAs treatment (Chaung et al., 2012; Chi et al., 2015; Seto et al., 2015). Hence, we need comprehensive indicators to guide safe discontinuation of NAs treatment for CHB patients.

Hepatitis B core-related antigen (HBcrAg) and pregenomic RNA (pgRNA) are two novel serological indicators for patients with CHB. Studies have shown that HBcrAg is a surrogate marker of both intrahepatic cccDNA and its transcriptional activity (Testoni et al., 2019). pgRNA is a direct transcription product of HBV cccDNA, and is further reverse transcribed by the reverse transcriptase activity of the polymerase into a new relaxed circular DNA (rcDNA) genome, eventually leading to replenishment of the cccDNA pool and formation of HBV DNA-containing virions (Lin et al., 2020). NAs act mainly on reverse transcriptase and do not interfere with pgRNA synthesis, so pgRNA is a good indicator of cccDNA activity in the liver (Trépo et al., 2014). The new serum biomarkers pgRNA and hepatitis B core-associated antigen (HBcrAg) have a role in quantifying cccDNA (Ghany et al., 2021), so they can be used to evaluate the risk of viral reactivation after discontinuation of NAs.

In this study, we established a retrospective cohort to examine VR rates up to 1 year after discontinuation of NAs in CHB patients and to explore the factors associated with successful discontinuation of NAs treatment, particularly end of treatment (EOT) HBsAg, pgRNA, and HBcrAg levels.

MATERIALS AND METHODS

Patients

This is a retrospective study, the study subjects were all CHB patients who discontinued NAs treatment at West China Hospital of Sichuan University between June 2020 and January 2021, including those who were lost to follow-up. HBeAg-positive and HBeAg-negative patients who meet the Asia-Pacific guidelines of NAs discontinuation criteria are eligible for inclusion. HBeAg positive patients were required to achieve HBeAg seroconversion and undetectable HBV DNA for at least 12 months of consolidation therapy. HBeAg negative patients were required to achieve undetectable HBV DNA and then at least 18 months of intensive treatment (Liaw et al., 2012; Terrault et al., 2016). Additional eligibility criteria were age $\geq$18 years, HBsAg positivity and undetectable HBV DNA at the time of NAs discontinuation. Exclusion criteria included coinfection with hepatitis C virus, hepatitis D virus, or human immunodeficiency virus, immunocompromised status or malignancy, autoimmune liver disease, alcohol abuse, previous history of liver transplantation, and other severe or active disease. History of decompensated liver disease or presence of decompensated cirrhosis (**Figure 1**).

This study was conducted in accordance with the 1975 Declaration of Helsinki. The study protocol was approved by the West China Hospital Ethics Committee, and informed consent was obtained from each patient.

Follow-Up, Endpoint

When NAs treatment was discontinued, each patient was tested for pgRNA, HBcrAg and other indicators. Patients were followed up every 3 months after discontinuation for a minimum of 12 months or VR, with biochemical and virological tests performed at each follow-up visit. The primary study endpoint was VR within 1 year of discontinuation, defined as HBV DNA levels >2,000 IU/mL with or without elevated alanine aminotransferase (ALT). Patients with VR had been restarted on NAs therapy and were managed according to CHB guidelines.

Laboratory Testing

Serum biochemical indexes were measured according to standard procedures (Olympus AU5400, Olympus Corporation, Tokyo, Japan). Serum HBsAg levels were quantitatively measured using an Elecsys® HBsAg II Quant Assay (Roche Diagnostics, Penzberg, Germany). Serum HBeAg status was assessed using electrochemiluminescence immunoassay (Roche Diagnostics, Indianapolis, IN, United States). Serum HBV DNA concentration was quantitatively determined using a Cobas TaqMan assay kit (Roche Diagnostics, Branchburg, NJ, United States), with a lower limit of detection of 20 IU/mL.

Serum Pregenomic RNA Assay

The pgRNA was detected by RNA simultaneous amplification testing method (HBV-SAT) based on real-time fluorescence detection of isothermal RNA amplification using HBV-SAT kit (Shanghai Rendu Biotechnology Co, Ltd., China) according to the manufacturer's recommendations. The detailed procedure for serum pgRNA measurement has been described in detail in our previous article (Chen et al., 2019). The linear range was established by testing panels of armored HBV RNA diluted in HBV-negative human serum. The linear concentration ranged from 2 to 8 log copies/mL. The R^2 value

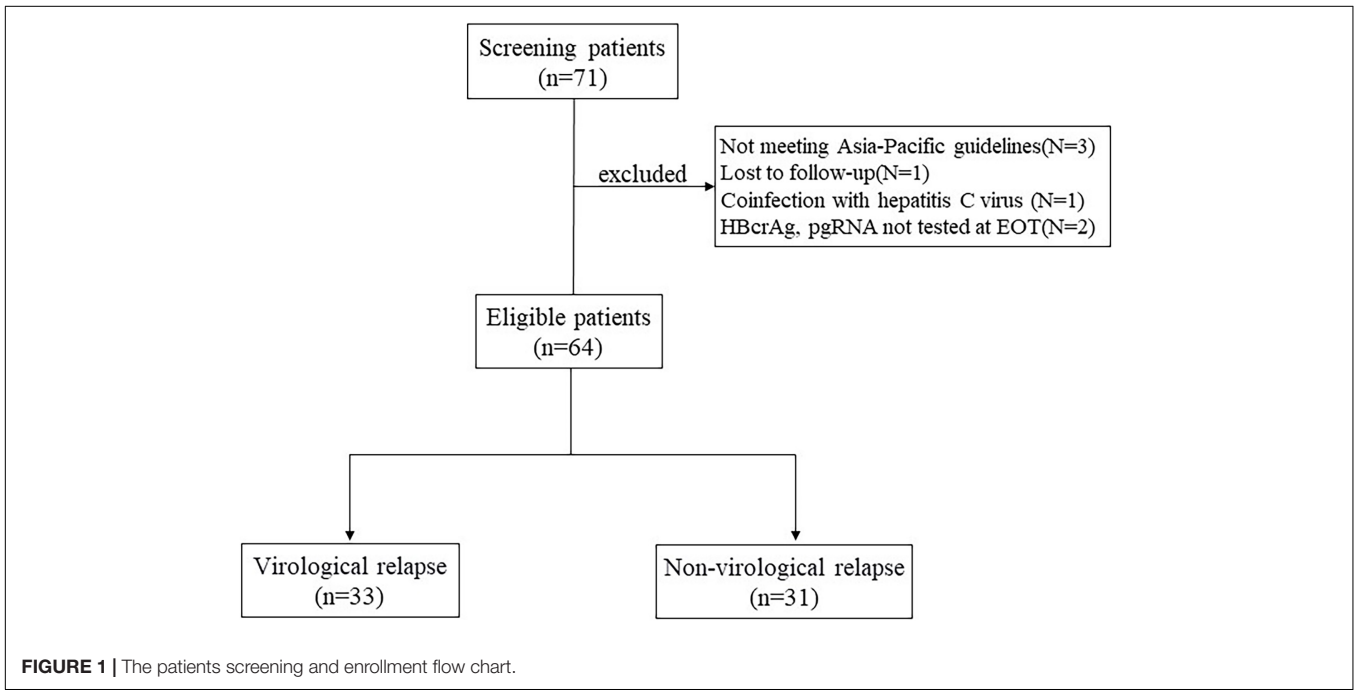

FIGURE 1 | The patients screening and enrollment flow chart.

TABLE 1 | Patient characteristics according to baseline HBeAg status.

Factors	HBeAg positive (*N* = 36)	HBeAg negative (*N* = 28)	*P-value*
Start of treatment			
Age, years	47.28 ± 6.86	47.39 ± 6.45	0.946
Gender, male/female, *n* (%)	19 (52.8)/17 (47.2)	13 (46.4)/15 (53.6)	0.614
Family history of HBsAg with/not *n* (%)	20 (55.6)/16 (44.4)	16 (57.1)/12 (42.9)	0.889
ALT IU/L	167 (139–216)	190.5 (149–262)	0.180
HBV-DNA log10 IU/mL	7.34 ± 0.14	5.56 ± 0.15	<0.001
HBsAg log10 IU/mL	3.97 ± 0.05	3.71 ± 0.07	0.007
End of treatment			
ALT IU/L	22.31 ± 1.46	20.93 ± 1.36	0.505
HBsAg log10 IU/mL	3.41 (2.97–2.47)	3.05 (2.77–3.42)	0.046
HBcrAg log10 U/mL	3.34 ± 0.10	3.08 ± 0.09	0.072
pgRNA positive/negative, *n* (%)	4 (14.3)/32 (85.7)	8 (22.2)/20 (77.8)	0.420
Duration of treatment (month)	72.44 ± 3.85	51.42 ± 2.74	<0.001
1 year after withdrawal			
HBeAg changes from negative to positive yes/no *n* (%)	13 (36.1)/23 (63.9)	0/28 (100)	<0.001
VR/non-VR *n* (%)	21 (58.3)/15 (41.7)	12 (42.9)/16 (57.1)	0.219
ALT >40 IU/L	12 (33.3)/24 (66.8)	9 (32.1)/19 (67.9)	0.920

TABLE 2 | Characteristics comparison between the patients in the VR and non-VR groups.

Factors	All (*n* = 64)	VR (*n* = 33)	Non-VR (*n* = 31)	*P-value*
Age, years	47.33 ± 6.36	47.57 ± 7.52	47.06 ± 5.64	0.759
Gender male /female, *n* (%)	32 (50)/32 (50)	19 (57.6)/14 (42.4)	13 (41.9)/18 (58.1)	0.211
Family history of HBsAg with/not *n* (%)	36 (56.3)/28 (43.7)	18 (54.5)/15 (45.5)	18 (58.1)/13 (41.9)	0.777
Start of treatment				
ALT IU/L	188.73 ± 71.48	187.63 ± 70.9	189.90 ± 73.2	0.900
HBV-DNA log10 IU/mL	6.56 ± 1.21	6.68 ± 1.09	6.43 ± 1.33	0.411
HBsAg log10 IU/mL	3.86 ± 0.39	3.87 ± 0.34	3.85 ± 0.43	0.855
HBeAg positive/negative, *n* (%)	36 (56.3)/28 (43.7)	21 (63.6)/12 (36.4)	15 (48.4)/16 (51.6)	0.219
First-line agents/others, *n* (%)	33 (51.6)/31 (48.4)	16 (48.5)/17 (51.5)	17 (54.8)/14 (45.2)	0.611
End of treatment				
Duration of treatment (month)	60 (42.75–83.25)	63 (42–85.5)	60 (45–81)	0.397
ALT IU/L	21.70 ± 8.1	21.84 ± 8.14	21.55 ± 8.20	0.884
HBsAg >100/≤100 IU/mL, *n* (%)	57 (89.1)/7 (10.9)	31 (93.9)/2 (6.1)	26 (83.9)/5 (16.1)	0.197
HBcrAg log10 U/mL	3.3 (2.8–3.57)	3.4 (3.3–3.8)	2.8 (2.6–3.2)	<0.001
HBcrAg >3.3/≤3.3 log10 U/mL *n* (%)	33 (51.6)/31 (48.4)	26 (78.8)/7 (21.2)	7 (22.6)/24 (77.4)	<0.001
pgRNA positive/negative *n* (%)	12 (18.8)/52 (81.3)	11 (33.3)/22 (66.7)	1 (3.2)/ 30 (96.8)	0.002
pgRNA positive+ HBcrAg >3.3 log10 U/mL yes/no *n* (%)	9 (14.1)/55 (85.9)	8 (88.9)/25 (45.5)	1 (11.1)/30 (54.5)	0.027
1 year after withdrawal				
ALT >40 IU/L	21 (32.8)/43 (67.2)	21 (63.6)/12 (36.4)	0 (0)/31 (100)	<0.001
HBeAg changes from negative to positive yes/no *n* (%)	13 (20.3)/51 (79.7)	13 (39.4)/20 (60.6)	0 (0)/31 (100)	<0.001
HBV-DNA log10 IU/mL		5.85 ± 1.32		

TABLE 3 | Risk factors for virological relapse after discontinuation of NAs.

Univariate analysis				Multivariable analysis		
Parameter	**OR**	**95% CI**	***p***	**OR**	**95% CI**	***p***
Age, per year	1.01	(0.93–1.09)	0.750			
Sex (male)	1.88	(0.70–5.07)	0.213			
Family history of HBsAg	0.87	(0.32–2.33)	0.777			
First-line drugs	0.78	(0.29–2.07)	0.611			
Start of treatment						
HBeAg positive	1.87	(0.69–5.07)	0.221			
HBV-DNA, per log10 IU/mL	1.19	(0.79–1.80)	0.405			
HBsAg, per log10 IU/mL	1.13	(0.32–4.03)	0.852			
End of treatment						
Duration of treatment (month)	1.01	(0.96–1.03)	0.515			
HBcrAg per log10 U/mL	15.91	(3.76–70.72)	<0.001	14.14	(3.08–65.03)	0.001
HBcrAg >3.3 log10 U/mL	12.73	(3.89–41.67)	<0.001			
PgRNA positive	15.00	(1.80–124.93)	0.012	14.59	(1.38–153.89)	0.026

of linear equation is more than 0.95. The limit of detection is 50 copies/mL.

Serum Hepatitis B Core-Related Antigen Assay

The serum HBcrAg level was quantitatively measured using the fully automated CLEIA system (Fujirebio Inc., Tokyo, Japan), and the detailed process of serum HBcrAg measurement is as previously reported (Nassal, 2015). Since the assay's validated measurement range is from 100 U/mL (2 log10 U/mL) to 10,000,000 U/mL (7 log10 U/mL), serial dilutions of the serum sample are required when serum HBcrAg level is above the detection limit.

Statistical Analysis

Continuous numerical variables are expressed as the mean ± SD or median (interquartile range, IQR) and categorical variables are expressed as ratios. For categorical variables, the χ^2 test was used to compare the characteristics of patients with VR and sustained virological response. A *t*-test or nonparametric test was used for continuous variables, as appropriate. Logistic regression analysis was used to assess the association between variables and study endpoints. The predictive accuracy of the risk score model was assessed by the index of concordance and the receiver

operating characteristic (ROC) curve over time. *p*-Value less than 0.05 was considered to indicate statistical significance. Statistical analyses were performed using IBM SPSS software version 26.0 and GraphPad Prism 8.0 software.

RESULTS

Patient Characteristics

A total of 64 patients were included in this study, with a mean age of 47.33 ± 6.36 years, no patients experienced loss of HBsAg at EOT. All patients were treated for a duration of 60 (42.75–83.25) months with NAs, and 33 (51.6%) patients were treated with first-line agents, including entecavir and tenofovir disoproxil fumarate. **Tables 1**, **2** show the clinical characteristics of all patients at the start of NAs treatment and at EOT. At the start of NAs treatment, 36 patients (56.3%) were HBeAg positive and 28 (43.7%) were HBeAg negative, HBeAg-positive patients have higher levels of HBV-DNA and HBsAg before the NAs treatment and are treated with NAs for a longer period of time. There was no significant difference in serum HBcrAg levels and pgRNA positivity between the two groups at EOT (**Table 1**).

Comparing Influencing Factors Between the Virological Relapse Patients and Non-virological Relapse Patients

Within 1 year of stopping NAs treatment, 33 patients (51.6%) experienced VR, including 21 (58.3%) patients who were HBeAg positive before the start of treatment and 12 (42.9%) patients who were HBeAg negative before the start of treatment. Of all patients with VR, 21 patients were accompanied by biochemical relapse, the mean HBV-DNA level of the virological relapsed patients was 5.85 ± 1.32 log10 IU/mL. We compared the VR and non-virological relapse (non-VR) patients and found no significant differences between the two groups in terms of age, sex, family history of HBsAg, duration of treatment, and pretreatment ALT. Baseline HBV-DNA level (6.68 ± 1.09 vs. 6.43 ± 1.33 log10 IU/mL, $p = 0.411$) and HBsAg level at the EOT [3.32 log IU/mL (IQR 2.95–3.50) vs. 3.21 log U/mL (IQR 2.80–3.54), $p = 0.687$] had no significant effect on VR. In the VR group, EOT HBcrAg levels were significantly higher than those in the non-VR group [3.4 log U/mL (IQR 3.3–3.8) vs. 2.8 log U/mL (IQR 2.6–3.2), $p < 0.001$]. In addition, EOT pgRNA positive patients were prone to VR (11/12 vs. 22/52, $p = 0.002$). Specific comparisons between the two groups are shown in **Table 2**.

Univariate and Multivariable Analysis of Risk Factors for Virological Relapse After Cessation of Nucleos(t)ide Analogs in Patients With Chronic Hepatitis B

To identify independent risk factors for VR, univariate and multivariate logistic regression analyses were performed (**Table 3**). Sex, age, duration of treatment, EOT HBsAg level, and baseline HBV-DNA level and HBeAg status were not associated with VR. Two variables were found to be independent risk factors for VR: EOT pgRNA positivity (OR = 14.59, $p = 0.026$) and higher levels of EOT HBcrAg (OR = 14.14, $p = 0.001$). We used the Youden index to predict the optimal cut-off value of EOT HBcrAg for VR, which measured was 3.25 (3.3) log10 U/mL (sensitivity of 78% and specificity of 77%). Virologic relapse rates were significantly lower in patients with low HBcrAg levels (≤3.3 log10 U/mL) than in those with high levels of HBcrAg (>3.3 log10 U/mL) (7/31 vs. 26/33, $p < 0.001$). Patients with EOT pgRNA positivity and EOT HBcrAg > 3.3 log10 U/mL were more likely to experience VR after discontinuation NAs (88.9 vs. 45.5%, $p = 0.027$). To further assess the value of EOT pgRNA, HBcrAg, and their combination in predicting VR, the area under the receiver operating characteristic curve (AUROC) was calculated for each parameter. The results showed that EOT pgRNA positivity plus EOT higher HBcrAg level had an AUROC value of 0.857 ($p < 0.001$), which was of good predictive value higher than the other parameters (pgRNA positivity, AUC = 0.651, $p = 0.039$. EOT HBcrAg, AUC = 0.817, $p < 0.001$) (**Figure 2**).

Characteristics of Patients Whose Serum Hepatitis B e Antigen Changes From Negative to Positive After Discontinuation of Nucleos(t)ide Analogs

A total of 13 (20.3%) patients experienced a conversion in serum HBeAg from negative at EOT to positive within 1 year of discontinuation of NAs, and those patients were HBeAg positive before the start of treatment. We analyzed the characteristics of these patients and found that baseline HBV-DNA levels (7.29 ± 0.61 vs. 6.38 ± 1.26 log10 IU/mL, $p = 0.01$), EOT HBcrAg levels (3.88 ± 0.49 vs. 3.06 ± 0.46 log10 U/mL, $p < 0.001$) (**Figures 3A,B**) were significantly higher than patients with continued HBeAg negative status. But we found no significant

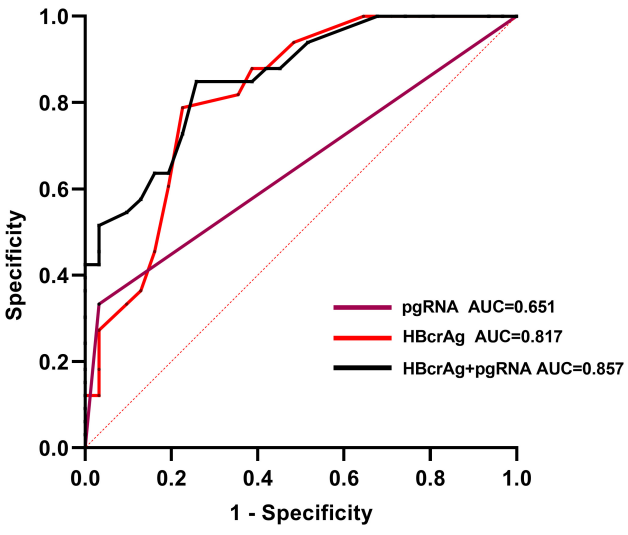

FIGURE 2 | Area under the receiver-operating characteristic curves (AUROC) of EOT pgRNA, EOT HBcrAg and their combination for predicting virological relapse in the cohort. The optimal cut-off value for the EOT HBcrAg for the Youden index to predict virological relapse is 3.3 log10 U/mL (sensitivity of 78% and specificity of 77%).

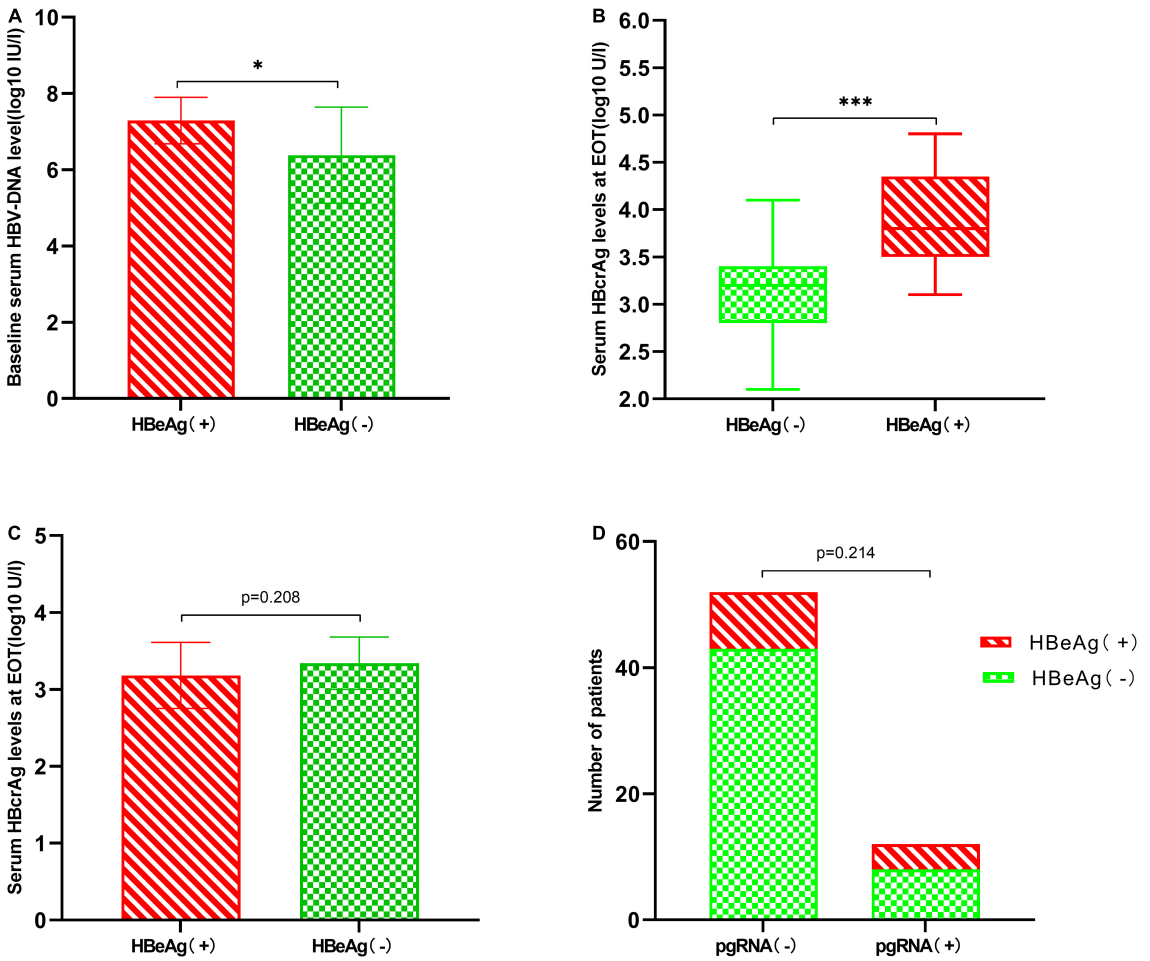

FIGURE 3 | Characteristics of patients whose serum HBeAg changes from negative to positive after 1 year of discontinuation of NAs. **(A)** Patients who experienced HBeAg changes from negative to positive have higher baseline HBV-DNA levels (7.29 ± 0.61 vs. 6.38 ± 1.26 log10 IU/mL, $p = 0.01$). **(B)** Patients who experienced HBeAg changes from negative to positive had higher EOT HBcrAg levels [3.80 log U/mL (IQR 3.50–4.35) vs. 3.20 log U/mL (IQR 2.80–3.40), $p < 0.001$]. **(C)** Comparison of EOT HBsAg levels between two groups (3.18 ± 0.43 vs. 3.34 ± 0.34 log10 IU/mL, $p = 0.208$). **(D)** EOT pgRNA status has no significant effect on HBeAg conversion (4/12 vs. 9/52, $p = 0.214$). *$P < 0.05$ and ***$P < 0.001$.

differences in age, gender, family history of HBsAg, duration of treatment, EOT HBsAg levels, and EOT pgRNA status between the two groups (**Table 4** and **Figures 3C,D**).

DISCUSSION

Discontinuation of NAs for HBV patients remains a difficult clinical issue. Although NAs significantly inhibit HBV replication and reduce serum HBV DNA to undetectable levels, complete elimination of HBV virus from hepatocytes is difficult due to the persistence of cccDNA in the nucleus of infected cells (Wang et al., 2016). After discontinuation of NAs, cccDNA has the risk of reactivation, resulting in a higher rate of VR, with a virological recurrence rate of 51.6% 1 year in our study. Dynamic monitoring of intrahepatic cccDNA levels can predict sustained virological response after cessation of NAs therapy. However, the invasive nature of dynamic liver biopsy and the potential for sampling error have greatly limited the use of intrahepatic cccDNA in actual clinical practice. Therefore, we need to identify the key factors of VR. Influencing factors such as age, sex, baseline HBV DNA, EOT HBsAg, and duration of treatment have been demonstrated to be associated with VR in previous studies (Chen et al., 2015; Jung et al., 2016; Hsu et al., 2019). However, our study showed no significant relationship between these factors and VR, suggesting that there are discrepancies between studies. We need more accurate and consistent indicators to guide safe drug discontinuation, serum HBcrAg and PgRNA are novel virological indicators, this prospective study demonstrates the significant role of two novel indicators in patients with chronic HBV infection, especially in predicting VR after drug discontinuation, with an AUROC value of 0.857 ($p < 0.001$). which is in line with most studies (Jaroenlapnopparat et al., 2021).

HBV-DNA is the most commonly used virological marker in clinical practice and has long been widely used to assess the activity of viral replication and the efficacy of NAs in treating

TABLE 4 | Characteristics of patients whose serum HBeAg changes from negative to positive after 1 year of discontinuation of NAs.

Factors	HBeAg converted to positive (*N* = 13)	HBeAg persistently negative (*N* = 51)	*P-value*
Age, years	49.69 ± 8.71	46.73 ± 5.95	0.120
Gender, male/female, *n* (%)	9 (69.2)/4 (30.8)	23 (45.1)/28 (54.9)	0.614
Family history of HBsAg with/not *n* (%)	4 (30.8)/9 (69.2)	24 (47.1)/27 (52.9)	0.291
Start of treatment			
HBeAg positive/negative *n* (%)	13 (100)/0	28 (54.9)/23 (45.1)	<0.001
HBV-DNA log10 IU/mL	7.29 ± 0.61	6.38 ± 1.26	0.01
HBsAg log10 IU/mL	3.97 (3.17–4.97)	3.85 (3.55–3.98)	0.161
End of treatment			
HBsAg log10 IU/mL	3.18 ± 0.43	3.34 ± 0.34	0.208
HBcrAg log10 U/mL	3.88 ± 0.49	3.06 ± 0.46	<0.001
pgRNA positive/negative, *n* (%)	4 (30.8)/9 (69.2)	8 (15.7)/43 (84.3)	0.214
Duration of treatment (month)	70.92 ± 22.61	61.29 ± 22.04	0.167

patients with CHB. However, NAs act on only a limited number of steps in the viral replication cycle and do not affect cccDNA. Therefore, HBV-DNA does not reflect the true status of cccDNA in hepatocytes in the presence of long-term antiviral drugs. A Korean study showed that HBV DNA >2,000,000 IU/mL before starting treatment in HBeAg-positive patients was an independent risk factor for VR (Jung et al., 2016) (OR = 9.285, p = 0.036). A Taiwan study also demonstrated that baseline HBV-DNA was a risk factor for VR in patients with HBsAg below 100 IU/mL (Tseng et al., 2020). However, in line with some studies (Tsuge et al., 2013), no significant effect of baseline HBV-DNA on VR was shown in our study.

Serum HBsAg levels correlate with hepatic cccDNA activity and the loss of serum HBsAg is an ideal indicator to evaluate safety discontinuation. Previous studies have shown that EOT serum HBsAg levels are an independent risk factor for VR, but predominantly at low levels, with EOT HBsAg >100 or >40 IU/L (Hsu et al., 2019; Fan et al., 2020; Tseng et al., 2020; Xie et al., 2021). In our study, although EOT HBsAg was not significantly associated with VR, patients with EOT HBsAg ≤100 IU/mL had a lower rate of VR than those with EOT HBsAg >100 IU/mL [28.6% (2/7) vs. 54.4% (31/57), p = 0.197], which is consistent with the results of previous studies. This result may be due to the low number of patients with low EOT HBsAg levels in our cohort.

Intrahepatic pgRNA is transcribed from cccDNA and correlates strongly with intrahepatic cccDNA activity, but detection of intrahepatic pgRNA is difficult and relies on liver puncture biopsy. Therefore, serum HBV RNA is a promising marker of cccDNA transcriptional activity. Especially during NAs treatment, serum HBV DNA levels are often undetectable and serum pgRNA is a better reflection of the status of intrahepatocellular cccDNA (Giersch et al., 2017). It has been shown that serum pgRNA is an independent predictor of viral rebound after discontinuation of treatment (Tsuge et al., 2013; Wang et al., 2016). A prospective study from China showed that 6 years after stopping treatment with NA, all patients with EOT pgRNA levels ≥20,000 copies/mL experienced biochemical relapse, compared to 23.8% of patients with HBV RNA levels <1,000 copies/mL (p < 0.001) (Xia et al., 2021). In our study it was shown that patients who were negative for EOT pgRNA were more likely to have a sustained virological response, while those who were EOT pgRNA positive were more likely to have a VR (11/12 vs. 22/54, p = 0.002). This result was consistent with previous studies. In addition, EOT pgRNA positivity was found to be an independent risk factor for VR in a multivariate regression analysis (OR = 14.59, p = 0.026) with an AUROC value of 0.651.

Hepatitis B core-related antigen is a novel serum complex viral protein that consists of three related proteins sharing the same 149 amino acid sequence, including HBcrAg, HBeAg and a truncated 22 kDa precore protein (p22Cr) (Mak et al., 2018). In our previous study, it was demonstrated that serum HBcrAg is a better indicator of cccDNA than pgRNA and HBsAg (Chen et al., 2019). Studies have also reported that HBcrAg can be detected even when serum HBV DNA becomes undetectable or HBsAg is lost (Seto et al., 2014). In a Taiwanese study, the independent risk factor of VR was EOT HBcrAg >4 log U/mL (Huang et al., 2021). Jung et al. (2016) showed that EOT HBcrAg >3.7 log U/mL was independently associated with more than three-times higher risk of VR in HBeAg-negative CHB patients. In our study, HBcrAg levels in the VR group were higher than those in N-VR group. In multivariate regression analysis, EOT higher HBcrAg level was an independent risk factor, and optimal cut-off value of EOT HBcrAg for VR is 3.3 log10 U/mL, Patients with EOT HBcrAg >3.3 log10 U/mL are more likely to experience VR. This suggests that the lower the EOT HBcrAg level, the lower the risk of VR after drug discontinuation.

Our study has a number of limitations. Firstly, the sample size was relatively small and the follow-up period was short, limited to 1 year. Secondly, this is a single-center study and multiple studies are needed to confirm our main findings. Thirdly, our study only included Asian populations. Fourth, the small sample size did not allow for separate analysis of E antigen-positive and E antigen-negative patients.

CONCLUSION

According to current discontinuation criteria, higher relapse rates occur after cessation of NAs. EOT HBcrAg and PgRNA levels have the potential to predict VR in patients with chronic HBV infection who discontinue NAs therapy. Low HBcrAg levels at the EOT and negative PgRNA are associated with a lower risk of relapse. Combining these two novel indicators could guide CHB patients in choosing the appropriate time to stop NAs.

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 West China Hospital Ethics Committee. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

E-QC contributed to the study concept and design. F-DW, JZ, L-QL, M-LW, Y-CT, Y-HW, and D-MZ contributed to the data acquisition. F-DW contributed to the data analysis and manuscript drafting. All authors approved the final version of the manuscript.

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In vivo Delivery Tools for Clustered Regularly Interspaced Short Palindromic Repeat/Associated Protein 9-Mediated Inhibition of Hepatitis B Virus Infection: An Update

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Chronic hepatitis B virus (HBV) infection remains a major global health problem despite the availability of an effective prophylactic HBV vaccine. Current antiviral therapies are unable to fully cure chronic hepatitis B (CHB) because of the persistent nature of covalently closed circular DNA (cccDNA), a replicative template for HBV, which necessitates the development of alternative therapeutic approaches. The CRISPR/Cas system, a newly emerging genome editing tool, holds great promise for genome editing and gene therapy. Several *in vitro* and/or *in vivo* studies have demonstrated the effectiveness of HBV-specific clustered regularly interspaced short palindromic repeat (CRISPR)/associated protein 9 (CRISPR/Cas9) systems in cleaving HBV DNA and cccDNA. Although recent advances in CRISPR/Cas technology enhance its prospects for clinical application against HBV infection, *in vivo* delivery of the CRISPR/Cas9 system at targets sites remains a major challenge that needs to be resolved before its clinical application in gene therapy for CHB. In the present review, we discuss CRISPR/Cas9 delivery tools for targeting HBV infection, with a focus on the development of adeno-associated virus vectors and lipid nanoparticle (LNP)-based CRISPR/Cas ribonucleoprotein (RNP) delivery to treat CHB. In addition, we discuss the importance of delivery tools in the enhancement of the antiviral efficacy of CRISPR/Cas9 against HBV infection.

Keywords: hepatitis B virus, cccDNA, CRISPR/Cas9, delivery, gene therapy

INTRODUCTION

Genome editing is a novel approach used for manipulating target genes in various cell types and organisms using engineered nucleases. Zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and clustered regularly interspaced short palindromic repeat (CRISPR)/associated protein 9 (CRISPR/Cas9) nuclease systems are some of the genome-editing tools that have been extensively studied (Gaj et al., 2013). The CRISPR/Cas9 nuclease system

has emerged as a potent genome editing tool and has revolutionized genome editing (Jinek et al., 2012; Cong et al., 2013; Doudna and Charpentier, 2014; Knott and Doudna, 2018; Pickar-Oliver and Gersbach, 2019). CRISPR/Cas9, a naturally occurring RNA-guided endonuclease, has been primarily discovered in bacteria and serves as a defense tool for adaptive immunity against bacteriophages (Bolotin et al., 2005; Mojica et al., 2005; Pourcel et al., 2005; Barrangou et al., 2007). Compared to ZFNs and TALENs, the CRISPR/Cas9 system is simple, efficient, and readily reprogrammable, and different DNA sequences can be targeted simply by redesigning guide RNAs (gRNAs) (Cong et al., 2013; Hu et al., 2016).

A gRNA has two components, including CRISPR RNA (crRNA) and trans-activating CRISPR RNA (tracrRNA). crRNA contains a 20-nucleotide (nt) RNA sequence complementary to the target DNA sequence, and tracrRNA serves as a Cas nuclease-binding scaffold (Marx, 2020). A single guide RNA (sgRNA) can be generated by combining crRNA and tracrRNA for targeting the gene sequence in the gene-editing tool (Allen et al., 2020). The targeting specificity of Cas9 enables avoidance of off-target effects, which is facilitated by the gRNA sequence (20 nt) (Zhang et al., 2015). Cas9 also requires a specific protospacer adjacent motif (PAM) localized on the non-target strand of DNA directly downstream of the target sequence (**Figure 1**). However, the possibility of off-target effects due to Cas9 binding to unintended genomic sites for cleavage cannot be entirely ruled out (Zhang et al., 2015; Naeem et al., 2020).

The Cas9 enzyme cuts both complementary and non-complementary strands, causing a double-strand DNA break (DSB), in turn initiating the DNA repair mechanism via error-prone non-homologous end-joining (NHEJ) or precise homology-directed repair (HDR) (Doudna and Charpentier, 2014; **Figure 1**). NHEJ-induced repair can cause undesirable errors at the target DNA locus, leading to mutagenic insertions and deletions (indels). In contrast, in the presence of a homologous donor template, HDR can cause site-specific insertions, deletions, nucleotide substitutions, or genomic sequence rearrangements, highlighting its potential application for accurate genetic editing (**Figure 1**). CRISPR/Cas9-based genome editing in eukaryotic cells was first reported in 2013 (Cong et al., 2013). The CRISPR/Cas9 system has become a widely applicable genome editing tool, and its use is not limited to cellular organisms, as it is frequently used in acellular organisms, such as viruses (White et al., 2015; Xiao et al., 2019; Teng et al., 2021).

Hepatitis B virus (HBV) infection is a major global public health concern. HBV can cause a spectrum of illnesses in humans, including acute hepatitis, chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma (HCC) (Liang, 2009). HBV is a partially double-stranded relaxed circular DNA (rcDNA) virus with a genome of 3.2 kb, with four overlapping open reading frames encoding seven viral proteins, including polymerase/reverse transcriptase, core, precore, three related envelope proteins [HBV surface proteins (HBs); large, middle, and small], and a regulatory X protein (Hu and Seeger, 2015; Seeger and Mason, 2015). Despite the availability of an effective preventive HBV vaccine, chronic HBV infection remains a major global health problem affecting millions of people globally (Mastrodomenico et al., 2021; World Health Organization, 2021). A recent estimate indicated that 296 million people were chronically infected with HBV in 2019 (World Health Organization, 2021). Current therapies consisting of pegylated interferon alpha and nucleos(t)ide analogs are only effective at suppressing HBV replication and reducing the risk of cirrhosis, liver failure, and HCC development; however, they cannot cure HBV infection owing to the persistent nature of covalently closed circular DNA (cccDNA) and/or integrated HBV DNA in hepatocytes (Suk-Fong Lok, 2019; Ezzikouri et al., 2020). cccDNA, a replicative template for HBV, must be fully eradicated to completely cure HBV (Nassal, 2015). Therefore, novel alternative therapeutic strategies need to be developed to eradicate cccDNA with minimal side effects, and the CRISPR/Cas system appears to be a promising tool for achieving the goal.

With the advent of the CRISPR/Cas technology, several studies have reported varying degrees of inhibition of HBV replication and/or cccDNA formation using the CRISPR/Cas9 system with different delivery tools (**Figure 2**; Lin et al., 2014; Seeger and Sohn, 2014; Dong et al., 2015; Lin et al., 2015; Liu et al., 2015, 2018; Ramanan et al., 2015; Wang et al., 2015; Zhen et al., 2015; Li H. et al., 2016; Seeger and Sohn, 2016; Zhu et al., 2016; Li et al., 2017, 2018; Kostyusheva et al., 2019; Kayesh et al., 2020; Suzuki et al., 2021; Yan et al., 2021; Martinez et al., 2022; Wang D. et al., 2022). Notably, it is difficult to efficiently quantify cccDNA (Wang Z. et al., 2022), and accurate quantification of intrahepatic cccDNA is important for assessing the efficiency of anti-HBV therapy. Furthermore, in addition to xenografted mice, murine models are extremely inefficient in cccDNA production (Ortega-Prieto et al., 2019; Lai et al., 2021).

In the case of *in vivo* clinical applications, genome editing using the CRISPR/Cas system requires an efficient and reliable delivery tool for transporting CRISPR/Cas into target cells or organs. Different delivery tools, such as physical, chemical, or biological methods, can be used to deliver CRISPR/Cas9 to the host target site. The components of the CRISPR/Cas9 genome editing system are delivered in one of three forms: DNA, RNA, or ribonucleoprotein (RNP) (Luther et al., 2018; Eoh and Gu, 2019; Yip, 2020). However, a practical, safe, and effective method of delivering genome-editing components for *in vivo* genome editing and gene therapy against HBV infection has not yet been developed. Although different delivery tools, such as viral and non-viral tools, are under development (Xu et al., 2021), extensive investigations on potential *in vivo* animal models are required to devise suitable delivery tools to address the issues impeding the clinical application of the CRISPR/Cas system in the treatment of chronic hepatitis B (CHB). Therefore, CRISPR/Cas9 delivery systems with low immunogenicity and high efficiency are required. Moreover, the low efficiency of *in vivo* delivery must be improved before it can be effectively used in therapy (Behr et al., 2021; Taha et al., 2022). Delivery plays an important role in the CRISPR/Cas-mediated inhibition of HBV replication (Suzuki et al., 2021). Against such a background, in the present review, we provide an overview of CRISPR/Cas9-mediated inhibition of HBV, with major focus on delivery systems, particularly adeno-associated virus (AAV) vector-based delivery of CRISPR/Cas9 targeting HBV. In addition, we discuss other tools, including non-viral delivery tools.

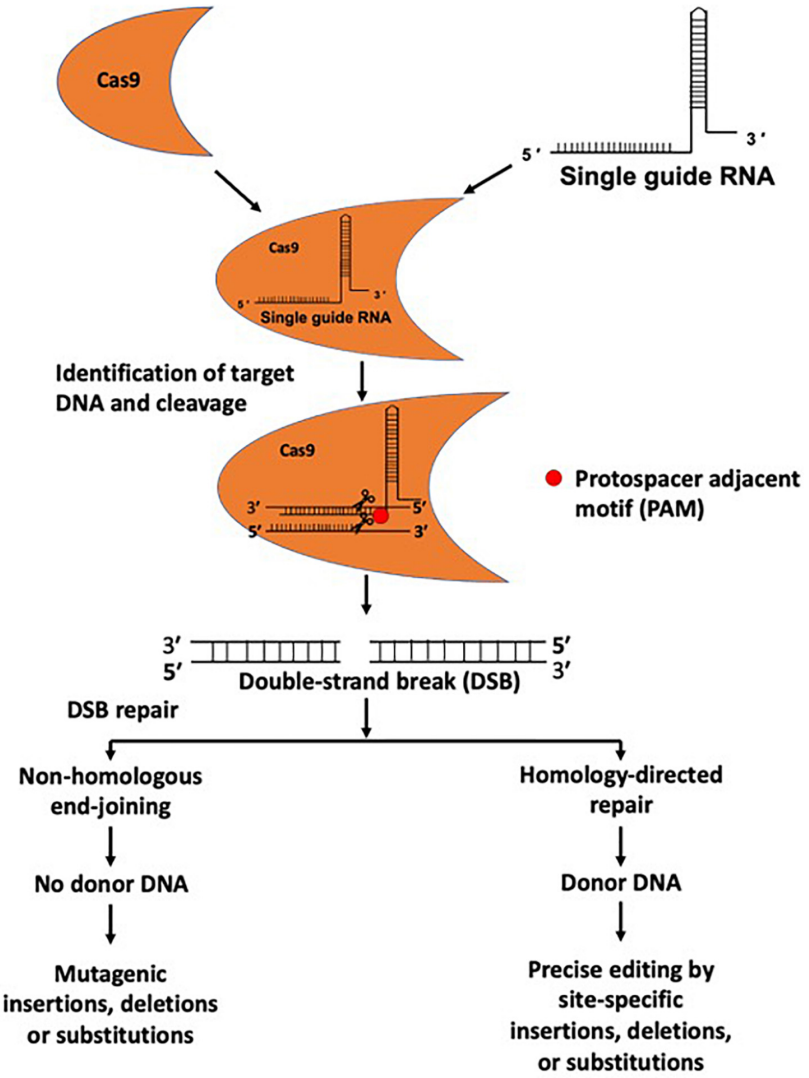

FIGURE 1 | Genome editing using CRISPR/Cas9 system. The CRISPR/Cas9 system is composed of a gRNA and Cas9 protein. gRNAs provide target specificity by sequence complementarity. gRNA and Cas9 proteins form a complex and cleave target DNA at a specific site and produce a double-strand DNA break (DSB). DSBs are repaired through non-homologous end joining (NHEJ) and homology-directed repair (HDR) mechanisms, and during the repair process insertions, deletions, nucleotide substitutions, or gene insertion may occur.

VIRAL VECTORS FOR CLUSTERED REGULARLY INTERSPACED SHORT PALINDROMIC REPEAT/ASSOCIATED PROTEIN 9 DELIVERY

Viral vectors are considered some the most promising delivery tools for gene therapy considering their relatively small genome size, genome organization, and evolutionary plasticity (Lukashev and Zamyatnin, 2016). They have shown great promise for application in the delivery of CRISPR/Cas9 for gene editing (Taha et al., 2022). Different viral vectors, including AAV, full-sized adenovirus, and lentivirus vectors, have been investigated for CRISPR/Cas9 delivery (Lino et al., 2018), with varying degrees of success. However, viral vector applications are limited by a number of issues, such as their oncogenic effects, toxicity, immunogenicity, and insertional mutagenesis, which should be addressed in future investigations.

ADENO-ASSOCIATED VIRUS VECTOR-BASED DELIVERY OF CLUSTERED REGULARLY INTERSPACED SHORT PALINDROMIC REPEAT/ASSOCIATED PROTEIN 9 TO TARGET HEPATITIS B VIRUS

AAVs are the most common viral vectors currently being investigated for application in *in vivo* gene therapy, owing

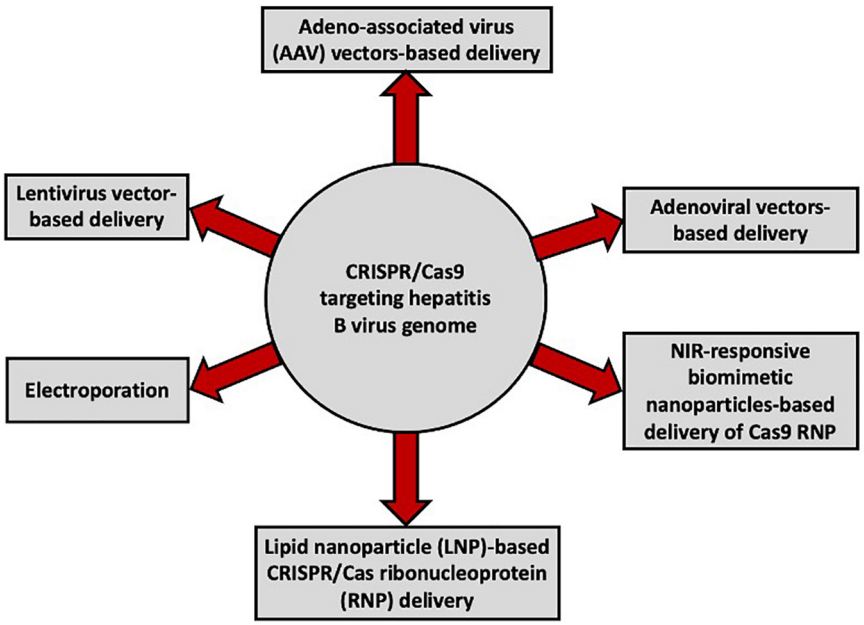

FIGURE 2 | Vectors used for the delivery of CRISPR/Cas9 targeting the HBV genome in different *in vitro* and *in vivo* studies.

to their properties, such as high titer, low immunogenicity, transduction of a broad range of target tissues, and low genomic integration rate (Colella et al., 2018). AAV is one of the most commonly used viral vector systems to date and has attracted considerable attention for use in *in vivo* CRISPR/Cas delivery. AAVs are small viruses that require the presence of a helper virus, including adenoviruses, herpes simplex virus, vaccinia virus, or human papillomavirus, to achieve a productive replication cycle (Geoffroy and Salvetti, 2005). AAV belongs to the family *Parvoviridae* and genus *Dependovirus* (Daya and Berns, 2008). They are single-stranded DNA viruses with a genome of approximately 4.7 kilobases (Wu et al., 2010). The AAV genome encodes several proteins, including four non-structural Rep proteins (Rep78, Rep68, Rep52, and Rep40) required for viral replication, three capsid proteins (VP1–VP3), and an assembly-activating protein (Sonntag et al., 2010). To date, 12 human serotypes of AAV, designated AAV-1 to AAV-12, and over 100 serotypes in non-human primates, have been identified. Nearly all AAV serotypes exhibit natural hepatic tropism and efficiently accumulate in the liver following intravenous administration (Palaschak et al., 2019). AAV vectors have been successfully used to treat patients with bleeding disorders and blindness (Nathwani et al., 2011; Tuddenham, 2012; Vandenberghe and Auricchio, 2012; George et al., 2017; Li and Samulski, 2020). Although AAV vectors are restricted by their limited packaging capacity (Grieger and Samulski, 2005), by using dual AAV vectors or triple AAV vectors, the transfer capacity of AAV can be expanded from 4.7 kb to approximately 9 or 14 kb, respectively (Yang et al., 2016; Bak and Porteus, 2017; Maddalena et al., 2018). One strategy of overcoming the size limit of AAV vectors is splitting large transgenes into two or three parts to generate dual or triple AAV vectors (Akil, 2020). Transduction of target cells with these two or three AAVs, through homologous recombination or other mechanisms, results in the transcription of a full-length Cas9 mRNA (Bak and Porteus, 2017; Akil, 2020). In our previous study, full-length *Streptococcus pyogenes* Cas9 (SpCas9) was obtained by splitting the SpCas9 transgene using dual AAVs (**Figure 3**) modified from a previous study (Kayesh et al., 2020). Moreover, a smaller Cas9, *Staphylococcus aureus* Cas9 (SaCas9), has been discovered, containing 1,082 amino acid residues, with 286 residues less than that of SpCas9, and has been used for delivery by AAV vectors. However, delivery with dual AAVs requires a high viral dose, which raises potential safety concerns and can reduce the editing potential (Hayashi et al., 2020; Fang et al., 2021). Although most AAV vectors exist in an extrachromosomal state, a fraction of AAV vectors can integrate into pre-existing DSBs (Miller et al., 2003, 2004). Notably, a recent study has reported high levels of AAV integration (up to 47%) in Cas9-induced DSBs (Hanlon et al., 2019). However, genome integration is greatly reduced in recombinant AAVs (rAAVs), which are devoid of the rep gene, and ITRs are the only viral origin sequences used to guide genome replication and packaging during vector production (Wang et al., 2019). As prolonged expression of Cas9 may increase the possibility of off-target effects, which can cause safety concerns, delivery in DNA form is advantageous if sustained Cas9 expression is required (Ishida et al., 2015; Yip, 2020).

Cas9 is an endonuclease that contains two nuclease domains, RuvC and HNH. The RuvC domain cleaves non-complementary DNA strands and the HNH domain cleaves complementary DNA strands (Liu et al., 2017). The SpCas9 is the most widely used endonuclease and has been demonstrated to effectively inactivate HBV sequences (Hille et al., 2018; Moyo et al., 2018). However, different Cas9 orthologs, such as SaCas9,

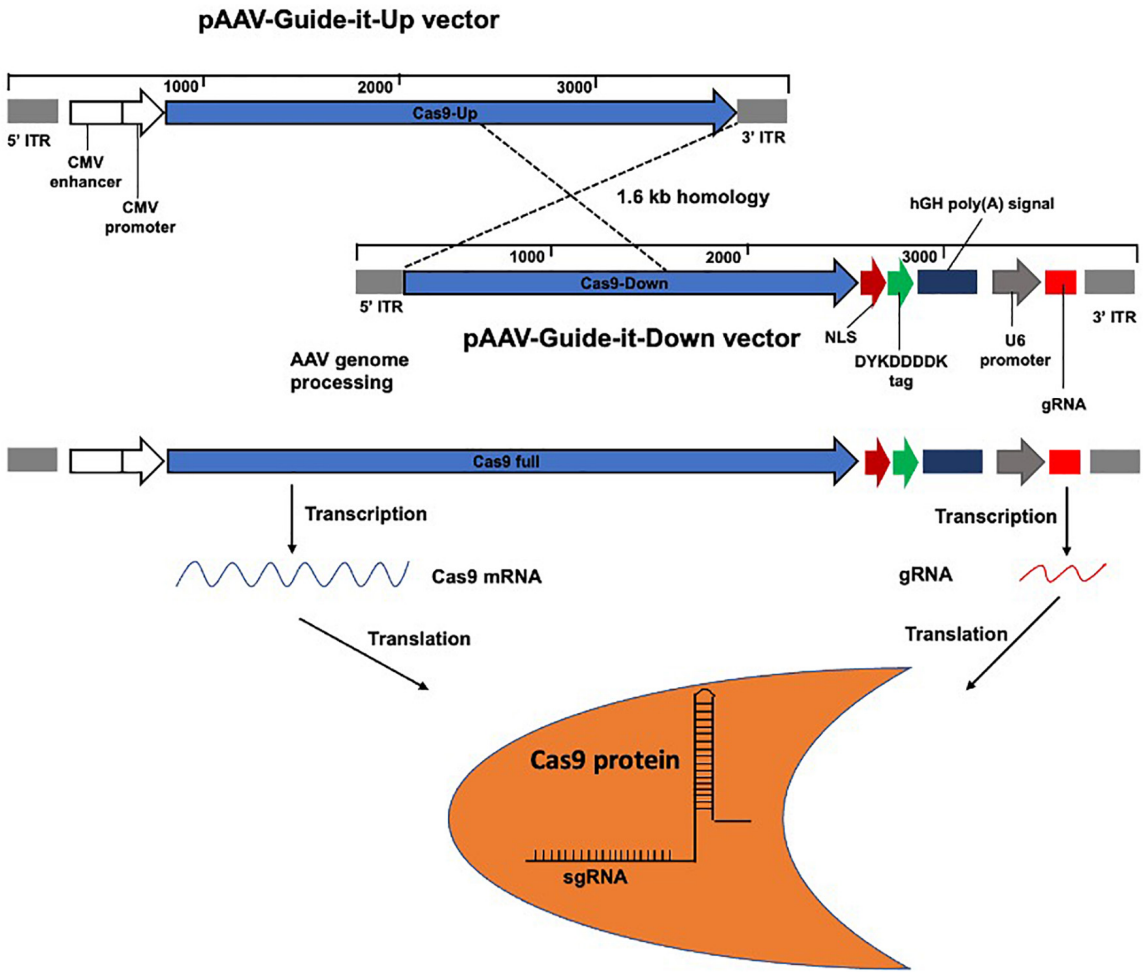

FIGURE 3 | Schematic representation of the larger transgene *Streptococcus pyogenes* Cas9 (SpCas9) incorporation by splitting into AAV vectors. Two vectors pAAV-Guide-it-Up and Guide-it-Down were used to express full-length SpCas9 and gRNA. gRNA sequence is located downstream of the U6 promoter of the Guide-it-Down vector for binding to the target DNA sequence.

Streptococcus thermophilus Cas9 (StCas9), *Neisseria meningitidis* Cas9 (NmCas9), and SpCas9 variants, have been optimized (Kleinstiver et al., 2015). A recent study reported preexisting adaptive immune responses in humans against SaCas9 and SpCas9 proteins (Simhadri et al., 2018; Charlesworth et al., 2019; Wagner et al., 2019), suggesting previous exposure of these bacterial Cas9 proteins from microbes in humans. Pre-existing immunity against Cas9 may negatively impact its clinical use by affecting its efficacy and posing significant safety issues (Mehta and Merkel, 2020). Therefore, it is essential to fully characterize the impact of pre-existing immunity against Cas9 for its successful use in *in vivo* genome editing. SaCas9 and sgRNA-8 delivered using single-stranded adeno-associated viral vectors (ssAAVs) resulted in HBV DNA and cccDNA suppression in HepG2.2.15 and HepG2-hNTCP cells (Scott et al., 2017). gRNA/SaCas9 delivered by AAV inhibited HBV antigen, pgRNA, and cccDNA in different cell lines, including Huh7, HepG2.2.15, and HepAD38 cells (Liu et al., 2018). As expected, the dual expression of gRNAs/SaCas9 was more efficient for HBV genome cleavage (Liu et al., 2018). Although not significant, the levels of HBsAg, HBV DNA, and pgRNA were decreased in mice with persistent HBV replication and a higher titer of AAV injection when compared with that in controls (Liu et al., 2018). In addition, a recent study showed inhibition of HBV by CRISPR/SaCas9 delivered using hepatotropic AAV8 in C57BL/6 mice (Yan et al., 2021). In another study, C57BL/6 mice were administered 2×10^{11} AAV8 vector genomes (vg) via tail vein injection to deliver SaCas9 in a volume of 200 μL (Yan et al., 2021).

CRISPR generates knockouts at the DNA level, whereas RNA interference (RNAi) silences genes by generating knockdowns at the mRNA level. RNAi has been shown to exert antiviral effects against HBV (Mccaffrey et al., 2003). A single administration of a double-stranded AAV8-pseudotyped vector (dsAAV2/8) carrying HBV-specific shRNA reportedly effectively suppressed HBV protein, mRNA, and replicative DNA in the liver of HBV transgenic mice, and the effect was sustained for at least 120 days after vector administration (Chen et al., 2007). However,

the suitability of the dsAAV2/8 vector for CRISPR/Cas9-based therapies and the treatment of chronic HBV infection remains to be investigated. Notably, AAV vectors obtained using the pseudotyping method are often referred to as AAV2/n, where the first number refers to the ITRs and the second to the capsid. Capsids are responsible for different transduction abilities (i.e., cell tropism and kinetics of transgene expression), and users can choose accordingly (Asokan et al., 2012; Balakrishnan and Jayandharan, 2014). Varying hepatocyte transduction efficiencies have been reported among AAV serotypes, and an engineered AAV3 capsid has been reported to exhibit much greater efficiency for human hepatocytes than AAV5, AAV8, and AAV9 (Vercauteren et al., 2016). Another recent study reported a mutant AAV vector candidate, AAV3B, with greater hepatocyte tropism and reduced seroreactivity (Biswas et al., 2020). Development of mutant AAV variants with human hepatocyte tropism and neutralizing antibody escape capacity is paramount, and further investigation is required to obtain a suitable vector for CRISPR/Cas9-mediated inhibition of HBV infection (Pei et al., 2020).

In our previous study, we investigated the potential of AAV2-mediated delivery of guide (g) RNAs/Cas9 to inhibit HBV replication both *in vitro* and *in vivo*. For *in vivo* experiments, 1×10^{12} vg copies of AAV2-WJ11/Cas9 were injected through the tail vein into persistent HBV genotype C-infected humanized chimeric mice. We observed that AAV2-WJ11/Cas9 significantly inhibited HBV DNA replication and reduced cccDNA levels both *in vitro* and *in vivo* (Kayesh et al., 2020); however, the effect required high multiplicity of genome (MOG) copies of the AAV2 vector, suggesting low transduction efficiency of the AAV2 vector. Using an immunocompetent tree shrew model of HBV infection (Kayesh et al., 2021), we observed a much lower transduction efficiency of the AAV2 vector in liver tissues (unpublished data) than in humanized chimeric mice (Kayesh et al., 2020). Stone et al. demonstrated the antiviral efficacy of SaCas9 in humanized FRG mice chronically infected with HBV genotype C, which resulted in decreased total liver HBV DNA and cccDNA levels (Stone et al., 2021). Mice were administered with 5×10^{11} AAV vector genome copies generated with capsid LK03 via tail vein injection to deliver SaCas9 in a volume of 100 μL (Stone et al., 2021). Although AAV is one of the most commonly used vector systems, a very high dose is required for its transduction (Kayesh et al., 2020). Despite years of research, AAV production is still expensive and remains one of the main barriers for AAV-based gene therapy (Naso et al., 2017; Wang et al., 2019), making it impractical to implement programs to treat HBV-infected individuals with AAVs globally. Moreover, problems such as liver toxicity at high doses, immunogenicity, off-target effects, and genomic integration remain to be resolved.

ADENOVIRAL VECTORS AS A DELIVERY TOOL FOR GENE THERAPY

Adenoviruses belong to the *Adenoviridae* family, and comprise a wide group of human and animal viruses that share functional, genetic, and structural characteristics (Smith et al., 2010). Adenoviruses have a linear double-stranded DNA genome that ranges from 26 to 46 kb in length (San Martin, 2012). The adenovirus genome encodes more than 40 different proteins, 12 of which are mature viral particles (Lehmberg et al., 1999). The episomal nature, large cloning capacity, high titers, and transducing ability of dividing and non-dividing cells make adenoviral vectors (AdVs) interesting candidates for exploitation in RNA-guided nuclease (RGN) delivery (Goncalves and De Vries, 2006). High-capacity adenoviral vectors (HCAdVs) lacking all the coding genes are considered powerful tools for the delivery of large amounts of DNA cargo into cells (Ehrke-Schulz et al., 2017). HCAdVs possess high packaging capacity (up to 35 kb), low immunogenicity, and low toxicity (Xu et al., 2019). Schiwon et al. (2018) demonstrated that co-delivery of multiple gRNA expression cassettes along with the Cas9 expression cassette through one HCAdV resulted in a significant reduction in HBV Ag and HBV cccDNA production.

LENTIVIRUS VECTOR-BASED DELIVERY OF CLUSTERED REGULARLY INTERSPACED SHORT PALINDROMIC REPEAT/ASSOCIATED PROTEIN 9 FOR HEPATITIS B VIRUS INHIBITION

Lentiviral vector (LV) is a single-stranded RNA virus with a packaging capacity of approximately 8 kb (Vogt and Simon, 1999). Although LVs can mediate potent transduction and stable expression in dividing and non-dividing cells both *in vitro* and *in vivo*, their use in clinical research is limited by several issues related to safety, ethics, and public health concerns, and significantly lower transduction efficiency in non-dividing cells that are quiescent in the G0 state (Connolly, 2002; Kuhn et al., 2002). To address such challenges, non-integrating lentiviral vectors (NILVs) are under development (Uchida et al., 2021).

LV-mediated delivery of CRISPR/Cas9 has been shown to efficiently cleave viral DNA and suppress HBV in HepG2.2.15 and HepG2-hNTCP cells (Ramanan et al., 2015). Dual expression of gRNA/CRISPR/Cas9 results in greater reductions in HBsAg and HBV RNA when compared with the expression of single guide RNAs (Ramanan et al., 2015). An anti-HBV effect in a mouse model was observed upon introduction of HBV and Cas9/sgRNA plasmids into the liver of immunodeficient mice (NRG) by hydrodynamic injection (HDI) (Ramanan et al., 2015), which suggests the efficacy of CRISPR/Cas9 system-based targeting of the HBV genome. Another study identified conserved HBV sequences in the S and X regions of the HBV genome, which were targeted for precise and effective cleavage by Cas9 nickase (Karimova et al., 2015). Base editing has advanced CRISPR/Cas-based technologies, and can be used to directly initiate point mutations in cellular DNA without a DSB (Kantor et al., 2020). The CRISPR/Cas9-mediated non-cutting editing strategy in the base-editing system has been shown to have the potential to cure CHB by permanent inactivation of integrated HBV DNA and cccDNA without off-target effects (Yang et al., 2020). Another recent study also reported efficient silencing of HBV following

targeting of the HBV S gene using CRISPR-mediated base editing (Zhou et al., 2022).

NON-VIRAL VECTORS FOR CLUSTERED REGULARLY INTERSPACED SHORT PALINDROMIC REPEAT/ASSOCIATED PROTEIN 9 DELIVERY

Although viral vector-mediated gene delivery results in higher transduction efficiency and long-term gene expression, viral vectors suffer from a number of limitations, including oncogenic effects, toxicity, immunogenicity, poor target cell specificity, inability to transfer large genes, insertional mutagenesis, and high costs (Wang et al., 2013). Non-viral vectors are based on the collective delivery of naked RNA or DNA using chemical or physical methods, resulting in efficient delivery of nucleic acids into the target cells (Wang et al., 2013; Jin et al., 2014). Non-viral vectors, particularly cationic lipid-based biomaterial approaches, show high potential because of their relative safety, ease of preparation, cell/tissue targeting, and low immunogenicity. However, the clinical application of non-viral methods is limited by their low transfection efficiency and poor transgene expression (Wang et al., 2013). In recent years, non-viral vectors have been found to be effective for CRISPR/Cas9 delivery to cells and tissues *in vivo* and *in vitro*. Various promising non-viral tools for CRISPR/Cas9 gene therapy have been developed, including liposomes (Liu et al., 2019), nanocarriers (Miller et al., 2017; Finn et al., 2018), and cell-penetrating peptides (Suresh et al., 2017). Nanoparticles targeting hepatocytes have been developed using endogenous and exogenous targeting ligand-based mechanisms using glycans, proteins, or modifications of the nanoparticle surface (Barrett et al., 2014; Detampel et al., 2014; Li M. et al., 2016). In the case of CRISPR/Cas9 gene therapy against HBV using a non-viral vector as a delivery tool, strong hepatotropism of the vector is essential for targeting the liver. In addition, lipid nanoparticles (LNPs) have been found to be efficient carriers of short-interfering RNAs (siRNAs) to hepatocytes *in vivo* (Akinc et al., 2010), which suggests the hepatotropism of LNPs.

A previous study showed that CRISPR/Cas9-delivered by lipid-like nanoparticles (LLNs) (Li et al., 2015) could suppress HBV DNA in a mouse model (Jiang et al., 2017). In a previous study, we also developed LNP as a non-viral delivery tool to deliver the CRISPR/Cas9 system and guide RNA, and investigated its usefulness in CRISPR/Cas9-mediated inhibition of HBV in HBV-replicating HepG2.2.15 cells (Suzuki et al., 2021). Furthermore, LNP-based CRISPR/Cas RNP delivery has been found to significantly enhance the inhibition of *in vitro* HBV replication (Suzuki et al., 2021) when compared with viral vector (AAV2) delivery (Kayesh et al., 2020), highlighting the relevance of the delivery tool in enhancing antiviral effects. Another recent study showed that the RNP delivery of CRISPR/Cas9 in HBV-infected HepG2-NTCP cells induced DSBs in cccDNA, which affected HBV replication. Moreover, Cas9-induced effects were sustained even after RNP degradation/loss of detection, suggesting stable changes due to transcriptional interference (Martinez et al., 2022). However, the efficacy of LNP-based CRISPR/Cas RNP delivery targeting the HBV genome remains to be investigated using a bona fide *in vivo* HBV infection model.

A previous study reported the synthesis and development of zwitterionic amino lipids (ZALs) that can co/deliver long RNAs, including Cas9 mRNA and sgRNAs (Miller et al., 2017). However, their use in the delivery of CRISPR/Cas9 to target the HBV genome remains to be investigated. A near-infrared (NIR)

TABLE 1 | Advantages and disadvantages of the various delivery tools used for CRISPR/Cas9 delivery.

Delivery tools	Advantages	Disadvantages
Adeno-associated virus vectors	• Widely studied • Safe • Broad tissue tropism, and some AAV serotypes, including AAV8, AAV8-pseudotyped vector (dsAAV2/8), and AAV3B exhibit high hepatocyte tropism	• Difficulty to produce • Limited packaging capacity • Low transduction efficiency • Serotype-dependent preexisting immunity • Repeated injections may be required • Liver toxicity at high dose ($>10^{14}$ vg/kg)
Lentivirus vectors	• High transduction efficiency • Long-term gene expression	• Can cause insertional mutagenesis • Do not efficiently transduce quiescent (G0) cells in the adult liver
Adenovirus vectors	• High infection efficiency • Large cloning capacity • Ability to transduce both dividing and non-dividing cells • Can be produced at high titer	• Transient expression • Toxicity at high dose • Serotype-dependent preexisting immunity • Repeated injections may be required • Induction of both innate and adaptive immune response
Lipid nanoparticle (LNP)-based CRISPR/Cas ribonucleoprotein (RNP)	• Easy scalable production • High delivery efficiency • Low toxicity • Transient expression resulting in lower off-target risk	• Repeated injections may be required • Poor efficiency in penetrating into the nucleus
NIR-responsive biomimetic nanoparticles	• Minimal off-target effects • Good biocompatibility	• Multiple interactions are required • Increased concentrations can cause cytotoxicity
Electroporation	• Suitable for all cell types • High transfection efficiency • Suitable for all CRISPR/Cas9 strategies	• Can cause significant cell death • Non-specific transfection

light-responsive nanocarrier, CRISPR/Cas9, was developed to target cancer therapeutics using upconversion nanoparticles (UCNPs) (Pan et al., 2019). NIR-responsive biomimetic nanoparticle (UCNPs-Cas9@CM)-based delivery of Cas9 RNP was found to inhibit HBsAg, HBeAg, HBV pgRNA, HBV DNA, and cccDNA both *in vitro* (HBV-infected cells) and *in vivo* (HBV-Tg mice) (Wang D. et al., 2022). A recent study reviewed the suitability and efficiency of nanoparticle-based delivery of CRISPR/Cas9 for genome editing (Duan et al., 2021), and the study could facilitate the selection of nanoparticle-based delivery of CRISPR/Cas9 to target HBV infections. Although CRISPR/Cas9 has been employed in clinical trials targeting host genes or viruses, to date, no study has been conducted to target the HBV genome using CRISPR/Cas9 (ClinicalTrials.gov; accessed on May 20, 2022), suggesting that further expanded preclinical investigations are still required before engaging in clinical trials.

PHYSICAL METHODS FOR CLUSTERED REGULARLY INTERSPACED SHORT PALINDROMIC REPEAT/ASSOCIATED PROTEIN 9 DELIVERY

Electroporation

Electroporation is widely used to deliver nucleic acids and proteins to mammalian cells (Mali et al., 2013; Tebas et al., 2014). Although electroporation can be used to deliver all types of CRISPR/Cas9 systems, including plasmid-based CRISPR/Cas9, Cas9 mRNA and sgRNA, and Cas9/sgRNA RNP, its use is limited because of the low plasmid DNA integration (approximately 0.01% of the target cells) and induction of significant cell death (Liu et al., 2017). Recently, Zhen et al. (2021) reported that combination therapy with anti-HBV and anti-PD1 gRNA/cas9 delivered using electroporation produces a synergistic antiviral effect against HBV infection.

Based on the available data and findings, the main advantages and disadvantages of various tools that have been investigated to date for the delivery of CRISPR/Cas9 targeting the HBV genome are summarized in **Table 1**.

CONCLUSION

A suitable delivery tool is crucial for the achievement of the desired CRISPR/Cas9 effects against HBV infection. Therapeutic translation of the CRISPR/Cas system remains a challenge because of the lack of a suitable delivery tool (Tong et al., 2019; Wilbie et al., 2019). Researchers are actively pursuing the development of efficient CRISPR/Cas delivery systems, which may address the issue in the near future. Although AAVs are the most widely investigated delivery tools for CRISPR/Cas9 targeting of the HBV genome, no clinical trials have been conducted to date with AAVs. Non-viral nanoparticle-based delivery tools, which may supersede the use of viral vectors in the near future, can be considered for extensive future investigations and could open new avenues for nanoparticle-based effective delivery of CRISPR/Cas9 against HBV infection.

AUTHOR CONTRIBUTIONS

MEHK, MK, and KT-K: conceptualization. MEHK and KT-K: writing—original draft preparation. MEHK, MH, MK, and KT-K: writing—review and editing. All authors have read and agreed to the published version of the manuscript.

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Hepatitis B Virus Reactivation Increased the Risk of Developing Hepatic Failure and Mortality in Cirrhosis With Acute Exacerbation

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Background and Aims: Hepatitis B virus (HBV) reactivation is a serious condition and has been extensively described in chemotherapeutic immunosuppressive population. However, little is known about HBV reactivation in immunocompetent patients with chronic hepatitis B (CHB). In this study, we evaluated the prevalence and the clinical significance of HBV reactivation in CHB patients with acute exacerbations.

Method: Patients were screened from two prospective multicenter observational cohorts (CATCH-LIFE cohort). A total of 1,020 CHB patients with previous antiviral treatment history were included to assess the prevalence, risk factors, clinical characteristics of HBV reactivation, and its influence on the progression of chronic liver disease.

Results: The prevalence of HBV reactivation was 51.9% in CHB patients with acute exacerbations who had antiviral treatment history in our study. Among the 529 patients with HBV reactivation, 70.9% of them were triggered by discontinued antiviral treatment

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and 5.9% by nucleos(t)ide analogs (NUCs) resistance. The prevalence of antiviral treatment disruption and NUCs resistance in patients with HBV reactivation is much higher than that in the patients without (70.9% vs. 0.2%, and 5.9% vs. 0, respectively, both $p < 0.001$). Stratified and interaction analysis showed that HBV reactivation was correlated with high short-term mortality in cirrhosis subgroup (HR = 2.1, $p < 0.001$). Cirrhotic patients with HBV reactivation had a significantly higher proportion of developing hepatic failure (45.0% vs. 20.3%, $p < 0.001$), acute-on-chronic liver failure (ACLF; 31.4% vs. 21.8%, $p = 0.005$), and short-term death (14.0% vs. 5.9% for 28-day, and 23.3% vs. 12.4% for 90-day, both $p < 0.001$) than those without. HBV reactivation is an independent risk factor of 90-day mortality for cirrhosis patients (OR = 1.70, $p = 0.005$), as well as hepatic encephalopathy, ascites, and bacterial infection.

Conclusion: This study clearly demonstrated that there was a high prevalence of HBV reactivation in CHB patients, which was mainly triggered by discontinued antiviral treatment. The HBV reactivation strongly increased the risk of developing hepatic failure, ACLF and short-term death in HBV-related cirrhotic patients, which may suggest that HBV reactivation would be a new challenge in achieving the WHO target of 65% reduction in mortality from hepatitis B by 2030.

Keywords: acute-on-chronic liver failure, cohort study, Hepatitis B virus reactivation, cirrhosis, mortality

INTRODUCTION

Chronic hepatitis B (CHB) remains a significant global health burden, and affects an estimated 250 million people worldwide (Smith et al., 2019). CHB is a potentially life-threatening disease which is mainly caused by HBV persistence infection, and can lead to liver irreversible injury, cirrhosis. Cirrhosis typically has two phases: an asymptomatic phase (compensated cirrhosis) followed by a rapidly progressive phase (decompensated cirrhosis) signaled by the development of complications of portal hypertension and liver dysfunction, which can progress to acute decompensation (AD), or AD with multi-organ failure known as acute-on-chronic liver failure (ACLF; Moreau et al., 2013). Patients with cirrhosis are susceptible to a range of acute hepatic insults, including HBV reactivation, bacterial infection, recent alcohol intake, hepatotoxic drugs, and acute variceal bleeding. Acute hepatic insult is the main cause of hospitalization in patients with CHB in the Asian region (Sarin et al., 2019).

HBV reactivation, defined by the abrupt reappearance or rise of HBV DNA in the serum of a patient with previously inactive or resolved HBV infection, is considered as the main cause of acute hepatic insult in CHB patients in East (Sarin et al., 2019). HBV reactivation is predominantly due to the unbalanced state between host immunity and viral replication (Shi and Zheng, 2020). This event can be caused by a variety of factors including anticancer agents, immunosuppressive, biological therapies, and occur spontaneously. Previous studies suggested that the HBV reactivation rate was 25% (ranged 4%–68%) in cancer patients with previously HBV infection after chemotherapy or immunosuppressive therapy (Paul et al., 2015). Approximately 65% of these patients had disease progressed and even could reach hepatic failure, which required liver transplantation or death (Karvellas et al., 2017). A recent study from Egypt in HBsAg-positive patients who was undergoing direct-acting antivirals (DAAs) against HCV showed that 28.6% of the patients appeared HBV reactivation and only 1(10%) with liver hepatitis (El Kassas et al., 2018).

However, many previous studies were retrospective (Terrault et al., 2018) and most of them were focused on HBV reactivation in the settings of patients underwent chemotherapy or immunosuppressive therapy, therefore the data was lacking to fully describe the incidence of HBV reactivation and its related characteristics in immunocompetent patients with CHB. In the current study, we evaluated the prevalence and the clinical significance of HBV reactivation in CHB patients with acute exacerbation in a prospective multicenter cohort.

MATERIALS AND METHODS

Study Population

Patients were screened from two prospective multicenter observational cohorts (Investigation and Validation cohort of CATCH-LIFE study; Gu et al., 2018; Qiao et al., 2021), recruited from 15 hospitals in HBV high-endemic areas of China from January 2015 to December 2016 and July 2018 to January 2019, respectively. Briefly, there were 2600 patients with acute exacerbation of chronic liver disease in the investigation cohort and 1397 patients under the same inclusion criteria in the

Abbreviations: CHB, Chronic hepatitis B; HBV, Hepatitis B virus; AD, Acute decompensation; UGIB, Upper gastrointestinal bleeding; ALT, Alanine aminotransferase; AST, Aspartate aminotransferase; ALP, Alkaline phosphatase; γ-GT, Gamma-glutamyltransferase; TBIL, Total bilirubin; ACLF, Acute-on-chronic liver failure; NUCs, Nucleos(t)ide analogs; Cr, Creatinine; WBC, White blood cell; INR, International normalized ratio.

validation cohort. The patients with immunosuppressive therapies history were excluded at the enrollment stage of the cohort. The detailed cohort design protocol and basement characteristics of the patients were described previously (Gu et al., 2018; Qiao et al., 2021). All participants were followed up for at least 90 days by telephone call and/or clinic visit with a predesigned standard case report form (CRF) to obtained and recorded the 28-day and 90-day severe outcomes. Within these patients, we further identified the CHB patients with an antiviral treatment history as the subset to assess the influence of HBV reactivation on the progression and outcomes of disease in this study. Totally, 1,020 CHB patients with acute exacerbation who had antiviral treatment history were included in this study (**Figure 1**). The study was approved by the Ren Ji Hospital Ethics Committee of Shanghai Jiaotong University School of Medicine. Written informed consent were obtained from every participant or their legal surrogate.

Definitions

Cirrhosis was diagnosed based on liver biopsy or clinical presentation with typical ultrasound or computed tomography imaging. Acute exacerbation of CHB was defined as nonmalignant CHB with AD or acute liver injury. Diagnostic criteria of AD: (1) acute development of at least one of the gastrointestinal hemorrhage, hepatic encephalopathy (HE), overt ascites or bacterial infection, or (2) jaundice [total bilirubin (TB) > 5 mg/dl]

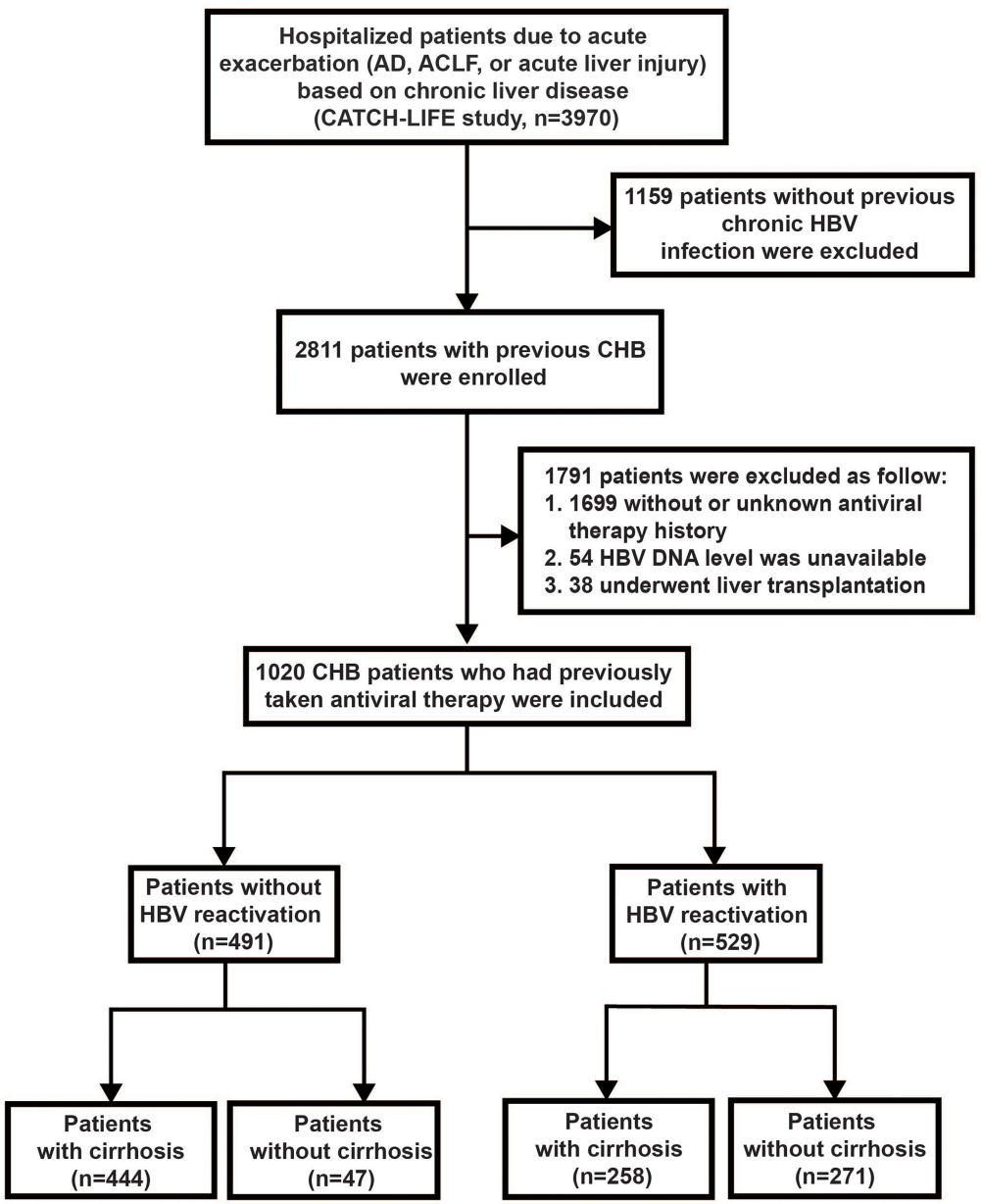

FIGURE 1 | Screening, enrollment, and flow of patients. AD, acute decompensation; ACLF, acute-on-chronic liver failure; and CHB, chronic hepatitis B.

within 1 month before enrollment. Diagnostic criteria of acute liver injury: (1) serum level of alanine aminotransferase (ALT) or aspartate aminotransferase (AST) was three times higher than the upper limit of normal, or (2) serum level of TB was two times greater than the upper limit of normal within 1 week. ACLF was defined according to the EASL-CLIF criteria (Moreau et al., 2013) regardless of cirrhosis. We evaluated whether ACLF existed at admission (day 0) or ACLF newly developed at day 4, 7, 14, 21 and day 28 during hospitalization.

HBV Reactivation Assessment and Data Collection

While the EASL-CLIF criteria did not provide recommendations for the diagnosis of HBV reactivation, the occurrence of HBV reactivation was assessed according to the recommendation (Terrault et al., 2018) of AASLD in 2018: (1) For HBsAg-positive and anti-HBc positive patients, an increase of more than 2 Log_{10} IU/ml in HBV DNA compared to baseline, or 4 Log_{10} IU/ml increase if the baseline level is not available; or (2) For HBsAg-negative and anti-HBc positive patients, HBV DNA is detectable or reappearance of HBsAg (Terrault et al., 2018); and further limited as (3) presenting with a definite antiviral treatment history more than 6 months, to decrease the heterogeneity of HBV reactivation population in this study. The data of clinical characteristics, laboratory tests and complications were obtained with standardized case report forms (CRF) from the medical records of each patient. All data were checked by a physician and two clinical assistants.

Statistical Analysis

The data were analyzed with SPSS software (version 25.0, SPSS Inc., Chicago, United States). Continuous variables are presented as mean ± standard deviation (SD) or median with interquartile range (IQR) while comparisons were performed between groups by Student's *t*-test or Mann–Whitney *U* test, respectively. Categorical variables are presented as proportions while comparisons were performed between groups using the χ^2 test or Fisher's exact test where appropriate. Univariate comparisons were performed using the Kruskal–Wallis test. Multivariable COX hazard analysis was applied to determine the independent factors for 90-day mortality. Survival rates were estimated by Kaplan–Meier analysis and Log-rank test. Statistical significance was set at $p < 0.05$ of two-tailed.

RESULTS

Prevalence and Risk Factors of HBV Reactivation in CHB Patients With Acute Exacerbation

Out of the total 2811 CHB patients with acute exacerbation from the CATCH-LIFE cohort, we identified 1,020 subjects with antiviral therapy history and available HBV DNA level to set as sub-population in this study (**Figure 1**). Among these 1,020 patients, 529 of them experienced HBV reactivation and 491 of them did not, the prevalence of HBV reactivation in CHB patients with previous antiviral therapy history was 51.9%, while that was 18.8% (529/2,811) in the total 2,811 CHB patients with acute exacerbation regardless of antiviral history. The baseline of 1,020 patients are shown in **Supplementary Table 1**.

Among the 529 patients with HBV reactivation, 375 patients (70.9%) were triggered by discontinued antiviral treatment and 31 patients (5.9%) by nucleos(t)ide analogs (NUCs) resistance (**Figure 2**). The prevalence of antiviral treatment disruption and NUCs resistance in patient with HBV reactivation is much higher than that in the patients without HBV reactivation (70.9% vs. 0.2%, and 5.9% vs. 0, respectively, both $p < 0.001$, **Figure 2**). The ratio of the other hepatitis virus (HAV, HCV, and/or HEV) co-infection and the hepatotoxic drugs taken within the past 3 months were similar between the two groups (3.02% vs. 3.26%, and 3.8% vs. 2.0%, respectively, both $p > 0.05$). The events of bacterial infection (15.3% vs. 21.4%, $p = 0.012$), ascites (35.4% vs. 55%, $p < 0.001$), and gastrointestinal bleeding (2.7% vs. 27.3%, $p < 0.001$) were less frequently happened in patients with HBV reactivation than those without, which may due to the higher proportion of cirrhosis in non-HBV reactivation group (90.4% vs. 48.8%, $p < 0.001$; **Figure 2**; **Supplementary Table 1**). Notably, the rate of serum HBeAg-positive was significantly higher in patients with HBV reactivation than that without (63.2% vs. 28.8%, $p < 0.001$, **Figure 2**; **Supplementary Table 1**).

Stratified and Interaction Analysis Showed That HBV Reactivation Was Correlate With Critical Outcome in Cirrhosis Subgroup

Within the 1,020 patients, no significant difference was observed for the 28-day and 90-day mortality between CHB patients with HBV reactivation and that without ($p > 0.05$). Considering the proportion of underlying cirrhosis was unbalance between two groups (**Supplementary Table 1**) which may resulted in bias and confusion, we further performed stratified analysis by dividing individuals into subgroups to evaluate and control confounding factors. We applied 90-day mortality as the event to compare the difference between CHB patients with or without HBV reactivation within these subgroups. As shown in **Figure 3**, there were 133 events of death within 90-days totally, which was 72 in HBV reactivation group and 61 in non-HBV reactivation group [13.6% vs. 12.4%, $p = 0.57$, hazard ratio (HR) = 1.11]. Remarkably, in cirrhosis subgroup the events of death within 90-days in CHB patients with HBV reactivation were significantly higher than that in CHB patients without HBV reactivation (23.3% vs. 12.4%, $p < 0.001$, HR = 2.10, 95% CI = 1.40–3.14, **Figure 3**). What is more, a significant interaction between HBV reactivation and cirrhosis ($P_{interaction} = 0.004$) was observed by the test for interaction under the Cox proportional hazards model. It suggested that the combination of HBV reactivation and cirrhosis strongly increased the risk of short-term death. No similar significance or interaction was observed in non-cirrhotic subgroup or any other subgroups ($p > 0.05$).

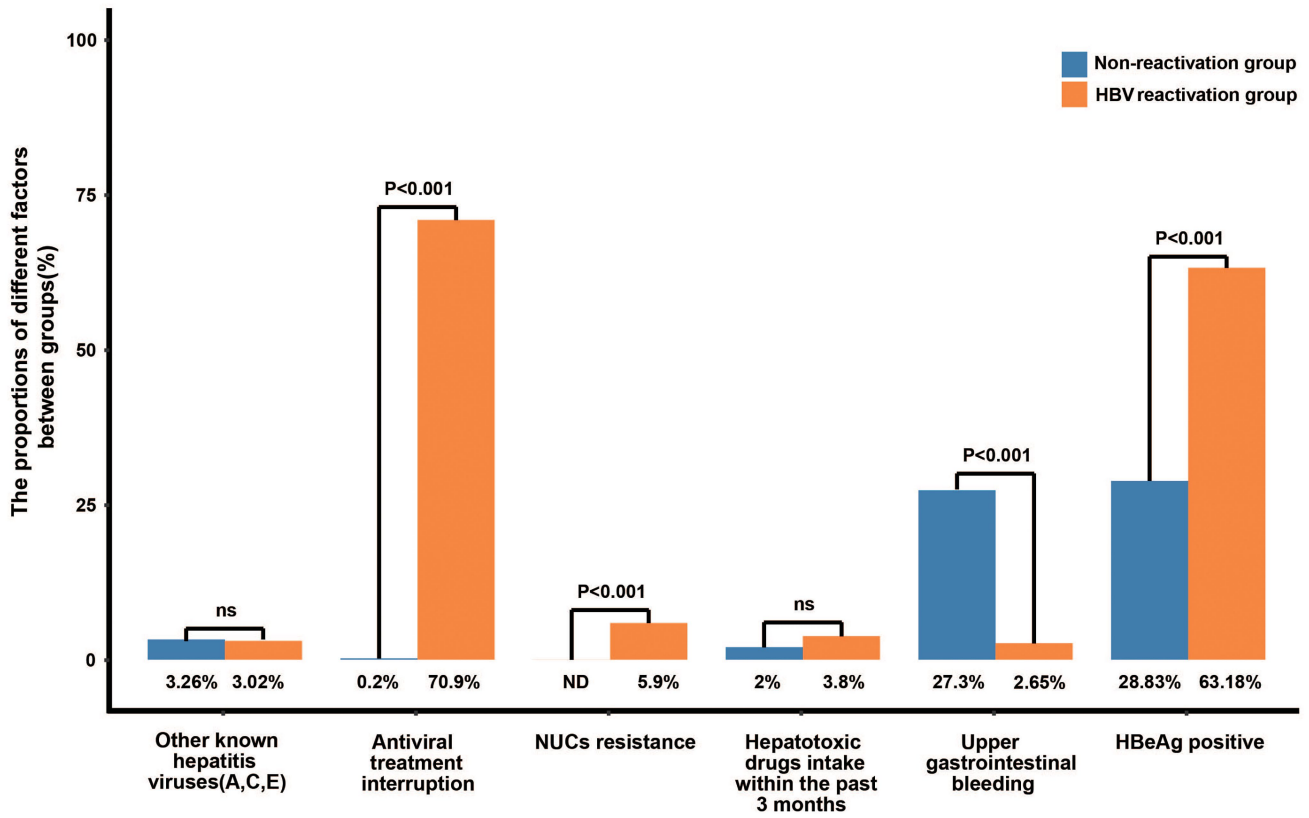

FIGURE 2 | Risk factors of HBV reactivation in CHB patients.

Characteristics and Influence of HBV Reactivation in Cirrhotic CHB Patients With Acute Exacerbation

In order to explore the influence of HBV reactivation on the disease progression of cirrhosis, we further compared the characters and the short-term mortality between cirrhotic patients with and without HBV reactivation in the cirrhosis subgroup.

All 702 cirrhotic patients with acute exacerbation were included in this analysis and the baseline characteristics are shown in **Table 1** (while the baseline of 318 non-cirrhotic patients is shown in **Supplementary Table 3**). Compared to the patients without HBV reactivation, HBV reactivation patients were a little bit younger (49.0 ± 10.0 vs. 50.6 ± 10.5 years, $p = 0.04$) and had a significantly lower incidence of gastrointestinal bleeding (5.4% vs. 30.2%, $p < 0.001$), while there was no significant difference in the incidence of bacterial infection ($p = 0.92$), ascites ($p = 0.31$), and others hepatitis virus co-infection ($p = 0.84$) between the two groups. However, cirrhotic patients with HBV reactivation had a significantly higher ALT, AST, alkaline phosphatase (ALP) and γ-glutamyltransferase (γ-GT) level (both $p < 0.001$), as well as bilirubin level and international normalized ratio (INR; both $p < 0.001$), than the non-HBV reactivation group, which suggested much more heavy liver injury when cirrhosis patient suffered from HBV reactivation. Overall, 45.0% of HBV reactivation patients developed liver failure, which was significantly higher than that of 20.3% in the non-HBV reactivation cirrhotic patients ($p < 0.001$, **Table 1**).

Furthermore, the 28-day mortality was significantly higher in HBV reactivation patients than that in the non-HBV reactivation patients (14.0% vs. 5.9%, $p < 0.001$, **Figure 4A**), as well as 90-day mortality (23.3% vs. 12.4%, $p < 0.001$, **Figure 4B**), which suggested a worse outcome when cirrhosis patient suffered from HBV reactivation.

HBV Reactivation Increased the Risk of Developing ACLF in Cirrhosis Patients and the Mortality of ACLF

To discover whether HBV reactivation plays a role in the development of liver failure in cirrhotic patients with acute exacerbation, we further analyzed the accumulative incidence and characteristics of ACLF. Among the 702 cirrhotic patients, 178 of them were diagnosed as ACLF within 28 days after admission. The cumulative incidence of ACLF within 28 days was significantly higher in cirrhotic patients with HBV reactivation than that without HBV reactivation (31.4% vs. 21.8%, $p = 0.005$, **Figure 4C**), which may suggest that HBV reactivation increased the risk of developing ACLF in cirrhosis patient with acute exacerbation.

Notably, among the 178 patients who developed ACLF, the incidence of hepatic failure was significantly higher when patients underwent HBV reactivation (87.7% vs. 53.6%, $p < 0.001$;

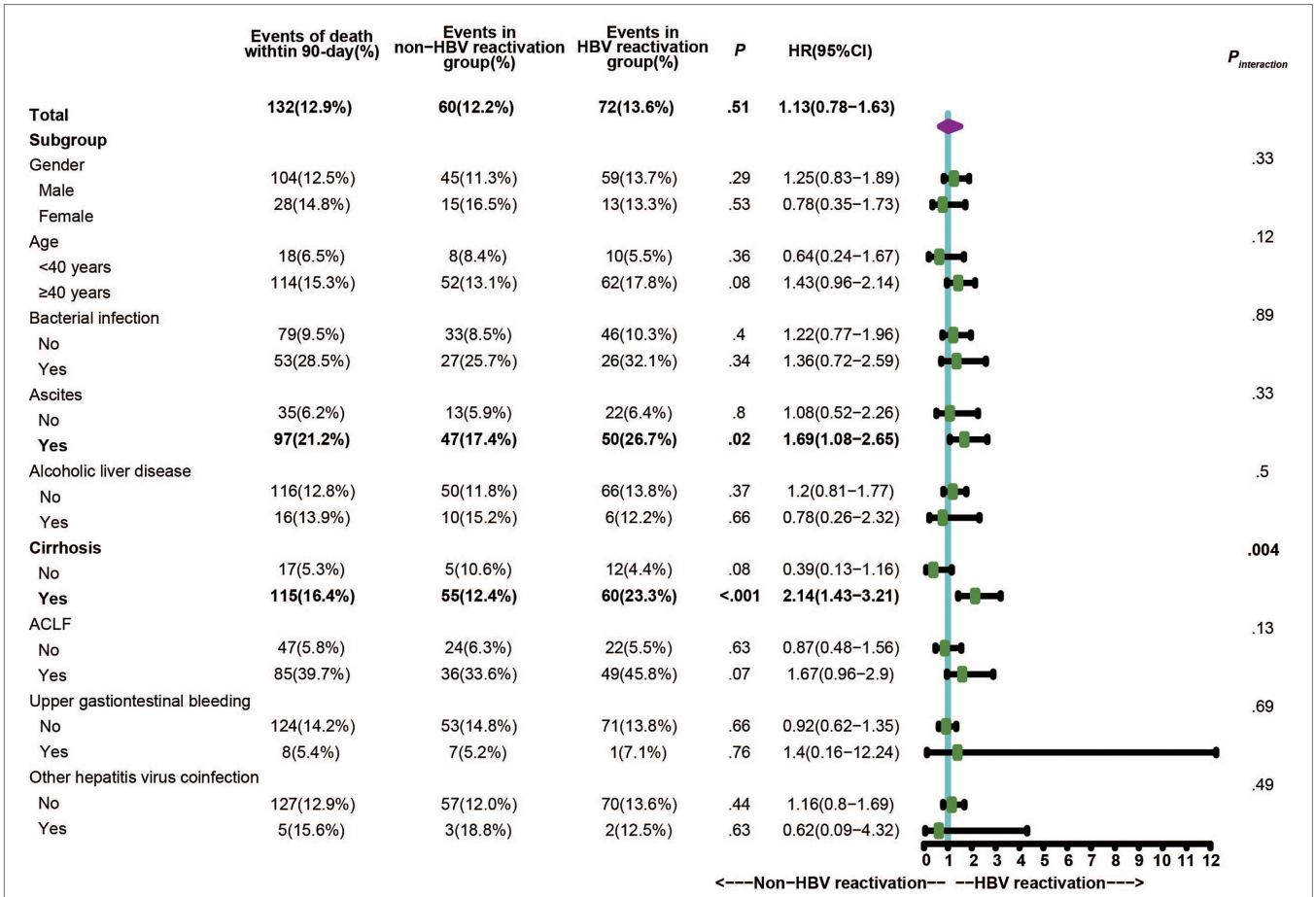

FIGURE 3 | Stratified and interaction analysis to identify the subgroup that interacted with HBV reactivation on the critical outcome of CHB.

Supplementary Table 2), while that of coagulation failure and extrahepatic organ failure (kidney, cerebral or lung) seemed to be similar between HBV reactivation ACLF and non-HBV reactivation ACLF ($p > 0.05$). For those cirrhotic ACLF patients without HBV reactivation, more precipitant events of alcohol consumption and gastrointestinal bleeding were observed ($p = 0.039$ and $p = 0.002$, respectively; **Supplementary Table 2**).

Furthermore, for those 178 ACLF patients, the 28-day and 90-day mortality was much higher in those with HBV reactivation than those without HBV reactivation (35.8% vs. 18.6%, $p = 0.009$; 51.9% vs. 33.0%, $p = 0.011$, respectively, **Figure 4D**; **Supplementary Table 2**), however similar difference was not observed in non-ACLF patients (10.2% vs. 6.9%, $p = 0.19$).

HBV Reactivation Was an Independent Risk Factor of 90-Day Mortality for Cirrhosis Patients

To discover the risk factors of mortality for cirrhosis patients with acute exacerbation and whether HBV reactivation have an independent influence on the short-term death, we performed the univariate and multivariate analysis among the 702 cirrhotic patients. The results of the univariate analysis are showed in **Table 2**. HBV reactivation [odds ratio (OR) = 2.01, 95% CI = 1.40–2.90, $p < 0.001$] played a role in the 90-day mortality, as well as bacterial infection (OR = 2.51, 95% CI = 1.73–3.63, $p < 0.001$), ascites (OR = 2.75, 95% CI = 1.74–4.34, $p < 0.001$) and HE grades III-IV (OR = 5.22, 95% CI = 2.29–11.89, $p < 0.001$).

Furthermore, in the multivariate analysis we included the variables with values of p less than 0.05 in the univariate analysis to identify whether HBV reactivation perform an independent influence on the severe outcome of cirrhosis. The results showed that HBV reactivation (OR = 1.70, 95% CI = 1.17–2.49, $p = 0.005$) was really an independent risk factor of 90-day mortality for cirrhosis patients besides the risk factors bacterial infection (OR = 1.98, $p = 0.001$), ascites (OR = 2.09, $p = 0.002$), HE grades III-IV (OR = 4.71, $p < 0.001$), and sepsis (OR = 2.84, $p = 0.006$), all details are listed in **Table 2**.

However, serum level of HBV DNA was not a predictive factor for the 90-martality (OR = 1.08, 95% CI = 1.00–1.18, $p = 0.13$). Among the laboratory variables listed in **Table 2**, only baseline level of INR was a significant strong predictor for the mortality of cirrhosis patient with acute exacerbation (OR = 1.68, 95% CI = 1.29–2.19, $p < 0.001$) rather than liver enzymes (ALT, AST, ALP, Y'-GT), bilirubin, albumin, creatinine, white blood cells count or platelet.

TABLE 1 | Characteristics of patients with cirrhosis between HBV reactivation and non-reactivation.

	Total ($n = 702$)	Non-HBV reactivation ($n = 444$)	HBV reactivation ($n = 258$)	p
Demographic, *n* (%)				
Male sex	565 (80.5)	360 (81.1)	205 (79.5)	0.60
Age, *y*, mean ± SD	50.0 ± 10.4	50.6 ± 10.5	49.0 ± 10.0	0.04
Alcohol consumption	93 (13.3)	61 (13.7)	32 (12.4)	0.62
Other hepatitis virus	23 (3.3)	15 (3.4)	8 (3.1)	0.84
Complication, *n* (%)				
Bacterial infection	166 (23.7)	98 (22.1)	68 (26.4)	0.92
Ascites	429 (61.1)	265 (59.7)	164 (63.6)	0.31
Gastrointestinal bleeding	148 (21.1)	134 (30.2)	14 (5.4)	<0.001
Hepatic encephalopathy				0.72
Grade I	27 (3.9)	19 (4.3)	8 (3.1)	
Grade II	28 (4.0)	19 (4.3)	9 (3.5)	
Grade III	6 (0.9)	4 (0.9)	2 (0.8)	
Grade IV	5 (0.7)	2 (0.5)	3 (1.2)	
HBV markers				
HBeAg positive, n/N (%)	226/624 (36.2)	102/394 (25.9)	124/230 (53.9)	<0.001
HBeAb positive, n/N (%)	180/625 (28.8)	135/393 (34.4)	45/232 (19.4)	<0.001
HBV DNA, log_{10} IU/ml, median (IQR)	2.7 (1.7–5.0)	2.0 (1.2–2.7)	5.7 (4.6–6.8)	<0.001
Laboratory test, median (IQR)				
Alanine transaminase, U/L	49.1 (25.2–158.0)	31.1 (20.0–55.4)	172.0 (72.6–456.0)	<0.001
Aspartate transaminase, U/L	67.0 (35.0–169.0)	43.0 (28.0–81.0)	182.0 (87.1–386.0)	<0.001
Alkaline phosphatase, U/L	114.0 (80.0–161.0)	98.2 (66.8–145.0)	136.0 (108–171.0)	<0.001
γ-glutamyltransferase, U/L	48.8 (24.0–96.2)	32.0 (18.5–66.0)	84.0 (49.0–136.0)	<0.001
Albumin, g/L	31.0 (27.1–34.7)	31.1 (27.1–35.0)	30.8 (27.3–34.4)	0.43
Total bilirubin, mg/dL	3.9 (1.5–14.1)	2.6 (1.1–9.6)	10.0 (3.3–21.0)	<0.001
White blood cell, 10^9/L	4.6 (3.2–6.6)	4.4 (2.9–6.5)	5.0 (3.7–6.9)	0.016
Platelet, 10^9/L	66.0 (44.0–101.0)	63.0 (42.0–99.0)	73.0 (51.0–106.0)	0.71
International normalized ratio	1.5 (1.3–1.9)	1.5 (1.3–1.8)	1.6 (1.4–2.1)	<0.001
Creatinine, mg/dl	0.8 (0.7–1.0)	0.8 (0.7–1.0)	0.8 (0.6–1.0)	0.35
Sodium, mmol/L	138 (134–140)	138 (134–140)	138 (135–140)	0.52
Organ failures, *n* (%)				
Liver failure	206 (29.3)	90 (20.3)	116 (45.0)	<0.001
Coagulation failure	88 (11.4)	46 (10.4)	34 (13.2)	0.26
Kidney failure	22 (3.1)	15 (3.4)	7 (2.7)	0.63
Cerebral failure	11 (1.6)	6 (1.4)	5 (1.9)	0.55
Adverse outcome, *n* (%)				
28-day death	62 (8.8)	26 (5.9)	36 (14.0)	<0.001
90-day death	115 (16.4)	55 (12.4)	60 (23.3)	<0.001
Diagnosed as ACLF within 28 days*	178 (25.4)	97 (21.8)	81 (31.4)	0.005

**ACLF was evaluated at admission (day 0) and day 4, 7, 14, 21, and 28 during hospitalization.*

SD, standard deviation and IQR, interquartile range. The identification of organ failure was based on CLIF-OF (Moreau et al., 2013).

DISCUSSION

It was reported that HBV reactivation could occur spontaneously or conditionally (Shi and Zheng, 2020), especially it was likely to occur when chemotherapeutic immunosuppressive agents were given for patients with cancer, immunity disease and organ transplantation (Paul et al., 2015; Karvellas et al., 2017), and when DAAs treatment was given for patients with HBV/HCV coinfection (El Kassas et al., 2018). HBV reactivation was a serious condition which could cause significantly higher mortality in these patients. However, little is known about the reactivation of HBV in widely immunocompetent patient with CHB. Here we provide new sight on this issue.

In our present study, the prevalence of HBV reactivation was 51.9% in CHB patients with acute exacerbations who had an antiviral treatment history. It seems that the HBV reactivation becomes a new remarkable burden after two decades of effort on widespread antiviral treatment of hepatitis B in China. Although the World Health Organization (WHO) has set the global targets to eliminate hepatitis B as a public health threat by 2030 through vaccination, screening and treatment (WHO, 2016), researchers still worry about that the elimination goal is highly unlikely to be reached (Cox et al., 2020) given the low average diagnosis rate of hepatitis B, which is only about 8% globally (Thomas, 2019). From the result of this study, HBV reactivation in CHB patients may be another factor would prevent the reach of the global goal and need to be concerned more.

Antiviral drug withdrawal or interruption was the predominant reason of the HBV reactivation in our present study. Among the 529 patients with HBV reactivation, 70.9% of them were triggered by cessation of antiviral treatment. Although both guidelines of APASL, EASL, and AASLD have put forward elaborate recommendations on NUCs treatment and withdrawal, it is still common in clinical practice that patients have poor

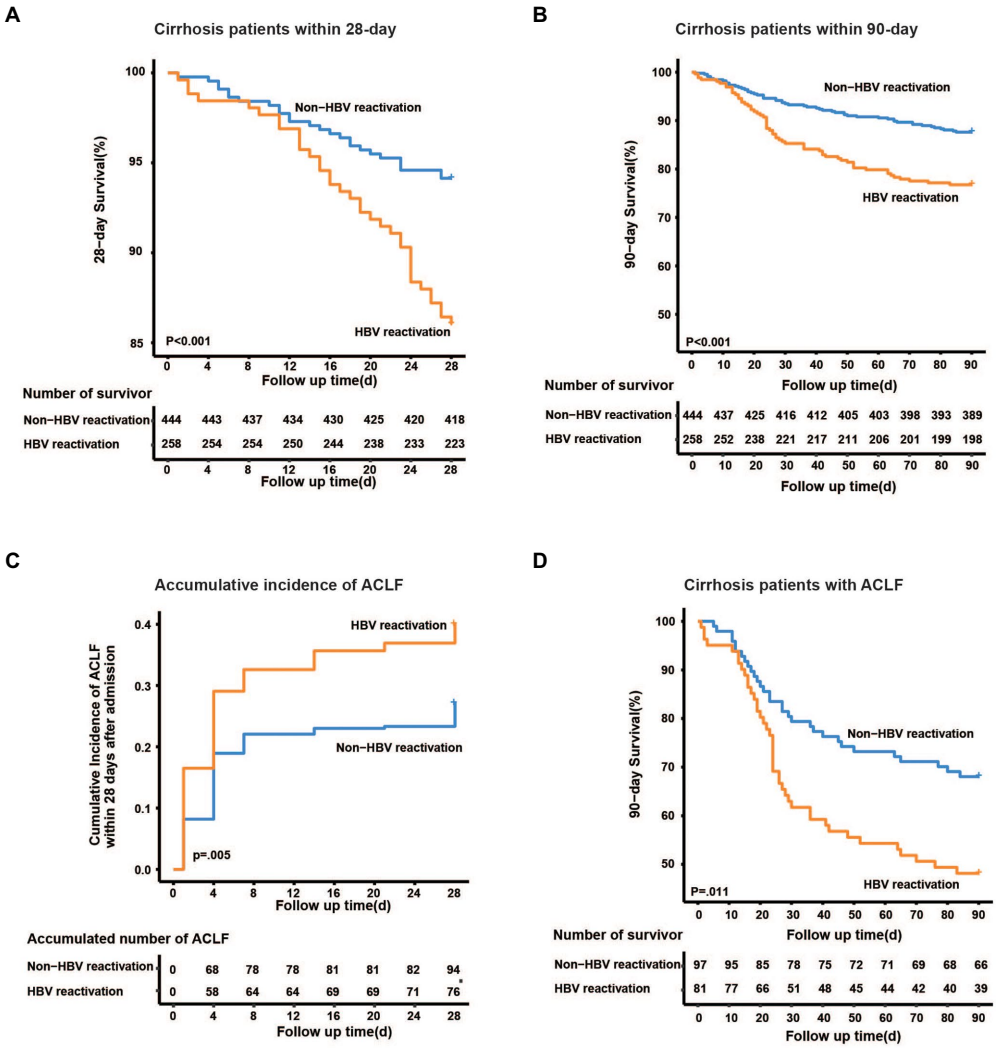

FIGURE 4 | Kaplan–Meier analysis of survival rate and accumulative developing of ACLF. Kaplan–Meier curves are shown for the cumulative incidence of 28-day death **(A)**, 90-day death **(B)** and developing of ACLF **(C)** in cirrhotic patients with and without HBV reactivation. **(D)** Cumulative 90-day mortality in ACLF patients between groups.

adherence and stop NUCs treatment under various reasons, with a reported treatment adherence about 46.4%–74.6% (Ford et al., 2018; Abu-Freha et al., 2020), which may results in virological breakthrough and increasement of mortality (Abu-Freha et al., 2020). Taken together, it suggests that more attention should be paid on the adherence of antiviral treatment in CHB patients to avoid HBV reactivation and its related critical outcomes.

In our study, stratified and interaction analysis showed that HBV reactivation was strongly increased the risk of short-term death in cirrhosis subgroup. The mortality was 14.0% for 28-day and 23.3% for 90-day in cirrhotic patients with HBV reactivation, which was significantly higher than that of 5.9% and 12.4%, respectively, in cirrhotic patients without HBV reactivation. There is limited research or cohort data about the mortality in immunosuppressive-therapy-induced HBV reactivation, it was reported to be 3.7% in HBsAg-positive patients and 0% in HBsAg-negative patients who received cytotoxic therapy for malignant lymphoma in an earlier article (Lok et al., 1991), the mortality is less than that of cirrhotic patients from our study which may due to the underlying conditions. Previous case reports described the HBV reactivation death due to liver failure despite the introduction of lamivudine at the onset of HBV reactivation (Lok et al., 1991; Gwak et al., 2007). To discover the possible reason of high mortality in cirrhosis with HBV reactivation, we further found that HBV reactivation strongly increased the risk of developing liver injury, liver failure and ACLF in cirrhotic CHB patients. ACLF is a critical syndrome distinguished from AD, characterized by high-grade inflammation and high 28-day mortality (Moreau et al., 2013). The cumulative incidence of ACLF within 28 days in our study was significantly higher in cirrhotic patients with HBV reactivation and consequently a higher 28-day and 90-day mortality than that without HBV reactivation, which suggested that HBV reactivation could increase the risk of developing ACLF and related death in cirrhosis patient

TABLE 2 | Univariate and multivariate analysis of the risk factors of the 90-day death in cirrhosis patients.

Variable	Univariate analysis			Multivariate analysis		
	p	OR	95% CI	*p*	AOR	95% CI
Risk factors						
Male sex	0.58	0.88	0.57–1.37			
Age	0.28	1.01	0.99–1.03			
Bacterial infection	<0.001	2.51	1.73–3.63	0.001	1.98	1.34–2.92
Ascites	<0.001	2.75	1.74–4.34	0.002	2.09	1.30–3.33
HE grades III-IV	<0.001	5.22	2.29–11.89	<0.001	4.71	2.01–11.05
Alcoholic liver disease	0.96	0.99	0.57–1.7			
Gastrointestinal bleeding	<0.001	0.26	0.13–0.53	0.041	0.48	0.24–0.97
HBV reactivation	<0.001	2.01	1.40–2.9	0.005	1.70	1.17–2.49
Other viral hepatitis	0.67	0.78	0.25–2.45			
Sepsis	<0.001	4.72	2.30–9.70	0.006	2.84	1.35–5.97
Predict markers						
Alanine transaminase	0.011	1.00	1.00–1.00			
Aspartate transaminase	0.003	1.00	1.00–1.00			
Alkaline phosphatase	<0.001	1.01	1.00–1.01			
γ-Glutamyltransferase	0.72	1.00	1.00–1.002			
Albumin	0.001	0.95	0.92–0.98	<0.001	0.93	0.89–0.97
Total bilirubin	<0.001	1.08	1.07–1.09	<0.001	1.07	1.05–1.09
International normalized ratio	<0.001	2.92	2.41–3.55	<0.001	1.68	1.29–2.19
Creatinine	0.017	1.19	1.03–1.38			
White blood cell	<0.001	1.06	1.04–1.09			
Platelet	0.94	1.00	1.00–1.00			
Log_{10} HBV DNA	<0.001	1.17	1.08–1.26			

OR, odds ratio; AOR, adjusted odds ratio; CI, confidence interval; and HE, hepatic encephalopathy.

with acute exacerbation. To discover whether the selection of different ACLF criteria could make a difference to the conclusion, we further analyzed the accumulative incidence of ACLF in our cohort under APASL (Sarin et al., 2019) and COSSH criteria (Wu et al., 2018), the results (data were not shown) was consistent with this study. As we and other studies have reported, liver and coagulation failures were the two most common types of organ failure in patients with HBV-related ACLF (Tan et al., 2018). Among the 178 patients who developed ACLF in this study, the incidence of hepatic failure was up to 87.7% in ACLF patients with HBV reactivation, which was significantly higher than that without, while that of extrahepatic organ failure (kidney, cerebral or lung) was similar between two groups.

In addition, we found that HBV reactivation was an independent risk factor for the 90-days mortality of CHB patients with cirrhosis, remained a high risk (1.7-fold) after adjusting by other factors as age, sex, bacterial infection, ascites, hepatic encephalopathic, alcoholic assumption, gastrointestinal bleeding, sepsis and other viral hepatitis. Previous study reported that half of HBeAg-positive patients had the chance of HBeAg seroconversion within 3 months after hepatitis flare (Yuen et al., 2003). In a proof-of-concept study, stopping antiviral therapy in non-cirrhotic patients with HBeAg-negative CHB resulted in HBV rebound and subsequent significantly HBsAg decline, with 20% HBsAg loss in 48-week follow-up (Höner Zu Siederdissen et al., 2016), suggesting the immune activation in those patients and a potential therapeutic strategy to accelerate HBsAg loss and functional cure (Höner Zu Siederdissen et al., 2016; van Bommel and Berg, 2021). However, this antiviral-stopping therapeutic strategy seems to be not feasible in cirrhosis patients considering the high incidence of HBV reactivation and its related high risk of liver failure and death from the data of our present study.

Similar correlation between short-term death and HBV reactivation was not observed in non-cirrhotic subgroup. Adversely, it showed a lower (4.4%) but not significant mortality in non-cirrhotic patients with HBV reactivation than that without (10.6%). One of the possible explanations might be that the non-cirrhosis patient in our population was younger (median age 39.8 years, data were shown in **Supplementary Table 3**). Moreover, there were only 47 non-cirrhotic patients without HBV reactivation, the volume was too small which might lead to bias when calculated the mortality. Thus, more evidence is need to be carried out to clarify whether HBV reactivation plays a role or not in non-cirrhotic patients.

Our study has several strengths. Firstly, in this prospective multicenter observational cohort, the HBV reactivation were observed in common CHB population who were not under an immunosuppressive condition, which provides a better understanding of the association between HBV reactivation and the progression of disease in HBV-related cirrhosis patients. Secondly, participants in our study belong to 15 hepatology or infectious disease centers in a high-endemic area of HBV, and the prospective multicenter design of the cohort with low rate of loss in follow-up, which ensured that the results were representative and reliable. Thirdly, it is an evidence-based data showed a high prevalence rate of HBV reactivation which major duo to antiviral treatment withdrawal in general CHB patients. Finally, it is a unique and evidence-based study in demonstrating the independent correlation between HBV reactivation and critical outcomes in cirrhotic patients with acute exacerbation.

This study has the following limitations. Untreated patients were excluded in this study due to lacked data of medical history or unclear antiviral treatment history. HBV flares in these patients will deserve attention in the future studies. Furthermore, despite we showed that antiviral treatment withdrawal accounted for the most of the HBV reactivation in CHB, however the details of antiviral drugs (NUCs or IFNs) and the discontinuation reason (stopped under the guidance of doctor when met the endpoint or stopped themselves due to personal reason) were not distinguished in this study. Additionally, it is hard to obtained the baseline level of HBV DNA before reactivation since most of them would not visit clinic or hospital before hepatitis flare. However, we had applied the widely-accepted AASLD definition (Terrault et al., 2018) which had a definite criterion when baseline HBV DNA level is not available, to maximum precision of the study as possible. Finally, we were not able to correlate the HBV reactivation with the clinical outcome in non-cirrhotic patients due to our limited number of patients.

In conclusion, the HBV reactivation in CHB was highly prevalent and mainly triggered by antiviral treatment withdrawal. The HBV reactivation could strongly increase the risk of developing liver injury, hepatic failure, ACLF and short-term death in cirrhosis patients, which may suggest that HBV reactivation would be a new challenge in achieving the WHO target (WHO, 2016) of 65% reduction in mortality from hepatitis B by 2030. Therefore, the antiviral-stopping therapeutic strategy, which aim to activate immune response and accelerate HBsAg loss, was not feasible in cirrhosis patients considering the high incidence of HBV reactivation and its related high risk of liver failure and death from the data of our present study.

TRANSPARENCY STATEMENT

The lead authors affirm that the manuscript is an honest, accurate, and transparent account of the study being reported; that no important aspects of the study have been omitted; and that any discrepancies from the study as planned have been explained.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ren Ji Hospital Ethics Committee of Shanghai Jiaotong University School of Medicine. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

HL, JiC, WT, and GD obtained the funding. HL, WT, and GD designed the study. YZu, HL, XW, XZe, YHu, JiC, ZM, YG, ZQ, FL, XL, YS, JS, HY, YZe, LQ, YZa, XX, YD, SS, YHo, QZ, YX, SLi, JuC, ZH, BL, XJ, SLu, YC, NG, CL, LJ, WY, JL, TL, RZ, XZo, HR, YZo, BX, WT, and GD screened, enrolled, and clinically managed all the participated patients and collected the data. YZu, RY, and WT performed the statistical analysis. YZu, WT, and GD drafted the manuscript. GD and WT contributed to the critical revision of the manuscript for important intellectual content. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2022.910549/full#supplementary-material

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Relationship between hepatitis C and kidney stone in US females: Results from the National Health and Nutrition Examination Survey in 2007–2018

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Background: The main objective of this study is to explore the effects of hepatitis C (HCV) on the prevalence rate of kidney stones in US women.

Method: Dates for HCV infection and kidney stones were collected from National Health and Nutrition Examination Survey (NHANES) database, a cross-sectional study. The analysis samples included adults aged ≥20 years and women from six consecutive cycles of the NHANES 2007–2018. The association between HCV infection and kidney stones was performed by using logistic regression models. Subgroup analyses were conducted to find sensitive crowds.

Results: A total of 13,262 participants were enrolled, including 201 infected with HCV. After adjustment for potential confounders, we revealed a positive relationship between HCV and kidney stones (OR = 1.70, 95%CI:1.13–2.56). The crowds' statistically significant difference was characterized by other races (OR = 8.17, 95%CI:1.62–41.22) and BMI within 25–29.9 kg/m2 (OR = 2.45, 95%CI:1.24–4.83).

Conclusions: HCV infection may affect the prevalence of urolithiasis in US women, even the causal relationship remains unclear, the relation deserves special attention. We considered such a study an ideal way to begin exploring the effects of HCV on kidney stones.

KEYWORDS

kidney stone, hepatitis C virus (HCV), National Health and Nutrition Examination Survey(NHANES), nephrolithiasis, cross-section study

Introduction

When it comes to the urinary system, nephrolithiasis (NL), also known as kidney stones, is a prevalent problem in the study field, with high rates of occurrence and recurrence (1–3). The morbidity of patients with nephrolithiasis is increasing as a result of global warming, including a fast-paced lifestyle and an unhealthy or irregular diet, among other factors (4). Kidney stones have become increasingly widespread all around the globe over the years, regardless of gender, race, or age of the patient. Note that the rate of kidney stone production varies significantly across different geographical locations and nations. The prevalence of kidney stones in the United States (5) was more than 10%, according to a recent multi-country survey study, although the rates were 9 percent and 5.8 percent in Europe (6) and China (7), respectively, according to the same study. We also found that gender plays a very important role in the epidemiology of renal calculi. According to a 2020 NHANES-based survey study (5), it was found that the prevalence of kidney stones increased significantly in women during 2008–2018, while it did not increase significantly in men. In another retrospective study that surveyed 500,000 Navy personnel, it was found that the prevalence of kidney stones was way higher in women than in men (8). To treat it, minimally invasive endoscopic procedures, such as percutaneous nephrolithotomy, flexible ureteroscopy lithotripsy, and other endoscopic procedures, are routinely performed. Although therapy has been completed, patients still have a substantial risk of recurrence of their condition (9). If it is not treated effectively, it may progress to serious consequences, such as irreversible kidney damage and end-stage renal disease. Nephrolithiasis has grown in importance as a public health concern, as well as a significant financial burden on medical healthcare systems (10).

Hepatitis C virus (HCV) is a single-stranded RNA virus with an envelope that belongs to the Flaviviridae viral family (11). It is responsible for the transmission of the disease. HCV infection is a serious public health hazard in many parts of the globe, including the United States. Worldwide, around 130–150 million individuals are estimated to be chronically infected with HCV (12), according to the WHO. HCV infection is diagnosed with the use of anti-HCV antibodies and HCV RNA (13). It is one of the most significant contributors to liver fibrosis, organ failure, and even liver cancer (14). Not only that but there is also a gender gap in HCV infection rates. HCV surveillance data typically show lower HCV detection rates in women than in men (15). However, a recent survey of HCV prevalence among drug users found a higher prevalence among women drug users (15). A Pakistani study also found significantly higher rates of HCV infection in women than in men (16). Recent investigations have shown that HCV may infect macrophage cells, which may explain the extrahepatic symptoms. Chronic HCV infection has the potential to harm other organs, including the lung (17), heart (18), and kidney. Almost all of the research conducted so far has focused only on the impact of HCV on renal function (19). The most typically documented kidney ailment linked with HCV is HCV-associated glomerular disease, which is characterized by the presence of mixed cryoglobulinemia. Although glomerular damage is often believed to be the cause of kidney disease, tubular injury has also been identified as a contributing factor. It

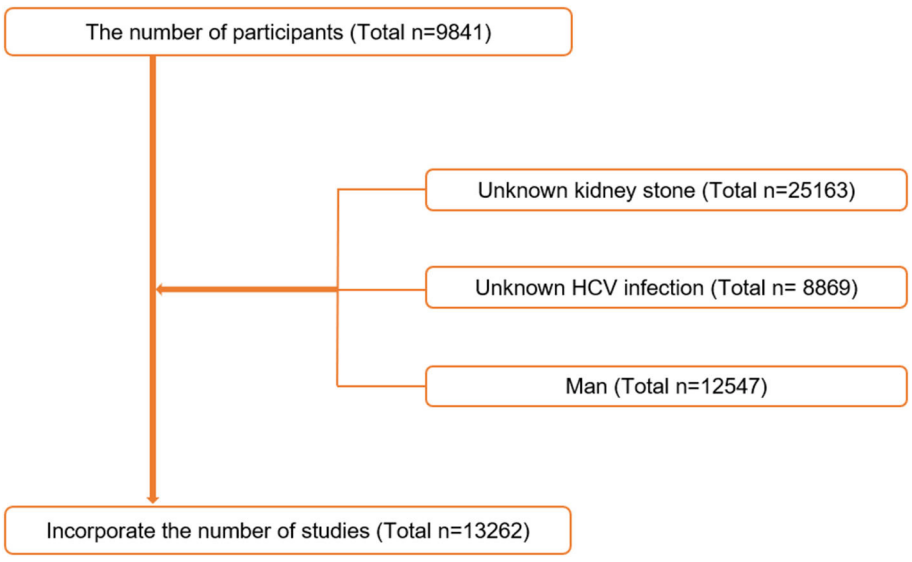

FIGURE 1
Flowchart of the sample selection from National Health and Nutrition Examination Survey (NHANES) 2007–2018.

has been found that individuals with HCV infection suffer from both glomerular and tubule damage, respectively (20). One of the most serious risks associated with kidney stone prevalence is tubular epithelial cell injury (2). Is there a correlation between the increased prevalence of kidney stones and HCV infection in women?

As a consequence, we undertook a large population-based cross-sectional investigation using the National Health and Nutrition Examination Survey database to determine if there is a link between HCV and the occurrence of kidney stones in the general population. Subgroup studies were conducted at the same time to identify the most vulnerable population. All of the studies aimed to provide some kind of proof for the prevention of renal illness on some level.

Materials and methods

Study design

Data for this study were obtained from the NHANES database based on big data mining methods and were conducted by the Centers for Disease Control and Prevention (CDC) (21, 22). NHANES is a nationally representative, cross-sectional survey meant to produce nationally representative estimates of the population's health and nutritional status. It is utilized for cross-sectional investigations. From 2007 through 2018, survey data was used six times in a row. The NHANES website (www.cdc.gov/nchs/nhanes/) has further information on the data.

Participants

In this research, all of the subjects were between the ages of 20 and 80. The current research has a total of 59,841 participants. The following were the criteria for exclusion: (1) unknown kidney stone (n = 25,163); (2) undetermined HCV infection status (n = 8,869); and (3) men (n = 12,547) (Figure 1). A total of 13,262 participants were included in the final analysis. The Institutional Review Committee of the National Center for Health Statistics(NCHS) gave its approval to the procedure.

Variables

To identify kidney stones, the results from the KIQ026 (Do you have kidney stones?) questionnaire were used. To

Abbreviations: HCV, Hepatitis C virus; NHANES, National Health and Nutrition Examination Survey; HCV-Ab, Hepatitis C virus antibody; HCV-RNA, Hepatitis C virus RibonucleicAcid; OR, Odds ratio; BMI, Body mass index; US, United States; PIR, Ratio of family income to poverty.

TABLE 1 Baseline characteristics of US female participants in NHANES from 2007–2018, weighted.

Characteristic	Nonstone formers $N = 12,197$	Stone formers $N = 1,065$	*P*-value
Age(years)	47.69 ± 17.10	51.61 ± 15.81	<0.0001
Total calcium(mg/dl)	9.37 ± 0.37	9.38 ± 0.45	0.214
Serum creatinine(mg/dl)	0.77 ± 0.27	0.84 ± 0.66	<0.0001
BMI(kg/m^2)	28.99 ± 7.48	31.00 ± 7.85	<0.0001
Race(%)			<0.0001
Mexican American	7.97	6.82	
Other hispanic	6.03	4.5	
Non-hispanic white	66.38	75.87	
Non-hispanic black	11.78	6.96	
Other race	7.85	5.84	
Infection status(%)			<0.0001
No	98.81	97.1	
Yes	1.19	2.9	
Blood pressure(%)			<0.0001
Yes	69.53	52.13	
No	30.38	47.74	
Unclear	0.09	0.14	
Education level(%)			0.4675
Less than high school	15.56	16.85	
High school	22.06	23.05	
More than high school	62.3	59.99	
Unclear	0.08	0.11	
Marital status(%)			0.6637
Cohabitation	60.16	61.51	
Solitude	39.8	38.46	
Unclear	0.04	0.03	
PIR(%)			0.0717
<1.39	31	34.27	
1.39–3.49	23.54	23.23	
≥3.49	38.03	34.63	
Unclear	7.43	7.87	
Ever receive blood transfusion(%)			<0.0001
Yes	12.1	21.53	
No	86.95	77.55	
Other	0.95	0.92	
Total water(%)			0.0562
<2000 ml	51.47	52.22	
2000–2500 ml	7.82	9.81	
More than 2500 ml	26.3	25.12	
Unclear	14.41	12.86	
Physical activity(%)			0.001
Never	32.89	37.69	

(Continued)

TABLE 1 Continued

Characteristic	Nonstone formers $N = 12,197$	Stone formers $N = 1,065$	*P*-value
Moderate	36.74	37.04	
Vigorous	30.35	25.24	
Unclear	0.02	0.03	
Alcohol (%)			0.0854
Yes	22.72	25.07	
No	50.2	46.6	
Unclear	27.08	28.33	
Diabetes (%)			<0.0001
No	89.69	79.54	
Yes	8.37	16.67	
Borderline	1.87	3.43	
Unclear	0.07	0.36	

Statistically significant, p < 0.05; Mean+SD for continuous variables. P-value was calculated by weighted linear regression model.
% for categorical variables, P-value was calculated by weighted chi-square test.
HCV, Hepatitis C virus; BMI, Body mass index (kg/m2); PIR, Ratio of family income to poverty.

confirm the diagnosis of HCV infection, laboratory tests were completed. The methodologies for detecting HCV-Ab and HCV-RNA were developed from the laboratory data column's "Description of Laboratory Methodology." HCV-Ab(+)or HCV-RNA(+) were used to define positive HCV infection. Age, race, education level, marital status, PIR (Ratio of Family Income to Poverty), physical activity, total water consumption, high blood pressure, diabetes, and alcohol users were all included in the questionnaires. The laboratory test yielded the BMI.

Statistical analysis

Regarding the selection of weights, the principle of the official guidelines provided by NHANES is to first specify the variable that examines the smallest population and then proceed with selecting the weights corresponding to that variable. In this study, our data included MEC examination data, and according to the recommendations of the weight selection guidelines, we selected the sub-weights corresponding to MEC. According to the NHANES analysis guidelines, the new sampling weights for the combined survey cycles were constructed by dividing the 2-year weights for each cycle by six (23).

Categorical, dichotomous, and continuous variables were used to record the data. Continuous variables were expressed as mean standard deviation (SD), and dichotomous and categorical variables were expressed as counts proportions. Weighted Chi-square tests (categorical variables) and weighted

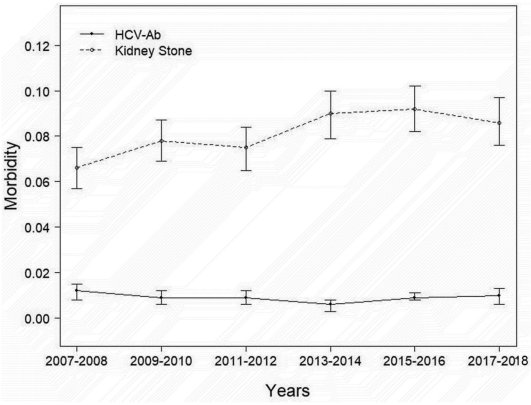

FIGURE 2
Prevalence of kidney stone and HCV infection from 2007–2018.

one-way analysis of variance (ANOVA) (normal distribution continuous variables) or weighted Kruskal-H Wallis's test (skewed distribution continuous variables) were used to evaluate differences in clinical characteristics between groups. When <10% of the data were missing, the missing item value was replaced by means. For variables with an additional category, missing indications were produced. Because HCV-Ab data was unavailable from 2015–2016, the infection status gap was filled by the total HCV infection prevalence from 2007 to 2018.

We employed machine learning to predict the effect of each research variable on kidney stone prevalence to assess the risk of the factors linked to kidney stone prevalence (Figure 3). To investigate the independent connection, three logical regression models were created in our study: (1) unadjusted; (2) adjusted for age and race; and (3) adjusted for all factors. In addition, we used stratified multivariate logistic regression to conduct subgroup analyses to find acceptable groups. Statistical analyses were carried out using R 3.5.3 (http://www.r-project.org/) and Empower Stats software (http://www.empowerstats.com), with a *P*-value of 0.05 being considered statistically significant.

Results

A total of 13,262 people were included in our study, and the necessary variables are listed in Table 1. In general, the morbidity rate associated with kidney stone development has been increasing (*P* for trend<0.001). However, this tendency did not hold for HCV infection prevalence (*P* for trend >0.05). In comparison to the nonstone former group, the stone former group had a greater prevalence of HCV infection ($p<0.0001$). The results are depicted in Figure 2, as well as in Supplementary Tables 1, 2. Additionally,

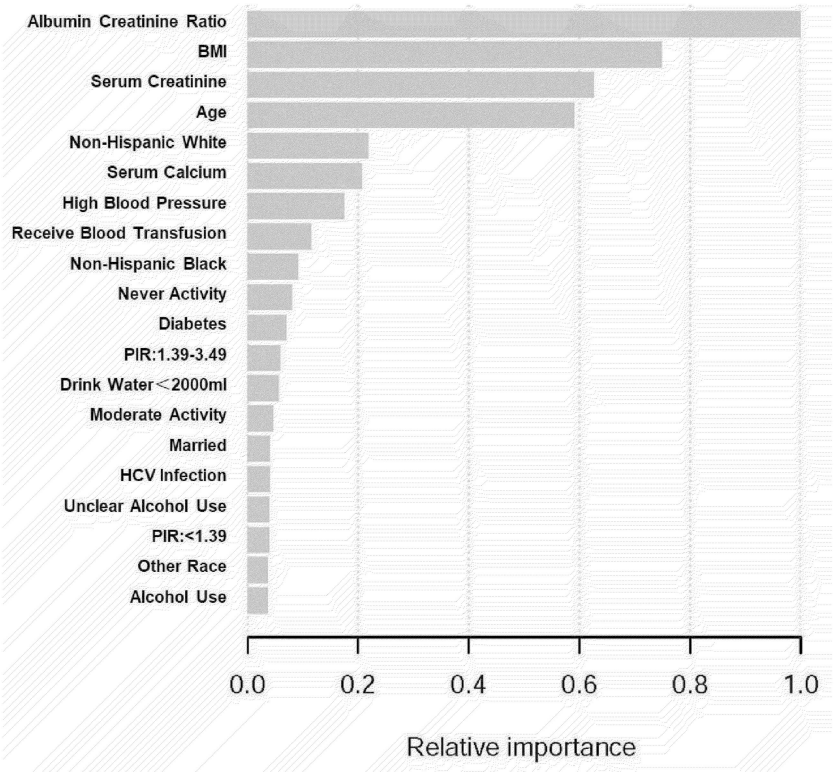

FIGURE 3
Importance of the variables in the machine learning model, scaled to a maximum of one.

we applied machine learning (24) to predict the effect of each research variable on the production of kidney stones. The result indicates that HCV infection is one of the several factors influencing the production of calculi (Figure 3).

The major objective of this study was to establish a causal relationship between HCV infection status and the prevalence of kidney stone development. We conducted multivariate logistic regression analysis. Three models were developed in accordance with the guidelines of the Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) statement (25). In an unadjusted model, kidney stone development was associated with HCV infection in US women (OR = 1.96, 95%CI:1.31–2.92). Following that, after adjusting for age and race, we developed a second model, the pattern was similar to the first. Surprisingly, the results were substantially equal before and after adjustment for all variables (OR = 1.70, 95%CI:1.13–2.56). Subgroup analysis revealed that this negative connection trend was more evident in subgroups that comprised other races (OR=8.17, 95% CI:1.62–41.22) and with BMI between 25 and 29.9 kg/m^2 (OR = 2.45, 95% CI:1.24–4.83) (Table 2).

Discussion

Nephrolithiasis is characterized by a high prevalence, a high recurrence rate, and a varied prognosis (4, 26). Procrastinating the start of treatment can result in serious impairment of renal function. Earlier research suggested that kidney stones are a complicated chronic systemic condition (27). At the moment, the treatment regimen for renal calculi includes surgery, rehabilitation, and medication. Rapid advancements in science and technology aided in the evolution of the surgical approach from open surgery to a variety of less invasive endoscopic procedures. Despite this, current treatment for kidney stones focuses mostly on clinical symptoms, with no commonly available etiological medication. The basic cause of kidney stone production and recurrence remains unknown (28). As a result, understanding important risk factors for kidney stones is of critical clinical importance.

Our investigation indicated that the prevalence of kidney stones was increasing every 2 years, and the findings in the literature corroborated our findings (3, 5). However, because the morbidity of HCV-Ab did not follow the epidemiology

TABLE 2 Analysis between confounders and renal stone prevalence.

Characteristic	Model 1 OR(95% CI)	Model 2 OR(95% CI)	Model 3 OR(95% CI)
HCV(−)	1	1	1
HCV(+)	1.96(1.31, 2.92)	1.96(1.31, 2.93)	1.70(1.13, 2.56)
Subgroups			
Race			
Mexican American	2.24(0.65, 7.73)	2.07(0.58, 7.40)	1.75(0.47, 6.54)
Other hispanic	2.36(0.68, 8.21)	1.90(0.52, 6.94)	1.92(0.51, 7.28)
Non-hispanic white	1.60(0.88, 2.91)	1.50(0.82, 2.76)	1.49(0.81, 2.75)
Non-hispanic black	1.90(0.85, 4.22)	1.49(0.65, 3.40)	1.41(0.61, 3.25)
Others	10.83(2.54, 46.13)	5.40(1.08, 26.94)	8.17(1.62, 41.22)
BMI (kg/m^2)			
<25	2.60(1.16, 5.83)	1.95(0.84, 4.49)	2.10(0.91, 4.86)
25–30	2.53(1.31, 4.88)	1.99(1.01, 3.93)	2.45(1.24, 4.83)
>30	1.38(0.73, 2.62)	1.39(0.72, 2.67)	1.24(0.64, 2.40)

Model 1, no covariates were adjusted.
Model 2, Model 1+age, race were adjusted.
Model 3, Model 2+diabetes, blood pressure, education, marital status, BMI, serum calcium, PIR, ever receive blood transfusion, total water, physical activity, alcohol use, serum creatinine were adjusted. The subgroup analysis was stratified by race and BMI, not adjusted for the stratification variable itself.

of nephrolithiasis from 2007 to 2018, we believe that it may eventually come down to effective prevention and treatment measures (29). Although HCV infection is curable, due to the large number of people who are ignorant of their illness, HCV infection continues to be a significant public health problem (30).

The purpose of this initiative was to investigate the association between HCV infection and renal calculi. The NHANES database was mined and analyzed for massive, organized, population-based cross-sectional data. We discovered that the number of HCV-infected people in the kidney stone group was significantly higher than the number of HCV-infected subjects in the non-stone group. The most important finding of our current investigation is the identification of HCV virus status as a risk factor for kidney stone prevalence using multivariate logistic models. This connection persisted after correcting for all the confounding variables. According to the link between HCV(+) and nephrolithiasis, the most significant crowds were those with BMI of 25–29.9 kg/m^2 and other races. Overweight (BMI 25–29.9 kg/m2) and obesity (BMI ≥ 30 kg/m2) are risk factors for the development of kidney stones, and Parvin (31) found a higher probability of kidney stones in the overweight and obese population in a study in 2021. The results of a cohort study on BMI and prevalence of kidney stones by Korean scholar Kim (32) in 2019 showed that in the metabolically healthy population, overweight and obesity have OR = 1.12, 95%: 13–1.22 and OR = 1.72, 95%:1.21–2.44, while in the metabolically unhealthy population, overweight and obesity have OR = 1.27, 95%. 1.20–1.34 and OR = 1.36, 95%:1.22–1.51. The results of this paper showed that in the female population with BMI of 25–29.9 kg/m2, OR = 2.45, 95%CI:1.24–4.83, suggesting that HCV infection is positively associated with the prevalence of kidney stones, and in the female population with BMI > 30 kg/m2, OR = 1.24, 95%CI:0.64–2.40, suggesting that HCV infection was positively associated with the prevalence of kidney stones, but not significantly, which may be related to the small sample size and needs to be verified in a large sample multicenter ready control trial.

Simultaneously, machine learning was utilized to determine whether certain variables have an effect on the occurrence of kidney stones in this study. Data may be ranked using machine learning according to their influence. The most relevant factors in the model of kidney stone development were the albumin creatinine ratio, BMI, serum creatinine, age, non-Hispanic white, high blood pressure, HCV infection status, and so on. Some research have been carried out to compare the advantages and disadvantages of machine learning approaches to traditional regression techniques. As of yet, however, the findings have been considerably disparate. Studies have revealed that logistic regression may be as accurate as, or, perhaps, more accurate than other machine learning algorithms in certain situations (20). Other research, on the other hand, have shown that machine learning approaches are more trustworthy than traditional regression analysis (24). Consequently, according to Wolpert's "No Free Lunch Theorem," no single strategy will be the most accurate in every situation, thus, comparisons of strategies across various study topics and datasets may provide different findings. Both machine learning and logistic regression models in this study indicated HCV infection as a risk factor for kidney stone development, which is a strong start in this area of research.

Our research shows for the first time that HCV infection may have an effect on kidney stone development, utilizing a cross-sectional study. As a result, there has been no research on its putative mechanism. We hypothesize the following causes based on past research on its correlation: It is widely established that renal tubular epithelial cell injury is a critical stage in the processing of kidney stones prevalence. Although chronic kidney disease (CKD) is now recognized as an extrahepatic manifestation of hepatitis C virus, it is often advanced. Researchers are reporting that tubulointerstitial damage is the early stage of renal manifestation rather than glomerular damage (20), which is positive. An Italian study of 98 cirrhotic individuals found tubular involvement to be the most prevalent kidney abnormality (33). The HCV core protein has also been shown to be more prevalent in the renal tubules of patients with HCV infection (34). In the proximal renal tubular epithelial cells, activation of caspases 3, 8, and 9 by HCV has been found to impact tubular barrier function directly in renal

epithelial cells, favoring apoptotic cascades (35). As a result of the damage to the renal tubular cells, crystals are readily formed on them. Crystals adhering to the surface of renal tubular cells are taken up by the cells. In due course, a stone is created from the crystals and crystal aggregates that have grown in the preceding step.

The following are some of our study's limitations: (a) Because this was a cross-sectional research, it cannot prove a causal link between HCV infection and the likelihood of kidney stone development; (b) It should be noted that the NHANES only included "self-reported" data on kidney stone history, which excluded asymptomatic stones. Another drawback is that we were unable to determine the sort of renal calculi that were present; and (c) Eating habits may have an impact on kidney stone development. However, no dietary information or characteristics related with kidney stone production were collected.

Conclusions

Participants with HCV infection had a higher chance of acquiring kidney stones. Infection with HCV may be a risk factor for the production of kidney stones in US women.

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 NCHS's Institutional Review Board reviewed and approved the study protocol. The patients/participants provided their written informed consent to participate in this study.

Author contributions

YC: conceptualization, methodology, and software. XS: data curation and writing—original draft. HL and GL: visualization and investigation. KH: supervision and software. CL and ZH: writing—review and editing. All authors contributed to the article and approved the submitted version.

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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.2022.940905/full#supplementary-material

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Acute decompensation events differentially impact the risk of nosocomial infections and short-term outcomes in patients with cirrhosis

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Aims: This research aimed to evaluate the influence of acute decompensation (AD) events upon admission on the subsequent risk of nosocomial infections (NIs) and the synergy between AD and the following NIs on the short-term outcome.

Methods: A total of 419 hospitalized individuals with cirrhosis and AD participated in the current study. Various AD events at admission and outcomes in patients with or without NIs were compared. The logistic regression and Cox proportional hazards models were designed for NIs development and liver transplant (LT)-free mortality at 28 and 90 days, respectively.

Results: During hospitalization, 91 patients developed NIs. Notably, a higher proportion of patients with NIs had jaundice (52.7 vs. 30.5%; $p < 0.001$) and bacterial infections (37.4 vs. 20.7%; $p = 0.001$) at admission compared to patients without NIs, while a lower proportion suffered gastrointestinal hemorrhage (16.5 vs. 36.6%; $p < 0.001$). Multivariate analysis revealed that jaundice was independently linked with the development of NIs (OR, 2.732; 95% CI: 1.104–6.762). The 28-day (16.5 vs. 7.3%; $p = 0.008$) and 90-day (27.5 vs. 15.9%; $p = 0.011$) LT-free mortality rates of patients with NIs were significantly higher than those without NIs. According to the Cox proportional hazards model, jaundice remained an independent risk factor for 90-day death (HR, 5.775; 95% CI: 1.217–27.397). The connection between total bilirubin and 90-day mortality was nonlinear, and a 6 mg/mL threshold was proposed.

Conclusion: The types of AD events differentially predispose to risk of NIs. Presenting jaundice at admission is independently associated with NIs occurrence and increased 90-day mortality of patients with NIs. Antibiotic prophylaxis may benefit this specific subset of patients.

KEYWORDS

acute decompensation, cirrhosis, nosocomial infections, jaundice, prognosis, antibiotic prophylaxis

Introduction

Liver cirrhosis is one of the major diseases worldwide (1), and its natural history can be classically divided into compensated and symptomatic decompensated phases (2). In the latter stage, ascites, hepatic encephalopathy (HE), gastrointestinal hemorrhage (GIH), non-obstructive jaundice, or bacterial infections (BIs) herald the onset of decompensated cirrhosis and often result in hospitalization and increased liver transplant (LT)-free mortality (3). Patients with cirrhosis and acute decompensation (AD) are more vulnerable to getting BIs due to the interaction of intestinal flora imbalance, bacterial translocation, and immune dysfunction (4). BIs have been shown to independently accelerate the natural course of cirrhosis (5) and increase mortality in compensated and decompensated liver cirrhosis patients (6–8). Moreover, BIs may be a preventable cause of acute-on-chronic liver failure (ACLF), organ damage, and death (4, 9).

For individuals with cirrhosis, the incidence of nosocomial infections (NIs) is an essential aspect (10). In a large, prospective, multicenter cohort study, 15% of enrolled patients with cirrhosis developed NIs, and the prognosis was worse (10). In contrast, the incidence of NIs is much higher in patients with cirrhosis and AD (11, 12). A recent Europe study found that NIs account for 52.5% of BIs in all enrolled patients with decompensated cirrhosis (12). Another prospective study reported that NIs developed in 36% of patients with cirrhosis and AD on admission (11). More importantly, NIs are related to a higher risk of ACLF, severe sepsis, and 28-day mortality in patients with decompensated cirrhosis (11, 12). It is worth noting that patients with decompensated cirrhosis have different AD events, which may lead to differences in the incidence of NIs. For instance, patients with variceal bleeding or low ascitic fluid protein levels are at a significantly increased risk of developing BIs (13). However, current studies have not elucidated how decompensation events differentially predispose to the occurrence of NIs and affect the outcome of patients complicated with NIs. Hence, this study aimed to investigate the incidence of NIs in cirrhosis with differential AD events, the relationship between the type of AD at admission and the development of NIs, and the synergy between AD and subsequent NIs on the outcome.

Materials and methods

Patients and diagnosis

This study enrolled patients with AD from a prospective cohort of patients with cirrhosis hospitalized for any reason between February 2014 and March 2015. In patients with chronic liver disorders, cirrhosis was diagnosed using a combination of AD, radiographic, or endoscopic findings. AD was defined as the acute onset of overt ascites (grade 2 or 3), HE, GIH, BIs, or jaundice [total serum bilirubin (TB) > 5 mg/dL] within 1 month before enrollment or any combination of these conditions (2, 14, 15). Depending on the quantity of fluid, ascites was graded from 1 to 3: (i) grade 1: only identified by ultrasonography; (ii) grade 2: medium symmetric abdomen distension; (iii) grade 3: considerable abdominal distension (3, 15). The West Haven criteria were used to evaluate HE (16). The criteria for defining ACLF and organ failures were established by the European Association for the Study of the Liver (EASL)-Chronic Liver Failure Consortium (CLIF) (3, 17). The exclusion criteria included the following: (i) age less than 18; (ii) hepatocellular carcinoma; (iii) pregnancy; (iv) human immunodeficiency virus (HIV) infection; (v) severe chronic extrahepatic disease; (vi) recent use of immunosuppressant medications; and (vii) liver transplantation recipient.

The diagnostic criteria for BIs were as follows: (i) spontaneous bacterial peritonitis (SBP): polymorphonuclear cells in ascitic fluid more than or equal to 250/mm^3 with or without a positive fluid culture (18); (ii) urinary tract infection: leukocytes > 10/high power-field in urine and positive cultures (19); (iii) pneumonia: new pulmonary infiltrate with clinical signs of infection (20); (iv) spontaneous bacteremia: positive blood cultures without a source of bacteremia; (v) secondary bacteremia: occurring within 24 h of an invasive procedure or catheter-related infection.; (vi) skin and soft tissue infections (SSTI): skin swelling, erythema, heat, and pain; (vii) cholangitis: intense right upper quadrant pain and radiological evidence; (viii) *Clostridium difficile* infection: diarrhea and positive *C. difficile* assay; (ix) spontaneous bacterial empyema (SBE): polymorphonuclear cells in pleural fluid > 500/mm^3 (250/mm^3 if positive culture).

The screening and treatment of NIs

A panel of screening and confirmatory tests for bacterial infections, including leukocyte count, serum c-reactive protein (CRP), lung CT scan, urine and stool analysis, ascites fluid examination, and microbiological culture, was established. Specific tests were selected based on the presenting signs of patients and the decision of senior physicians. NIs are infections acquired during the process of receiving heath care that was not present during the time of admission. Infections identified within 48 h of hospitalization were considered nosocomial.

The specific empirical antibiotics regimen for different types of NIs was as follows (4, 21): (i) SBP or SBE: 3rd generation cephalosporin, piperacillin-tazobactam or carbapenem; (ii) pneumonia: piperacillin-tazobactam, 3rd generation cephalosporin, levofloxacin, or moxifloxacin; (iii) urinary tract infection: 3rd generation cephalosporin; (iv) SSTI: piperacillin-tazobactam, daptomycin or linezolid;

(v) cholangitis: 3rd generation cephalosporin, piperacillin-tazobactam or carbapenem; (vi) *C. difficile* infection: Metronidazole or vancomycin (22). If the causative organism was identified, empirical antibiotic therapy was continued or adjusted depending on antimicrobial susceptibility testing and efficacy evaluation.

Data collection

Data for all variables were collected at the time of admission or the first laboratory test after admission, and this was certainly before the development of NIs. Variables included demographics, etiology of cirrhosis, cirrhosis complications (ascites, HE, GIH, BIs, and jaundice), comorbidities (e.g., hypertension and diabetes), laboratory data for Model for End-Stage Liver Disease (MELD) score and CLIF-C organ failure score, antibiotic therapy within 2 weeks before enrollment, and systemic inflammation biomarkers (e.g., leukocyte count, neutrophil count, and CRP). In addition, the data concerning the site of infection and pathogenic bacterium of NIs were collected. The primary research outcome was LT-free mortality at 28 and 90 days after enrollment. Prognostic information was collected through continuous medical services or telephonic interviews.

Statistical analysis

Statistical analyses were carried out using R (version 4.1.0) and SPSS (version 26.0). The chi-squared test was used to compare categorical data expressed as frequencies or percentages. Continuous variables with normal distribution were summarized as means and standard deviations (SDs) and compared using the Student's *t*-test. The non-normally continuous variables were shown as medians with interquartile ranges and compared using the Mann–Whitney U or Kruskal Wallis tests. The logistic regression model was used to detect possible correlations between cirrhosis complications and NI development. The Box-Tidwell test confirmed the assumption of linearity in the logit for continuous variables. Multicollinearity was assessed by checking the Variance Inflation Factor on a multiple regression model with the same variables. Cox proportional hazard regression was performed to identify risk variables related to 28- and 90-day LT-free mortality in enrolled patients who developed NIs. The assumption of proportional hazard models was validated by correlation studies between the weighted Schoenfeld residuals and incident timings. The Kaplan-Meier method was used to estimate the mortality of patients and subgroups. The associations between levels of serum TB and 90-day mortality of cirrhotic patients with AD and NIs were assessed on a continuous scale using restricted cubic spline curves based on the Cox proportional hazards model. The two-sided significance level was set at 0.05.

Results

Between February 2014 and March 2015, 1248 hospitalized patients with cirrhosis were identified (Figure 1). The number of enrolled patients with AD on admission was 419 (33.4%), and 829 (66.6%) patients were excluded. Out of total patients, 91 (21.7%) developed nosocomial infections (NIs) during hospitalization.

Clinical characteristics of enrolled patients

Patients with and without NIs had similar demographic data, etiology of cirrhosis, and comorbidities as described in Table 1. However, patients with NIs showed significantly higher levels of systemic inflammatory markers [leukocyte count, neutrophil count, and serum c-reactive protein (CRP)], international standard ratio (INR), and total serum bilirubin (TB) (Table 1). The remaining laboratory values on admission, including platelet count, hemoglobin, serum albumin, alanine aminotransferase, serum creatinine, serum sodium, and blood ammonia, were similar between the two groups. Notably, antibiotic medication within 2 weeks before enrollment (30.8 vs. 13.7%, respectively, $p < 0.001$) and ACLF on admission (22.0 vs. 13.7%, respectively, $p < 0.05$) were considerably more frequent in patients with NIs than in patients without NIs. As expected, patients who acquired NIs while hospitalized had significantly higher 28-day (16.5 vs. 7.3%, respectively, $p < 0.05$) and 90-day (27.5 vs. 15.9%, respectively, $p < 0.05$) LT-free mortality rates than patients who did not develop NIs, as patients with NIs had higher prognosis ratings, including MELD score, MELD-Na score, and CLIF-C organ failure score ($p < 0.05$).

Profile of AD events at admission

Among the 419 patients with AD, overt ascites (44.4%) was the most common AD event, followed by jaundice (35.3%) and GIH (32.3%) (Table 2). However, among those patients who acquired NIs during hospitalization, the most common event upon admission was jaundice (52.7%), followed by overt ascites (44.0%) and BIs (37.4%). The order in patients without NIs is overt ascites (44.5%), GIH (36.6%), and jaundice (30.5%). More importantly, jaundice (52.7 vs. 30.5%, $p < 0.001$) and BIs (37.4 vs. 20.7%, respectively, $p < 0.05$) occurred more frequently in patients with NIs than in those without NIs. In comparison, the incidence rate of GIH was significantly lower in patients who developed NIs (16.5 vs. 36.6%, respectively, $p < 0.001$). Regarding the number of events, the frequency of one or two complications was similar between the groups (Table 2). Patients with NIs had a higher percentage of individuals

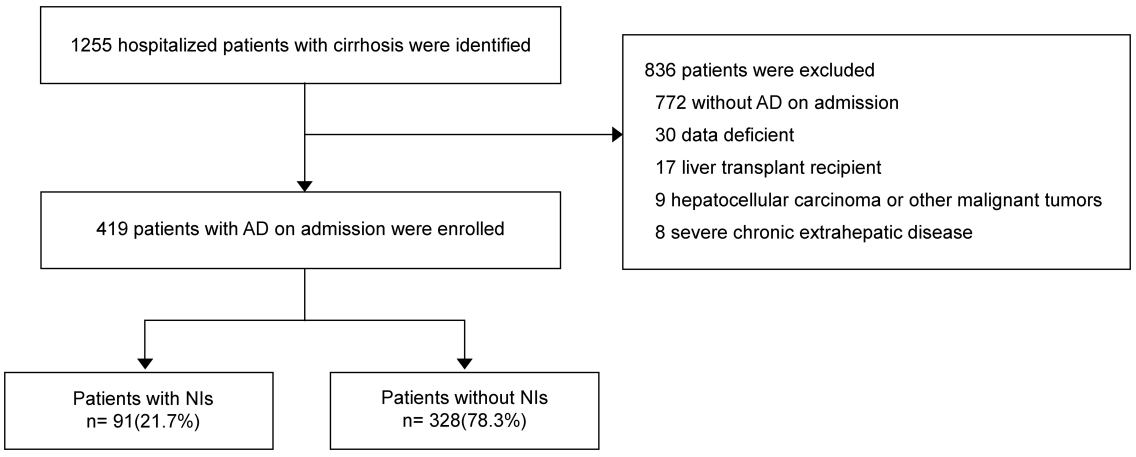

FIGURE 1
Flow chart for patient selection.

with three complications (17.6 vs. 7.6%, respectively, $p < 0.05$), but almost none had four or more complications. Details of patients with combined AD events are shown in Figure 2A and Table 2. The prevalence of specific AD events was statistically different between the groups. Notably, patients with jaundice or BIs at admission had a significantly greater incidence of NIs, while patients with GIH had a lower incidence of NIs.

Types of NIs and isolated bacteria

As shown in Table 3, the most common type of bacterial infections in the 91 patients who developed NIs during hospitalization was SBP (39.6%), followed by pneumonia (38.4%), spontaneous or secondary bacteremia (6.6%), cholangitis (5.5%), *C. difficile* infection (4.4%), spontaneous bacterial empyema (SBE) (3.3%), and skin and soft tissue infection (SSTI) (2.2%). A total of 18 pathogens were identified in these patients. *Klebsiella pneumoniae* was the most frequently isolated bacterium (27.8%), followed by *C. difficile* (22.2%) and *Acinetobacter baumannii* (11.1%).

Risk factors of NIs

Supplementary Table S1 shows variables associated with the occurrence of NIs in the univariate analysis. BIs and jaundice were linked to the occurrence of NIs in all types of AD events, while GIH was linked to a lower incidence of NIs. Furthermore, patients with three kinds of events simultaneously were more likely to develop NIs than those with only one [odds ratio (OR), 2.778; 95% CI: 1.375–5.611; $p = 0.004$] or two complications (OR, 2.240; 95% CI: 1.053–4.766; $p = 0.036$). In terms of laboratory tests, neutrophil count and serum CRP levels were related to the NIs development. In addition, other potential risk factors include antibiotics usage, ACLF, and MELD score. The ALCF and MELD scores were excluded from multivariate analysis because specific laboratory data were calculated in these prognostic scores. In the multivariate analysis, only jaundice (OR, 2.732; 95% CI: 1.104–6.762; $p = 0.030$), neutrophil count (OR, 1.080; 95% CI: 1.004–1.163; $P = 0.039$), and antibiotics usage within 2 weeks before enrollment (OR, 2.095; 95%CI: 1.127–3.896; $p = 0.019$) were independent predictors of NIs (Table 4).

Overall and events-specific LT-free mortality

Overall, 39 (9.3%) and 77 (18.4%) deaths occurred within 28 and 90 days, respectively. Among all types of cirrhosis complications, jaundice resulted in the highest LT-free mortality at 28 and 90 days (20.3 and 35.8%, respectively), followed by HE (12.3 and 26.3%), BIs (12.7 and 24.5), ascites (7.4 and 17.6%), and GIH (4.4 and 8.1%) (Figure 2B). One essential aspect is to determine the influence of NIs on prognosis. Our findings confirmed that LT-free mortality was higher in the NIs group than in the non-NIs group, reaching 16.5% at 28 days and 27.5% at 90 days (Figures 2C,D and Table 1). Furthermore, patients who developed NIs with jaundice were more prone to suffer poorer outcomes than others (28-day, 29.2%; 90-day 45.8%; Figure 2E). Moreover, patients with GIH were associated with the lowest 90-day mortality in both the overall population and the NIs group.

TABLE 1 Baseline characteristics of the enrolled patients with and without NIs.

Variables	Total ($n = 419$)	Patients with NIs ($n = 91$)	Patients without NIs ($n = 328$)	*P*-value
Demographic data				
Age, year, mean ± SD	54.12 ± 12.36	53.95 ± 13.10	54.17 ± 12.17	0.878
Male sex, n (%)	323 (77.1)	72 (79.1)	251 (76.5)	0.602
Etiology of cirrhosis, n (%)				
HBV	257 (61.3)	51 (56.0)	206 (62.8)	0.241
Alcohol	79 (18.9)	17 (18.7)	62 (18.9)	0.962
HBV & Alcohol	3 (0.7)	2 (2.2)	1 (0.3)	0.120
Others	80 (19.1)	21 (23.1)	59 (18.0)	0.274
Comorbidities, n (%)				
Hypertension	63 (15.0)	12 (13.2)	51 (15.5)	0.577
Diabetes	44 (10.5)	10 (11.0)	34 (10.4)	0.864
Others	35 (8.4)	8 (8.8)	27 (8.2)	0.864
Laboratory data				
Biomarkers of systemic inflammation, median (IQR)				
Leukocyte count, $x10^9$/L	4.65 (2.90–7.20)	6.20 (3.75–10.43)	4.25 (2.80–6.60)	<0.001
Neutrophil count, $x10^9$/L	2.90 (1.76–4.77)	4.24 (2.40–7.32)	2.60 (1.72–4.14)	<0.001
Serum CRP, mg/L	10.00 (4.10–19.08)	15.00 (8.10–29.80)	8.00 (3.40–17.45)	<0.001
Platelet count, $x10^9$/L, median (IQR)	69.50 (43.00–111.25)	75.50 (50.25–133.00)	68.00 (43.00–104.00)	0.162
HB, g/L, median (IQR)	96.00 (78.00–117.00)	100.00 (83.75–119.00)	95.00 (75.25–116.00)	0.110
ALB, g/L, mean ± SD	29.38 ± 5.46	28.59 ± 5.98	29.60 ± 5.29	0.118
ALT, U/L, median (IQR)	28.00 (18.00–52.00)	29.00 (18.00–66.00)	28.00 (18.00–50.00)	0.292
TB, mg/dL, median (IQR)	2.46 (1.17–9.88)	5.91 (1.52–16.02)	2.16 (1.17–8.17)	0.002
Cr, mg/dL, median (IQR)	0.81 (0.67–1.06)	0.84 (0.72–1.12)	0.81 (0.67–1.05)	0.278
Serum sodium, mmol/L, mean ± SD	138.44 ± 5.04	138.38 ± 7.01	138.46 ± 4.35	0.924
Blood ammonia, μmol/L, median (IQR)	52.00 (33.50–78.00)	47.00 (29.00–74.50)	55.00 (34.00–78.00)	0.300
INR, median (IQR)	1.34 (1.19–1.65)	1.57 (1.86–2.38)	1.32 (1.18–1.61)	0.001
Recent antibiotic usage[†], n (%)	73 (17.4)	28 (30.8)	45 (13.7)	<0.001
ACLF, n (%)	62 (14.8)	20 (22.0)	42 (12.8)	0.029
Prognostic scores, mean ± SD				
MELD score	15.43 ± 7.90	17.67 ± 8.49	14.81 ± 7.62	0.004
MELD-Na score	16.60 ± 9.28	19.33 ± 10.08	15.84 ± 8.92	0.003
CLIF-C organ failure score	7.05 ± 1.38	7.47 ± 1.64	6.93 ± 1.27	0.004
LT-free Mortality from enrollment, n (%)				
28-day	39 (9.3)	15 (16.5)	24 (7.3)	0.008
90-day	77 (18.4)	25 (27.5)	52 (15.9)	0.011

[†] Antibiotic therapy within 2 weeks before enrollment.
NIs, nosocomial infections; HBV, Hepatitis B virus; CRP, C-reactive protein; ALT, alanine aminotransferase; TB, total bilirubin; INR, international standard ratio; ACLF, acute-on-chronic liver failure; MELD score, Model for End-Stage Liver Disease score; CLIF-C: Chronic Liver Failure Consortium.

Risk factors of 28- and 90-day death

Next, we explored the risk factors for short-term outcomes in patients who developed NIs. The results of the univariate analysis are presented in Supplementary Table S2. Multivariate analysis revealed neutrophil count [HR 1.159; 95% CI: 1.077–1.248; $p < 0.001$] and INR (HR 6.948; 95% CI: 2.602–18.555; $p < 0.001$) were independently associated with the 28-day death (Table 4). The independent risk factors for 90-day death were jaundice (HR 5.775; 95% CI: 1.217–27.397; $p = 0.027$), neutrophil count (HR 1.115; 95% CI: 1.037–1.199; $p = 0.003$) and, INR (HR 3.409; 95% CI: 1.439–8.073; $p = 0.005$). Jaundice is generally defined as the TB > 5 mg/dL. To determine a TB threshold beyond which jaundice impacted the outcome, we used a restricted cubic spline to flexibly model and visualize the relationship between levels of TB and the probability of 90-day death on a continuous scale (Figure 3). The multivariable-adjusted hazard ratio was below the reference line (at a hazard

TABLE 2 Prevalence of AD events in enrolled patients with and without NIs.

Variables	Total ($n = 419$)	Patients with NIs ($n = 91$)	Patients without NIs ($n = 328$)	*P*-value
AD events of cirrhosis, n (%)				
BIs	102 (24.3)	34 (37.4)	68 (20.7)	0.001
Overt ascites	186 (44.4)	40 (44.0)	146 (44.5)	0.925
Grade 2	172 (41.1)	41 (45.1)	131 (39.9)	0.300
Grade 3	16 (3.8)	1 (1.1)	15 (4.6)	
Jaundice	148 (35.3)	48 (52.7)	100 (30.5)	<0.001
GIH	135 (32.2)	15 (16.5)	120 (36.6)	<0.001
HE	57 (13.6)	12 (13.2)	45 (13.7)	0.896
Grade I	42 (10.0)	10 (11.0)	33 (10.1)	0.979
Grade II	11 (2.6)	2 (2.2)	9 (2.7)	
Grade III∼IV	3 (0.7)	0 (0)	3 (0.9)	
Number of AD events, n (%)				
One	251 (59.9)	47 (51.6)	204 (62.2)	0.029
Two	126 (30.1)	28 (30.8)	98 (29.9)	
Three	41 (9.8)	16 (17.6)	25 (7.6) †	
≥Four	1 (0.2)	0 (0)	1 (0.3)	
Multiple AD events, n (%)				
Only GIH	85 (20.3)	8 (8.8)	77 (23.5) †	0.001
Only overt ascites	78 (18.6)	12 (13.2)	66 (20.1)	
Only jaundice	56 (13.4)	18 (19.8)	38 (11.6) †	
Overt ascites and BIs	27 (6.4)	7 (7.7)	20 (6.1)	
Overt ascites and GIH	25 (6.0)	3 (3.3)	22 (6.7)	
Overt ascites and jaundice and GIH	24 (5.7)	10 (11.0)	14 (4.3) †	
Overt ascites and jaundice	20 (4.8)	7 (7.7)	13 (4.0)	
Only HE	16 (3.8)	3 (3.3)	13 (4.0)	
Only BIs	16 (3.8)	6 (6.6)	10 (3.0)	
Jaundice and HE	16 (3.8)	2 (2.2)	14 (4.3)	
Jaundice and BIs	13 (3.1)	6 (6.6)	7 (2.1) †	
Others	43 (10.3)	9 (9.9)	34 (10.4)	

† Significantly different from the group with NIs and group without NIs.
AD, acute decompensation; NIs, nosocomial infections; BIs, bacterial infections; HE, hepatic encephalopathy; GIH, gastrointestinal hemorrhage.

ratio of 1.0) until around 6 mg/dL of serum TB, representing a breaking point for risk of death in patients with NIs.

Discussion

There is a need to understand how AD events differentially impact the risk of NIs and short-term outcomes in patients with cirrhosis. In the present study, we investigated the epidemiological and microbiological features of NIs in patients with cirrhosis and AD and the relationship between NIs and cirrhosis complications. In this study, the prevalence of NIs in patients with cirrhosis and AD was 21.7%, and SBP was the most common infection. These results were consistent with previously published works (10, 23, 24). The isolated bacteria correspond to the type of infection. It has been suggested that *Enterobacteriaceae* were the most common gram-negative bacteria that cause SBP in patients with cirrhosis, including *Escherichia coli* and *Klebsiella pneumoniae* (23, 24). A worldwide study showed that these two bacteria were the most frequently isolated bacteria in cirrhotic patients with infection (24). However, *Klebsiella pneumoniae* was the most commonly isolated bacteria in our study. This difference can be due to the epidemiological characteristics of each region. It is noteworthy that the results of incidences for *Escherichia coli* and *Klebsiella pneumonia* infections in Asia were close to each other in the first infection (25 and 24%, respectively) (24). In addition, *Klebsiella pneumonia* was more common in the second

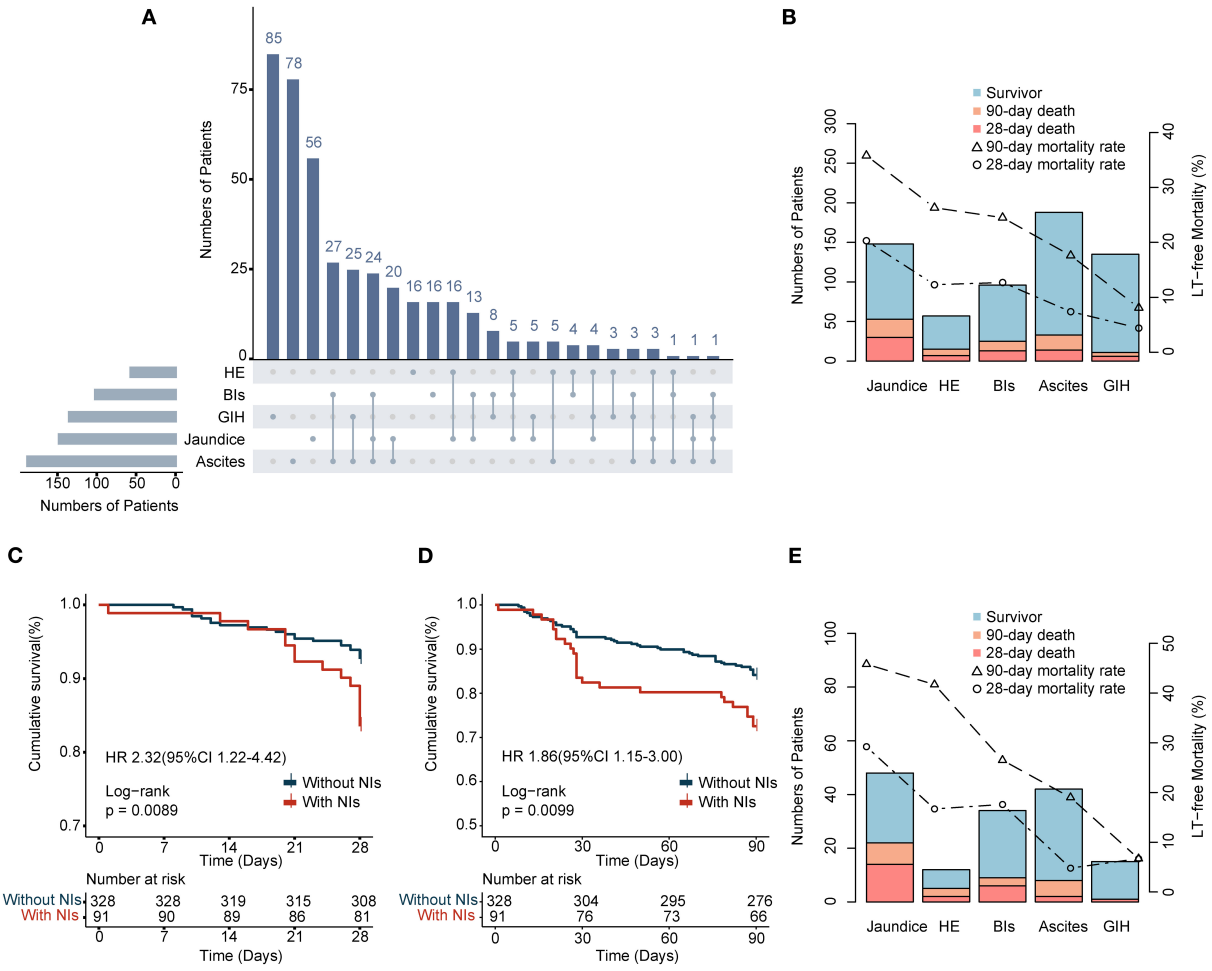

FIGURE 2
(A) Stacked column chart depicting combinations of AD events in enrolled patients. **(B,E)** Pareto charts showing the characteristics of AD events in two cohorts and the events-specific LT-free mortality. **(C,D)** 28- and 90-day survival curves of enrolled patients with or without NIs.

infection than *Escherichia coli* (19 and 14%, respectively) (24). More importantly, the report of NIs surveillance in our center found that the prevalence of *Klebsiella pneumonia* infection was roughly 17%, ranking first (data not provided).

Among all types of AD events of cirrhosis, jaundice was the most common complication in patients with NIs on admission (Table 2). Specifically, the median level of serum TB in patients with NIs was 5.91 mg/dL, statistically higher than 2.16 mg/dL in patients without NIs. This result is in accordance with earlier published studies (10). Further, an earlier report by Field et al. showed that patients in the surgical intensive care unit with serum TB >3 mg/dL had a 3-fold higher risk of infection than patients with serum TB ≤3 mg/dL (25). Corroborating this report, our results also demonstrate that jaundice was not only an independent risk factor for developing NIs but also a predictor of 90-day mortality in cirrhotic patients with AD and NIs. However, the underlying mechanism by which jaundice increases the risk of BIs has not been clarified.

In recent years, growing evidence has gradually revealed the potential immunomodulatory effects of bilirubin *in vitro* and *in vivo*, which may help us delve deeper into the above issue (26–29). Liu et al. reported that bilirubin significantly suppressed antigen-specific and polyclonal T cell responses through various mechanisms, including inhibition of costimulatory molecule activities, interference with immune transcription factor activation, and suppression of the expression of class II Major Histocompatibility Complex molecules in Antigen Presenting Cells (26). Similarly, Khan et al. demonstrated that unconjugated bilirubin not only resulted in DNA strand breaks and oxidative stress in lymphocytes but induced apoptosis of various immune cells, including mouse splenic $CD4^+$ T cells, $CD19^+$ B cells, peritoneal exudates cells, and human peripheral blood mononuclear cells (27). For

macrophage, early studies have confirmed that bilirubin may affect the expression of Fc receptors by altering the lipid environment of the cytoplasmic membrane (28). Besides, bilirubin has also been proven to inhibit the vascular cell adhesion molecule-1-dependent lymphocyte migration and reduce airway inflammation in a murine asthma model (29). These findings revealed the specific mechanism by which bilirubin inhibits the function of crucial immune cells involved in innate and adaptive immunity. Accordingly, we hypothesized that bilirubin's immunosuppressive effects might result in organisms being more susceptible to pathogens. Indeed, Khan et al. have confirmed that bilirubin pretreatment increased the susceptibility of mice to develop BIs and significantly reduced the median survival time of mice with infection (27). These findings were thought to have close relevance to bilirubin promoting the release of serum pro-inflammatory cytokines (TNF-α, IL-1β, and IL-6) and inhibiting various immune cell responses (27).

Clinically, once the serum TB level is higher than 2.5–3 mg/dL, patients will show signs of jaundice (yellowing of skin, sclera, and mucous membranes) (30). As mentioned, a study divided patients into high or low bilirubin groups with 3 mg/dl as the threshold level and revealed that the risk of infection

TABLE 3 Types of nosocomial infection and isolated bacteria.

Variables	Frequency, n (%)
Types of infection ($n = 91$)	***n* = 91**
SBP	36 (39.6)
Pneumonia	35 (38.4)
Spontaneous or secondary bacteremia	6 (6.6)
Cholangitis	5 (5.5)
Clostridium difficile infection	4 (4.4)
SBE	3 (3.3)
SSTI	2 (2.2)
Isolated bacteria	***n* = 22**
Klebsiella pneumoniae	5 (22.7)
Clostridium difficile	4 (18.2)
Acinetobacter baumannii	2 (9.1)
Candida albicans	2 (9.1)
Candida krusei	1 (4.5)
Enterococcus faecium	1 (4.5)
Aeromonas hydrophila	1 (4.5)
Escherichia coli	1 (4.5)
Pseudomonas aeruginosa	1 (4.5)
Stenotrophomonas maltophilia	1 (4.5)
Streptococcus pluranimalium	1 (4.5)
Vibrio vulnificus	1 (4.5)

SBP, spontaneous bacterial peritonitis; SBE; spontaneous bacterial empyema; SSTI; skin and soft tissue infections.

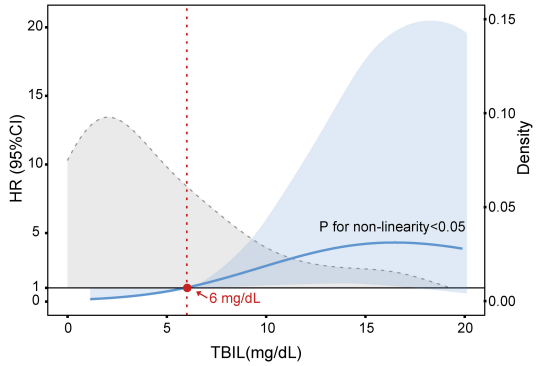

FIGURE 3
Multivariable adjusted hazard ratios (HR) for 90-day LT-free mortality according to the levels of serum total bilirubin (TB) on a continuous scale in cirrhotic patients with AD and NIs. The blue line is multivariable adjusted HR, with light blue shaded area represent 95% CI. Dashed gray curve shows the fraction of population with different TB levels. Serum TB 6 mg/dL was selected as the reference level. Analyses were adjusted for overt ascites, neutrophil count, ALT, and INR at baseline.

TABLE 4 Multivariate analysis of the risk factors for the development of NIs in patients with cirrhosis and AD and short-term outcome in cirrhotic patients with AD and NIs.

Variables	Risk factors for NIs		Risk factors for 28-day mortality		Risk factors for 90-day mortality	
	OR (95% CI)	*P*-value	HR (95% CI)	*P*-value	HR (95% CI)	*P*-value
Jaundice vs. non-jaundice	2.732 (1.104–6.762)	0.030	-	0.125	5.775 (1.217–27.397)	0.027
Neutrophil count	1.080 (1.004–1.163)	0.039	1.159 (1.077–1.248)	<0.001	1.115 (1.037–1.199)	0.003
Recent antibiotic usage[†]	2.095 (1.127–3.896)	0.019	-	-	-	-
INR	-	-	6.948 (2.602–18.555)	<0.001	3.409 (1.439–8.073)	0.005

[†] Antibiotic therapy within 2 weeks before enrollment.
The binary logistic regression model was carried out to identify the potential links between complications of cirrhosis and NI development. Cox proportional hazard regression was used to identify risk factors associated with 28- and 90-day LT-free mortality in enrolled patients who developed NIs. Variables statistically significant at $p < 0.10$ on univariate analysis were applied to final models.
AD, acute decompensation; NIs, nosocomial infections; INR, international standard ratio.

was significantly increased in the high bilirubin group (25). Furthermore, a recent study by Patel et al. demonstrated that serum bilirubin levels are closely related to the increased risk of death in patients with severe sepsis and septic shock (31). In our study, we defined jaundice as TB > 5 mg/dL according to the guideline proposed by the Asian Pacific Association for Liver Research (APASL) (32). Our results indicated that the risk of NIs in patients with jaundice was significantly increased and is associated with significantly greater LT-free mortality. At present, however, no research has clearly proposed the threshold for predicting the outcomes of patients with NIs and cirrhosis based on serum bilirubin. Based on the restricted cubic spline model, we first demonstrated that serum TB at ∼6 mg/mL was a threshold for predicting 90-day death in individuals with cirrhosis and NIs (Figure 3). This finding provides a theoretical basis for establishing a more reasonable and personalized therapeutic schedule, especially for applying the anti-infective agents. However, since our study is a single-center and retrospective cohort with a small sample size, additional prospective and multicenter studies are needed to confirm these conclusions fully.

Interestingly, our data indicate that patients with GIH had a low incidence of NIs, which is inconsistent with the previous studies (23). Past research has demonstrated that the presence of BIs is an independent predictor of failure to control bleeding and death (33). Clinical practice guidelines recommended that empirical antibiotic therapy should be started as soon as GIH is suspected (3). Therefore, the reason for this discrepancy is thought initiatively be associated with antibiotic prophylaxis in our center. Nevertheless, the remarkable thing is that empiric antibiotic treatment is not routinely used in patients with jaundice, which got us thinking: can antibiotic prophylaxis reduce the risk of NIs and short-term mortality in this subset of patients?. The pity is that no randomized controlled trial or multicenter retrospective study has investigated this issue so far.

Furthermore, the study provided evidence for the "immunoparesis hypothesis," according to which BIs are a complication of immunoparesis to limit the vigorous proinflammatory response (34, 35). In our study, patients who developed NIs during hospitalization had significantly higher levels of certain substances regarded as the biomarkers of systemic inflammation, such as leukocyte count, neutrophil count, and serum CRP on admission compared to those without NIs. Utilizing Cox proportional hazard and binary logistic regression models, we confirmed that neutrophil count was an independent predictor for NIs acquisition as well as an independent risk factor of 28- and 90-day mortality in cirrhotic patients with AD and NIs. These findings supported previous studies highlighting the role of systemic inflammation in disease progression (34, 36, 37).

Notably, our findings showed that patients who received antibiotic therapy within 2 weeks before enrollment had approximately 2-fold higher risk of NIs compared to those without antibiotic therapy. The use of antibiotics before admission is typically indicative of concomitant infections. Previous studies have found significantly higher prevalence of secondary infections in cirrhotic patients with first infection, which was considered to be related to the immunosuppression in severely infected patients (20). At present, it is believed that sepsis can lead to immunosuppression characterized by lymphocyte apoptosis, which is clearly related to the susceptibility to NIs. (38, 39). Besides, antibiotic overuse and failure of antibiotic strategies are closely associated with infection in cirrhotic patients, especially infections caused by multidrug-resistant organisms in the healthcare setting (23, 40, 41). Lacking appropriate prophylactic or therapeutic antibiotics usage may deteriorate the organ damage and adverse outcomes caused by infection (41). Therefore, we suspected that the correlation between NIs and antibiotic usage revealed by our study might be related to the antibiotic issues stated above. It is now generally agreed that appropriate use of antibiotics (i.e., avoid overusing antibiotics, restrict antibiotic use population, and timely adjustment to sensitive antibiotic drugs based on a bacterial culture and sensitivity test results) and nonantibiotic prophylactic strategies (i.e., probiotics) can help reduce the incidence of drug resistance and increase the clinical curative effects (41). Consequently, we strongly recommend that prophylactic use of antibiotics should be directed at initial infections and that rational use of antibiotics should be taken more seriously.

This study has certain limitations. First, our study is a single-center cohort with a limited number of patients who developed NIs and lacking of external validity to support widespread changes in practice; thus, large and multicenter studies are required to corroborate our findings. Second, we only included variables at the time of admission, not at subsequent time points, which may introduce bias. Third, the hepatitis B virus was the major etiology of cirrhosis in this cohort, and the impact of etiology on the findings could not be ruled out. For instance, previous studies showed that the number of patients admitted due to jaundice was exceptionally high among those with hepatitis B virus-related cirrhosis (42, 43). Fourth, we only take short-term prognosis as the primary outcome, and the impact of acute decompensation events on long-term prognosis of cirrhotic patients with AD and NIs remains to be further explored.

In conclusion, we confirmed the high prevalence and negative short-term outcomes of NIs in hospitalized patients with cirrhosis and AD. Cirrhotic patients admitted for different types of AD events had a differential risk of developing NIs. To the best of our knowledge, we first revealed that presenting jaundice at admission is independently associated with NIs occurrence and increased 90-day mortality in NIs patients. Serum TB levels above ∼6 mg/dL may be used to predict 90-day death in individuals with cirrhosis and NIs. Appropriate

antibiotic prophylaxis may benefit this specific subset of cirrhotic patients.

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 Ethics Committee of the First Affiliated Hospital, School of Medicine, Zhejiang University. The patients/participants provided their written informed consent to participate in this study.

Author contributions

XX, XY, YS, and JS designed the research study. XX, XY, KG, HT, JY, YL, SY, and HW performed the study and collected the data. XX and XY analyzed the data and wrote the manuscript. All authors have read and approved the final manuscript.

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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/fmed.2022.962541/full#supplementary-material

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Hepatitis B virus DNA integration as a novel biomarker of hepatitis B virus-mediated pathogenetic properties and a barrier to the current strategies for hepatitis B virus cure

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Chronic infection with Hepatitis B Virus (HBV) is a major cause of liver-related morbidity and mortality worldwide. HBV-DNA integration into the human genome is recognized as a frequent event occurring during the early phases of HBV infection and characterizing the entire course of HBV natural history. The development of refined molecular biology technologies sheds new light on the functional implications of HBV-DNA integration into the human genome, including its role in the progression of HBV-related pathogenesis and in triggering the establishment of pro-oncogenic mechanisms, promoting the development of hepatocellular carcinoma. The present review provides an updated and comprehensive overview of the current body of knowledge on HBV-DNA integration, focusing on the molecular mechanisms underlying HBV-DNA integration and its occurrence throughout the different phases characterizing the natural history of HBV infection. Furthermore, here we discuss the main clinical implications of HBV integration as a biomarker of HBV-related pathogenesis, particularly in reference to hepatocarcinogenesis, and how integration may act as a barrier to the achievement of HBV cure with current and novel antiviral therapies. Overall, a more refined insight into the mechanisms and functionality of HBV integration is paramount, since it can potentially inform the design of *ad hoc* diagnostic tools with the ability to reveal HBV integration events perturbating relevant intracellular pathways and for identifying novel therapeutic strategies targeting alterations directly related to HBV integration.

KEYWORDS

HBV-DNA integration, HBV cure, HBV biomarkers, chronic HBV infection, hepatocellular carcinoma

Introduction

Hepatitis B virus (HBV) is a major global health problem and a leading cause of death. According to recent WHO estimates, 270 million people have a chronic HBV infection, resulting in 800,000 deaths every year, attributed to cirrhosis and liver cancer (World Health Organization [WHO], 2017; Razavi-Shearer et al., 2018). In particular, hepatocellular carcinoma (HCC), which remains associated with a poor prognosis, is the fourth leading cause of cancer death worldwide (Sung et al., 2021). The lifetime risk of developing HCC is 10- to 100-fold greater for patients with chronic HBV infection than non-infected individuals, and this risk (although reduced) persists even with successful antiviral therapy and notably, also in patients with clinically resolved infection (El-Serag, 2012; Shi et al., 2012; Mak et al., 2020). In contrast with other aetiologies, a substantial number of HBV-infected individuals develop HCC without signs of liver damage, highlighting the existence of direct HBV pro-oncogenetic potential (Fattovich et al., 2004; Levrero and Zucman-Rossi, 2016). HBV-DNA integration in the hepatocytes' genome is under intensive investigation for its role in promoting enhanced cell proliferation by chromosomal genome instability or producing chimeric viral-human RNAs/proteins with transactivating properties (Lau et al., 2014; Jin et al., 2019; Alvarez-Benayas et al., 2021). Furthermore, in the setting of HBeAg-negative chronic hepatitis B (CHB), HBV-DNA integration can represent a source for the production of HBsAg that can occur even when cccDNA is completely silenced, thus challenging the rationale for HBV functional cure, whose surrogate marker is HBsAg loss (Ringlander et al., 2020; Rydell et al., 2020; Meier et al., 2021). Considering this, the current review is dedicated to providing a comprehensive overview related to the issue of HBV-DNA integration during the course of HBV infection and its role in modulating HBV pathogenetic/oncogenetic properties. Novel insights on the methodological aspects for detecting HBV-DNA integrants and their potential role as an early biomarker for HCC development are also presented.

Overall, this review will assist in deciphering the current knowledge and identifying areas of future research to better understand the role of HBV-DNA integration in disease progression and the development of HCC.

Replication cycle of hepatitis B virus and integration of viral DNA into the host genome

Hepatitis B Virus (HBV) is a member of Hepadnaviridae family. It is an enveloped virus with a relaxed circular double-stranded DNA (dsDNA) genome of about 3.2 kbp. The genome is composed by four overlapping open reading frames (ORFs), encoding seven proteins. In particular, the ORF S encodes the three isoforms of HBV surface antigen (HBsAg), referred to as Large-, Middle-, and Small-HBsAg, the ORF C encodes the HBV capsid antigen (HBcAg) and the secreted HBV "e" antigen (HBeAg), the ORF P encodes the reverse transcriptase (RT) and lastly the ORF X the regulatory protein HBx with transactivating properties (**Figure 1A**; Liang, 2009; Urban et al., 2010; Tu et al., 2017).

Hepatitis B virus replication cycle starts with attachment and entry into hepatocytes through low specificity interactions between HBsAg and heparan sulfate proteoglycans on the surface of hepatocytes and then through highly specific interaction between viral pre-S1 domain of HBsAg and cellular sodium taurocholate co-transporting polypeptide (NTCP) (Beck and Nassal, 2007; Liang, 2009; Urban et al., 2010; Tu et al., 2017).

After entry, the nucleocapsid is released into the cytoplasm and reaches the nucleus, where the HBV relaxed circular DNA (rcDNA) is converted into the so-called covalently closed circular DNA (cccDNA). This process is mediated by the nuclear host cell components of DNA repair machinery and leads to the synthesis of the episomal template used for the transcription of both forms of HBV messenger RNAs (mRNAs): subgenomic mRNAs and pregenomic RNA (pgRNA) (Urban et al., 2010; Tu et al., 2017). The former contain the information to produce the three HBsAg isoforms and the HBx protein while the latter is mainly used as a template for the synthesis of HBV-DNA and for the translation of proteins such as the RT, HBcAg, and HBeAg. All the viral mRNA molecules share the same 3′ terminus and are polyadenylated near position 1931, using the conventional polyA signal located at positions 1916–1921 (Liang, 2009; Urban et al., 2010; Tu et al., 2017).

Reverse transcription occurs within the newly synthetized nucleocapsids where the pgRNA and the polymerase are translocated and so the new genome is produced inside the viral progeny (Beck and Nassal, 2007; Urban et al., 2010; Tu et al., 2017). This mechanism of replication makes HBV a particularly interesting virus, as its genome is constituted by DNA, it still requires an RNA-intermediate for genome replication.

Most nucleocapsids, produced by HBV-infected cells, contain relaxed circular DNA. These can be enveloped and secreted as virions or migrate to the nucleus and re-constitute the intranuclear cccDNA pool. Conversely, a small proportion of nucleocapsids contains double-stranded linear DNA (dslDNA), that can be released as enveloped virions or can be transported to the nucleus contributing to the further replenishment of the cccDNA pool *via* homologous recombination (Tu et al., 2017).

Notably, intranuclear HBV-dslDNA can also integrate into the host cell genome (Tu et al., 2017). HBV-DNA integrations occur at the site of cellular double-stranded DNA breaks by exploiting cellular repair mechanisms such as non-homologous or microhomology mediated end-joining (NHEJ and MMEJ)

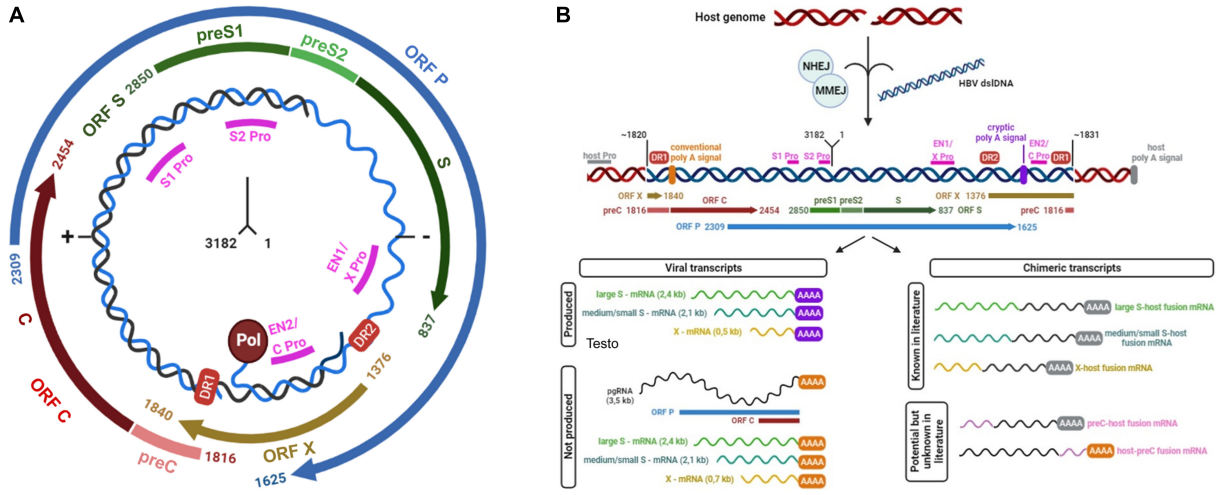

FIGURE 1

(A) Schematic representation of HBV genome. HBV genome is represented in relaxed circular form (rcDNA). The numbers at the center of the figure indicate the localization of first and last nucleotides. The four open reading frames or ORFs (S, P, X, C) are depicted. Pol circle indicates viral reverse transcriptase, linked to 3'-end of HBV-DNA. DR1 and DR2 red boxes indicate direct repeat regions 1 and 2: DR1 is present in both strands of HBV-rcDNA while DR2 is present only in the complete negative strand (–). Magenta lines show promoters (indicated as Pro) of different ORFs: in particular, enhancer 1 (EN1) acts as promoter of ORF X while enhancer 2 (EN2) acts as promoter for the expression of both ORF C and ORF P. **(B)** HBV double-strand linear DNA (dslDNA) integrated in host genome and its derived transcripts. The figure depicts the integration of HBV double-strand linear DNA (blue) into the host genome (red). The left-hand side of HBV-dslDNA is expected to be located at or near position 1820 of HBV genome, while the right-hand side at or near position 1831. These sites are not exclusive due to the integration of fragments of viral genome. Magenta lines show viral promoters (Pro), while gray line indicates a generic host promoter (host Pro). Viral open reading frames (ORFs) are depicted. DR1 and DR2 red boxes indicate viral direct repeat regions 1 and 2. Viral conventional poly A signal is showed as an orange bar (nucleotide positions: 1916–1921) while the cryptic poly A signal is represented by a violet bar (nucleotide positions: 1788–1793). The figure reports viral or viral-human transcripts produced from integrated dslDNA. The former derives from the stop of transcription at the site of the cryptic poly A signal, while the latter from the stop of transcription at a human poly A signal. In both panels, the numbering of nucleotides was based on the HBV DNA sequence with Genbank Accession #AB241115—genotype A.

(Bill and Summers, 2004; Tu et al., 2018; **Figure 1B**). The left-hand side of HBV-dslDNA is expected to be located at or near position 1820 of HBV genome, while the right-hand side is expected to be located at or near position 1831 (Mason et al., 2016; **Figure 1B**). However, these positions can vary since the error-prone host DNA repair pathways can introduce terminal truncations during the process of integration (Freitas et al., 2018).

Due to this organization of dslDNA, integrated HBV-DNA cannot support the synthesis of pgRNA, HBcAg, and RT, and thus it cannot represent a source for the production of new viral particles (**Figure 1B**; Tu et al., 2017). Conversely, in integrated HBV-DNA, the promoters of ORF S are intact and functional, thus allowing the synthesis of mRNAs for the L-, M-, and S-HBsAg (Shamay et al., 2001; Tu et al., 2017; **Figure 1B**). However, in integrated HBV-DNA, the conventional poly-A signal is located upstream of the promoters of ORF S (positions 1916–1921) and thus cannot be used (**Figure 1B**). For this reason, the transcription of ORF S can be terminated at a recently identified viral cryptic poly A signal (located at positions 1788–1793) (Freitas et al., 2018) or can pass the virus-host junction and continue into the host sequences until a host poly A signal is reached (**Figure 1B**). This process can give origin to chimeric virus-host mRNAs (Schutz et al., 1996; Kairat et al., 1999; Freitas et al., 2018; **Figure 1B**). Very limited information is known about the viral cryptic polyA signal and its activation during the transcription of dslDNA (Kairat et al., 1999).

Similarly, the promoter of the ORF X is functional in integrated HBV-DNA and can induce the production of C-terminal truncated HBx proteins that can retain their transactivation properties (Kumar et al., 1996; Tu et al., 2017; **Figure 1B**). Furthermore, there is evidence for an uninterrupted transcription by the cellular RNA-polymerase that can favor the production of chimeric HBx-host transcripts (Tu et al., 2017; Ruan et al., 2019; **Figure 1B**). Both truncated HBx forms and chimeric transcripts are currently being studied for their role in HBV-driven hepatocarcinogensis (Sung et al., 2012; Tu et al., 2017; Ruan et al., 2019).

Beyond HBV- dsl DNA, a recent study has also shown the integration of fragments of viral genome that could give origin to the production of truncated HBV proteins (Li et al., 2022).

The understanding of the above-mentioned mechanisms, underlying the process of HBV-DNA integration, has required several efforts and in particular a constant improvement of molecular techniques with the ability to properly identify and to evaluate the localization of HBV-DNA integration in the

human genome. Since the first studies in the 1980s [based on Southern blotting, *in situ* hybridization (ISH) and Polymerase Chain Reaction (PCR)] significant progress has been made. In particular, the recent introduction of innovative next-generation sequencing technologies has allowed to sequence the entire human genome, providing a strong and rapid enlargement in the current knowledge regarding the occurrence of HBV integration, its localization, as well as its functional impact on the human genome (cite paragraph 8 for an overview of the main techniques utilized for revealing HBV-DNA integration).

HBV-DNA integration during the natural history of hepatitis B virus infection

HBV-DNA integration has been shown to be present from the very early stages of HBV infection. It is observed not only in HCC and cirrhotic patients with chronic hepatitis (Brechot et al., 1980; Shafritz et al., 1981; Mason et al., 2010), but also in patients with acute HBV infection (Kimbi et al., 2005). These observations are consistent with the results obtained in woodchuck and duck animal models with hepatitis B. Indeed, viral DNA integration into the host cellular genome is a common characteristic of the Hepadnaviridae family (Tu et al., 2017).

HBV-DNA integration in the setting of acute hepatitis B virus infection

There is a paucity of studies evaluating HBV-DNA integration in acute infections, mainly due to ethical issues in sampling liver tissue in this setting (Pollicino and Caminiti, 2021). Previous studies in patients developing fulminant hepatitis, have shown that HBV-DNA integration can occur in the first weeks of infection and involve multiple sites of the host genome (Scotto et al., 1983; Lugassy et al., 1987), in line with what is observed in animal and *in vitro* models (Yang and Summers, 1999; Summers and Mason, 2004; Tu et al., 2018).

Evaluation of viral integration events in HBeAg-positive phases of chronic infection

The natural history of chronic HBV infection has been categorized in different phases (Lampertico et al., 2017; Pollicino and Caminiti, 2021) on the basis of specific biochemical, serological, and virological characteristics, including HBeAg status, serum HBV-DNA, and alanine aminotransferase (ALT) levels.

The first phase, defined as "HBeAg-positive chronic infection," is characterized by high levels of HBV-DNA ($\sim 10^{10}$ virions per mL), reflecting high rates of viral replication, while ALT levels remain normal despite the presence of an HBV-specific-T-cell response (Bertoletti and Kennedy, 2015; Gish et al., 2015; Mason et al., 2016; Park et al., 2017). This phase is then followed by HBeAg-positive chronic hepatitis, characterized by elevated ALT and reduction in serum HBV-DNA, reflecting the activation of an antiviral immune response that can progressively constrain viral replication (Pollicino and Caminiti, 2021). The establishment of a vigorous host immune response can promote necroinflammation, accelerating the progression toward cirrhosis (Pollicino and Caminiti, 2021).

Recent studies have focused on the issue of HBV-DNA integration in the setting of HBeAg-positive phases of chronic HBV infection (Mason et al., 2016; Budzinska et al., 2018b; Rydell et al., 2020). Notably, we have previously demonstrated the presence of HBV-DNA integration events in all patients with HBeAg-positive chronic infection and hepatitis (Mason et al., 2016). In particular, by using an inverse PCR approach, we identified 500 unique HBV-DNA integrants; 246 of which were randomly located in transcribed regions (231 map to introns and 13 to exons, 1 at an intron/exon boundary) (Mason et al., 2016). These results have been confirmed in a recent metanalysis showing a higher frequency of HBV-DNA integrants in HBeAg-positive than HBeAg-negative patients (Budzinska et al., 2018a). It is plausible that the high levels of HBV replication, characterizing HBeAg-positive phases, can promote the abundant production of HBV-DNA intermediates that can undergo the integration process, posing the basis for the initiation of mechanisms underlying HBV oncogenic potential (**Figure 2**). This concept challenges the notion of HBeAg-positive infection as a quiescent disease phase (Bertoletti and Kennedy, 2015), raising the question about early treatment initiation in this subset of patients.

Evaluation of viral integration events in HBeAg-negative phases of chronic infection

During the HBeAg-positive chronic hepatitis phase, the activation of an efficient immune response against HBV can lead to a progressive decline of HBeAg and serum HBV-DNA, reflecting a decrease in the burden and/or transcriptional activity of cccDNA (Kennedy et al., 2017; Pollicino and Caminiti, 2021). This determines the entry into the "HBeAg-negative chronic infection" phase, which is characterized by HBeAg-negativity, low serum HBV-DNA (usually < 2,000 IU/ml) and normal serum ALT (Pollicino and Caminiti, 2021). As this immune-control state is maintained, patients have a low risk of liver disease progression (Lampertico et al., 2017). Nevertheless, approximately one third of

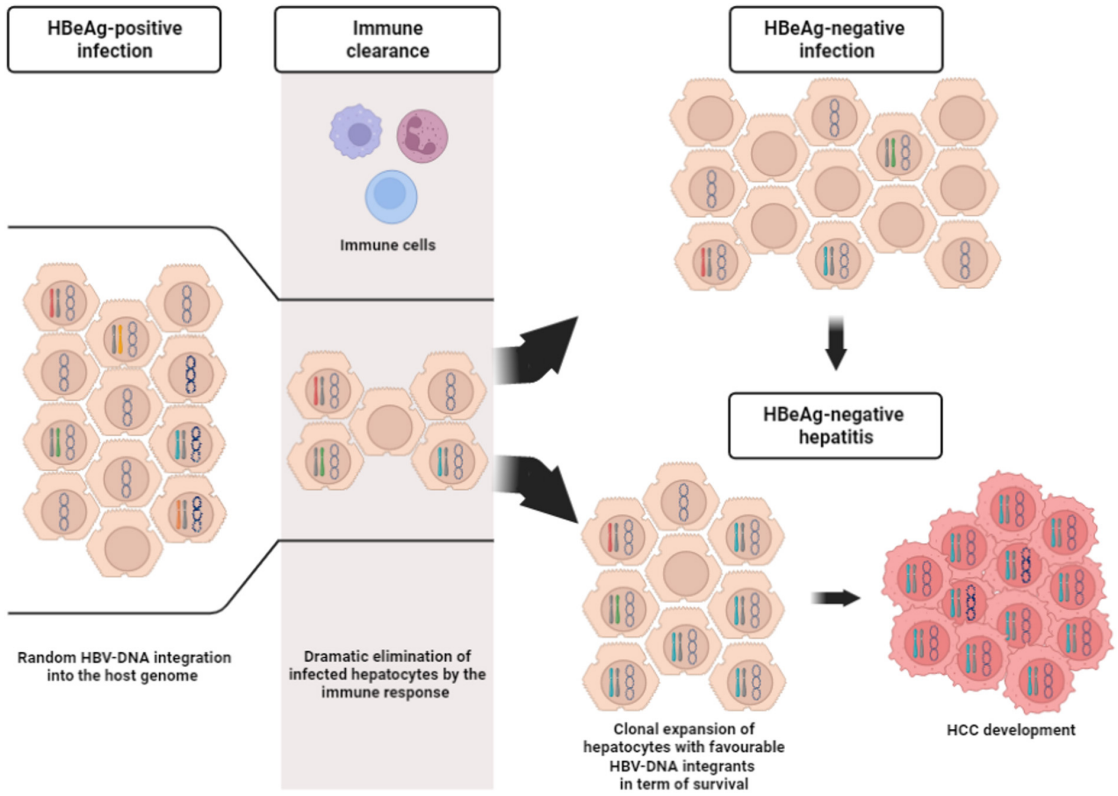

FIGURE 2
Description of the course of hepatocytes during chronic HBV infection. HBeAg-positive infection is characterized by a huge number of infected hepatocytes, as a consequence of high viral replication levels. The infected hepatocytes can harbor only cccDNA inside the nucleus (represented by the circular molecule) or also HBV-DNA randomly integrated into the host genome (represented by differently colored chromosomes). Not infected hepatocytes are indicated by cells with empty nucleus. Immune responses (depicted by the three different cells at the top of the gray stripe) reduce the number of the infected hepatocytes. From this stage the infection can run into two different outcomes. The former is represented by HBeAg-negative infection, where the pool of infected hepatocytes (with/without HBV-DNA integrants) remains limited, while the latter is represented HBeAg-negative hepatitis. In this stage, the immune response can induce the clonal selection and expansion of infected hepatocytes with favorable HBV-DNA integrants in term of survival. This process can hesitate in HCC development.

patients lose this "immune control" and progress to the "HBeAg-negative chronic hepatitis" phase (Lampertico et al., 2017; Pollicino and Caminiti, 2021). This disease phase is characterized by fluctuating or increasing serum HBV-DNA followed by elevations in serum ALT that can exacerbate liver damage thus accelerating the progression toward cirrhosis and HCC (Raimondo et al., 1990; Hsu et al., 2002). This phase is characterized by HBeAg-negativity due to the emergence of specific mutations in the pre-core and/or basal core promoter regions of the HBV genome that abolish or downregulate HBeAg production (Laras et al., 1998; Revill et al., 2020).

A lower rate of HBV-DNA integration has been observed in HBeAg-negative than HBeAg-positive patients, presumably reflecting a more limited pool of infected hepatocytes (Budzinska et al., 2018a; Rydell et al., 2020; **Figure 2**). Indeed, it has been hypothesized that, during the immune clearance phase and HBeAg seroconversion, the development of a strong immune response may favor the selection of those hepatocytes in which HBV-DNA integrations have conferred a selective advantage in terms of survival and escape from cytotoxic immune response (Budzinska et al., 2018a; **Figure 2**). Furthermore, this survival advantage is a key event since it can promote the progressive accumulation of chromosomal aberrations paving the way to the neoplastic transformation of the hepatocytes and in turn HCC development (Tu et al., 2017; Svicher et al., 2021; **Figure 3**).

We have recently addressed the issue of HBV-DNA integration in the setting of HBeAg-negativity (Svicher et al., 2021). By analyzing the whole exome of hepatocytes, this study has shown that HBV-DNA integration occurs not only in a notable proportion of patients with high HBV-DNA levels (55.6%), but also at significant frequency in patients with low (25%) and moderate viremia (14.3%), despite a more limited HBV reservoir (Svicher et al., 2021). Furthermore, by applying ddPCR, we found that HBV-DNA integrants occurred with a prevalence ranging from 0.5 up to 158 events per 1,000 hepatocytes, potentially suggesting a potential clonal expansion of hepatocytes harboring these HBV-DNA

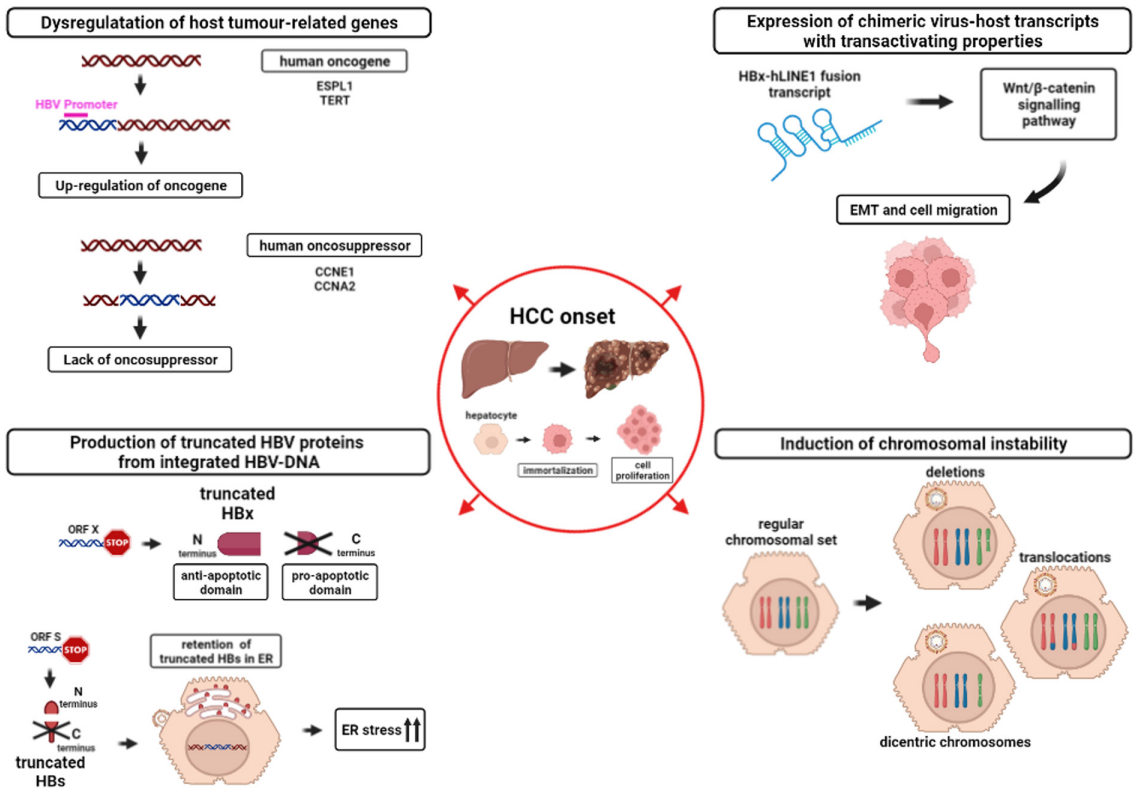

FIGURE 3
Mechanisms involved in HCC onset caused by HBV-DNA integration into the host genome. The figure depicts the downstream effects of HBV-DNA integration favoring HCC onset: (i) *Dysregulation of host tumor-related genes (top-left)* due to the up-regulation of host oncogenes (red) or to the lack of oncosuppressors. Magenta line indicates a generic HBV promoter; (ii) *Expression of chimeric virus-host transcripts with transactivating properties (top-right).* HBx-human long interspersed nuclear element 1 (hLINE1) fusion transcript, derived from integrated HBV-DNA, can activate the Wnt/β-catenin signaling pathway, leading to epithelial-mesenchymal transition (EMT) and cell migration; (iii) *Production of truncated HBV proteins from integrated HBV-DNA (bottom-left).* HBV ORF X integration into the host genome can lead to the synthesis of a truncated HBx protein, retaining the anti-apoptotic N-terminal domain and lacking the pro-apoptotic C-terminal domain. Similarly, HBV ORF S integration can lead to the synthesis of truncated HBs proteins that are retained in the membrane of endoplasmic reticulum (ER), triggering ER stress, and in turn activating pro-oncogenic pathways; iv) *Induction of chromosomal instability (bottom-right).* The hepatocyte with a regular chromosomal set can develop chromosomal aberrations, such as deletions, translocations or dicentric chromosomes, as a consequence of HBV-DNA integration into the genome.

integrations (Svicher et al., 2021). This is supported by gene ontology analysis revealing the localization of HBV-DNA integration in human genes (NUP85, ANKRD52, ELAC2, and AGBL5) known to be involved in the regulation of cell proliferation and also in promoting the neoplastic transformation of different cell types including hepatocytes (Noda et al., 2006; Yu et al., 2017). Interestingly, the rate of HBV-DNA integration varies according to HBV genotypes, with genotype D characterized by the highest prevalence (38.9%) followed by genotype E (33.3%) and genotype C (22.2%) (Svicher et al., 2021). No results were available for the other genotypes due to very limited samples size.

Furthermore, the evidence of HBV-DNA integration in HBeAg negative low viremic patients with a limited HBV reservoir can be a reminder that even patients, not meeting treatment criteria, remain at risk of disease progression, supporting a re-evaluation of treatment candidacy (Svicher et al., 2021). This is in line with a recent study showing that treatment with NUCs can reduce the number of transcriptionally active HBV integrations, suggesting that this NUC-mediated effect should be considered in patients' management (Hsu et al., 2022).

HBV-DNA integration in the setting of occult HBV infection

Occult HBV infection (OBI) is defined as the presence of replication-competent HBV-DNA in the liver and/or in the blood of HBsAg-negative individuals (Raimondo et al., 2019). In particular, OBI is characterized by the long-lasting persistence of cccDNA in hepatocytes, whose transcriptional activity is strongly suppressed by the host's defense mechanisms (Raimondo et al., 2019).

Based on the HBV-specific antibody profiles, OBI is mainly characterized by positivity to hepatitis B core antibody (anti-HBc) with or without hepatitis B surface anti-body (anti-HBs) (Raimondo et al., 2019).

Occult HBV infection can occur either following the resolution of acute hepatitis B or after decades of chronic HBV infection. Although the risk of disease progression is particularly low, there is evidence that HBV-DNA integration can occur also in the setting of OBI and can contribute to HBV-mediated carcinogenesis (Saitta et al., 2015; Chen et al., 2019). In particular, a previous study showed that the prevalence of HBV-DNA integrations in hepatic tumor tissues from OBI patients is quite high and can involve regulatory and functional host genes modulating cell proliferation (Saitta et al., 2015), highlighting a potential oncogenic risk also in this phase.

HBV-DNA integration as a biomarker in mediating hepatitis B virus-related tumorigenesis

HBV-DNA integration is known to result in the dysregulation of genes in the neighborhood of the insertion site (Bailey and Murnane, 2006; Feitelson and Lee, 2007; Péneau et al., 2022; Ramirez et al., 2021; Álvarez et al., 2021). In the setting of hepatocarcinogenesis, the insertional mutagenesis of HBV-DNA can enhance expression of oncogenes, inactivate oncosuppressor expression, generate chimeric or truncated transcripts. Furthermore, HBV-DNA integration can also promote genome instability leading to the accumulation of chromosomal aberrations even at long distance from the integration site (Bailey and Murnane, 2006; Feitelson and Lee, 2007; Zhao et al., 2016; Yu et al., 2017; Álvarez et al., 2021; Bousali et al., 2021; Péneau et al., 2022; Ramirez et al., 2021). These mechanisms can trigger the clonal selection of hepatocytes with enhanced survival and proliferative properties, causing their neoplastic transformation (Ding et al., 2012; Fujimoto et al., 2012; Zhao et al., 2016; Bousali et al., 2021; Lin et al., 2021).

Hereafter, we discuss in more details the downstream effects of HBV-DNA integration in (i) dysregulating host tumor-related genes, (ii) inducing the expression of chimeric virus-host transcripts with transactivating properties, and (iii) producing truncated HBV proteins, and (iv) causing chromosomal instability (**Figure 3**).

Dysregulation of host tumor-related genes

HBV-DNA integration can target directly genes regulating crucial intracellular pathways as cell cycle regulation, cell immortalization, cell-to-cell interaction, and cell signaling, thus inducing their altered gene expression, a process that can mediate the acquisition of pro-oncogenic properties by the involved hepatocytes (Paterlini-Bréchot et al., 2003; Murakami et al., 2005; Saigo et al., 2008; Sung et al., 2012; Kawai-Kitahata et al., 2016; **Figure 3**).

Despite the fact that HBV-DNA integration during chronic HBV infection is found to be randomly distributed across the host genome in non-tumor tissues (Budzinska et al., 2018b; Péneau et al., 2022), a peculiar recurrence of HBV-DNA integrations at the level of specific genes involved in carcinogenic pathways has been revealed in studies analyzing tumor liver samples. Among them, the most frequent targets of HBV-DNA integration found to be associated with HCC are represented by: TERT (telomerase reverse transcriptase), MAPK1 (mitogen-activated protein kinase 1), MLL2 and MLL4 (myeloid/lymphoid or mixed-lineage leukemia 2 and 4), CCNE1 and CCNA2 (cyclin E1 and A2), TP53 (tumor protein p53), CTNNB1 (Catenin Beta 1) FAR2 (fatty acyl-coA reductase 2), ITPR1 (inositol 1,4,5-trisphosphate receptor type 1), and IRAK2 (interleukin 1 receptor associated kinase 2) (Paterlini-Bréchot et al., 2003; Murakami et al., 2005; Saigo et al., 2008; Sung et al., 2012; Kawai-Kitahata et al., 2016). Among them, the enrichment of HBV-DNA integration in TERT, CCNE1, and CCNA2 genes in tumoral samples has also been confirmed by analyzing the publicly available database VISDB, collecting 20558 HBV-DNA integration sites in tumor/peritumor/non-tumor liver samples from 45 publications (Tang et al., 2020). In particular, $\geq$90% of HBV-DNA integration occurring in TERT, CCNE1, or CCNA2 is revealed in liver tumor samples compared to $\leq$10% in non-tumoral ones reported in VISDB, corroborating their role in mechanisms underlying HBV-driven carcinogenesis.

HBV-DNA integration in the TERT gene is considered the most important hotspot of HBV integration in tumor samples (Sze et al., 2021) and has been associated with a more aggressive tumor behavior and a significantly poorer survival (Zhao et al., 2016; Cui et al., 2020). In particular, the integration of the HBV enhancer 1 upstream of the gene encoding TERT has been recently shown to upregulate TERT expression (Péneau et al., 2022), resulting in its increased capability to maintain telomere integrity and stability of damaged hepatocytes. This is a critical event in permitting hepatocytes to overcome the mechanisms of cell senescence and, in turn, driving neoplastic transformation (Jang et al., 2021; Sze et al., 2021).

Expression of chimeric virus-host transcripts with transactivating properties

As previously mentioned, the un-interrupted transcription by the cellular RNA-polymerase can lead to the production of fusion HBV-human transcripts (**Figure 3**).

The most frequently described HBV-human fusion transcripts are those containing HBx linked to the "long interspersed nuclear element 1" (LINE1), that is usually silent in the hepatocytes (Heikenwalder and Protzer, 2014; Lau et al., 2014; Liu et al., 2021). The integration of ORF X upstream human LINE1 (hLINE1) can induce the expression of a fusion HBx-hLINE1 transcript, whose detection was correlated with a shorter patients' survival (Heikenwalder and Protzer, 2014; Lau et al., 2014; Zhang Y. et al., 2021). This fusion HBx-hLINE1 transcript is suggested to act as a long non-coding RNA, capable of activating the Wnt/β-catenin signaling pathway and to influence the epithelial-mesenchymal transition and in turn to enhance cell migration (Heikenwalder and Protzer, 2014; Lau et al., 2014; Zhang Y. et al., 2021).

Production of truncated hepatitis B virus proteins from integrated HBV-DNA

HBV integration can also promote the production of viral proteins (both HBx and HBs) and/or their altered forms, which may contribute to viral persistence, to continuous liver damage and ultimately to HCC development (Shamay et al., 2001; Tu et al., 2001; Wang et al., 2003, 2006, 2010; Ning-Fang et al., 2008; Warner and Locarnini, 2008; Sze et al., 2013; Ng et al., 2016; Salpini et al., 2017; Zhang Y. et al., 2021; **Figure 3**).

Indeed, as previously mentioned, as a result of HBV integration, the promoter of the ORF X is functional in integrated HBV-DNA and can induce the production of HBx proteins, lacking the C-terminal domain known to have pro-apoptotic properties (Shamay et al., 2001; Zhang Y. et al., 2021; **Figure 3**). The resulting overexpression of the C-terminal truncated HBx can inhibit the apoptosis and induce the acquisition of stem cell-like properties, thus promoting the neoplastic transformation of the hepatocytes (Tu et al., 2001; Ning-Fang et al., 2008; Wang et al., 2010; Sze et al., 2013; Ng et al., 2016).

Similarly, recent studies have highlighted that the ORF S can be frequently involved in HBV-DNA integration events in liver tumor tissues (Hu et al., 2020; Jang et al., 2020). In particular, it has been observed that the integration of fragments of viral genome can lead to the production of truncated HBsAg, lacking the C-terminal domain. These C-terminal truncated HBsAg forms cannot be properly secreted, but are retained in the membrane of the endoplasmic reticulum, thus activating intracellular signaling known to promote cell proliferation and to represent a factor contributing to HCC onset (Wang et al., 2003, 2006; Warner and Locarnini, 2008; Salpini et al., 2017; **Figure 3**). Furthermore, the accumulation of these truncated forms of HBsAg in the endoplasmic reticulum can cause cellular oxidative stress, known to augment DNA damage with consequent double-stranded breaks in which dsl HBV-DNA can be integrated. Interestingly, it has also been demonstrated that these aberrant HBsAg forms may inhibit DNA double-stranded break repair thus contributing genomic instability (Hsieh et al., 2015) further promoting the neoplastic transformation of hepatocytes (Wang et al., 2006; Hsieh et al., 2015).

Induction of chromosomal instability

Lastly, there is increasing evidence showing that HBV-DNA integration in the human genome can promote a status of generalized genomic instability, that increases the risk of accumulating genomic rearrangements, further promoting the neoplastic transformation of hepatocytes (Ding et al., 2012; Zhao et al., 2016; Tu et al., 2017; **Figure 3**).

Recently, by analyzing HBV-DNA integration profiles of 296 liver tumor samples, a recent study unraveled that the insertion of HBV-DNA into the human genome can cause dramatic genetic aberrations, including non-homologous chromosomal fusions, dicentric chromosomes and long telomeric deletions, that may also lead to loss of tumor suppressor genes (such as TP53, ARID1A, RB1, RPS6KA3, and IRF2) (Álvarez et al., 2021; **Figure 3**). Furthermore, by applying *ad hoc* mathematical models, these genomic rearrangements have been estimated to occur several years (up to 20 years) before cancer diagnosis, supporting their crucial role as early drivers of hepatocarcinogenesis (Álvarez et al., 2021). In a similar direction, recent studies showed that HBV-DNA integration can profoundly modify the architecture of human genome by altering cancer-related genes even at long distance from integration site (Péneau et al., 2022; Ramirez et al., 2021; Li et al., 2022).

Hepatitis B virus integration as a diagnostic biomarker of hepatocellular carcinoma occurrence and recurrence

The increasing data on the role of HBV-DNA integration in promoting pro-oncogenic mechanisms, together with the relevant advancement in next-generation sequencing technologies, have raised growing interest in the possibility to detect HBV-DNA integration as a potential novel prognostic marker for HCC.

A recent metanalysis, including >18,000 HBV-DNA integration sites from tumor samples, identified a total of 396 recurrently targeted genes, of which 28 recurred in at least 10 HCC patients (Lin et al., 2021). This metanalysis is particularly relevant since it paves the way for the identification of key cellular genes, representing hot spots of HBV-DNA integration associated with HCC onset. The ultimate goal of these studies

will be the generation of diagnostic gene panels, based on refined next generation sequencing methods, that could reveal the presence of HBV-DNA integrations involving those genes associated with oncogenesis, potentially acting as an early biomarker for HCC development (**Table 1**).

Promising data have also emerged on the utility of circulating HBV-human chimeric DNA, resulting from HBV-DNA integration events, as a useful non-invasive biomarker for early identification of HCC development and its recurrence (Li et al., 2018; Liu et al., 2021; Zhang D. et al., 2021; **Table 1**). In 2019, a study demonstrated, for the first time, the presence of cell-free chimeric HBV-human DNA from blood samples of 20 patients with chronic HBV infection. These chimeric DNAs derived from 87 different HBV integration sites and were particularly enriched in tumor-related genes, thus suggesting, the possibility to use chimeric human-HBV DNA in blood as circulating biomarker for HCC (Li et al., 2018). In 2021, by applying a novel approach of Circulating Single-Molecule Amplification and Resequencing, another study confirmed that most recurrent integration events detected in blood cell-free DNA have originated from tumor tissues, corroborating the potential utility of non-invasive detection of HBV-DNA integration as a circulating tumor marker for HBV-related HCC (Zheng et al., 2021).

In keeping with these findings, a recent study detected HBV-human DNA in 97.7% of patients with HBV-related HCC (Li C. L. et al., 2020). Notably, by analyzing cell-free DNA from blood samples 2 months following HCC resection, the same HBV-human chimeric DNA were also found in 10 cases (23.3%), nine of whom (90%) experienced HCC recurrence within a year (Li C. L. et al., 2020). These data demonstrate that chimeric DNA resulting from HBV-DNA integration can persist despite resection and can also represent a potential novel tool for early detection of HCC recurrence.

Hepatitis B virus integration in lymphoid cells and potential role in lymphomagenesis

Growing evidence highlights that an alternative active HBV reservoir is represented by immune cells, providing relevant sites for HBV persistence. Specifically, productive HBV infection has been demonstrated to occur in hematopoietic stem cells (HSCs) from HBV chronically infected patients (Romet-Lemonne et al., 1983; Elfassi et al., 1984; Ma et al., 2012) as well as *in vitro* experiments (Zeldis et al., 1986; Steinberg et al., 1990). Furthermore, HBV-DNA and its replicative intermediates (cccDNA, HBV-RNAs) were also found in circulating peripheral blood mononuclear cells (PBMCs), as well as singularly in B and T cells subsets, monocytes and NK cells from patients with acute or chronic HBV infection (Stoll-Becker et al., 1997; Trippler et al., 1999; Lee et al., 2015).

Moreover, peripheral lymphoid cells have also been recognized as an active site of HBV-DNA integration, detected with high frequency in all phases of HBV infection, including acute and occult hepatitis B (Pontisso et al., 1984; Laure et al., 1985; Pasquinelli et al., 1986; **Table 2**).

More recently, other studies, based on more sensitive and refined molecular assays, have strongly supported the frequent occurrence of HBV-DNA integration, revealing their localization into the genome of PBMCs of HBV chronically infected patients (Laskus et al., 1999; Murakami et al., 2004; Umeda et al., 2005; Wang et al., 2008).

Nevertheless, the pathogenic and clinical implications of HBV lymphotropism and of the related HBV-DNA integrations in lymphoid cells have not yet been fully elucidated. Recently, some studies have supported a potential contribution of HBV-DNA integration in the pathogenesis of immunoproliferative diseases. In particular, HBV has been suggested to contribute to the development of hematological malignancies such as non-Hodgkin Lymphoma (NHL) (Coffin et al., 2021) and more recently it has been revealed that HBV-DNA integration is a common phenomenon in NHL (Li M. et al., 2020). Indeed, the authors identified multiple HBV-DNA integration events in half of the NHL patients analyzed, occurring both in coding and non-coding human regions. Notably, HBV-DNA integration involved the exonic regions (crucial for mRNA synthesis) of four specific genes (FAT2, SETX, ITGA10, and CD63), determining their altered expression and potentially perturbating relevant intracellular pathways. HBV-DNA integration was also found to be recurrent in seven coding genes (ANKS1B, CAPZB, CTNNA3, EGFLAM, FHOD3, HDAC4, and OPCML), which may have potential functions in NHL development. In line with these data, a recent study has shown that HBV-DNA integration profiles in PBMCs are superimposable to those observed in tumor liver tissue, further supporting their role in paving the way toward lymphomagenesis (Lau et al., 2020).

Beyond PBMCs, HBV-DNA integrations have also been detected in HSCs of HBV chronically infected patients (Shi et al., 2014). Such integrations could pave the basis for uncontrolled cell proliferation and in turn for the onset of leukemia.

Overall, further studies are necessary to better elucidate the role of HBV-DNA integration in hematological malignancies.

Hepatitis B virus integration as a barrier to the achievement of hepatitis B virus cure

The current treatment goal for novel anti-HBV therapies is HBV functional cure, defined as sustained HBsAg loss together with undetectable serum HBV-DNA off-therapy,

reflecting the silencing of cccDNA (Lampertico et al., 2017; Lok et al., 2017). Indeed, HBV functional cure represents an optimal therapeutic endpoint, associated with a significantly decreased HCC incidence and no progression to HBV-related cirrhosis (Liu et al., 2016; Yip et al., 2019; Vittal et al., 2022). Unfortunately, this therapeutic goal is rarely achieved by currently available treatment options [nucleos(t)ide analogs (NUCs) and peg-interferon], having a limited effect on cccDNA pool and its activity. However, more recently several new compounds have been developed (immune modulators, capsid assembly modulators, RNA-interference, antisense molecules, entry inhibitors, and HBsAg-release

TABLE 1 Diagnostic application of HBV-DNA integration as a novel biomarker for HCC occurrence and recurrence.

Main findings	Potential application of HBV DNA integration in HCC diagnosis	References
Specific cellular genes involved in tumorigenic pathways have been recognized as hot spots of HBV integration in patients with HCC	Development of diagnostic gene panels, based on refined next-generation sequencing methods, that could reveal, in liver biopsies or in blood, the presence of HBV integrations involving those genes associated with oncogenesis, thus potentially acting as an early biomarker of HCC onset	Zhao et al., 2016; Li et al., 2018; Hu et al., 2020; Lin et al., 2021; Zheng et al., 2021
Chimeric HBV-human DNA resulting from HBV integration in genes involved in tumorigenic pathways can persist despite resection and anticipate HCC recurrence	Development of molecular assays for detecting and quantifying circulating HBV-human chimeric DNAs in blood, deriving from HBV integrations already recognized in primary HCC, as non-invasive biomarker of HCC recurrence	Li C. L. et al., 2020

TABLE 2 Current knowledge on HBV integration in blood cells.

Findings on HBV-DNA integration in blood cell	Study population	Frequency of HBV integration in blood cells	Technique for detecting HBV integration	References
First evidence of HBV-DNA integration in PBMCs of HBV-infected patients	16 CHB patients	PBMCs (25%)	Southern blot	Pontisso et al., 1984
	4 HIV + patients (1 CHB and 3 OBI)	PBMCs (100%)	Southern blot	Laure et al., 1985
HBV-DNA integration in PBMCs occurs in all phases of HBV infection, including acute and occult infection	38 HBsAg + patients (8 AHB; 21 CHB, 6 CI, 3 HCC) and 34 OBI patients	PBMCs (100% AHB, 85.7% CHB; 33.3%; 66.6% HCC and 35.2% OBI)	Southern blot	Pasquinelli et al., 1986
First sequencing analysis of HBV-human junctions in the setting of HBV-DNA integration of PBMCs	10 CHB patients	PBMCs (20%)	Nested-PCR	Laskus et al., 1999
Accumulating evidences on HBV-DNA integration as a frequent event at level of PBMCs of patients with ongoing or past HBV infection	7 CHB patients; 9 OBI	PBMCs (42.8% CHB; 22.2% OBI)	Alu-PCR	Murakami et al., 2004
	21 OBI	PBMCs (14.2%)	Inverse-PCR	Umeda et al., 2005
	30 CHB adults and 19 CHB children	PBMCs (86% adults; 65% children)	Sanger Sequencing	Wang et al., 2008
Multiple HBV-DNA integration events revealed in PBMCs of CHB patients with non-Hodgkin lymphoma (NHL)	12 CHB with NHL diagnosis	Lymphoid malignant tissues (50%)	Next generation sequencing	Li M. et al., 2020
HBV-DNA integration in PBMCs is enriched at level of genes associated with tumorigenesis. Patterns of HBV integration are sheared by tumoral liver cells and PBMCs.	42 CHB patients	PBMCs (81%) A total of 271 integration events of which 58 in coding genes involved in oncogenetic pathway	Alu-PCR; Next-generation sequencing	Lau et al., 2020
Evidence of HBV-DNA integration in bone marrow hematopoietic stem cells (HSC)	8 CHB patients	CD34 + HSC (62.5%)	FISH	Shi et al., 2014

AntiHBc-positive patients were defined as OBI.
PBMCs, Peripheral Blood Mononulear Cells; CHB, chronic hepatitis B; HIV, human immunodeficiency virus; HBsAg, Hepatitis B surface antigen; AHB, Acute Hepatitis B; CI, HBV Chronic Infection; HCC, hepatocellular carcinoma; HSC, Hematopoietic Stem cells; PCR, polymerase chain reaction; FISH, fluorescence *in situ* hybridization.

inhibitors), showing greater promise in terms of achieving functional cure.

In this regard, the occurrence of HBV-DNA integration and the conclusive evidence of HBsAg production derived from integrated HBV-DNA has challenged functional cure, or HBsAg loss, as the ideal therapeutic endpoint (**Figure 4**). Indeed, the persistence of HBsAg in serum can reflect the continuous production of HBsAg from integrated HBs-encoding regions even despite a completely silenced cccDNA. In keeping with this concept, a recent study estimated that in HBeAg-negative patients, ∼80% of HBsAg transcripts derived from intrahepatic integrated HBV-DNA and did not reflect cccDNA transcriptional activity (Podlaha et al., 2019). Similarly, other studies support a significant contribution of integrated HBV-DNA to serum HBsAg levels in HBeAg-negative patients (Ringlander et al., 2020; Rydell et al., 2020; Meier et al., 2021), as well as in chimpanzees (Wooddell et al., 2017). In these studies, most HBsAg transcripts were characterized by the lack of the conventional HBV polyA signal, typically reflecting transcription of HBs derived from integrated HBV-DNA (**Figure 1B**).

These findings are in keeping with a study showing that, after 2 years of NUC therapy, HBeAg-negative patients with no evidence of HBV-DNA integration experienced a relevant decay of HBsAg levels, in contrast to those with integrated HBs region, who maintained constant HBsAg levels despite antiviral therapy (HBsAg decline: 2.53 log IU/ml vs. 0.1 log IU/ml, $P = 0.002$) (Hu et al., 2018). Notably, even more minimal change in serum HBsAg levels were observed despite the achievement of undetectable cccDNA in patients receiving long term NUC treatment (Lai et al., 2017).

Overall, these findings support the need for novel biomarkers to better identify patients who have silenced cccDNA, particularly in the light of the multiple novel HBV therapies currently in clinical trials aimed at achieving HBV functional cure.

Furthermore, the production of HBsAg derived from integrated HBV-DNA can contribute to the elevated burden of viral antigens and the subsequent exhaustion of anti-HBV immune response, typically observed in chronic HBV infection (Loggi et al., 2013; Ferrari, 2015; Kim et al., 2020). The role of this phenomenon in jeopardizing the achievement of HBV functional cure merits further investigation (**Figure 4**).

Beyond functional cure, the ultimate endpoint of anti-HBV treatment is represented by complete sterilizing cure, implying the elimination of both cccDNA and integrated HBV-DNA (Lok et al., 2017; Testoni et al., 2017).

So far, the only approach that has been proposed to achieve this ideal endpoint relies on advanced genome editing strategies such as CRISPR/Cas (clustered regularly interspaced short palindromic repeat/CRISPR associated Cas) (Lin et al., 2014). Although further studies are required, CRISPR/Cas has been shown to be a promising approach to reduce the burden of cccDNA and of integrated HBV-DNA paving the way for a potential sterilizing HBV cure (Wang et al., 2015, 2017; Chen et al., 2021). However, the clinical application of this approach deserves further clarification.

Overview on different techniques utilized for the analysis of hepatitis B virus integration events

Hereinafter, we provide an overview of the first-, second-, and third- generation techniques utilized to detect HBV-DNA integration into human genome with their main advantages and limitations (**Figure 5**).

First generation techniques

Southern blot

Southern blot was the first technique used for revealing the presence of HBV integration in both tumor tissue and HCC cell lines, in first studies lead in 1980s.

Southern Blot is based on total DNA extraction from HBV-infected cells or tissues, followed by its digestion with a restriction enzyme and a final labeling by 32P-marked HBV hybridization probes. Lastly the digested and labeled DNAs fragments are separated through gel electrophoresis and autoradiographed on films, showing the presence of HBV-DNA integration (Brechot et al., 1980; Chakraborty et al., 1980). This method does not provide any information on the localization of HBV-DNA integration and it suffers a very low sensitivity, since it can detect only HBV integrations present in at least 10^3–10^5 copies, biasing HBV integration detection only toward hepatocytes that have undergone clonal expansion (Budzinska et al., 2018a).

In situ hybridization

In situ hybridization (ISH) is a technique detecting HBV integration directly in cells or tissues using a probe, labeled radioactively in first experiments or with a fluorochrome in its more recent evolution [Fluorescent *in situ* hybridization (FISH)]. This technique confers the advantage to identify the location of HBV integration, showing the chromosomal sites of HBV DNA integration (Tokino and Matsubara, 1991; Huang et al., 2005). However, similarly to Southern Blot, ISH is characterized by poor sensitivity and can also be associated with high error rate in HBV integration recognition, due to potential unspecific binding of probes, resulting in a high signal noise, sometimes difficult to interpret.

Arthrobacter luteus PCR

Alu-PCR is a modified version of the classical PCR applied for studying HBV integration on the basis of its

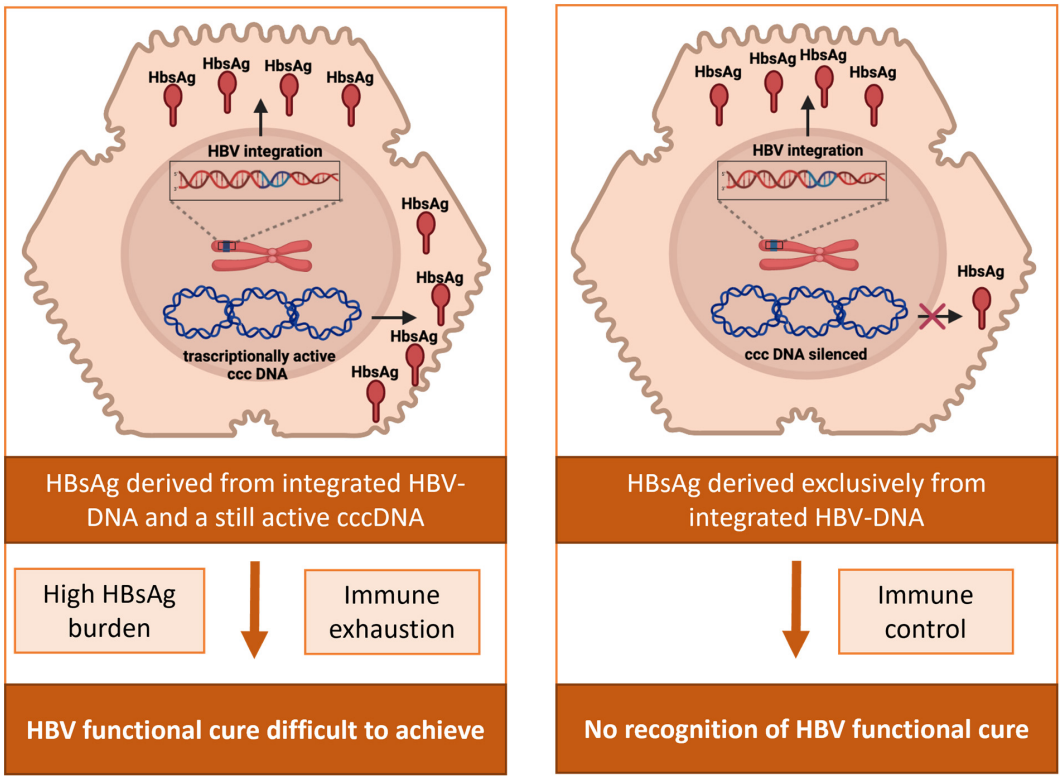

FIGURE 4
Impact of HBV-DNA integration in the achievement and assessment of HBV functional cure. In patients with an active HBV reservoir, HBsAg can derive from the transcriptional activity of cccDNA and from HBV-integrated DNA. HBsAg from integrated HBV-DNA can consequently contribute to the elevated burden of viral antigens and the subsequent exhaustion of anti-HBV immune response, potentially jeopardizing the achievement of HBV functional cure. In patients with a silenced HBV reservoir, HBsAg in serum can still be present deriving exclusively from integrated HBs-encoding regions, thus hampering the proper recognition of the achievement of HBV functional cure.

occurrence close to Alu elements, which are transposable short stretches of DNA recognized by Arthrobacter luteus (Alu) restriction endonuclease, interspersed throughout the entire human genome at a mean interval of about 4 kb (Minami et al., 1995). This technique uses primers pairs, one matching with Alu elements while the other matching with HBV sequence, in order to amplify HBV-human junctions, representing the sites of HBV DNA integrations. Alu-PCR is associated with a higher sensitivity in detecting HBV integration respect to the previously described methods, however, its main bias lies in the possibility to detect only those integrations occurring close to Alu sequences, thus losing the remaining HBV integration events (Murakami et al., 2005; Saitta et al., 2015).

Inverse PCR

Inverse PCR (InvPCR) is a refined *ad hoc* designed technique, permitting to amplify the unknown human DNA regions that are adjacent to integrated HBV sequence. InvPCR includes a first step of DNA digestion by using specific restriction enzymes, followed by DNA circularization of cleavage products through self-ligation and amplification by using specific outward HBV-matching primers (Tsuei et al., 1994). This strategy allows to obtain the left, right, or both ends of the HBV-human junction, representing the integrated HBV DNA sequence (Tu and Jilbert, 2017). The major advantage of this method relies in its high sensitivity, permitting to detect HBV integrations, even when occurring as single copy (Tu and Jilbert, 2017). Moreover, it enables to characterize the localization of HBV integration and to quantify their absolute numbers. Unfortunately, invPCR is quite time-consuming and technically demanding, limiting its large use in many laboratories. Moreover, the use of restriction enzymes may limit the detection of some HBV integration junctions due to the lack of the corresponding cutting sites upstream and downstream of the HBV-human DNA junctions (Tu and Jilbert, 2017).

Second and third generation techniques

The development and refinement of Next-generation sequencing (NGS) methods, characterized by a high sequencing

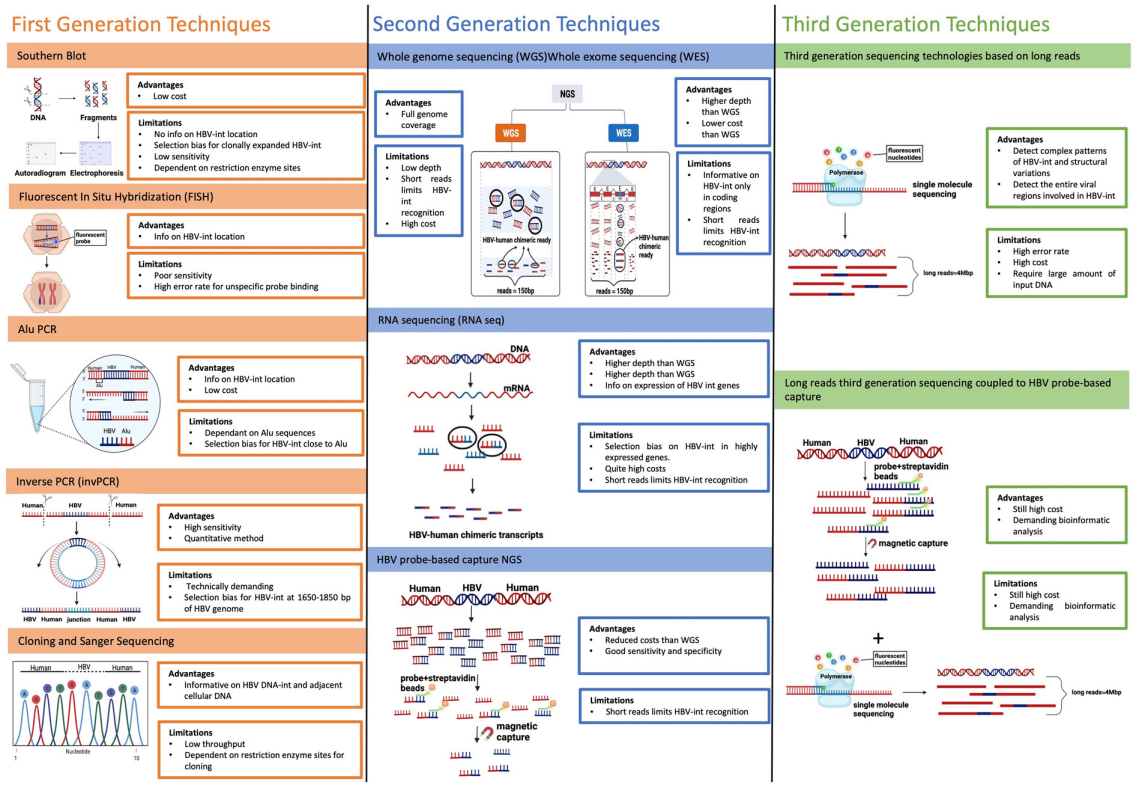

FIGURE 5
Main techniques utilized to detect HBV-DNA integration. The figure reports an overview of the first-, second-, and third- generation techniques utilized to detect HBV-DNA integration into human genome with their main advantages and limitations. First generation techniques include: Southern Blot, Fluorescent *In situ* Hybridization (FISH), Alu PCR and Inverse PCR (invPCR). Second-generation techniques are based on Next-generation sequencing (NGS) and are based on short reads (with a length of 150 base pair in most used NGS platform). They include: whole-genome sequencing (WGS), whole-exome sequencing (WES), and RNA sequencing (RNA-seq). Third-generation techniques rely on the use of long-read sequencing, a technology based on single-molecule sequencing without pre-PCR amplification, producing long-reads (up to 4 Mbps) in a real-time sequencing process.

throughput, has given the great opportunity to generate large amount of data on HBV integration occurring in the entire human genome in a high number of samples and in a relatively short time (Ding et al., 2012; Budzinska et al., 2018a).

In NGS-based technologies, the extracted total DNA is randomly fragmented and then amplified and sequenced as millions of short reads (with a length of 150 base pair in most used NGS platform). Afterward, HBV integrations are recognized by specific bioinformatics approaches capable to reveal the presence of HBV-human chimeric reads, containing HBV-human breakpoints, that represents the sites of HBV integration into human genome (Budzinska et al., 2018a; Svicher et al., 2021).

In particular, NGS technologies can be applied for analyzing HBV integrations occurring in the entire human genome by whole-genome sequencing (WGS) or those restricted to human coding regions (exons) by whole-exome sequencing (WES). Furthermore, it is possible to evaluate only the transcriptionally active HBV integrations by RNA sequencing (RNA-seq) (Fujimoto et al., 2012; Jiang et al., 2012; Sung et al., 2012; Shiraishi et al., 2014; Svicher et al., 2021). However, it should be considered that it is crucial to use a deep sequencing coverage in order to achieve a high sensitivity in the detection of HBV integrations by NGS approaches. Unfortunately, this high sequencing coverage necessary to guarantee a high sensitivity in detecting HBV integrations is also associated with high cost. This represents the main disadvantage of these approaches, that constrains their large-scale utilization. Recently, in order to overcome this limitation, a novel NGS approach was developed, based on the preliminary enrichment of HBV-containing sequence fragments by using a set of capture probes, *ad hoc* designed to cover the entire HBV genome. The resulting HBV-enriched sequence library is then sequenced by NGS platform, reducing the necessary sequencing volume to 2 GB per sample and, thus, limiting the relative costs. Overall, HBV probe-based capture technology is more cost-effective while still providing similar specificity and sensitivity to detect viral integration throughout the human genome (Yang et al., 2018; Ishii et al., 2020).

Third-generation techniques to detect HBV-DNA integration rely on the use of long-read sequencing, a technology based on single-molecule sequencing without the pre-PCR amplification step, producing long-reads (up to 4 Mbps) in a real-time sequencing process. This has allowed to overcome the limited length of the DNA reads produced by most NGS platform, to strongly increase the probability to detect HBV integration and, in turn, to analyze extensively the entire HBV regions involved in integration events and the resulting complex interchromosomal genomic rearrangements as fusions and translocations (Alvarez-Benayas et al., 2021; Ramirez et al., 2021; van Buuren et al., 2022). More recently, the long-read sequencing approach has been coupled to the enrichment of HBV sequences by HBV targeting probes, further optimizing the sensitivity of this assay in revealing HBV integrations reducing, at the same time, the costs.

Conclusion

The availability of more advanced molecular techniques has enabled a deeper understanding of the role of HBV-DNA integration in modulating viral pathogenetic properties. It has been demonstrated that HBV-DNA integration is an early event in HBV infection that can even occur during acute infection and can persist throughout the different phases of chronic infection including occult infection. Integrated HBV-DNA cannot support the production of virions but can represent an important source of viral proteins such as HBsAg. In particular, the sizeable production of HBsAg from HBV-DNA integrants, even in the presence of a transcriptionally silenced cccDNA, has challenged the concept of HBV functional cure, defined as HBsAg-loss, as the ideal therapeutic endpoint. In the light of the multiple novel HBV therapies currently in clinical trials, this has led to much debate in the field about the need for novel biomarkers to better identify patients who have silenced cccDNA.

The events of HBV-DNA integration are particularly abundant during the HBeAg-positive phases of chronic infection, as a consequence of intensive HBV replication and occur with no preferential hotspot in the viral genome. During the process of HBeAg seroconversion, the activation of an efficient anti-HBV immune response can induce a bottleneck, favoring the selection of those hepatocytes in which HBV-DNA integrations have conferred a selective advantage in terms of survival and capability to escape the cytotoxic immune response. This can lead to an enrichment of HBV-DNA integrants in genes involved in the modulation of cell proliferation and apoptosis, promoting the clonal expansion of hepatocytes and representing a first event in mechanisms underlying the neoplastic transformation of the hepatocytes and thus HCC development.

Overall, HBV-DNA integration represents a fascinating and critical element of HBV pathogenetic potential. Moreover, it is emerging as the single most important barrier to achieving both HBV functional cure and sterilizing cure and all novel therapeutic approaches will have to address the effects of HBV-DNA integration to have a meaningful impact on the field. This is an area which will continue to challenge scientists and clinicians and the progress we make will ultimately determine our success in the HBV cure program.

Author contributions

RS and VS: review conceptualization and writing. SD'A: literature searching, figure preparation, and review writing. LB: literature searching and figure preparation. LP: literature searching. UG: review revision. PK: review conceptualization and revision. 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.

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Advances in multi-omics research on viral hepatitis

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Viral hepatitis is a major global public health problem that affects hundreds of millions of people and is associated with significant morbidity and mortality. Five biologically unrelated hepatotropic viruses account for the majority of the global burden of viral hepatitis, including hepatitis A virus (HAV), hepatitis B virus (HBV), hepatitis C virus (HCV), hepatitis D virus (HDV), and hepatitis E virus (HEV). Omics is defined as the comprehensive study of the functions, relationships and roles of various types of molecules in biological cells. The multi-omics analysis has been proposed and considered key to advancing clinical precision medicine, mainly including genomics, transcriptomics and proteomics, metabolomics. Overall, the applications of multi-omics can show the origin of hepatitis viruses, explore the diagnostic and prognostics biomarkers and screen out the therapeutic targets for viral hepatitis and related diseases. To better understand the pathogenesis of viral hepatitis and related diseases, comprehensive multi-omics analysis has been widely carried out. This review mainly summarizes the applications of multi-omics in different types of viral hepatitis and related diseases, aiming to provide new insight into these diseases.

KEYWORDS

viral hepatitis, genomics, proteomics, transcriptomics, metabolomics

Introduction

Viral hepatitis is a major global public health problem that affects hundreds of millions of people and is associated with significant morbidity and mortality. Five biologically unrelated hepatotropic viruses account for the majority of the global burden of viral hepatitis, including hepatitis A virus (HAV), hepatitis B virus (HBV), hepatitis C virus (HCV), hepatitis D virus (HDV), and hepatitis E virus (HEV). Despite that HAV does not develop into a chronic infection, HBV, HCV, HDV and occasionally HEV may cause chronic infections, of which HBV and HCV have a significant association with

chronic incidence. Most deaths from viral hepatitis are due to HBV and HCV infections. According to the statistics, it was estimated that 296 million people were infected with hepatitis B, 58 million people were infected with hepatitis C and 1.1 million people died as a consequence of viral hepatitis infections in 2019 (Tanaka et al., 2022). In 2015, the United Nations adopted a resolution to combat viral hepatitis as part of the agenda to achieve the 2030 sustainable development goals. Subsequently, the first global strategy was developed in 2016 for the elimination of viral hepatitis (Vo Quang et al., 2021).

The advances in technology have created a variety of new fields of study, commonly referred to as omics. Omics is defined as the comprehensive study of the functions, relationships and roles of various types of molecules in biological cells. The multi-omics analysis has been proposed and considered key to advancing clinical precision medicine, including genomics, transcriptomics and proteomics, metabolomics and so on (Olivier et al., 2019). Genomics focuses on genomic DNA, the genome is usually divided into small fragments and then iteratively assembled by bioinformatics algorithms, along with gene annotation and other data analysis (Low et al., 2019). Transcriptomics can be performed to study the sum of mRNAs at a certain time point, and can also use known gene probe for specific genes (Kalisky et al., 2018). Besides, proteomics is oriented to the whole protein based on 2D-Gel and mass spectrometry. It is divided into top–down and bottom–up analysis methods. Similar to the genomics, the protein is decomposed into small peptide segments, and the known and unknown protein sequences are thus identified (Aslam et al., 2017). Through the liquid phase and mass spectrometry, metabolomics can analyze a mixture of metabolites, such as macromolecules and small molecules (Li B. et al., 2017). Technological innovation has promoted the awareness of several diseases (Wu et al., 2015, 2017a,b; Crooke et al., 2021). In general, the applications of multi-omics have provided new insights into the diagnosis, prognosis and treatment of these diseases.

To better understand the pathogenesis of viral hepatitis and related diseases, comprehensive multi-omics analysis has been widely carried out. Hence, this review summarizes the applications of multi-omics in different types of viral hepatitis and related diseases, aiming to throw light on the development of these diseases.

Multi-omics in hepatitis A and related diseases

Hepatitis A virus is a positive-strand RNA virus, which is transmitted through the fecal oral route. HAV outbreaks are often associated with poor sanitation, overcrowding or contamination of food and water (Abutaleb and Kottilil, 2020). HAV infections in children are usually asymptomatic, but adults will present symptoms with jaundice, abdominal pain and hyperbilirubinemia.

Using genomics, Wassenaar et al. (2020) compared the intraspecific genome diversity of the single-stranded RNA(+) viruses of HAV, HCV, and HEV, and they found that these viruses all can cause hepatitis, but have no genetic similarity. Heydari et al. (2021) performed whole-genome sequencing of two patients with acute hepatitis A and plotted an HAV genome-wide phylogenetic tree. Whole-genome sequencing can clearly reveal HAV sequence. After sequence alignment, the researchers can explore its origin and spread history.

To better understand the biogenesis of quasi-enveloped HAV (eHAV) virions, McKnight et al. (2017) used proteomics quantitative analysis to successfully identify surface markers for eHAV vesicles and supported exosome-like mechanisms of eHAV outflow. In the study of duck HAV genotype 3 (DHAV-3), Liang et al. (2020) collected DHAV-3-infected duck livers for proteomic analysis, and they found that type I interferon plays an extremely important role in the pathogenic mechanism of DHAV-3. Similarly, by proteomics, DHAV-1 infection was considered to cause endoplasmic reticulum stress-induced duck embryo fibroblast cell autophagy, and proteins involved in the DHAV-1 infection process or endoplasmic reticulum stress-induced autophagy process were successfully identified (Lan et al., 2019). Infection process of HAV in the hosts associated with specific proteins will be shown using proteomics.

Kanda et al. (2015) demonstrated that epigenetic control is involved in HAV internal ribosomal entry site-dependent translation and HAV replication. It was suggested that in the clinical application of epigenetic therapy for malignant tumors, special attention should also be paid to the underlying viral disease (Kanda et al., 2015).

Multi-omics in hepatitis B and related diseases

Hepatitis B virus is the most common cause of acute and chronic liver diseases worldwide, and approximately 4 million people are infected with HBV every year, especially in Asia and Africa (Khan A. et al., 2021). About 10% of patients infected with HBV will develop chronic infections, including liver fibrosis and cirrhosis. Each year, about one million people die from hepatitis B-related chronic liver diseases (Lu et al., 2018; Asrani et al., 2019). Most chronic hepatitis B (CHB) patients show no obvious symptoms, but as the disease progresses, they eventually develop liver cirrhosis and hepatocellular carcinoma (HCC) (Khan A. A. et al., 2021; Su et al., 2022). The applications of multi-omics can show the molecular and functional maps of HBV and related diseases.

Genomics

By comparing the genomes of ancient African strains and HBV, Guzmán-Solís et al. (2021) found a high degree of similarity between the two viruses, which suggested that HBV may originate on the African continent and was transported to America during the transatlantic slave trade and subsequently introduced to New Spain. Next-generation sequencing methods are used to sequence concurrently, enabling us to detect any pre-existing mutations before antiviral therapy. Hence, drug resistance mutations were detected in CHB patients receiving nucleos(t)ide analog therapy using genomics (Widasari et al., 2014). Genome comparisons can enrich the discussion of HBV origin and transmission. Besides, genomics also allows us to understand HBV genotypes, quasi-species, splicing, defective HBV, virus evolution within a single host and so on.

As the DNA virus, HBV is different from other RNA hepatitis viruses since its viral genome can be integrated into the host liver cell genome. HBV integration is considered to lead to the occurrence of HCC, and the study of its structure is of great significance to the occurrence and development of HBV-related HCC. Ramirez et al. (2021) successfully fabricated a panel of HBV-targeting biotinylated oligonucleotide probes, and they described the structure and transcriptional signatures of integrated HBV in different HCC cell lines. At the junctions between chromosomes, five chromosomal translocations integrating HBV DNA were found, and many integrations and translocations were transcriptionally silent, which further revealed the possible mediating mechanism of HBV-related HCC (Ramirez et al., 2021). To clearly describe the structure of HBV integration, Péneau et al. (2022) found that clonal selection for HBV integration may be associated with two mechanisms that lead to HCC through long-read sequencing or Bionano whole genome mapping. The first possible mechanism is that the integration of viral enhancers near the cancer driver gene may lead to overexpression of the oncogene, and the second possible mechanism is that frequent chromosomal rearrangements at the HBV integration site can cause changes in the distance of the cancer driver gene. Therefore, HBV integration is thought to have the ability to predict HBV-associated patients with HCC and has a certain clinical value (Péneau et al., 2022). In addition, the structure of HBV isolated from HCC patients was also determined, and it was found that HBV immune escape mutants may be an important factor in the occurrence and development of HCC (Lin et al., 2002). The applications of genomics in HBV-related diseases mainly focus on HBV-induced HCC. Gene expression profiling can facilitate the discovery of diagnostic and prognostic markers for HBV-related HCC.

Advances in genomics have deepened the understanding of the diagnosis, prognosis of HBV-related HCC. The detailed genetic analysis of liver tissue provides important information for tumorigenesis and progression (Dhanasekaran et al., 2019; Wu Y. et al., 2020; Wei et al., 2022). The findings of genomics research may promote the progress of individualized management of HCC, thereby innovating therapeutic methods.

Proteomics

The applications of proteomics are of great significance in hepatitis B and related diseases (**Table 1**). Proteomics can help reveal the origin and development of HBV. Krause-Kyora et al. (2018) showed that HBV has been circulating in European populations for over 7,000 years through proteomics.

The applications of proteomics provide new strategies for the occurrence, progression and replication of HBV. Based on yeast proteomics, Zeyen et al. (2020) found that hepatitis B subviral enveloped particles utilize the coat protein complex II component for intracellular transport by selectively utilizing Sec24A and Sec23B. Based on isobaric tags for relative and absolute quantitation (iTRAQ) quantitative comparative proteomics, RSK2 was identified as a novel host protein that plays a role in HBx enhancing HBV replication (Yan et al., 2018). Using the substrate capture proteomics, Murphy et al. (2016) showed that the main function of HBx is to degrade SMC5/6, which can suppress HBV replication by inhibiting HBV gene expression. Xie et al. (2011) found that HBx has a promoting effect on HBV replication, while they confirmed that cyclosporine A has an inhibitory effect on HBV replication. Pin1 is considered to be an interactor that binds to the transactivation domain of HBx, suggesting the potential relationship between Pin1 and the function of HBx in HBV replication (Zhou et al., 2021). In addition, through iTRAQ proteomic analysis, Zhao et al. (2020) found that the high expression of S100 proteins is related to the transmission of HBV in the placenta, which provides new insight into the mother-to-infant transmission of HBV.

In addition to the traditional HBV markers HBsAg and anti-HBs, the applications of proteomics have also expanded the development of diagnostic markers. Two differential proteins, VWF and C8B were considered to have the potential to distinguish HBV infection genotypes B and C and could provide precise guidance for HBV genotyping (Chen et al., 2021). The peptide YLWEWASVR derived from the hepatitis B surface antigen was confirmed as a biomarker for the diagnosis of hepatitis B virus infection (Tsai and Hsiao, 2017). Moreover, the use of proteomics/genomics databased in the identification of the HBV receptor in 2012, which is considered as one of the most important discoveries related to HBV in the last decade (Yan et al., 2012).

In CHB patients, fibronectin levels in plasma have been demonstrated to be a predictor of HBsAg clearance (Liu et al., 2019). Long-term HBV infection has been shown to lead to cellular proteome remodeling, which can mediate the pathological effect (Zai et al., 2022). Through the mass

TABLE 1 The applications of proteomics in hepatitis B and related diseases.

Authors	Diseases	Biomarkers	Role
Krause-Kyora et al., 2018	HBV infection	–	Proteomics showed the origin and development of HBV.
Zeyen et al., 2020	HBV infection	Coat protein complex II, Sec24A and Sec23B	Hepatitis B subviral enveloped particles utilize the coat protein complex II for intracellular transport by selectively utilizing Sec24A and Sec23B.
Yan et al., 2018	HBV infection	RSK2	RSK2 plays a role in HBx enhancing HBV replication.
Murphy et al., 2016	HBV infection	SMC5/6	SMC5/6 can suppress HBV replication by inhibiting HBV gene expression.
Xie et al., 2011	HBV infection	Cyclosporine A	Cyclosporine A has an inhibitory effect on HBV replication.
Zhou et al., 2021	HBV infection	Pin1	Pin1 is an interactor that binds to the transactivation domain of HBx.
Zhao et al., 2020	HBV infection	S100 proteins	High expression of S100 proteins is related to the transmission of HBV in the placenta.
Chen et al., 2021	HBV infection	VWF and C8B	VWF and C8B have the potential to distinguish HBV infection genotype B and genotype C.
Tsai and Hsiao, 2017	HBV infection	YLWEWASVR	The peptide YLWEWASVR derived from hepatitis B surface antigen was confirmed as a biomarker for the diagnosis of HBV infection.
Liu et al., 2019	CHB infection	Fibronectin	Fibronectin levels in plasma have been demonstrated to be a predictor of HBsAg clearance
McBrearty et al., 2021	CHB infection	Short-chain fatty acids	Short-chain fatty acids are able to prevent CHB from progressing to HCC.
He et al., 2016	HBV-related cirrhosis	ACY1	ACY1 autoantibodies were considered as biomarkers to differentiate HBV-related cirrhosis and CHB patients.
Dai et al., 2019	CHB with different fibrosis stages	Ficolin-2 and carboxypeptidase B2	The expression of ficolin-2 (FCN2) and carboxypeptidase B2 (CPB2) was different in CHB patients with different fibrosis stages.
Katrinli et al., 2016	Different stages of fibrosis	HIF-1α	The interaction between HBx and HIF-1α may be a novel target pathway for therapies.
Kan et al., 2017	HBV-related fibrosis	CAT, BLVRB, NXN, PRDX1 and IDH1	CAT, BLVRB, NXN, PRDX1, and IDH1 were also identified as possible drug and therapeutic targets for the detection of HBV-related fibrosis.
Ye et al., 2020	HBV-associated fibrosis	AAV shRNAs	AAV shRNAs can effectively regulate HBV-associated fibrosis by reducing oxidative stress, inflammation, and activating the PPAR signaling pathway.
Wu D. et al., 2020	HBV-ACLF	Plasminogen	Plasminogen can be used as a prognostic marker for HBV-ACLF.
Sun et al., 2019	HBV-ACLF	–	The hematological dysfunction of HBV-ACLF patients was revealed and a diagnostic and prognostic model established.
Zhou et al., 2017	HBV-ACLF	–	Six novel HBV-ACLF candidate biomarker may provide basic information for the study of HBV-ACLF biomarkers.
Wei et al., 2016	HBV-induced HCC	ARFIP2 and ANXA1	ARFIP2 and ANXA1 are potential biomarkers to differentiate HBV genotype B and C-induced HCC.
Xu et al., 2017	HBx-mediated HCC	–	HBx/CDC42/IQGAP1 signaling pathway may play an important role in HBx-mediated HCC.
Zhang et al., 2007	HBV-infected G1 tumors	Proteasome activator subunit 1 and DJ-1	Proteasome activator subunit 1 and DJ-1 were found to be downregulated in HBV-infected G1 tumors.
Lin et al., 2022	HBV-related HCC	–	An LGPI model was constructed by screening differential proteins in non-tumor liver tissue and HCC liver tissue.
Chai et al., 2021	HBV-related HCC	H2BK120ac, H3.3K18ac and H4K77ac	H2BK120ac, H3.3K18ac and H4K77ac were confirmed to be significantly associated with HBV-related HCC prognosis.
Gao et al., 2019	HBV-related HCC	PYCR2 and ADH1A	PYCR2 and ADH1A were associated with HBV-related HCC prognosis.
Li et al., 2019	HBV-related HCC	CREB1	HBx-CTTN interaction can promote HCC proliferation and migration through CREB1.

spectrometry-based proteomic analysis, McBrearty et al. (2021) found that short-chain fatty acids can prevent CHB from progressing to HCC.

In recent years, the applications of proteomics in HBV-related cirrhosis have also been reported. Autoantibodies recognized aminoacylase-1 (ACY1) were considered biomarkers

to differentiate HBV-related cirrhosis and CHB patients by serum proteomic detection (He et al., 2016). Proteomics also provides new ideas for the diagnosis, prognosis and treatment of HBV-induced fibrosis. Through serum proteomics analysis, Dai et al. found that the expression of ficolin-2 (FCN2) and carboxypeptidase B2 (CPB2) was different in CHB patients with different fibrosis stages, indicating the diagnostic value of FCN2 and CPB2 (Dai et al., 2019). At different stages of fibrosis, Katrinli et al. (2016) observed changes in the glycolytic pathway caused by the presence of HBx, so the interaction between it and HIF-1α may be a novel target pathway for therapies. Kan et al. (2017) screened 28 HBV-specific proteins by comprehensive proteomics and transcriptomics, and they emphasized the critical role of oxidative stress in HBV-related liver fibrosis. Catalase (CAT), Biliverdin Reductase B (BLVRB), Nucleoredoxin (NXN), Peroxiredoxin 1 (PRDX1), and Isocitrate Dehydrogenase [NADP(+)] 1 (IDH1) were also identified as possible drug and therapeutic targets for the detection of HBV-related fibrosis. AAV shRNAs were found to effectively regulate HBV-associated fibrosis by reducing oxidative stress, inflammation, and activating the PPAR signaling pathway (Ye et al., 2020).

Proteomics also plays an important role in HBV-related liver failure. Wu D. et al. (2020) used TMT-labeled quantitative proteomics to find that plasminogen can be used as a prognostic marker for HBV-ACLF. Sun et al. (2019) revealed the hematological dysfunction of HBV-ACLF patients through targeted proteomics and established a diagnostic and prognostic model. Quantitative proteomics analyses have identified six novel HBV-ACLF candidate biomarkers, which may provide basic information for the study of HBV-ACLF biomarkers (Zhou et al., 2017).

As HBV is a risk factor for the development of HCC, the applications of HBV-related HCC proteomics have also been paid more and more attention. Wei et al. (2016) concluded that ARFIP2 and ANXA1 are potential biomarkers to differentiate HBV genotype B and C-induced HCC through quantitative proteomic analysis. With the altered protein expression during the progression of HBV-related HCC, some proteins can be considered potential biomarkers for diagnosis and therapy (Jiang et al., 2019). Through the quantitative proteomics, Xu et al. (2017) found that the HBx/CDC42/IQGAP1 signaling pathway may play an important role in HBx-mediated HCC. Similarly, proteasome activator subunit 1 and DJ-1 were found to be downregulated in HBV-infected G1 tumors, revealing their possible mediating mechanisms (Zhang et al., 2007). Lin et al. (2022) constructed an LGPI model by screening differential proteins in non-tumor liver tissue and HCC liver tissue, predicting the overall survival and prognosis of patients with HBV-related HCC. By quantitative proteomics, H2BK120ac, H3.3K18ac and H4K77ac were confirmed to be significantly associated with HBV-related HCC prognosis (Chai et al., 2021). Gao et al. (2019) screened out two HCC metabolic reprogram prognostic markers associated with HBV-related HCC, PYCR2, and ADH1A. HBx-CTTN interaction can promote HCC proliferation and migration through CREB1, and the HBx/CTTN/CREB1 axis was considered a potential new therapeutic target for HCC (Li et al., 2019).

Metabolomics

Through the differential metabolomic analysis, Luteolin-7-*O*-glucoside was confirmed to inhibit HBsAg and HBV replication through mechanisms involving mitochondria (Cui et al., 2017). Yu et al. (2022) focused on metabolic changes during HBV replication and infection, and they found that the high levels of amino acids depletion and phosphatidylcholines and lysophosphatidylcholines biosynthesis play important roles in the pathogenesis of HBV infection. Through the metabolomic analysis, Hu et al. (2021) found that HBP-mediated *O*-GlcNAcylation can positively regulate the host's antiviral response to HBV.

Combined with serum-targeted metabolomics, the metabolic signature of CHB infection progression was further revealed (Schoeman et al., 2016). By analyzing metabolomics data at different stages in patients with CHB, Nguyen et al. (2021) found that ammonia detoxification, glutamine and glutamate metabolism, methionine metabolism, branched-chain amino acid imbalance, and disorders of the tricarboxylic acid cycle are influencing factors in the progression of patients with CHB. Combined with the gut microbiome and metabolome, Sun et al. (2021) provided new insights into bile acid metabolic pathways in patients with CHB.

Through serum metabolomics, Nie et al. (2014) found that 17 metabolites were associated with the prognosis of HBV-related acute-on-chronic liver failure (HBV-ACLF), providing information for markers for the diagnosis and prognosis of HBV-ACLF. Lian et al. (2016) analyzed serum samples from HBV-ACLF, HBV-related chronic liver failure (HBV-CLF) and healthy populations, and they found that phosphatidylcholines, lysophosphatidylcholines and conjugated bile acids (GCDCA, GUDCA) metabolites may act as markers for ACLF and CLF diagnosis and provide new insights into the pathogenesis of ACLF and CLF.

Through metabolomics, amino acid imbalance metabolism was thought to play an important role in the development and progression of HBV-related HCC (Huang et al., 2020). Compared with HBV-infected patients, HBV-related HCC patients have lower levels of metabolite lysophosphatidylcholines in their blood, which may serve as a clinical diagnostic marker for HCC (Li et al., 2021). Through serum metabolomics, Cai et al. (2020) discovered enzymes associated with HBV-related HCC diagnosis and prognosis. Through the genetic screening, combined with RNA-seq and metabolomic analysis, Chen et al. (2022) found

the joint effect of PSTK as a resistance medium for targeted therapy of HCC cells, suggesting an ideal treatment method for HBV-related HCC.

Transcriptomics

Transcriptomics functions in the study of HBV and related diseases. Using RNA-seq transcriptomics, König et al. (2019) demonstrated that HBV does not induce significant gene expression changes in HepG2-NTCPsec+ and that HepG2-NTCPsec + cells support a net amplification of the HBV genome, leading to the development of a new model of HBV infection. Hou et al. (2017) conducted an in-depth transfer group analysis of formalin-fixed paraffin-embedding liver biopsy in the clinical stage. They found that viral load and liver injury are associated with the fluctuations that coincided with those of the liver transcriptome (Hou et al., 2017). Using transcriptomic and proteomic methods, the RIG-I-like receptor signaling pathway was confirmed to be the main signaling pathway for changes in HBV-related fibrosis (Kan et al., 2017). In addition, Li et al. (2022) found that HBV exacerbation-induced immune dysregulation disorder is the underlying mechanism identified in HBV-ACLF through mRNA sequencing of peripheral blood mononuclear cells in patients.

Multi-omics in hepatitis C and related diseases

Hepatitis C virus virions are spherical and are single-stranded positive-stranded RNA viruses. HCV can often cause hepatitis C infection. According to estimates, approximately 71 million people worldwide suffer from chronic hepatitis C virus (CHC) infection (Rabaan et al., 2020). CHC infection is associated with advanced liver disease and can induce hepatocellular carcinoma, which will cause many extrahepatic manifestations. The applications of multi-omics also help researchers understand HCV and related diseases deeply.

Genomics

Through functional genomics, Li et al. (2016) found that *E*-cadherin is a mediator of HCV entry into host cells and is closely related to HCV-induced epithelial-mesenchymal transition. Takagi et al. (2021) revealed the sequence of HCV-G4-KM long clones by sequencing the HCV-G4-KM long clones in mouse serum, and they proved that the sequence of HCV-G4-KM long NAs plays an important role in infectious cloning. By combining proteomics and genomics, Ramage et al. (2015) demonstrated the role of HCV between the infectious process and the host, and they explored the mechanism by which HCV affects the function of the infected host. In addition, functional genomics has been used to explore the interaction between HCV and miRNA and also demonstrated the HCV-mediated pathogenesis (Li Q. et al., 2017). Genomics can reveal the process of HCV entering and infecting host cells.

Proteomics

Proteomics matters in hepatitis C and related diseases (**Table 2**). Proteomics can show the process of HCV assembly, host cell entry and replication. Kumar et al. (2019) reported that MARCH8 catalyzes polyubiquitination of K63-linked HCV non-structural 2 proteins, followed by ESCRT recruitment and HCV envelope. Proteomics of HCV virions determined an essential role for the nucleoporin Nup98 in virus morphogenesis (Lussignol et al., 2016). HCV was confirmed to enter hepatocytes through the CD81 receptor complex calpain-5 and CBLB (Bruening et al., 2018). Gerold et al. (2015) identified serum response factor binding protein 1 by quantitative proteomics, which can be recruited to CD81 during HCV uptake and support HCV infection in HCC cells and primary human hepatocytes. Borawski et al. (2009) demonstrated that both class III phosphatidylinositol 4-kinases α and β are novel host factor regulators of HCV replication. In addition, HCV was reported to induce lipid rafts to localize to autophagosomes, thereby mediating HCV RNA replication (Kim et al., 2017).

Gangadharan et al. (2012) identified 20 novel biomarkers of HCV-associated liver fibrosis to assess the degree of liver fibrosis. Cheung et al. (2010) found that G3BP can be used as a marker for HCV-related liver fibrosis and cirrhosis. Through the serum proteomics, C4-A and inter-α-trypsin inhibitor heavy chain H4 were screened to predict HCV-related liver fibrosis (Yang et al., 2011). The development of biomarkers for HCV-associated fibrosis and cirrhosis were also explored using genomics.

Diagnostic and prognostic biomarkers of HCV-related HCC can be discovered by proteomics. Lee et al. (2006) identified complement C3a as a candidate biomarker for HCV-related HCC by proteomics. Malov et al. (2021) confirmed that heparin binds to growth factors, glypican-3 and osteopontin could serve as HCV-associated HCC markers. The screening of specific proteins is conducive to accurate diagnosis and prognosis in patients with HCV infection and related diseases.

Metabolomics

Through the comprehensive metabolomics analysis, Fitian et al. (2014) identified overall metabolic disorders in patients with HCV-associated HCC and cirrhosis, and they hypothesized that abnormal dicarboxylic acid metabolism, enhanced bile

TABLE 2 The applications of proteomics in hepatitis C and related diseases.

Authors	Diseases	Biomarkers	Role
Kumar et al., 2019	HCV infection	MARCH8	MARCH8 catalyzes polyubiquitination of K63-linked HCV non-structural 2 proteins, followed by ESCRT recruitment and HCV envelope.
Lussignol et al., 2016	HCV infection	Nup98	Proteomics of HCV virions determined an essential role for the nucleoporin Nup98 in virus morphogenesis.
Bruening et al., 2018	HCV infection	CD81 receptor complex calpain-5 and CBLB	HCV was confirmed to enter hepatocytes through the CD81 receptor complex calpain-5 and CBLB.
Gerold et al., 2015	HCV infection	Serum response factor binding protein 1	Serum response factor binding protein 1 can be recruited to CD81 during HCV uptake.
Borawski et al., 2009	HCV infection	Class III phosphatidylinositol 4-kinases α and β	Both class III phosphatidylinositol 4-kinases α and β are novel host factor regulators of HCV replication.
Kim et al., 2017	HCV infection	–	HCV was reported to induce lipid rafts to localize to autophagosomes, thereby mediating HCV RNA replication.
Gangadharan et al., 2012	HCV-associated liver fibrosis	–	20 novel biomarkers of HCV-associated liver fibrosis were identified to assess the degree of liver fibrosis.
Cheung et al., 2010	HCV-related liver fibrosis and cirrhosis	G3BP	G3BP can be used as a marker for HCV-related liver fibrosis and cirrhosis.
Yang et al., 2011	HCV-related liver fibrosis	C4-A and inter-α-trypsin inhibitor heavy chain H4	C4-A and inter-α-trypsin inhibitor heavy chain H4 were screened to predict HCV-related liver fibrosis.
Lee et al., 2006	HCV-related HCC	C3a	Complement C3a as a candidate biomarker for HCV-related HCC.
Malov et al., 2021	HCV-related HCC	Glypican-3 and osteopontin	Glypican-3 and osteopontin, could serve as HCV-associated HCC markers.

acid metabolism, and elevated fibrinogen-cleaved peptides might be the signs of liver cirrhosis. Shanmuganathan et al. (2021) compared the ability of multisegment injection-capillary electrophoresis-mass spectrometry and nuclear magnetic resonance to characterize the serum metabolome, and they found that both instrumental techniques can quickly and reliably quantify serum metabolites in large-scale metabolomics research with the good overlap of biomarker replication. Therefore, metabolomics can show the metabolic process of HCV infection and its associated diseases.

Transcriptomics

Through the genome-wide miRNA functional screening and transcriptomic analysis, Sodroski et al. (2019) generated a comprehensive map of HCV-miRNA interactions. They found that inhibition of key host restriction factors mediates the proviral effects of miR-135a on HCV transmission (Sodroski et al., 2019). Through the comprehensive functional genomics analysis, miR-25, let-7, and miR-130 families were also proved to inhibit the necessary HCV cofactors, thus limiting HCV infection in multiple stages (Li Q. et al., 2017). Hence, cellular microRNAs have been shown to regulate HCV infection by acting directly on the viral genome or indirectly on virus-associated host factors.

Multi-omics in hepatitis D and related diseases

Hepatitis D virus is only found in humans currently. It is a satellite virus, which is assembled, released and entered by the envelope protein of HBV. It is the smallest known RNA virus, encoding a single protein.

Using meta-transcriptomics, Wille et al. (2018) identified the genome of a novel HDV in duck. Sequence analysis showed that HDVs share a common secondary structure. The predicted viral protein shares a 32% amino acid similarity with the small delta antigen of HDV, which contains a distinct phylogenetic lineage. The discovery of avian influenza virus-like pathogens helps us better understand the origin of HDV and subviral pathogens (Wille et al., 2018). Taking meta-transcriptomic data, Chang et al. (2019) found that highly differentiated HDV-like viruses also exist in fish, amphibians and invertebrates. None of these novel HDV-like infections is associated with other hepatitis virus infections, supporting the idea that the HDV-HBV association may be unique to humans

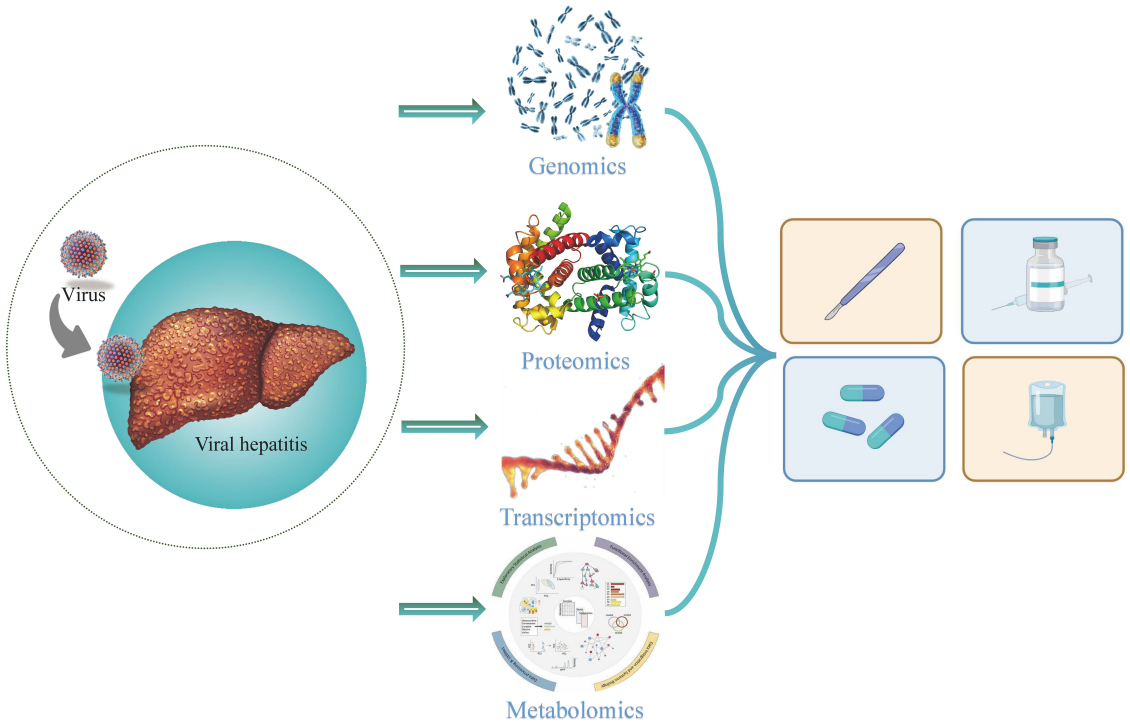

FIGURE 1
Multi-omics in viral hepatitis.

(Chang et al., 2019). To summary, transcriptomics can reveal the diversity and host range of HDV, and also indicate the origin and evolutionary history of HDV.

Multi-omics in hepatitis E and related diseases

Hepatitis E virus (HEV) is an important zoonotic virus that can infect various hosts. It has 7 main genotypes. Patients with HEV infection are mostly asymptomatic, some patients will present jaundice and symptoms of acute hepatitis (Desai, 2020). Besides, HEV infection can also cause many extrahepatic manifestations (European Association for the Study of the Liver, 2018; Wu et al., 2021).

The study by Shen et al. (2014) employed a comparative gel proteomics approach to investigate the changes in A549 cell proteins following *in vitro* HEV exposure, which was beneficial for the study of the interaction between HEV and host cells. Three different strains of porcine HEV were identified by Rogée et al. (2015). They revealed the process by which HEV damages cells, providing important evidence for the replication factors and related pathogenesis of HEV (Rogée et al., 2015). Through serum metabolomics, it was demonstrated that dynamic changes in serum metabolites were associated with AHE infection and severity (Wu et al., 2022b). Through the meta-transcriptomic, Zhang et al. (2019) determined the HEV virus subtypes in broilers and further proved by the phylogenetic analysis that the avian HEV identified in the study is a novel subtype of genotype 3 avian HEV. Thus, transcriptomics provides complete genomic data on the evolutionary relationships of avian HEV, which helps us further understand the evolution of HEV. Besides, Wu et al. (2022a) performed the 16S ribosomal ribonucleic acid gene sequencing, and they found that gut microbiota dysbiosis is associated with plasma levels of Interferon-γ and viral load in patients with acute hepatitis E infection. Overall, the investigation on HEV infection and related diseases using multi-omics are less, which requires more efforts.

Conclusion and perspectives

In conclusion, the applications of multi-omics have shown the origin and development of the hepatitis virus and provided new strategies for the diagnosis, prognosis and treatment of viral hepatitis and related diseases (**Figure 1**). There are many multi-omics studies on HBV infection, HCV infection and related diseases, several biomarkers were found and more correlations were revealed. Nevertheless, the accuracy of these screened biomarkers for the diagnosis and prognosis in patients still needs to be discussed. Moreover, multi-omics studies on other hepatitis are inadequate, and more efforts should be made.

Besides, multi-omics applications are not limited to genomics, proteomics, metabolomics and transcriptomics, and other omics are also developing, including radiomics, viromics, and so on. The joint application of these omics is believed to provide new insight into viral hepatitis and related diseases.

Author contributions

ZX and JL had the idea for the manuscript. ZX and DL performed the literature search and data analysis. XX and XW drafted and critically revised the work. All authors contributed to the article and approved the submitted version.

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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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Current treatment of chronic hepatitis B: Clinical aspects and future directions

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Hepatitis B virus (HBV) infection is a public health threat worldwide, and there is no direct treatment yet available. In the event of infection, patients may present liver cirrhosis and cancer, which threaten the patients' health globally, especially in the Asia-Pacific region and China. In 2019, Chinese hepatopathologists updated the 2015 Guidelines for the Prevention and Treatment of Chronic Hepatitis B as the clinical reference. The other versions formulated by the American Association for the Study of Liver Diseases (2018 AASLD guidelines) (AASLD, 2018), European Association for the Study of the Liver (2017 EASL guidelines) (EASL, 2017), and Asian-Pacific Association for the Study of the Liver (2015 APASL guidelines) (APASL, 2015) also provide clinical guidance. However, there are still some issues that need to be addressed. In the present study, the following aspects will be introduced successively: (1) Who should be treated in the general population according to the guidelines; (2) Treatment of specific populations infected with HBV; (3) Controversial issues in clinical practice; (4) Perspective.

KEYWORDS

HBV infection, ALT, treatment, immune tolerant, withdrawal, retreatment

Introduction

According to the World Health Organization (WHO) statistics, approximately 0.257 billion people are infected with the Hepatitis B virus (HBV) worldwide, with around 0.88 million deaths annually (Trépo et al., 2014; Valery et al., 2018), which causes a huge medical and economic burden. Among 70 million HBV-infected patients in China, 20–30 million people had CHB (chronic hepatitis B) (Liu et al., 2019). Viral hepatitis is expected to be eliminated by 2030. Therefore, significant efforts have been made worldwide to provide practical and standardized guidelines for preventing, diagnosing, and treating HBV infection. However, not all patients requiring treatment are within the guidelines of treatment criteria; thus, it is essential for all the candidates from the HBV-infected population.

Candidates in the general population

Antiviral treatment is an effective therapeutic strategy for CHB patients that efficiently suppresses HBV replication, decreases inflammatory necrosis in the liver, reduces the incidence of liver cirrhosis and related complications, and reduces the fatality rate associated with hepatocellular carcinoma (HCC) and other liver diseases. In the 2019 China guidelines (Chinese Society of Infectious Diseases, 2019), HBV infection is divided into four phases: immune tolerance, immune clearance, immune control, and immune reactivity,and it is different from the 2015 version (Hou and Lai, 2015; **Table 1**). Additionally, the 2019 China guidelines eased the restrictions on indications for antiviral therapy, and reducing the demand for HBV-DNA load. Conversely, the HBV-DNA load is considered for the performance of antiviral therapy in the 2018 guidelines (Terrault et al., 2018) updated by the 2018 AASLD guideline and the 2017 EASL guidelines (**Table 2**). For the treatment of HBV infection with normal ALT (alanine aminotransferase), antiviral therapy is recommended in patients > 30-years-old with a family history of liver cirrhosis or cancer in the 2019 China guidelines. In another case >30-years-old without a family history of liver cirrhosis or cancer, a hepatic biopsy was recommended. Although we can refer to many guidelines, there are many patients failed to fulfill the criteria for treatment at follow-up and eventually developed liver fibrosis, cirrhosis, and cancer (Wang et al., 2013; Alam et al., 2014).

Patients with a normal level of alanine aminotransferase

Hepatitis B virus infection is a dynamic process characterized by fluctuations in alanine ALT, which might hint toward immune-mediated virus clearance (Ghany et al., 2020). Since the ALT level is not always indicative of inflammation in the liver, patients with normal ALT levels can present inflammation and fibrosis on liver biopsy. Thus, ALT is used as a substitute for liver inflammation when liver histology is a failure (Wu et al., 2019, 2020; Fang et al., 2022). But the challenge in defining the ULN (upper limits of normal) of ALT is the difficulty of including totally healthy subjects without liver diseases, especially MAFLD (Metabolic-Associated Fatty Liver Disease), the leading cause of liver disease worldwide (Tampi et al., 2020). An Italy study reveals that Male sex, body mass index, glucose, lipids, ferritin, hypertension, and younger age are independent predictors of ALT (Valenti et al., 2021). Many hepatologists call for the adjustment of the ULN of ALT (Park et al., 2008; Chen et al., 2010). In 2019 China guidelines, the ULN remains constant at 50 U/L in males and 40 U/L in females; however, many studies have recommended rational values as 35 U/L in males and 23 U/L in females (Harbour and Miller, 2001; Zheng et al., 2012; Shang et al., 2013). In 2018 AASLD guidelines, the ALT ULN is modified as 35 U/L in males and 25 U/L in females, as described previously (Lee et al., 2010; Ruhl and Everhart, 2012; Terrault et al., 2018). In 2017 EASL guidelines and 2015 APASL guidelines, the ALT ULN is 40 U/L in both males and females (Ruhl and Everhart, 2012; Sarin et al., 2016; European Association for the Study of the Liver, 2017). Therefore, whether patients have normal ALT levels partially depends on the ULN. The ULN values mentioned in this study are consistent with those in the literature.

Hepatitis B virus-infected patients with normal alanine aminotransferase

Patients infected with HBV but have normal ALT levels may exhibit manifestations of liver fibrosis and inflammation upon histological examination (Chang et al., 2021; Liu et al., 2022). Previous studies have shown that people with normal ALT levels develop moderate liver tissue inflammation (16.8–40%) and moderate liver fibrosis (24.2–35.9%) (Ormeci et al., 2016; Tan et al., 2017; Choi et al., 2019; Liu et al., 2022), which are associated with a high risk of progression to liver cirrhosis and cancer. In a retrospective study consisting of 327 HBV DNA + CHB patients who underwent liver biopsy, significant differences were detected between high-normal ALT (20 U/L < ALT < ULN) and low-normal ALT (ALT ≤ 20 U/L) groups in liver inflammation and fibrosis ($P < 0.01$). The rate of significant liver tissue inflammation is 44.6 and 26.5% corresponding to 20 U/L < ALT < ULN and ALT ≤ 20 U/L, respectively (Duan et al., 2021). Another study (Choi et al., 2019) demonstrated that patients with normal ALT have a significantly decreased risk of long-term liver cancer and required liver transplantation after antiviral treatment compared to those without treatment. Therefore, in cases with normal ALT levels, a comprehensive examination is essential, especially in the condition of liver fibrosis and inflammation, to provide antiviral treatment in a time-dependent manner.

Immune tolerant chronic hepatitis B

Immune tolerance is a status presenting HBeAg+, high viral replication, and normal ALT. It was put forward as a lack of specific immune response to HBV due to the immature immune system during infancy. No treatment is recommended for such cases in the current guidelines, as it is speculated that patients in the immune tolerance phase have a low risk of progression to liver cirrhosis or cancer (Cornberg et al., 2019; Lee et al., 2020). Moreover, ineffective treatment may lead to a low e-antigen seroconversion rate (Wu et al., 2010; Jeng and Lok, 2021). Patients with immune tolerance are mostly teenagers and

TABLE 1 Comparison between 2015 (China) and 2019 (China) guidelines.

Guidelines	ALT level or Liver inflammation/fibrosis degree	Other	Timing of assessment for liver inflammation and fibrosis (liver biopsy or non-invasive examination)
2015	Continuous ALT > 2 ULN, otherwise, ≥Grade 2 liver inflammation or fibrosis	Liver cirrhosis (regardless of ALT/HBeAg), aggressive anti-viral treatment	Normal ALT, age > 30 years, a family history of liver cirrhosis or liver cancer; ALT = 1–2 ULN, age > 30 years
2019	Continuous ALT > 1 ULN, excluding other causes; Normal ALT, ≥Grade 2 liver inflammation and (or) fibrosis	Liver cirrhosis decompensation, HBV DNA+, anti-viral treatment; liver cirrhosis decompensation, HBsAg+, anti-viral treatment; age > 30 years, a family history of liver cirrhosis or HBV-related liver cancer, anti-viral treatment; HBV-related extrahepatic manifestation	Normal ALT, age > 30 years

TABLE 2 Indications for chronic hepatitis B (CHB) treatment in 2017 (EASL) and 2018 (AASLD) guidelines.

Guidelines	HBeAg+		HBeAg-	
	HBV DNA level	ALT	HBV DNA level	ALT
2017 (EASL) (ULN 40 U/L)	≥2,000	>ULN and (or) at least moderate liver necroinflammation or fibrosis	≥2,000	>ULN and (or) at least moderate liver necroinflammation or fibrosis
	≥20,000	>2 ULN, regardless of the degree of fibrosis	≥20,000	>2 ULN, regardless of the degree of fibrosis
2018 (AASLD) (Male, ULN 35 U/L; Female, ULN 25 U/L)	≥20,000	>2 ULN, or significant liver histologic transformation	≥2,000	>2 ULN, or significant liver histologic transformation

readily develop drug resistance because of poor compliance after long-term antiviral treatment (Dolman et al., 2018).

Definitions of IT-CHB differ among guidelines. In 2018 AASLD guidelines, IT-CHB is defined by HBV DNA > 10^6 IU/mL and normal ALT, while in 2017 and 2019 China guidelines, IT-CHB is defined by HBV DNA > 10^7 IU/mL and normal ALT. However, the ULN of ALT remains controversial with limited value in distinguishing patients experiencing immune tolerance, as the patients are still at risk of liver inflammation or fibrosis when their ALT levels are defined as normal under the lowest ULN standard (Ormeci et al., 2016; Tan et al., 2017; Choi et al., 2019; Chang et al., 2021; Duan et al., 2021; Liu et al., 2022). Hong et al. (2015) speculated that innate immune cell maturation and helper T lymphocyte development existed before intrauterine exposure to HBV during infancy, while another study discovered HBV-specific T lymphocytes in that period (Bertoletti and Ferrari, 2016). Presumably, the immune tolerance phase refers to a status of mild immune response that is difficult to be recognized, and thus, an accurate diagnosis was challenging. A previous study also found the presence of HBV-DNA integration into the host chromosome and the clonal expansion of stem cells in cases of IT-CHB (Mason et al., 2016). A high level of HBV replication is regarded as an independent risk factor for disease progression to cirrhosis and HCC. Additionally, some experts speculated that liver cirrhosis or cancer progresses dynamically and continuously (Kennedy et al., 2017; Sun et al., 2018). Given the fact that the age of CHB onset is mainly after 30 years, the disease progresses for many years before the onset. Furthermore, there is a decreased transmission in immunotolerant patients bearing a high viral load after antiviral treatment. In all guidelines, no treatment is recommended for immunotolerant patients; instead, antiviral therapy is clarified as alleviating liver tissue inflammation and fibrosis and delaying and decreasing the risk of cirrhosis, relevant complications, and cancer. In patients with IT-CHB, aggressive antiviral treatment is beneficial in lowering the risk of liver cirrhosis and cancer. Whether IT-CHB patient needs treatment requires both individualized and comprehensive assessment implicating multiple factors, such as patient age, willingness to receive treatment, risk of disease progression, virus genotype (Yang et al., 2008; Wong et al., 2013), family history, and lifestyle.

Treatment of specific populations infected with hepatitis B virus

As mentioned above, a family history of HBV-related cirrhosis or HCC is a major factor for treatment. Typically, when people develop cirrhosis or HCC, treatment should be positive,

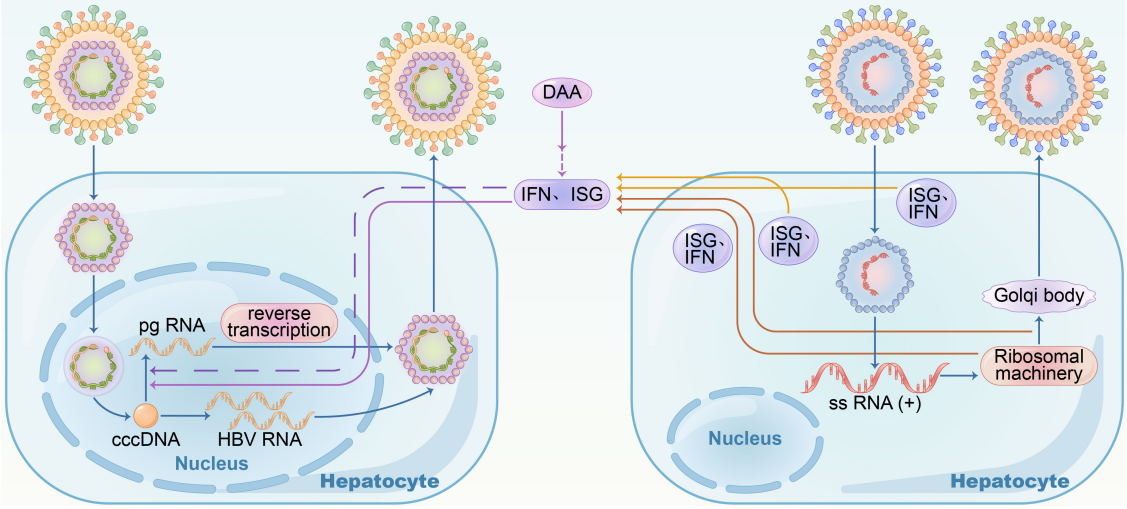

FIGURE 1
Hepatitis B virus– hepatitis C virus (HBV–HCV) coinfection. In HBV-HCV coinfection, HBV replication was suppressed by interferon response resulting from HCV replication; when treated with DAA, HBV RNA increased, and there was no direct virus-virus interference.

which has been described elsewhere. The following groups are highlighted in this review.

Coinfection

Hepatitis B virus, human immunodeficiency virus (HIV), and hepatitis C virus (HCV) share the same modes of transmission, and hence, superinfection is rather common. HBV coinfected with HCV is likely to progress to cirrhosis, chronic and fulminant hepatitis, and HCC in the liver (Squadrito et al., 2013; Pol et al., 2017). All HBsAg-positive patients should be screened for anti-HCV, especially those at a high risk of infection, such as drug injections and male homosexuality. In coexisting HBV and HCV cases, HBV replication differs from HBV mono-infection and is prevented by IFN (interferon) response from HCV replication compared to direct interactions between viruses (Cheng et al., 2020; Shih and Liu, 2020). In HBV-HCV superinfection, the presence of HBV infection does not affect HCV replication. Cheng et al. reported that ISG (Interferon-stimulated gene) expression mediated by HCV infection induced HBV suppression; on the contrary, both HBV and HCV viruses showed high levels of replication when ISG expression was blocked. Specifically, CXCL10 may be a marker of significant IFN response in HBV-HCV coinfection and HCV clearance. In clinical practice, CHB patients who are RNA-positive for HCV should be treated with DAA (direct-acting antiviral) plus anti-HBV therapy, according to almost guidelines (**Figure 1**). Moreover, monitoring serum HBsAg and HBV DNA levels monthly during the process in patients who receive DAA with HBsAg-negative and hepatitis virus core antigen-positive (anti-HBc+) is essential in case of HBV reactivation.

In HIV-HBV coinfection cases, early initiation of ARVT is recommended, irrespective of the CD4 count. According to the 2019 China guidelines, the therapy included two types of anti-HBV drugs to avoid HIV resistance to NAs (Chinese Society of Infectious Diseases, 2019).

Patients who receive immunosuppressive or cytotoxic therapy

While receiving immunosuppressive or cytotoxic therapy, patients with HBsAg-positive, anti-HBc-positive or HBsAg-negative, and anti-HBc-positive have the possibility of HBV reactivation. Thus, it is necessary to screen for HBsAg before therapy. In the case of HBsAg-positive, patients should receive anti-HBV prophylaxis before immunosuppression or cytotoxic therapy or consecutively. If HBsAg-negative, HBcAb+ patients undergoing anti-CD20 antibody therapy (for example, rituximab) or stem cell transplantation, should accept anti-HBV prophylaxis for at least 18 months (12 months suggested in 2018 AASLD) after immunosuppressive therapy, as the rate of HBV reactivation is high, according to 2019 Chinese guidelines.

Pregnancy-related situations

Screening for HBsAg in childbearing age women planning a pregnancy and pregnant women is strongly recommended. When they fulfill the treatment criteria, antiviral prophylaxis with tenofovir disoproxil fumarate (TDF) is a preferred agent.

NAs used for the prevention of HBV perinatal transmission in a pregnant woman at weeks 24–28 of gestation with normal ALT but positive HBeAg and high levels of viremia (HBVDNA $> 2 \times 10^5$ IU/mL) can be stopped immediately or within 3 months after delivery under timely monitoring (Chinese Society of Infectious Diseases, 2019; Kumar et al., 2022).

Organ transplantation

Solid organ transplant recipients are susceptible to HBV. Donor and recipient HBV infection status and perioperative management are the main determinants of susceptibility. Antiviral therapy and HBIG might reduce reactivation or reinfection after transplantation (Chinese Society of Organ Transplantation and Chinese Medical Association, 2019).

Treatment of chronic hepatitis B in children

Vaccination against HBV among young Chinese individuals decreased the HBV infection rate. In recent years, several studies reported gradually increasing the horizontal transmission of HBV in early childhood, and family members may be the main source of infection. Interferons and NAs are the potential treatment options according to the age of young people (Pan et al., 2020).

Controversial issues in clinical practice

Nucleo(t)ide analogs withdrawal and retreatment

Oral nucleo(t)ide analogs (NAs) used in first-line treatment, such as TDF, entecavir (ETV), and tenofovir alafenamide fumarate (TAF), have strong anti-virus effects, fewer side effects, convenience, and low resistance. However, long-term duration, especially >10 years of administration, requires intensive focus on drug safety and may result in reduced compliance in patients (Ford et al., 2018; Shin et al., 2018). Drug withdrawal can be considered in HBeAg+ patients after e-antigen seroconversion and consolidation therapy, as recommended by guidelines (2019 China, 2018 AASLD, 2017 EASL). Conversely, for HBeAg- patients, the 2019 China guidelines recommended drug withdrawal upon serum HBsAg disappearance and HBV DNA below the limit of detection, and the 2018 AASLD guidelines recommended at least 2 years of viral inhibition and consolidation therapy, while the 2017 EASL guidelines recommended a minimal 3-year viral inhibition. The 2015 APASL guidelines recommended drug withdrawal upon serum HBsAg disappearance, followed by 12-month consolidation therapy or undetectable HBV DNA and a minimum of 2 years of treatment.

Whether antiviral treatment can be discontinued in HBeAg- patients is yet controversial. Clinical practice has identified a potential risk of recurrence, decompensation of liver cirrhosis, liver failure, and death after drug withdrawal. The recurrence after drug withdrawal can be defined as a VR (virological relapse): viral rebound plus HBV DNA > 2,000 IU/mL; CR (clinical relapse): VR plus ALT $> 2 \times$ ULN; hepatitis flare: VR plus ALT $> 5 \times$ ULN. Due to the putative consequences, physicians face enormous pressure to discontinue the drugs in HBeAg- patients, while in recent years, the safety of the management has been promoted. For example, in a cohort study involving 691 CHB patients (including 308 patients with liver cirrhosis) (Jeng et al., 2018), 3-year follow-up witnessed CR in 419 (61%) patients, hepatitis flare in 280 (41%) patients, total bilirubin > 2 mg/dL in 72 (10%) patients, PT (prolonged anti-pertussis toxin), and INR (International Normalized Ratio) > 1.5 in 16 (2%) patients. The incidence of decompensated liver cirrhosis was reported as 0.28% at 155-week mean follow-up duration, including 0% in CHB patients and 2.85% in liver cirrhosis patients. In a randomized controlled trial of 67 patients (2:1 to stop or continue NA therapy) over 72 weeks, 21% of patients developed an ALT $> 10 \times$ ULN, and another 10% had ALT $> 5 \times$ ULN; most patients relapsed after discontinuation, but neither hepatic decompensation nor mortality occurred (Liem et al., 2019). Some studies indicated that drug withdrawal is feasible and safe in HBeAg- CHB patients (Berg et al., 2017; Liaw, 2019), and other studies put forth the positive clinical value of drug withdrawal for HBsAg clearance (Chen et al., 2018; Papatheodoridis et al., 2018). For instance, the small-scale, randomized controlled trial by Berg et al. (2017) observed a 19% HBsAg clearance rate during the 3-year follow-up period in CHB patients after discontinuation of TDF-based antiviral therapy, while this rate in patients persistently receiving TDF was 0%. This finding was consistent with the study by Jeng et al. (2018), wherein benefits of HBsAg clearance without retreatment were observed after drug withdrawal. In the study of Jeng et al., 42 patients were free from HBsAg seroclearance during a median off-therapy follow-up of 155 weeks. Approximately 1.78% of the annual incidence is attributed to the 6-year cumulative incidence. Clinically relapsed patients who did not receive treatment had a 7.34-fold higher incidence of HBsAg clearance than those who received treatment. Thus, it may be possible to achieve a functional cure after an untreated clinical relapse that triggers sufficient immune control. In a prospective study (Rinker et al., 2018), 15 HBeAg-negative CHB patients on long-term NA treatment underwent NA discontinuation. After ceasing HBV therapy, a relapse of active HBV replication might trigger an immunological environment that influences T cell phenotypes and increases HBV-specific T cell responsiveness

in vitro after ceasing HBV therapy. Additionally, blocking PD-L1 (programmed cell death protein 1) may further strengthen these T cell responses to HBV.

In 2021, APASL introduced Guidance on Stopping Nucleo(t)ide Analogs in Chronic Hepatitis B Patients (Kao et al., 2021), wherein NA withdrawal is recommended in HBeAg+ patients experiencing e-antigen seroconversion and consecutive 3-year treatment and in HBeAg- patients who have had undetectable HBV DNA and received treatment for at least 3 years. Accumulating evidence proposed that a finite course of antiviral therapy is a more viable option than pursuing HBsAg disappearance in HBeAg- patients. Nevertheless, these findings need to be substantiated further. In addition to HBsAg clearance, timely monitoring and examination for HBV DNA, ALT, and PT are necessary to actively assess the risk for recurrence and disease progression. Low HBsAg level after drug withdrawal (especially <100–150 U/mL), low baseline HBV DNA load, and prolonged antiviral therapy are recognized as low-risk factors for disease recurrence. For the past few years, several efforts have been made to identify new predictive factors for recurrence. Reportedly, low HBcAg and HBV RNA levels after drug withdrawal are predictive of a low risk of disease recurrence (Carey et al., 2020; Sonneveld et al., 2022). Fan et al. (2020) demonstrated that after 4 years of NA withdrawal, HBV RNA + patients were more likely to have CR and VR than those with HBV RNA below the limit of detection at the time of withdrawal. Moreover, HBcAb (<100 U/mL), host factors, and HBV genotype were also associated with CHB recurrence (Chen et al., 2018; Rivino et al., 2018; Tseng et al., 2018; Kuo et al., 2019). Currently, there is no guidance and consensus for retreatment of recurrence after drug withdrawal. Berg et al. (2017) suggested that patients who experienced recurrence after drug withdrawal and conformed to one of the following five criteria were recommended to undergo retreatment: (1) Two consecutive total bilirubin and ALT > ULN; (2) ALT > 10 × ULN; (3) 2 × ULN < ALT ≤ 5 × ULN for minimum 12 consecutive weeks; (4) 5 × ULN < ALT ≤ 10 × ULN for minimum four consecutive weeks; (5) 2 s (s) extension of PT, which cannot be corrected by vitamin K supply, and increased ALT. In clinical practice, the risk of disease progression should be highlighted before retreatment of recurrence after drug withdrawal to decrease the incidence of decompensated liver cirrhosis, liver failure, and death. Also, defining the potential predictors and retreatment criteria in order to optimize the benefits of HBsAg loss and minimize the adverse effects of severe hepatitis flare after stopping NA therapy is imperative.

Low level viremia

In the 2019 China guidelines, with the exclusion of patient compliance and test errors, poor therapeutic response is defined by HBV DNA > 10^3 IU/mL after 48-week oral first-line antiviral treatment in CHB patients without liver cirrhosis, while HBV DNA > 10^3 IU/mL after 24-week administration of first-line antiviral agents in CHB patients with liver cirrhosis. Drug regimens were adjusted according to the previous administrations and drug resistance (**Table 3**). Due to the advancement in the testing methods, HBV DNA < 10^3 IU/mL, even within 20–100 IU/mL, can be readily achieved in clinical practice. LLV first received attention in the 2018 AASLD guidelines. Some researchers recommended that patients with HBV DNA < 2 × 10^3 IU/mL after a minimal 48-week of first-line antiviral therapy are considered to have LLV, with the exclusion of patient compliance, viral resistance mutations, and test errors (Chen et al., 2006; Kim et al., 2017). The incidence of LLV might be associated with the cccDNA (covalently closed circular DNA) of HBV (Dandri and Petersen, 2016). cccDNA has a long half-life that cannot be completely eradicated from liver cells, leading to persistent low-level HBV DNA in the serum. A Korean study reported persistent or intermittent LLV in 37.9% of CHB patients in the cohort of 996 patients receiving initial first-line antiviral treatment (Kim et al., 2017). Studies from China also reported that the incidence of LVV was >30% (Sun et al., 2020; Zhang et al., 2021). Thus, it was confirmed that persistent LLV is associated with a high risk of disease progression to liver fibrosis or cancer (Kim et al., 2017; Sun et al., 2020). Currently, no medicine-based evidence explicates the utilization of the current anti-HBV agents to minimize the incidence of LLV and related side effects. In addition, no effective solutions have yet been proposed by the guidelines. As recommended by the Expert Opinion on Expanding Anti-HBV Treatment for CHB in China, the first-line antiviral agents should be adopted in a timely manner in CHB patients receiving non-first-line agents, while in patients who received the first-line agents, other first-line agents, or combination strategies (two first-line agents or combination with peginterferon) are recommended. A previous study demonstrated that LLV patients receiving ETV alone or in combination for 48 weeks achieved complete virologic suppression after replacement by TAF (HBV DNA < 20 IU/mL) (Ogawa et al., 2020).

TABLE 3 Recommendations for salvage therapy in resistant patients in 2019 (China) guideline.

Type of resistance	Recommended drugs
LAM or LdT-resistance	TDF or TAF
ADV-resistance, without a history of LAM or LdT administration	ETV, TDF, or TAF
ADV-resistance, LAM/LdT-resistance	TDF or TAF
ETV-resistance	TDF or TAF
ETV and ADV-resistance	Combination of ETV and TDF, or ETV and TAF

Similarly, another study suggested that ETV-naïve LLV patients could benefit from switching to TAF (Li et al., 2021). Nonetheless, all these studies are limited in experimental methods and sample size. Thus, in the future, prospective, double-blind, randomized controlled trials are required to substantiate these findings.

Conclusion

There are hundreds of millions of people infected with HBV worldwide which causes a huge medical and economic burden. In China, it is expected to eliminate viral hepatitis by 2030. To this end, great efforts have been made by scholars domestically and abroad. In this review, we review the guidelines about who should be treated and try to the most extent to provide some references about how to deal with some patients who fail to meet the treatment standards and the controversial problems during follow-up. There are still many researches need to be done to better address these issues. The controversy over when antiviral therapy should be discontinued in CHB patients is partly due to the lack of effective methods for evaluating cccDNA in the liver cell nucleus (Caviglia et al., 2018; Martinez et al., 2021; Wong et al., 2022). In recent years, growing evidence has identified HBcAg and HBV RNA as favorable biomarkers as an alternative in assessing antiviral efficacy, decision-making on NA withdrawal, and predicting the risk of recurrence (Giersch et al., 2017; Wu et al., 2017; Xie et al., 2021). Huang et al. (2021) reported that the duration for the replacement of the cccDNA pool ranged between several months to 1 year. This finding hinted that short-term cccDNA clearance could be achieved with the cooperation of NAs and cccDNA corepressor. Nevertheless, the sample size of the study was small; thus, some confounding factors, such as serum HBV DNA contamination, were excluded. Additional studies are required to mine the biological characteristics of cccDNA, including synthesis, renewal, and epigenetics, in order to develop a therapy for CHB that can directly target cccDNA.

Previous studies demonstrated that HBV-specific CD4+/CD8+ T cell dysfunction or depletion plays a vital role in persistent infection caused by HBV (Fisicaro et al., 2017; Polaris Observatory Collaborators, 2018; Yong et al., 2018; Pan et al., 2020; Huang et al., 2021; Fang et al., 2022; Li et al., 2022; Zhang et al., 2022). In a recent study by Zhang et al., the single-cell immune sequences of 0.243 million cells from 46 pairs of peripheral blood and liver samples of 23 patients were analyzed, and the dynamic alterations of T cell depletion after HBV infection were profiled (Zhang et al., 2022). In addition to T cell depletion, a decline in the functions and number of DCs and NKs/NKTs augmented the expression of negative regulators of immune checkpoint proteins (such as the PD-1 and cytotoxic T-lymphocyte antigen 4). The innate immunocompromise represented by Toll-like receptors also participated in persistent HBV infection. Thus, breaking immune tolerance and restoring the HBV-specific immune response might be conducive to facilitating HBV control and clearance (Rehermann, 2013). A large number of agents have been developed for different life stages of HBV, such as cccDNA-targeting agents, blockers for HBsAg efflux, Toll-like receptor agonists, immune checkpoint inhibitors, and therapeutic vaccines (Akbar et al., 2012; Park et al., 2016; Rivino et al., 2018; Salimzadeh et al., 2018). Therefore, we believe that drugs that can cure hepatitis B will be developed in the near future, owing to the continuous advancement of technologies.

Author contributions

TL, JZ, and PX collected the data. LL and YS contributed to figures and tables. MZ and HW edited the manuscript. YW reviewed the manuscript. All authors contributed to the article and approved the submitted version.

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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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Fc receptor-like 5 gene polymorphisms and mRNA expression are associated with liver fibrosis in chronic hepatitis B

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Objective: To investigate the associations of Fc receptor-like 5 (*FCRL5*) gene polymorphisms and mRNA expression with liver fibrosis in chronic hepatitis B (CHB).

Methods: A total of 114 CHB patients with liver fibrosis and 120 CHB patients without liver fibrosis were selected for this study. The gender, age, body mass index (BMI), alanine transaminase (ALT) value, aspartate aminotransferase (AST) value, aspartate aminotransferase-to-platelet ratio index (APRI), and fibrosis index based on 4 factors (FIB-4) were recorded. Two polymorphisms of the *FCRL5* gene (rs6427384 and rs6692977) were genotyped. The mRNA expression level of *FCRL5* in peripheral blood monocytes was determined.

Results: ALT, AST, APRI, and FIB-4 in patients with fibrosis were significantly higher than those in non-fibrosis patients. There was statistically significant difference between fibrosis and non-fibrosis groups in the genotype distribution (χ^2=7.805, p=0.020) and allele frequencies (χ^2=13.252, $p < 0.001$) at *FCRL5* rs6692977. When compared with CC genotype, the genotype CT or TT at rs6692977 was significantly associated with a increased risk of liver fibrosis in CHB patients (CT vs. CC: OR=1.921, 95% CI=1.093–3.375, p=0.023; TT vs. CC: OR=2.598, 95% CI=1.067–6.324, p=0.031). The mRNA relative expression levels of *FCRL5* in patients with liver fibrosis were significantly higher than those in the non-fibrosis group (t=13.456, p<0.001). The fibrosis patients carried TT or CT genotype of rs6692977 had significantly higher *FCRL5* mRNA expression levels than those carried CC genotype (t=2.859, p=0.005). The mRNA expression levels of *FCRL5*, APRI, and FIB-4 index showed predictive efficacy in liver fibrosis with cut-off values of 0.75 (AUC=0.896, 95% CI=0.856–0.935), 0.45 (AUC=0.852, 95% CI=0.802–0.902) and 1.84 (AUC=0.765, 95% CI=0.703–0.826), respectively.

Conclusion: *FCRL5* gene rs6692977 polymorphisms and mRNA expression levels are associated with liver fibrosis in CHB patients.

KEYWORDS

Fc receptor-like 5, gene polymorphisms, liver fibrosis, chronic hepatitis B, association study

Introduction

Hepatitis B virus (HBV) infection and chronic hepatitis B (CHB) caused by HBV are global public health problems (Jieanu et al., 2015). China has one of the highest HBV prevalence worldwide (Su et al., 2022). Liver fibrosis is the result of persistent inflammatory response and chronic scar healing response in the process of chronic liver injury. Hepatitis B-related fibrosis is an important progression stage of CHB (Zhao et al., 2022). If not timely and effective treatment, liver fibrosis may eventually develop into liver cirrhosis or even hepatocellular carcinoma (HCC), which seriously affects the health and quality of life of patients (Li et al., 2022). Therefore, screening populations and identifying individuals at a high risk of disease progression may be beneficial in translating potential therapeutic targets into clinical practice, thereby improving outcomes (Wu et al., 2015, 2017).

At present, the causes of liver fibrosis in patients with CHB are not yet fully understood. Genetic predisposition, innate immune aspects, and inflammatory response combined with environmental factors were recognized to exert a crucial role in the development of hepatitis B-related liver fibrosis (Yang et al., 2018; Campos et al., 2020; Ma et al., 2021; Naghib and Kariminik, 2022). As one of the most common heritable variations, single nucleotide polymorphisms (SNPs) have attracted the attention of many researchers in recent years. Up to now, many studies have shown abundant gene susceptibility loci associated with HBV infection and related diseases, including interleukin-10 (Guo et al., 2015), Sodium taurocholate co-transporting polypeptide (Su et al., 2022), and RNA-binding protein lin28 gene polymorphisms (Han et al., 2020). Nevertheless, the associations having been reported cannot completely explain the mechanism of hepatitis B-related fibrosis. Thus it is worthwhile to continue to explore the genetic variants and molecular markers associated with this disease.

Fc receptor-like molecules (FCRLs) are a class of proteins similar to Fc receptors that play an important role in maintaining the homeostasis balance of the immune system (Li et al., 2014b). The *FCRL* genes are members of the immunoglobulin gene superfamily, discovered by multiple teams using different strategies, and scholars eventually designated them as a uniform nomenclature (Liu et al., 2020). In recent years, a large of studies have found that FCRLs play a vital role in immunodeficiencies, virus infections, and autoimmune diseases (Ehrhardt and Cooper, 2011; Li et al., 2014a; Davis, 2020). FCRL5 is one of the FCRLs family and contains a long, highly acidic C-terminal tail, which mediates DNA binding in a non-sequence-specific manner. FCRL5 can regulate immune response by affecting the signal transduction of immune cell receptors (Zhu et al., 2013). Genetic mutations in the *FCRL5* gene may affect the transcription and translation processes, thus altering its protein expression levels and modifying its protein function (Liu et al., 2020). Previous studies have shown that *FCRL5* gene variants were associated with several immune and inflammatory diseases such as allergic rhinitis (Gu et al., 2019), Graves' disease (Simmonds et al., 2010), and ankylosing spondylitis (Liu et al., 2020).

In this study, we explored the associations of two *FCRL5* polymorphisms (rss6427384 and rs6692977) and *FCRL5* mRNA expression with liver fibrosis in CHB patients.

Patients and methods

Study subjects

A total of 114 CHB patients with liver fibrosis admitted to our hospital from January 2021 to March 2022 were selected as the study group, including 71 males and 43 females, with an average age of 52.35 ± 5.83 years. Additionally, 120 CHB patients without liver fibrosis who matched the age and gender distribution of the study group were incorporated into the control group, containing 75 males and 45 females, with an average age of 53.28 ± 6.37 years. Patients satisfying the following criteria were included: (1) the diagnosis of all subjects according to the 2019 "Guidelines for the Prevention and Treatment of Chronic Hepatitis B," and liver fibrosis was diagnosed based on histological evaluation of liver biopsy specimens; (2) aged between 18 and 70 years; (3) clinical and laboratory data were complete. Patients with the following conditions were excluded: (1) severe kidney, cardiovascular, and cerebrovascular diseases; (2) complicated with malignant tumors, endocrine diseases, or autoimmune diseases; (3) had other types of liver diseases, such as alcoholic liver disease or hepatitis C; (4) had other viral infections, such as human immunodeficiency virus, human papillomavirus or influenza virus infections. All procedures for this study were following the Declaration of Helsinki. This research was approved by the Hospital Ethics Committee (approval number: 2021263), and informed consent was obtained from all individual participants included in the study.

Methods

Collection of blood samples and clinical data

Two tubes of 5 ml fasting venous blood samples were taken from all the subjects. Refer to medical records and test sheets of

them, and record their gender, age, body mass index (BMI), alanine transaminase (ALT) value, aspartate aminotransferase (AST) value, and platelet value. In addition, the aspartate aminotransferase-to-platelet ratio index (APRI) and fibrosis index based on 4 factors (FIB-4) were calculated with the following formulas:

$$\mathrm{APRI} = \left(\begin{array}{l}\mathrm{AST\ value}\left(\mathrm{IU/L}\right)/\mathrm{upper\ limit\ of}\\ \mathrm{normal\ AST\ value}\left(\mathrm{IU=L}\right)\end{array}\right) / \mathrm{platelet\ count}\left(10^9/\mathrm{L}\right)\times 100.$$

$$\mathrm{FIB-4} = \left(\mathrm{AST\ value}\left(\mathrm{IU/L}\right)\times \mathrm{age}\left(\mathrm{years}\right)\right) / \left(\mathrm{platelet\ count}\left(10^9/\mathrm{L}\right)\times\sqrt{\mathrm{ALT}\left(\mathrm{U/L}\right)}\right).$$

DNA sample extraction and genotyping

By reviewing the previous literature and the HapMap databases for Chinese Han population in Beijing (HapMap Data Rel 28 PhaseII + III, on NCBI B36 assembly, dpSNP b126), we selected two SNPs (rs6427384 and rs6692977) in *FCRL5* gene. The two SNPs were genotyped through the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method. Genomic DNA was extracted from peripheral venous blood of all subjects using a QIAGEN kit (QIAGEN, Hilden, Germany) based on the manufacturer's instructions, and stored at −20°C before the genotyping detection. All SNPs were genotyped using the improved Multiplex Ligase Detection Reaction (iMLDR) Assay technology (Shanghai Genesky Bio-Tech Co, Ltd.; www.Geneskies.com). The primers are as follows: rs6427384, forward: 5′-GAGCATTCAGGGAA CTACTA-3′; reverse: 5′-TGGAGGAGGATATTAGGTTG-3′; rs6692977, forward: 5′-CGGTCTCACTGGGCTAAA-3′; reverse: 5′-TGACTTTGCTGGCTTTGG-3′.

Measurement of *FCRL5* mRNA expression

Peripheral blood mononuclear cells (PBMCs) from peripheral blood of all subjects were isolated by Ficoll–Hypaque density gradient centrifugation method. Total cellular RNA was extracted using miRNeasy Mini Kit (Qiagen, Germany). The quantification and concentration of RNA were determined using NanoDrop™ 2000 Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, United States), and RNA was reverse transcribed into complementary DNA using a PrimeScript™ RT reagent kit (Takara Bio Inc., Japan). The *FCRL5* mRNA expression levels were detected in the quantitative Real-Time PCR System (Applied Biosystems, Foster City, CA, United States) using the SYBR Green kit (Takara Bio Inc., Japan). The relative expression levels of *FCRL5* mRNA were normalized to the internal control U6 and were calculated by $2^{-\Delta\Delta CT}$. The primer sequences of FCRL5 and U6 are as follows: FCRL5, forward: 5′-GTGCAAGTGTAGA TGCCGACAA-3′; reverse: 5′- GTGCAAGTGTAGATGCCGA CAA-3′;U6,forward:5′-CTCGCTTCGGCAGCACA-3′;reverse:5′-AACGCTTCACGAATTTGCGT - 3′.

Statistical analysis

Statistical analyses were conducted using SPSS version 23.0 (SPSS Inc., Chicago, IL, United States). Continuous data with normally distribution were displayed as mean ± standard deviation (SD), and the comparison of normal distributed data between two groups was using the t-test. Continuous data with abnormal distribution were displayed as median (interquartile range, IQR), and data between two groups were compared using the Mann–Whitney *U* test. Categorical data were presented as absolute numbers and percentages. The distributions of genotype, genetic models, and allele frequencies in different groups were compared using the Chi-square test, and odds ratios (ORs) with 95% confidence intervals (CIs) were calculated. Hardy–Weinberg equilibrium (HWE) was assessed by the Chi-square test. The correlation between the two continuous variables was calculated by Pearson or Spearman correlation test. The receiver-operating characteristic (ROC) curve and the area under the curve (AUC) were applied to evaluate the predictive value of the variables for liver fibrosis, p-value <0.05 was considered as statistically significant.

Results

Comparison of demographic and clinical characteristics

As shown in Table 1, there were no statistically significant differences in gender, age, and BMI between fibrosis and non-fibrosis patients (all $p > 0.05$). ALT, AST, APRI, and FIB-4 in patients with fibrosis were significantly higher than those in non-fibrosis patients (all $p < 0.05$).

TABLE 1 Comparison of demographic and clinical characteristics between fibrosis and non-fibrosis group.

Characteristics	Fibrosis group ($n = 114$)	Non-fibrosis group ($n = 120$)	$\chi^2/t/Z$	p
Gender (male/female)	71/43	75/45	0.001	0.972
Age (year)	52.15 ± 6.21	53.27 ± 5.85	1.420	0.157
BMI (kg/m^2)	24.24 ± 3.06	23.83 ± 2.73	1.084	0.280
ALT (U/L)	51.10 ± 12.91	35.44 ± 7.97	11.097	<0.001
AST (U/L)	49.18 ± 12.59	29.66 ± 9.77	13.202	<0.001
APRI	0.61 (0.49, 0.85)	0.31 (0.22, 0.42)	9.299	<0.001
FIB-4	2.33 (1.66, 3.08)	1.34 (1.02, 1.96)	6.938	<0.001

TABLE 2 Comparison of genotype distribution and genetic models of *FCRL5* gene SNPs between fibrosis and non-fibrosis group.

Genotype	Fibrosis group ($n = 114$)	Non-fibrosis group ($n = 120$)	χ^2	p	OR (95% CI)	p
rs6427384						
TT	69 (60.53%)	85 (70.83%)	3.073	0.215	1.00 (reference)	
TC	35 (30.70%)	29 (24.17%)			1.487 (0.828–2.671)	0.183
CC	10 (8.77%)	6 (5.00%)			2.053 (0.711–5.931)	0.177
CC + TC vs. TT					1.584 (0.919–2.729)	0.097
CC vs. TC + TT					1.667 (0.584–4.753)	0.335
rs6692977						
CC	52 (45.61%)	76 (63.33%)	7.805	0.020	1.00 (reference)	
CT	46 (40.35%)	35 (29.17%)			1.921 (1.093–3.375)	0.023
TT	16 (14.04%)	9 (7.50%)			2.598 (1.067–6.324)	0.031
TT + CT vs. CC					2.059 (1.221–3.475)	0.006
TT vs. CT + CC					2.014 (0.852–4.762)	0.106

TABLE 3 Comparison of allele frequencies of *FCRL5* gene SNPs between fibrosis and non-fibrosis group.

Allele	Fibrosis group ($n = 114$)	Non-fibrosis group ($n = 120$)	χ^2	p	OR (95% CI)
rs6427384					
T	173 (75.88%)	199 (82.92%)	3.554	0.059	1.00 (reference)
C	55 (24.12%)	41 (17.08%)			1.543 (0.981–2.427)
rs6692977					
C	150 (65.79%)	219 (91.25%)	13.252	<0.001	1.00 (reference)
T	78 (34.21%)	21 (8.75%)			5.423 (3.209–9.165)

Comparison of genotype distributions and inheritance models

The distributions of the two polymorphisms rs6427384 and rs6692977 were all confirmed to Hardy–Weinberg equilibrium (HWE) in the two groups. The distribution of genotype of the two SNPs in fibrosis and non-fibrosis patients is displayed in Table 2. There was a statistically significant difference between fibrosis and non-fibrosis groups in the genotype distribution of *FCRL5* rs6692977 ($p = 0.020$). While no statistically significant difference in the genotype distribution was found between the two groups at rs6427384 ($p = 0.215$).

Dominant, recessive, and co-dominant genetic models in fibrosis patients were compared with that in non-fibrosis patients. The results displayed that when compared with CC genotype, the genotype CT or TT at rs6692977 was significantly associated with a increased risk of liver fibrosis in CHB patients (CT vs. CC: OR = 1.921, 95% CI = 1.093–3.375, $p = 0.023$; TT vs. CC: OR = 2.598, 95% CI = 1.067–6.324, $p = 0.031$). In addition, the dominant model was also statistically significant. That is, CHB patients who carried CT or TT genotype had a higher risk of liver fibrosis than those carried who CC genotype (OR = 2.059, 95% CI = 1.221–3.475, $p = 0.006$). Yet, for rs6427384, the dominant, recessive, and co-dominant genetic models were not statistically significant between fibrosis and non-fibrosis groups (all P>0.05).

Comparison of allele frequencies

We further compared the allele frequencies of *FCRL5* gene SNPs between the two groups. There was a statistically significant difference in the frequency of allele C and T at rs6692977 between fibrosis and non-fibrosis groups. The occurrence of allele T significantly increased the risk of liver fibrosis in CHB patients (OR = 5.423, 95% CI = 3.209–9.165, $p < 0.001$), as demonstrated in Table 3. However, no statistically significant difference in the allele frequency at rs6427384 was observed between fibrosis and non-fibrosis patients ($p > 0.05$).

Comparison of *FCRL5* mRNA expression levels

To confirm the results that *FCRL5* gene polymorphisms were associated with liver fibrosis, we measured the *FCRL5* mRNA expression levels in all subjects using the qRT-PCR assay. As shown in Figure 1A, the mRNA relative expression levels of *FCRL5* in patients with liver fibrosis were 0.83 ± 0.13, which were significantly higher than those in the non-fibrosis group (0.63 ± 0.09), with a statistically significant difference ($t = 13.456$, $p < 0.001$).

Additionally, to verify the influence of *FCRL5* SNPs on gene mRNA expression, we compared the *FCRL5* mRNA expression

levels between fibrosis patients with different genotypes. We found that the *FCRL5* mRNA expression levels were not statistically discrepant between the different genotypes of rs6427384 ($t = 0.012$, $p = 0.991$), as demonstrated in Figure 1B. But fibrosis patients who carried TT or CT genotype of rs6692977 had significantly higher *FCRL5* mRNA expression levels than those who carried CC genotype ($t = 2.859$, $p = 0.005$), as shown in Figure 1C.

Demographic and clinical characteristics and *FCRL5* gene variants

We compared the demographic and clinical characteristics of the fibrosis patients with different *FCRL5* gene genotypes. For rs6427384 locus, there were no significant differences in gender, age, BMI, ALT, AST, APRI, and FIB-4 between fibrosis patients who carried the C allele (CC or TC genotype) and only T allele (TT genotype) (all $p > 0.05$), as shown in Table 4. For rs6692977 locus, the ALT value of fibrosis patients with T allele (TT or CT genotype) was significantly higher than those with CC genotype ($p < 0.05$), as displayed in Table 5.

Demographic and clinical characteristics and *FCRL5* mRNA expression levels

We analyzed the associations of *FCRL5* mRNA expression levels and demographic and clinical parameters in fibrosis and non-fibrosis patients, respectively (Figure 2). In the fibrosis group, BMI was positively correlated with *FCRL5* mRNA expression levels ($r = 0.258$, $p = 0.006$), and age, ALT, AST, APRI, and FIB-4 had no significant correlation with FCRL5 gene mRNA expression levels (all $p > 0.05$). In the non-fibrosis group, no significant correlations were observed between the demographic and clinical characteristics and *FCRL5* mRNA expression levels (all $p > 0.05$).

Predictive effectiveness by ROC curve analysis

To evaluate the predictive value of *FCRL5* mRNA expression levels, APRI and FIB-4 index for liver fibrosis in CHB patients, ROC Curve analysis was conducted. Whether liver fibrosis occurred was considered as the state variable (non-fibrosis = 0, fibrosis = 1), and *FCRL5* mRNA expression, APRI and FIB-4 index were regarded as test variables, respectively. The mRNA expression levels of *FCRL5*, APRI and FIB-4 index showed significantly predictive value in liver fibrosis with cut-off values of 0.75 (AUC = 0.896, 95% CI = 0.856–0.935, sensitivity = 73.7%, and specificity = 93.3%), 0.45 (AUC = 0.852, 95% CI = 0.802–0.902, sensitivity = 82.5%, and specificity = 79.2%) and 1.84 (AUC = 0.765, 95% CI = 0.703–0.826, sensitivity = 70.2%, and specificity = 73.3%), respectively. The maximum of the Youden index for FCRL5 mRNA expression, APRI and FIB-4 in the prediction of liver fibrosis were 0.670, 0.617 and 0.435, respectively, as displayed in Figure 3.

Discussion

The outcome of HBV infection is influenced by the host immune system, and immunologic injury is an important factor influencing the occurrence and development of liver fibrosis in CHB patients (Bandopadhyay and Bharadwaj, 2020; Wu et al., 2020). FCRLs are a class of proteins similar to Fc receptors, which play an important role in maintaining the homeostasis of the immune system (Tolnay, 2022). *FCRL* gene belongs to the immunoglobulin gene superfamily, of which *FCRL5* is an important member and has been reported to play a crucial part in the regulatory mechanism of immune cells (Liu et al., 2020). A previous study demonstrated that FCRL5 exerts binary and compartment-specific influence on innate-like B-cell receptor signaling, and implied a specialized counterregulatory role for FCRL5 at the intersection of innate

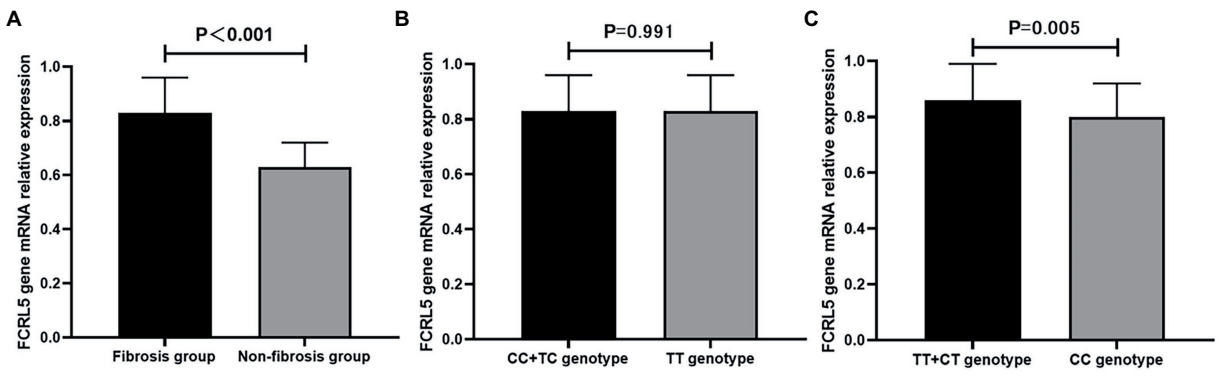

FIGURE 1
Comparison of *FCRL5* mRNA expression levels. **(A)** Comparison between fibrosis and non-fibrosis group. **(B)** Comparison between different rs6427384 genotypes. **(C)** Comparison between different rs6692977 genotypes. FCRL5, Fc receptor-like 5.

TABLE 4 Associations of rs6427384 genotypes with demographic and clinical characteristics.

Characteristics	CC + TC ($n = 45$)	TT ($n = 69$)	$\chi^2/t/Z$	p
Sex (male/female)	31/14	40/29	1.382	0.240
Age (year)	52.43 ± 6.01	51.97 ± 6.37	0.385	0.701
BMI (kg/m^2)	24.59 ± 2.78	24.01 ± 3.23	0.988	0.325
ALT (U/L)	50.47 ± 13.66	51.51 ± 12.48	0.419	0.676
AST (U/L)	50.89 ± 12.47	48.06 ± 12.64	1.175	0.242
APRI	0.60 (0.48, 0.82)	0.61 (0.50, 0.87)	0.638	0.524
FIB-4	2.27 (1.69, 3.04)	2.37 (1.66, 3.35)	0.330	0.741

TABLE 5 Comparison of rs6692977 genotypes with demographic and clinical characteristics.

Characteristics	TT + CT ($n = 62$)	CC ($n = 52$)	$\chi^2/t/Z$	p
Sex (male/female)	39/23	32/20	0.022	0.881
Age (year)	51.82 ± 6.14	52.55 ± 6.34	0.618	0.538
BMI (kg/m^2)	24.43 ± 3.21	24.08 ± 2.94	0.602	0.548
ALT (U/L)	53.81 ± 13.20	47.87 ± 11.88	2.504	0.014
AST (U/L)	49.85 ± 11.87	48.37 ± 13.47	0.627	0.532
APRI	0.66 (0.51, 0.85)	0.59 (0.43, 0.86)	0.999	0.318
FIB-4	1.83 (2.31, 3.01)	2.47 (1.53, 3.14)	0.188	0.851

and adaptive immunity (Zhu et al., 2013). Ogega and Skinner (2022) observed that B cell overexpression of FCRL5 was associated with low antibody titers in HCV infection, and limited upregulation of FCEL5 could potentially generate higher titers of protective antibodies against HCV or other pathogens. In addition, in recent years, many studies have found that FCRL5 was associated with several diseases. Zhang et al. (2022) revealed that FCRL5 was an independent risk factor for colorectal cancer by bioinformatics analysis. Guzeldemir-Akcakanat et al. (2016) found that the expression of FCRL5 was up-regulated in Generalized Aggressive Periodontitis. In addition, it has been suggested that FCRL5 may promote malignant cell growth in hairy cell leukemia and other tumors expressing FCRL5 (Dement-Brown et al., 2012). Additionally, Cell proliferation and downstream isotype expression were enhanced under FCRL5 stimulation, reflecting the physiological role of FCRL5 in antigen-initiated B cell expansion and development (Dement-Brown et al., 2012). The expression of a gene may be regulated by its genetic polymorphisms. Gene mutations may affect the binding of the region where the site is located with transcription factors, thus affecting the transcription process of mRNA. Previous studies have reported that SNPs of multiple genes are related to the occurrence, development, and prognosis of HBV-related fibrosis (Guo et al., 2015; Han et al., 2020; Su et al., 2022). The purpose of this study was to investigate the relationship between *FCRL5* gene variations and liver fibrosis in CHB patients and its effect on gene mRNA expression levels.

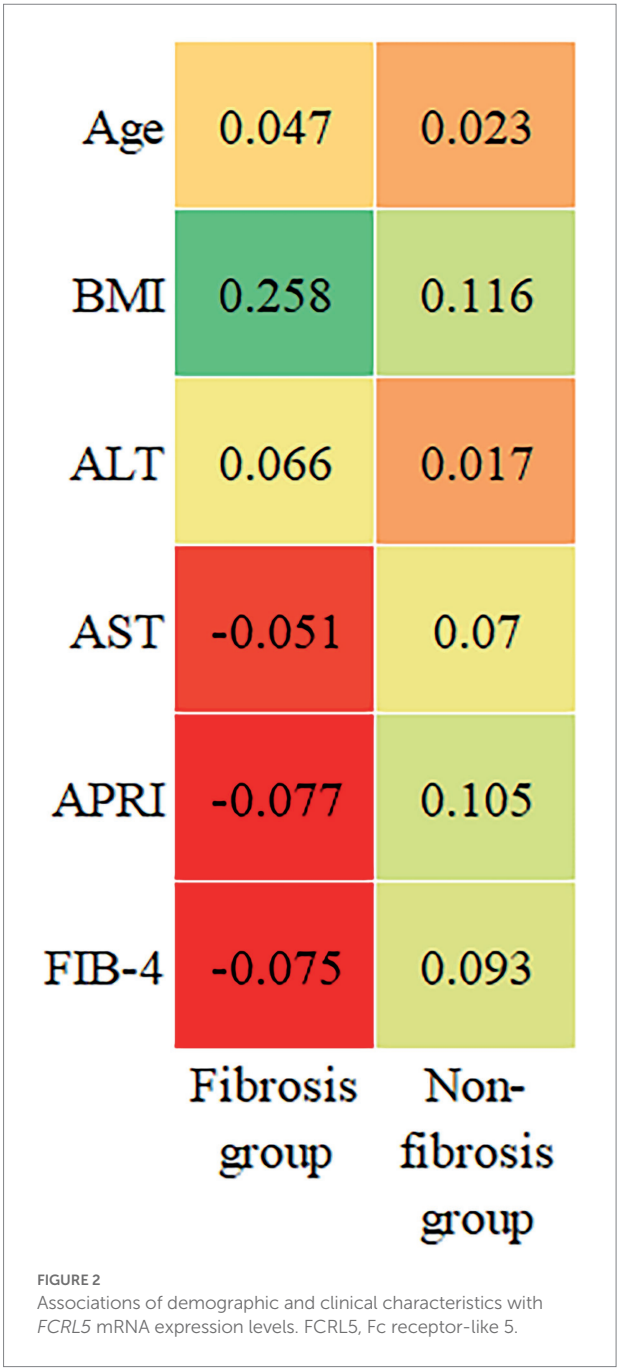

FIGURE 2
Associations of demographic and clinical characteristics with *FCRL5* mRNA expression levels. FCRL5, Fc receptor-like 5.

Previous studies indicated significant relationships of *FCRL5* gene variants with plenty of inflammatory or immunological disorders. Gu et al. (2019) showed that *FCRL5* gene polymorphism is closely related to the occurrence of allergic rhinitis combined with asthma in the Chinese population. Compared with healthy control group, the proportion of CT genotype and T allele frequency of rs6692977 in asthmatic patients with allergic rhinitis was significantly increased. Simmonds et al. (2010) found that rs6692977 mutation was associated with the occurrence of hyperthyroidism, with a higher frequency of the T allele in the case group compared to the control population. Liu et al. (2020) showed that rs6427384 polymorphisms of the *FCRL5* gene were

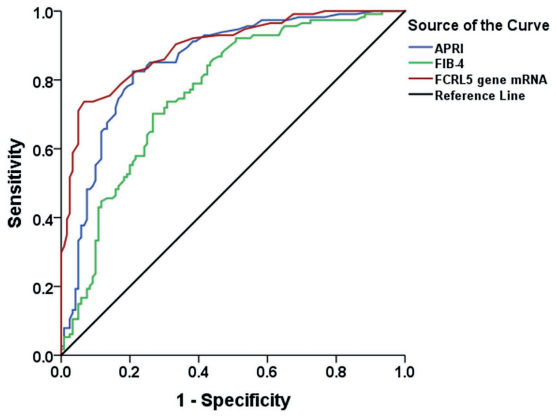

FIGURE 3
ROC curve of the *FCRL5* mRNA expression, APRI and FIB-4 index for predicting liver fibrosis in CHB patients. FCRL5, Fc receptor-like 5; ROC, receiver operator characteristic.

associated with the incidence of ankylosing spondylitis in the Chinese Han population, and CC genotype and C allele of rs6427384 locus were significantly reduced in the case group when compared with the healthy control population. The results of our study revealed the associations between rs6692977 but not rs6427384 mutations with liver fibrosis in CHB patients, which suggest that to a certain extent, hepatitis B-related fibrosis have similarity and differences in genetic background with other disorders with immune dysfunction.

In addition, this study observed that the mRNA expression levels of *FCRL5* in CHB patients with liver fibrosis were significantly higher than those in non-fibrosis patients. The *FCRL5* mRNA expression levels in patients with TT or CT genotype at rs6692977 were significantly higher than those in patients with CC genotype. This result reveals that the *FCRL5* mRNA expression levels are influenced by rs6692977 polymorphisms. Then we found that the ALT value of fibrosis patients with TT or CT genotypes was significantly higher than those with CC genotypes. Hence, *FCRL5* gene variants may be associated with liver function. The study of Sun et al. (2021) showed that the expression of FCRL5 protein was significantly up-regulated in patients with liver cancer, and it could promote the proliferation, invasion and migration of liver cancer cells. The mechanism may be related to the high expression of matrix metalloproteinase-9 and vascular endothelial growth factor induced by FCRL5 in liver cancer cells. Liver fibrosis is a developmental and evolutionary stage of liver cancer. Therefore, we speculated that rs6692977 TT or CT variants of the *FCRL5* gene could up-regulate gene expression levels, and thus participate in the pathogenesis of liver function injury and liver fibrosis.

Furthermore, we observed that in the fibrosis group, BMI was positively correlated with *FCRL5* mRNA expression levels. A study by Seto et al. (2016) revealed that an increased BMI hindered fibrosis regression during therapy in CHB. As we mentioned above, changes in FCRL5 expression levels are associated with immune dysregulation, which has been shown to lead to increased BMI. Hence, in the treatment of liver fibrosis, FCRL5 levels may also need to be controlled to achieve better outcomes. In recent years, APRI and FIB-4 have been widely used to diagnose liver fibrosis in CHB patients. The results of our study showed that the sensitivity and specificity of APRI for the prediction of liver fibrosis were 82.5% and 79.2%, respectively, and that of FIB-4 for the prediction of liver fibrosis were 70.2% and 73.3%, respectively. The ROC curve results of *FCRL5* mRNA expression levels for the predictive value of liver fibrosis showed that AUC was 0.896, with sensitivity and specificity of 73.7% and 93.3%. Compared with APRI and FIB-4, the specificity of *FCRL5* mRNA expression levels in predicting fibrosis was predominant, while the sensitivity was ordinary. Liver biopsy has been considered as the gold standard for the diagnosis of liver fibrosis. However, a series of defects in liver biopsy, such as large sample error, high bleeding risk, and heavy medical burden, have limited its wide application. In addition, repeated histopathology is impractical because of its inescapable invasiveness. Imaging techniques, including transient electrograph (TE), shear wave elastography (SWE), acoustic radiation pulse imaging (ARFI), and magnetic resonance elastography (MRE) have good diagnostic efficacy for liver fibrosis (Pfeifer et al., 2015; Ragazzo et al., 2017; Xiao et al., 2017; Herrmann et al., 2018). However, they are expensive, and the diagnostic accuracy is easily affected by a series of factors such as obesity, ascites, acute inflammation, liver congestion, portal hypertension and so on, which reduces the credibility of the diagnostic results (Tsochatzis et al., 2011; Yin et al., 2011). Compared to liver biopsy and imaging methods, laboratory tests are non-invasive, cheap and have better repeatability. Although the efficacy of *FCRL5* mRNA expression levels in predicting liver fibrosis was mediocre, it can provide a new perspective for predicting liver fibrosis by non-invasive method.

However, there are still some limitations in the current study. Firstly, the sample size of this study was small, so selection bias was inevitable. Secondly, the levels of laboratory indicators are closely related to the medication status of patients, which has not been analyzed. In the future, if the conditions permit, we will increase the sample size and conduct more rigorous and detailed analyses. Thirdly, we did not conduct functional experiments to investigate the exact mechanisms of FCRL5 gene variations in the occurrence and development of liver fibrosis. We will explore this aspect further in the future when conditions are available.

In conclusion, *FCRL5* gene rs6692977 polymorphisms and mRNA expression levels are associated with liver fibrosis in CHB patients.

Data availability statement

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

Ethics statement

The studies involving human participants were reviewed and approved by the Affiliated Suzhou Hospital of Nanjing Medical University. The patients/participants provided their written informed consent to participate in this study.

Author contributions

YW is the guarantor of this work. JG, HW, JS, LL, and WS: specimen and data collection. JY: statistical analysis and manuscript writing. JY and JG: article revising. All authors contributed to the article and approved the submitted version.

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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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Analysis of S gene characteristic sequences and changes in properties of protein expression in HBV ASCs with low-level HBsAg

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Objective: We detected the serum HBsAg immune complex (HBsAg-CIC) and sequenced the HBV S gene in these patients to reveal the association between sustained low-level expression of HBsAg and mutated S gene sequence characteristics, protein function changes, and HBsAg immune complex formation.

Methods: A total of 204 samples were collected and divided into high-level (n = 60, HBsAg level >10 IU/ml) and low-level (n = 144, HBsAg level ≤10 IU/ml) HBsAg groups. The clinical and epidemiological data of the two groups were statistically compared. According to different serological patterns and genotypes, the HBsAg-CIC results of the high-level and low-level HBsAg groups were divided into different subgroups, and then the HBsAg-CIC positive rates among different subgroups were compared. We sequenced the S gene of HBV from the two groups and identified the relevant mutations in the MHR of the S gene. In addition, we compared the changes in HBsAg protein properties and functions after hot spot mutation in the MHR of the S gene.

Results: Comparing the positive rates of HBsAg-CIC under different serological patterns and genotypes in the two groups, the HBsAg-CIC positive rate was higher in the low-level HBsAg group. Moreover, there was weak correlation between HBsAg-CIC and HBsAg or HBV DNA in both groups (r = 0.32, 0.27, 0.41, 0.48; P < 0.05). Sequencing of S gene in the two groups, showed that the hot-spot mutations were T126A, M133L/T/S, and F134L/T/I in MHR of S gene of genotype B, and hot-spot mutations were Q101R and I126S/T in MHR of S gene of genotype C. Additionally, the positive rate of MHR mutation in the S gene from HBsAg-CIC positive patients was higher in the low-level HBsAg group.

Conclusion: The host immune process of clearing HBV seems to have multiple site mutations in MHR, which changes the physicochemical properties and functions of HBsAg and intensifies the formation of HBsAg-CIC, thus avoiding the effective recognition of HBsAg by the host and resulting in immune tolerance between the host and HBV, which may be one of the formation mechanisms of sustained low-level expression of HBsAg in the serum of HBV-infected persons.

KEYWORDS

HBsAg, HBV S gene, HBV genotype, MHR, mutation site, HBsAg function

Introduction

Hepatitis B virus (HBV) infection is one of the most serious problems endangering human health. At present, approximately 240 million people in the world are infected with HBV (1, 2). HBV is mainly prevalent in Asia, the Pacific Islands, Africa, southern Europe and Latin America, and there are more than 100 million HBV carriers in China (3–5). Based on the interaction between the virus and host immunity, HBV infection presents a variety of clinical manifestations, including acute hepatitis, chronic hepatitis, liver dysfunction, liver cirrhosis and hepatocellular carcinoma (6). Some asymptomatic HBV-infected people have persistent low-level expression of HBsAg, and the literature reports show that the proportion is as high as 15.03–21.1% (7, 8). However, some patients with low-levels expression of HBsAg often miss detection in the process of clinical detection, which undoubtedly does not increase the risk of HBV transmission and poses a serious threat to the safety of clinical blood transfusion.

HBsAg is typically used in the clinical diagnosis and screening of HBV-infected persons as an important serological marker. In addition, it was reported that the level of HBsAg was closely related to the disease stage, disease progression and prognosis of HBV-infected patients (9, 10). The level of HBsAg in the serum of HBV-infected patients depends not only on the process of virus replication but also on the expression of the corresponding coding mRNA and the complex balance of the interaction between HBV and the host immune system (8, 11, 12). HBV-infected people with persistent low-level expression of HBsAg often have low replication of HBV DNA, and most of these people are asymptomatic HBV-infected people (13–19). Unlike occult hepatitis infection, the mechanism of chronic HBV infection with persistent low-level HBsAg expression and occult hepatitis infection partially intersect, but the low-level HBsAg population can detect the low concentration of HBsAg in the host serum, accompanied by low or no replication of HBV DNA, while the occult hepatitis infected people show negative HBsAg detection and positive HBV DNA (20). So far, the mechanism of sustained low-level expression of HBsAg in HBV-infected patients has not been fully clarified, but it is closely related to host and virus factors and virus gene mutation is an important factor (8, 12, 21, 22). HBsAg is composed of 226 amino acid (aa) residues encoded by the HBV S gene. The region from aa 99 to aa 169 is called the major hydrophilic region (MHR) and is an important antigen epitope to stimulate B cells to produce neutralizing antibodies (23, 24). Mutation of the HBV S gene, especially the MHR region, can cause immune escape by changing the antigenicity of HBsAg and reducing the binding force of neutralizing antibodies or affecting the secretion of HBsAg (25, 26). The existence of HBV-infected people with persistent low-level expression of HBsAg poses a new challenge to the prevention and treatment of HBV and has attracted extensive attention from experts in the field of infectious diseases (27–30). At present, there are few reports on the distribution of HBsAg-CIC in the serum of HBV-infected persons with sustained low-level expression of HBsAg. Moreover, we investigated whether the S gene mutation causes a change in HBsAg protein properties and whether the change in HBsAg protein properties will increase the formation of HBsAg-CIC in the serum of HBV-infected persons with sustained low-level expression of HBsAg. With these problems, we carried out the following research.

Materials and methods

Study design and samples

A randomized controlled study was conducted from February 2014 to December 2015 at the Clinical Laboratory Center at the 903rd Hospital of the PLA (Hangzhou, China). In total 244 serum samples of chronic asymptomatic HBV carriers (ASCs) from the 903rd Hospital of the PLA, the First Affiliated Hospital of the Medical College of Zhejiang University and Hangzhou Xixi Hospital were collected. The patients were all from the physical examination center of the hospital, and were found during the normal physical examination. Definition of chronic ASC is characterized by the presence of positive

serum HBsAg for more than 6 months, normal serum amino transferase levels and no evidence of liver cirrhosis (LC) or hepatocellular carcinoma (HCC) based on the clinical criteria and ultrasound examination (31). The study was carried out in accordance with the principles of the Declaration of Helsinki. All patients signed informed consent, and the project was approved by the medical ethics committee of the 903rd Hospital of the PLA service support force.

Based on laboratory test results, as well as more than 1 year of follow-up of the patients, their clinical data and their history of infection or natural history the exclusion criteria were established (32, 33).

A total of 204 samples (40 cases were excluded) were collected and divided into high-level ($n = 60$, HBsAg level >10 IU/ml) and low-level ($n = 144$, HBsAg level ≤10 IU/ml) HBsAg groups. The clinical testing center of the National Health Commission of China has provided a serum standard with a low-level fixed value, the CMIA method replaced the traditional ELISA method for quantitative detection of serum HBsAg, low-level HBsAg was defined as serum HBsAg <5.0 ng/ml or ≤10.0 IU/ml (34). According to HBV serum markers, HBV-infected patients were divided into three serological patterns: HBsAg/HBeAg/anti-HBc positive, HBsAg/anti-HBe/anti-HBc positive, and HBsAg/anti-HBc positive. Blood samples (5 ml) were collected with a sampling tube without coagulant, and 3 ml serum samples were centrifuged at 3,000 rpm. One milliliter of serum sample was placed in three 1.5 ml EP tubes and frozen at −70°C until use.

Experimental reagents and instruments

The following clinical and laboratory data of the included patients were recorded: age, sex, Hepatitis B surface antigen (HBsAg), Hepatitis B surface antibody (anti-HBs), Hepatitis B e antigen (HBeAg), Hepatitis B e antibody (anti-HBe), Hepatitis B core antibody (anti-HBc), HBV DNA, among other markers. Architect i2000 automatic chemiluminescence immunoanalyzer based on chemiluminescence immunoassay (CLIA) (Abbott Laboratories, USA) were used. HBV DNA fluorescence quantitative detection kit was purchased from ACON Biotechnology Co., Ltd (Hangzhou, China). HBV DNA was detected using ABI 7300plus real-time fluorescent PCR system (ABI Applied System, USA) and Np968 nucleic acid extraction system (Tianlong, China).

Circulating immune complex (CIC) detection

In this study, HBsAg-CIC was detected (HBsAg-CIC related detection results in this study were completed when the samples were collected) based on the self-developed patented immune complex dissociation Technology (which has the advantages of high efficiency, versatility, strong specificity and high sensitivity; protocol no. ZL201410034039.5 and ZL201410033277.4). The CIC detection method used in this study consists of the following steps: separation, washing, redissolution, detection, result judgment. HBsAg-CIC dissociation was performed in the serum of HBV-infected patients according to the literature and patented technology, and presence of HBsAg-CIC was judged according to the result judgement standard (35). The samples of the high-level and low-level HBsAg groups were grouped according to different serological models and genotypes. The positive rates of HBsAg-CIC were compared, and the correlation between the content of dissociated HBsAg in HBsAg-CIC and the content of free HBsAg in serum was evaluated.

Comparison and analysis of HBV gene mutations

Gene sequencing was performed on serum samples from 144 asymptomatic infected persons in the low-level group and 60 asymptomatic infected persons in the high-level group. Nested PCR was used to amplify HBV nucleic acids in high- and low-level HBsAg groups, and product recovery sequencing was performed (8, 12). Seqman, a subroutine of Laser gene software, was used to splice the sequencing results, and MEGA software was used to compare the spliced S genes with S genes of different genotypes to identify genotypes with different sequencing results (36). Based on the reference sequence of the S gene reported in the literature, the frequency of mutation at different sites of the S gene in the high and low HBsAg groups was counted, and the frequency of mutation at the same location between the two groups was compared and verified. In addition, the mutation of the HBV S gene in HBsAg-CIC samples between the two groups was statistically analyzed. A mutation rate >10% is defined as a hot spot mutation (8, 12, 37).

Functional analysis of HBsAg

Protean of Lasergene software (DNAstar, Inc., Madison, WI, USA) was applied to predict the effect of amino acid mutations in the MHR region in individuals in the low-level HBsAg group with genotype B on the following parameters: the HBsAg hydrophilicity using a Kyte-Doolittle hydropathy plot; the HBsAg antigenic index using the Karplus-Schultz method; and the HBsAg surface probability using the Jameson-Wolf and Emini method. Functional changes in HBV MHR ("a" antigenic determinant as a major detection site) of individuals in the low HBsAg group with genotype B were analyzed based on the reference sequences from individuals in the high-level HBsAg group with genotype B.

TABLE 1 Clinical data and virological characteristics of the low- and high-level HBsAg chronicASC groups.

Parameter	Low-level HBsAg group ($n = 144$)	High-level HBsAg group ($n = 60$)	*P*
Gender			
Male	92	39	>0.05
Female	52	21	
Age (years)	54.88 ± 14.93	42.67 ± 12.19	<0.05
Laboratory results			
HBsAg (IU/ml)	4.08 ± 3.56	5406.49 ± 12885.12	<0.05
HBV DNA-positive rate (%)	47.92 (69/144)	95.00 (57/60)	<0.05
HBV genotype			
B genotype (%)	36.11 (52/144)	40.00 (24/60)	<0.05
C genotype (%)	7.64 (11/144)	38.33 (23/60)	

TABLE 2 Results of HBsAg-CIC positive rate between different serological patterns and genotypes in the two groups.

Parameter	Low-level HBsAg group ($n = 144$)	High-level HBsAg group ($n = 60$)	*P*
HBsAg/HBeAg/anti-HBc positive	/	15 (21)[a]	/
HBsAg/anti-HBe/anti-HBc positive	105 (144)	7 (21)[a]	<0.05
HBsAg/anti-HBc positive	/	9 (21)[a]	/
Total	105 (144)	31 (60)	<0.05
Genotype B	32 (52[b])[d]	8 (24[c])[e]	<0.05
Genotype C	10 (11[b])[d]	18 (23[c])[e]	>0.05

"/": No relevant data; "a": In the high-level group, the difference in the HBsAg-CIC positive rate between different HBV serological modes was statistically significant (χ^2 =6.94, P<0.05); "b": The samples of 144 patients in the low-level group were successfully classified into 52 cases of B genotype and 11 cases of C genotype by HBV gene sequencing; "c": The samples of 60 patients in the high-level group were successfully classified into 24 cases of B genotype and 23 cases of C genotype by HBV gene sequencing; "d": There was no significant difference in the HBsAg-CIC positive rate between genotype B and genotype C in low-level group (χ^2 =3.52, P>0.05); "e": There was significant difference in the HBsAg-CIC positive rate between genotype B and genotype C in high-level group (χ^2 =11.37, P<0.05).

Statistical analysis

For statistical analysis of the data used, SPSS 19 software was used, and the data were imported into Graph Pad Prism5 software to complete the drawing of the bar graph. Canvas 11 image editing software was used to draw a Venn diagram. The continuous variables and categorical variables between the two groups were statistically analyzed by *t*-test, chi square test or Fisher exact test. Correlation analysis was performed using Pearson Product-Moment Correlation. All *P*-values were double tailed. When $P < 0.05$, the difference between two or more groups was considered statistically significant.

Results

Comparison of routine laboratory data between the two groups

In Table 1, the results showed that there were significant differences in HBV genotype (B genotype was the main genotype in the low-level HBsAg group), HBV DNA positive rate (was lower in the low-level HBsAg group) and age (was higher in the low-level HBsAg group) between the two groups ($P < 0.05$), but there was no significant difference in sex between the two groups ($P > 0.05$).

Distribution of HBsAg-CIC among asymptomatic HBV infections

Comparing the HBsAg-CIC positive rates in different genotypes and serological patterns of the two groups (Table 2), the results showed that the HBsAg-CIC positive rate between the two groups was statistically significant ($P < 0.05$), and in the low-level HBsAg group, the HBsAg-CIC positive rate was higher than in the high-level HBsAg group. Under the condition of the same B genotype, the HBsAg-CIC positive rates between the two groups were statistically significant (the HBsAg-CIC positive rate was higher in the low HBsAg group, $P < 0.05$). In addition, the difference in the HBsAg-CIC positive rate in different serological patterns was statistically significant in the high-level HBsAg group ($P < 0.05$), and the HBsAg-CIC positive rate was the highest among the serological patterns of HBsAg/HBeAg/anti-HBC positivity. In the high-level HBsAg group, the difference in the HBsAg-CIC positive rate in different genotypes was statistically significant ($P < 0.05$), and in the C genotype, the HBsAg-CIC positive rate was higher.

Evaluation of the correlation between HBsAg CIC and HBsAg or HBV DNA between the two groups

The correlation between HBsAg-CIC and HBsAg or HBV DNA between the two groups was evaluated as shown in Figure 1. In the low-level HBsAg group, HBsAg-CIC has a weak correlation with HBsAg and lg(HBV DNA) ($r = 0.32, 0.27$; $P < 0.05$). In the high-level HBsAg group, lg(HBsAg-CIC) had also

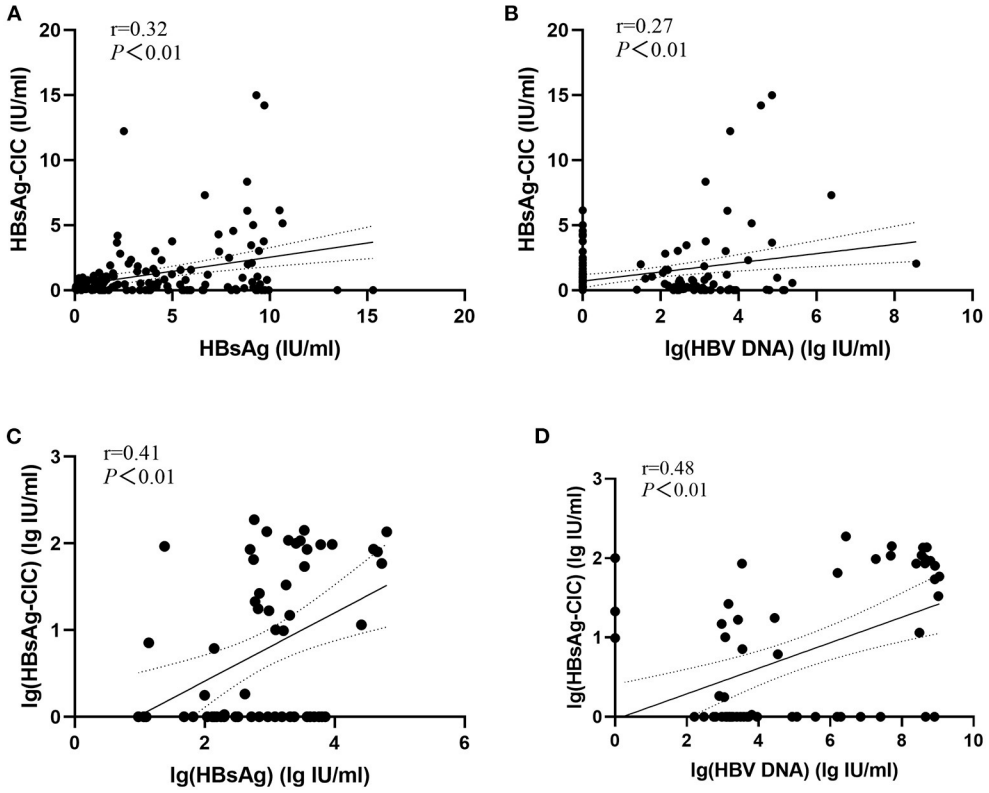

FIGURE 1
Evaluation of the correlation between HBsAg-CIC and HBsAg or HBV DNA between the two groups. **(A)** Linear regression model of HBsAg-CIC and HBsAg in patients with low-level HBsAg. **(B)** Linear regression model of HBsAg-CIC and lg(HBV DNA) in patients with low-level HBsAg. **(C)** Linear regression model of lg(HBsAg-CIC) and lg(HBsAg) in patients with high-level HBsAg. **(D)** Linear regression model of lg(HBsAg-CIC) and lg(HBV DNA) in patients with low-level HBsAg.

weak correlation with lg(HBsAg) and lg(HBV DNA) ($r = 0.41$, 0.48; $P < 0.05$).

Evaluation of the relationship between mutation in the MHR of the S gene and the HBsAg-CIC positive rate

The S gene sequencing results of the two groups were compared with the reference sequences of corresponding genotypes (8, 12), as shown in Figure 2. The results showed that the hot spot mutations in the S gene MHR of HBV genotype B patients were T126A, M133L/T/S, and F134L/T/I. Hotspot mutations in the MHR of the S gene of HBV genotype C in the two groups were Q101R and I126S/T.

In addition, 32 patients with genotype B in the low-level HBsAg group were found to be HBsAg-CIC positive, and 18 had MHR mutations of the S gene. Among the patients in the high-level HBsAg group, 8 cases were HBsAg-CIC positive for genotype B, and 1 case had an MHR mutation of the S gene. The positive rate of MHR mutation in the S gene in HBsAg-CIC-positive patients with the B genotype was significantly different between the two groups ($\chi^2 = 4.91$, $P < 0.05$). Ten cases were HBsAg-CIC positive for genotype C in the low-level HBsAg group, and 7 cases had MHR mutations. In the low-level HBsAg group, 18 cases were HBsAg-CIC positive for genotype C, and 6 cases had MHR mutations (Figure 3).

Functional analysis of HBsAg

In this study, the hydrophilicity, antigenic index, and surface probability of mutational sites and surrounding amino acid sites in the MHR region of individuals with HBV genotype B in the low-level HBsAg group were analyzed to predict the functions of these MHR sites (Genotype B: T126A, M133L/T/S, F134L/T/I, Genotype C: Q101R, I126S/T). Compared with the reference sequence, we found that in the low-level HBsAg group, the changes in multiple sites and multiple areas were related to hydrophilicity, the antigenic index and surface probability. It will not only affect the binding between HBsAg and the detection

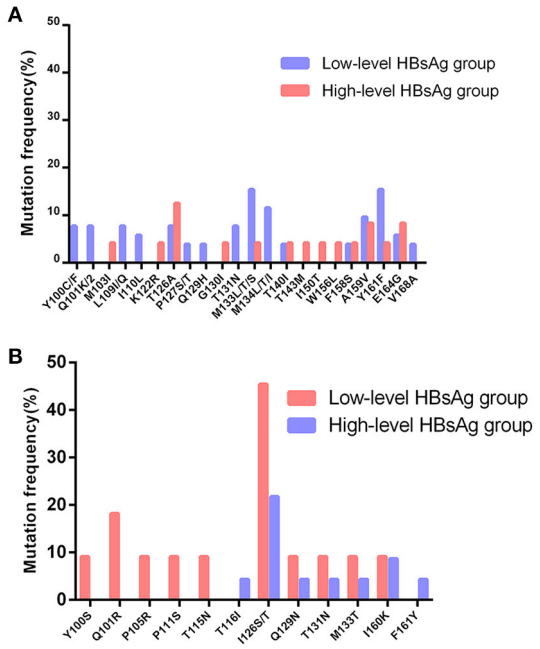

FIGURE 2
Distribution and rate of mutations within MHR in S gene of genotype B and C between the two groups. **(A)** Distribution and rate of mutations within MHR in S gene of genotype B between the two groups. **(B)** Distribution and rate of mutations within MHR in S gene of genotype C between the two groups.

antibody, resulting in the deviation of the detection results, but also affect the host's effective recognition of HBsAg, resulting in immune escape and continuous immune tolerance (Figure 4).

Discussion

At present, the preferred serological marker for the diagnosis of HBV infection is hepatitis B surface antigen (HBsAg) (38, 39). It is the earliest serological marker after HBV infection and can be detected 4–10 weeks after HBV infection. HBsAg mainly consists of large (L), medium (M) and small (S) surface antigens. The smallest surface antigen, S protein (24 kDa), is 226 amino acids in length. HBsAg is very important to monitor the natural process, evaluate the treatment response and predict stages of disease progression (40, 41). Previous studies have shown that some patients with HBV infection continue to express HBsAg at low levels, and this population has unique clinical manifestations and epidemiological characteristics. In this study, we grouped and compared the results of routine laboratory examinations of patients. It was found that HBV-infected patients with persistent low-level expression of HBsAg had the characteristics of older age, B genotype, HBsAg/anti-HBe/anti-HBC positivity, and HBV DNA low replication, which was consistent with previously reported studies (8, 12).

Based on immune complex dissociation technology independently established by our project team (the technology has the advantages of high efficiency, generality, strong specificity, and high sensitivity), HBsAg-CIC of high- and low-level HBsAg groups was detected in this study. The results showed that HBsAg/HBeAg/anti-HBC positive had a higher HBsAg-CIC positive rate than the other two serological patterns, which was consistent with Tsai et al. (42). The reason is that there is a positive correlation between the HBsAg-CIC positive rate and HBeAg. In other words, it is closely related to virus replication. In different genotypes of the high-level HBsAg group, the difference in the HBsAg-CIC positive rate was statistically significant, and the HBsAg-CIC positive rate of genotype C was higher than that of genotype B. In different genotypes of the low-level HBsAg group, the difference in the HBsAg-CIC positive rate was not statistically significant. This may be related to the fact that in the low-level HBsAg group, the number of samples of the C genotype was low. After reviewing many studies, no investigation report on different HBV genotypes of HBsAg-CIC has been found, but the literature clearly indicates that the proportion of HBV genotype C is significantly higher than that of HBV genotype B in HBeAg-positive patients. Consistent with this study, 70% (14/20) of HBV infections with serological pattern HBsAg/HBeAg/anti-HBc positive were C genotype. This indirectly indicates that investigating the HBsAg-CIC positive rate among HBV patients with different genotypes is reliable.

In addition, this study also evaluated the correlation between HBsAg-CIC and HBsAg or HBV DNA between the two groups. The results showed that in the low-level group and high-level group, the content of HBsAg-CIC was weak positively correlated with the content of serum HBsAg and HBV DNA, suggesting that the formation of HBsAg-CIC was closely related to virus replication and the effective recognition of antigen epitopes of serum HBsAg. However, this study also found that when the serological pattern was the same, the positive rate of HBsAg-CIC in the low-level HBsAg group was higher than that in the high-level HBsAg group. HBV infected persons with HBeAg positive and genotype C have active viral replication and high content of free HBsAg, which in turn stimulates the body to produce HBsAg-CIC in a higher level than other serological patterns and B genotypes. The elimination of HBV referred to in this discussion actually referred to the elimination of HBsAg-CIC. In other words, compared with the high-level HBsAg group, why is the HBsAg-CIC positive rate higher in the low-level HBsAg group than in the high-level HBsAg group under the condition of low replication. We think that in the high-level group, especially those with HBeAg positive and genotype C HBV infection, the body may play the role of HBsAg-CIC clearance normally, but its clearance capacity is insufficient compared with the amount of HBsAg-CIC formation. However, although the formation of HBsAg-CIC was low in the low-level HBsAg group, the body did not exert the function of clearing HBsAg CIC or the

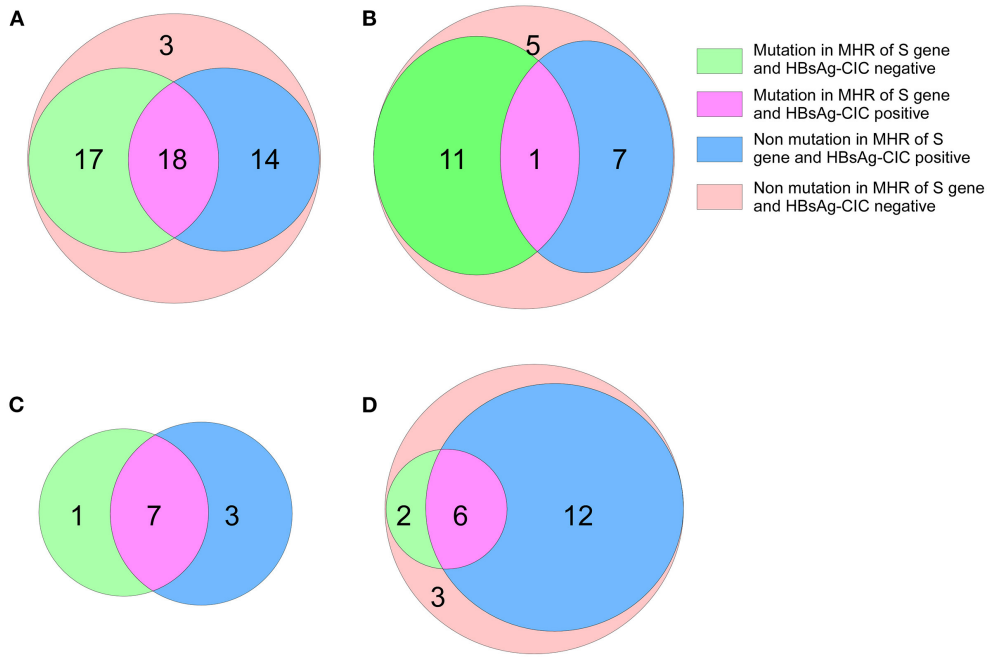

FIGURE 3
Mutation in MHR of S gene and distribution of HBsAg-CIC in patients with different genotypes between the two groups. **(A)** Mutation in MHR of S gene and distribution of HBsAg-CIC positive of genotype B in patients with low-level HBsAg. **(B)** Mutation in MHR of S gene and distribution of HBsAg-CIC positive of genotype B in patients with high-level HBsAg. **(C)** Mutation in MHR of S gene and distribution of HBsAg-CIC positive of genotype C in patients with low-level HBsAg. **(D)** Mutation in MHR of S gene and distribution of HBsAg-CIC positive of genotype C in patients with high-level HBsAg.

ability of effectively clearing HBsAg CIC decreased which led to the accumulation of HBsAg-CIC, resulting in a higher positive rate of HBsAg-CIC in the low-level group (43). This conclusion needs further experimental verification.

Relevant studies show (13, 17) that there is unexplained immune tolerance between HBV and the host, or the application of immunosuppressants enhances the immune tolerance of the body, so that HBsAg cannot be completely cleared, resulting in the expression of low-level HBsAg. In addition, S gene methylation, pre-core region (PC) and basal core promoter (BCP) mutations regulate the secretion of HBsAg, resulting in lower HBsAg levels in patients. To this end, we carried out further research, obtained the distribution of hotspot mutations in MHR by sequencing the S genes of the two groups, and then counted the number of HBsAg-CIC-positive patients with S gene MHR mutations in different genotypes in the two groups. After statistical comparison, we found that in the low-level HBsAg group, the frequency of MHR in-mutation in HBsAg-CIC-positive patients of the B genotype was higher than that in the high-level HBsAg group, while the difference in the frequency of MHR in-mutation in HBsAg-CIC-positive patients of the C genotype between the two groups was not significant. This may be related to fewer HBV infection cases of genotype C. In addition, based on the immune interaction between the virus and the host (immune surveillance and immune escape), we believe that the gene mutation in the low-level HBsAg group is higher than that in the high-level group in the S gene, may be that the formation of the low-level HBsAg is more conducive to the long-term coexistence between the virus and the host, which needs to be further demonstrated (7, 44).

From the above results, we can indirectly conclude that the HBsAg-CIC positive rate in the low-level HBsAg group was higher, which may be related to the mutation in the MHR of the HBV S gene. We evaluated the antigenicity, hydrophilicity and other related parameters of HBV genotypes B and C with hot spot mutations in the MHR of the S gene. The study found that mutations occurring within the MHR hotspot may change the antigenicity and hydrophilicity of HBsAg in the corresponding position, which increases the antigenicity and hydrophobicity of the HBsAg part locus mutation. There were also mutations that reduced antigenicity and hydrophobicity at some HBsAg sites. Combined with our previous studies, the low expression level of HBsAg in HBV-infected persons may be a dynamic balance between the host and HBV, thus limiting the further effective elimination of HBV by the host. In addition, it has been reported that mutation of the S gene in MHR may be related to HBV immune escape (45). We speculated that the formation of HBsAg-CIC may be the process of active elimination of HBV

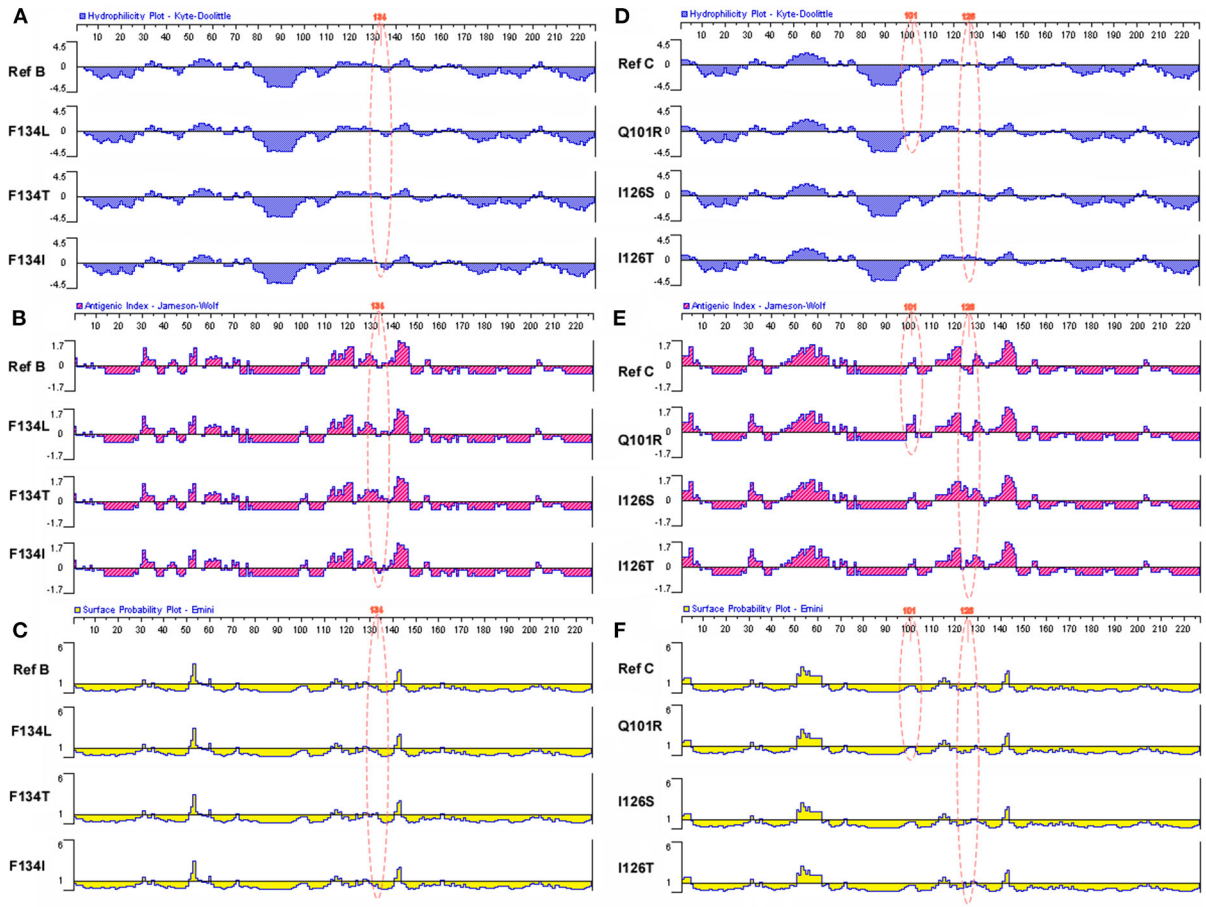

FIGURE 4
Related functional changes induced by amino acid mutations in MHR region. **(A)** Effects of hotspot mutations (T126A, M133L/T/S, and F134L/T/I) in the genotype B with low-level HBsAg group on HBsAg antigen index. **(B)** Effects of hotspot mutations (T126A, M133L/T/S, and F134L/T/I) in the genotype B with low-level HBsAg group on HBsAg hydrophilicity. **(C)** Effects of hotspot mutations (T126A, M133L/T/S, and F134L/T/I) in the genotype B with low-level HBsAg group on HBsAg surface probability. **(D)** Effects of hotspot mutations (Q101R and I126S/T) in the genotype B with low-level HBsAg group on HBsAg antigen index. **(E)** Effects of hotspot mutations (Q101R and I126S/T) in the genotype B with low-level HBsAg group on HBsAg hydrophilicity. **(F)** Effects of hotspot mutations (Q101R and I126S/T) in the genotype B with low-level HBsAg group on HBsAg surface probability.

by the host, but the virus escaped from the MHR mutation and was further cleared by the host. Multiple factors lead to a higher proportion of HBsAg-CIC in HBV-infected patients with low levels of HBsAg, which further promotes the continuous low level of HBsAg expression. Therefore, whether the HBsAg-CIC positive rate in the low-level HBsAg group was higher than that in the high-level HBsAg group may be related to the change in HBsAg protein properties caused by mutations in MHR. Whether the formation of HBsAg-CIC is an influential factor for HBV-infected patients with continuously low levels of HBsAg expression needs to be verified by subsequent experiments.

Although a large number of samples were collected and grouped in this study, we speculated that the sustained low-level expression of HBsAg may be related to the mutation of multiple sites in the MHR region of S gene and the formation of HBsAg-CIC, but this study still has some limitations. For example, (1) there are few samples of HBsAg-CIC positive patients with different genotypes in patients with continuous low-level expression of HBsAg, which cannot fully confirm the correlation between multi-site mutation in MHR region of S gene and the formation of HBsAg-CIC in patients with different genotypes of HBV, which needs to be verified by further experiments. (2) Part of this study is based on bioinformatics, which cannot be fully and truly reflect the changes of HBsAg related characteristics caused by S gene mutation, which needs to be verified by further experiments.

In conclusion, a large-scale grouping study was conducted on 204 patients with ASC. The results of these analyses show that the potential mechanism of sustained low levels of HBsAg expression, which is the process of host clearance of

HBV immunity, seems to involve multiple site mutations in MHR, which changes the physical and chemical properties and functions of HBsAg, intensifies the formation of HBsAg-CIC, avoids the effective recognition of HBsAg, and leads to immune tolerance between the host and HBV.

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 Medical Ethics Committee of the Hospital (the 903rd Hospital of the PLA) (Protocol No: PLA-117-20160309). The patients/participants provided their written informed consent to participate in this study.

Author contributions

CM participated in the project design and research, performed the statistical analysis, and was responsible for drafting and revision of the manuscript. YD participated in the project design and coordination, assisted in writing the manuscript, and helped with the statistical analysis. YQZ was responsible for sample collection. YY and JC performed the virologic analysis and helped draft the manuscript. XX and CJ performed the molecular genetic analysis and sample collection. YY performed the virologic analysis and sample collection and participated in the statistical analysis. All authors read and approved the final manuscript.

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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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Case Report: Chronic hepatitis E in a hematopoietic stem cell transplant recipient: The first report of hepatitis E virus genotype 4 causing chronic infection in a non-solid organ recipient

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Hepatitis E virus (HEV) is one of the most important public health issues around the world, and chronic HEV infection has been reported in immunosuppressed individuals. This study reported a male case, with very severe aplastic anemia (AA), who developed chronic hepatitis E after hematopoietic stem cell transplantation (HSCT). Abnormal alanine aminotransferase (ALT) appeared after HSCT and persisted for twenty-nine months. The case was seropositive for anti-HEV IgG and IgM after HSCT. Twenty-two months after HSCT, HEV RNA and antigen (Ag) testing were positive and persisted for five and seven months, respectively. Positive stains of HEV Ag were present in a liver biopsy sample. HEV Ag was present in bone marrow. The individual rapidly developed liver cirrhosis and was rescued by a regimen of oral ribavirin. These factors suggested there is a risk of HEV infection in HSCT recipients.

KEYWORDS

hepatitis E virus, chronic hepatitis, hematopoietic stem cells transplantation, cirrhosis, aplastic anemia

Introduction

Hepatitis E virus (HEV) infection usually causes acute viral hepatitis in developing and developed countries (1). In immunocompetent individuals, hepatitis E (HE) generally self-limits to acute hepatitis. However, HEV can cause ~30% mortality in pregnant women in the third trimester and also induces chronic HE infection in immunosuppressed patients (1).

Prior studies have shown that extra-hepatic manifestations, such as acute pancreatitis and musculoskeletal and hematological disorders, develop in HEV-infected individuals (2) (3). In addition, an increasing number of studies reported that individuals who received blood transfusion are at risk for HEV infection (4).

Chronic HEV infection was observed in individuals who receive immunosuppressive therapy following solid organ transplantation (SOT) or stem cell transplantation (SCT) and are HIV-positive (5). Moreover, acute and persistent HEV infection was also reported in patients with acute lymphoblastic leukemia (ALL) (6). The progressive cirrhosis caused by chronic infection has mainly been reported for genotype 3 (G3) of HEV, and occasionally for genotype 4 (G4) (5). Chronic HE (genotype unknown) in a child with ALL after SCT was reported in Canada (7). Chronic infection or fatal outcome caused by HEV G3 has been reported in some patients with hematological malignancy (8–10). Although HEV G3 has been identified as a potential burden among patients with hematological malignancy (11), including hematopoietic stem cell transplantation (HSCT) recipients, chronic infection of HEV G4 in patients undergoing HSCT has never been reported.

Here, we report a case of a 33-year-old male with very severe aplastic anemia (AA). After hematopoietic stem cell transplantation (HSCT), HEV RNA and antigen (Ag) persisted for five months and seven months, respectively. The individual rapidly developed liver cirrhosis induced by G4 (subtype 4a) chronic HE and was rescued by a regimen of ribavirin treatment.

Case presentation

In Jan 2017, a 33-year-old male (AM-C01 AA) who reported multiple bleeding for 4 days was diagnosed with very severe AA and was treated with HSCT from HLA-matched sibling donors (MSD) until Feb 2017. A routine investigation performed prior to HSCT showed hepatitis A virus (HAV), hepatitis B virus (HBV), hepatitis C virus (HCV), HEV, cytomegalovirus (CMV), and Epstein-Barr virus (EBV) negative. For MSD, routine investigation for HBV, HCV, CMV, and EBV returned before HSCT. The conditioning regimen for HSCT consisted of cyclosporine (50 mg/kg), antithymocyte globulin (ATG, 10 mg/kg), and Mesna (30 mg/kg). Two hundred and twenty-five milliliters of peripheral hematopoietic stem cells (HSCs, 9.30×10^8/kg mononuclear cells, and 6.23×10^6/kg $CD34^+$ cells) were given, and one hundred and fifty milliliter peripheral HSCs (6.17×10^8/kg mononuclear cells and 3.27×10^6/kg $CD34^+$ cells) administered on the second day. Prophylaxis of graft-versus-host disease (GvHD) was comprised of ciclosporin A (CsA), methotrexate (MTX), and immunosuppressant (MMF). Acyclovir and gamma-globulin maintained anti-infection. Twenty-two days after HSCT, BM and peripheral blood analysis showed a complete hematological remission, and no GvHD was observed.

Abnormal alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were observed after 21 days of HSCT with 85 U/L and 53 U/L in Mar 13rd 2017 (day -765), which persisted for twenty-nine months until Jul 29th 2019 (day +103) (Figure 1A). Non-specific viral symptoms were present, such as fever. Signs of jaundice and weight loss were not observed. Though complete hematological remission was observed, the case was treated with an anti-infective drug. Upon admission to our hospital in Jul 31st 2018 (day -260), abnormal liver enzymes were persistent, ALT 314 U/L, and AST 193 U/L (Figure 1A). Liver-directed autoantibodies were negative. Further anti-HEV IgM and IgG serological testing were performed using an enzyme-linked immunosorbent assay (ELISA) kit (Beijing Wantai, Beijing, China) according to the manufacturer's instructions. This revealed the patient to be anti-HEV IgM and IgG positive. Treatment of hepatitis consisted of oral drugs including compound glycyrrhizin, glutathione, and silibinin. HEV RNA detection was performed using real-time RT-PCR using QIAGEN OneStep RT-PCR Kit (Qiagen, Hilden, Germany) as previously reported (12), and HEV Ag-ELISA assay (Beijing Wantai, Beijing, China) was performed on serum and stool samples. Follow-up samples in serum and stool revealed HEV RNA and Ag positive in Dec 12nd 2018 (day -126), ALT 160 U/L, and AST 68 U/L (Figures 1A, B). Serological testing revealed anti-HEV IgM and IgG to be persistent (Figure 1B). Viral RNA was extracted from the patient's stool supernatant using QIAamp Viral RNA Mini Kit (Qiagen, Hilden,

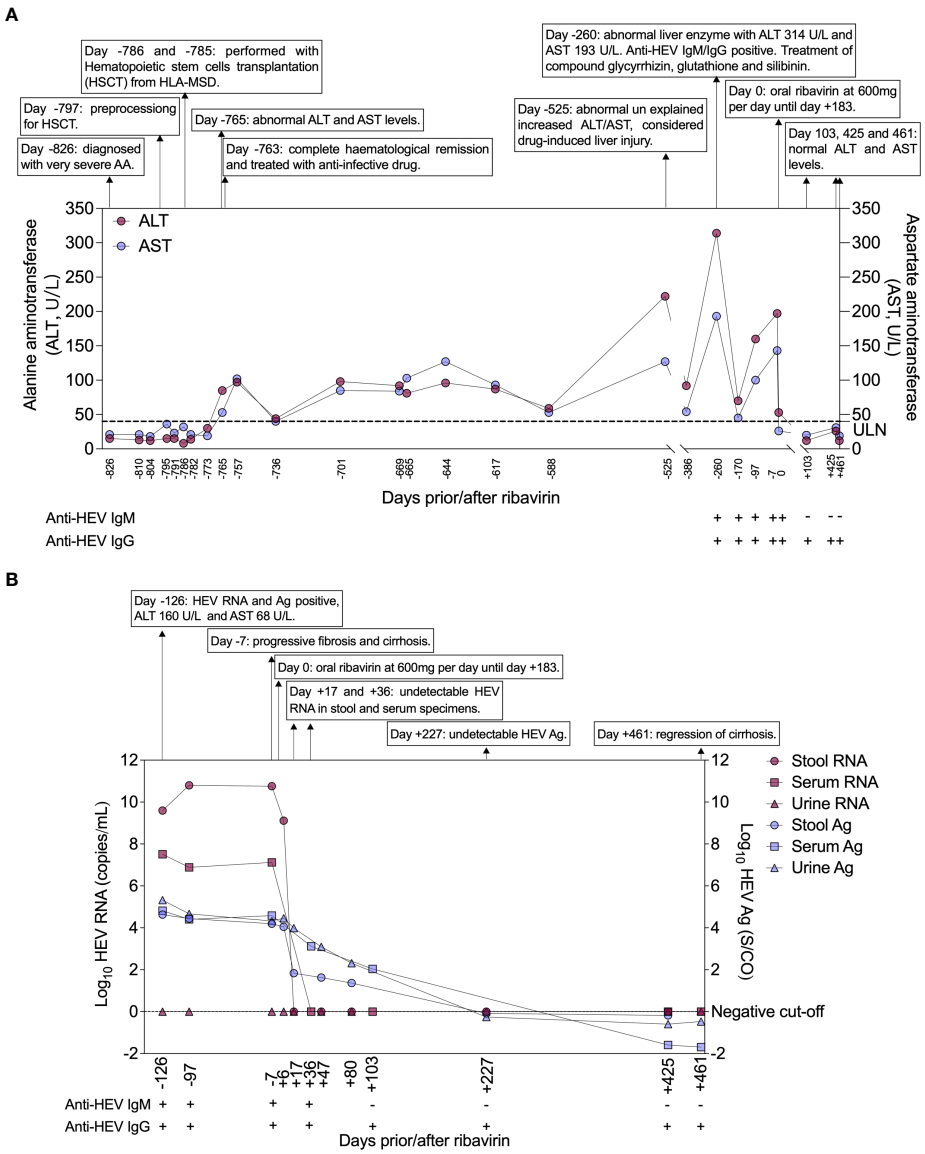

FIGURE 1
Clinical course of HEV infection prior to and after ribavirin. **(A)** Light red legend indicating ALT (circles) and light blue legend indicating AST (circles). The y-axis on the left represents levels of ALT (U/L) and the right represents levels of AST (U/L). **(B)** The light red legend indicates HEV RNA of stool (circles) and serum (squares). The light blue legend indicates HEV antigen of stool (circles), serum (squares), and urine (triangles). The y-axis on the left represents viral load of HEV (Log_{10} copies/mL) and the right represents levels of HEV Ag (Log_{10} S/CO). The x-axis of **(A)** and **(B)** are scaled to illustrate time intervals in relation to oral ribavirin. The presence and absence of anti-HEV IgM and anti-HEV IgG in serum are indicated by "+" and "−", respectively. AA, aplastic anemia; Ag, antigen; ALT, Abnormal alanine aminotransferase; AST, aspartate aminotransferase; antigen; IgM, immunoglobulin M; IgG, immunoglobulin G.

Germany), and viral genome library was built for the sample based on next-generation sequencing (NGS) results (GenBank no. OP185389). The sequence analysis based on a full-length genome revealed that the patient was infected with HEV G4, subtype 4a (Figure 2).

Bone marrow (BM) biopsy was performed for the case in Jan 10th 2019 (day -97) and moderate positive stains of HEV Ag were found in BM cells detected by immunohistochemistry (IHC) (Figure 3A). To further investigate the presence of HEV in BM, IHC of HEV Ag was performed in BM smears, and positive stains were detected (Figure 3B). HEV RNA (6.72×10^2 copies/mL) and HEV Ag (S/CO 11919.4) was detected in BM fluid. These results corresponded to a recent study reported by Wang et al. (13). They demonstrated the presence and persistence of HEV RNA and Ag in human BM and BM tropism of HEV both G3 and G4.

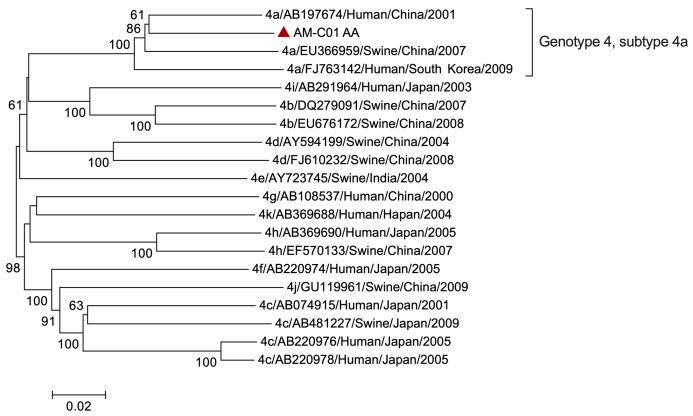

FIGURE 2
Phylogenetic tree analysis of HEV full-genome sequence in the case. The phylogenetic analysis was based on HEV full-genome. Viral genotype, GenBank accession number, virus host, country of origin, and year of detection are indicated for the reference sequences. The sequence obtained from the case is highlighted with a red triangle. The bootstrap values > 70 were shown.

Hematoxylin and eosin staining (H&E) of a liver biopsy sample collected on Apr 10th 2019 (day -7) showed a large number of focal lymphocytes aggregates, severe interface hepatitis, and lobular architectural disruption (Figure 3C). A biliary pattern was shown in the liver biopsy, and increased fibrosis was present in the portal area, with a medium amount of lymphocytes infiltrate (Figure 3D). Collapsed reticular fibers scaffold was shown in hepatic lobule (Figure 3E). The histopathological findings and the presence of HEV Ag in liver tissue were compatible with chronic HE (Figure 3F). Beginning on Apr 17th 2019 (day 0), treatment of chronic HE consisted of oral ribavirin at a dose of 600 mg per day until Oct 17th 2019 (day +183). No main adverse event (AA) was observed during the 6 months of antiviral therapy. Samples did not detect HEV RNA in stool and serum specimens at day +17 and +36 and HEV Ag was undetectable in urine and stool specimens on Nov 30th 2019 (day +227) (Figure 1B). The case was found with a normal ALT and AST at 12 U/L and 20 U/L on Jul 29th 2020 (day +103) (Figure 1A). On Jul 21st 2020 (day +461), a liver biopsy showed mild interface hepatitis, reduced portal and lobular inflammation, with few lymphocytes infiltrate and regression of cirrhosis and architectural disruption (Figures 3C–E). The absence of HEV Ag in liver tissue was detected by IHC (Figure 3F). The overview of the case's diagnosis, treatment, and follow-up process and timelines of major clinical events are summarized in Figure 4.

Discussion

Chronic HEV infection is most often observed in immunosuppressive individuals receiving SOT, with 57.1% to 65.9% of HEV-infected individuals developing chronic hepatitis in previous studies (14, 15). HEV infection appears to be a rather rare risk in HSCT recipients (non-SOT) (16, 17). However, as evidenced by a retrospective cohort of 328 patients with 2.4% (8/328) HEV incidence rate, chronic HEV infection was also observed in HSCT recipients, with 62.5% (5/8) patients of HEV G3 infection developing chronic hepatitis (18). Carré et al. reported the first case of Ph ALL with a HEV-induced fatal event with G3 after HSCT (19).

The European Center for Disease Prevention and Control (ECDC) expert panel weighed that the minimum criterion for HEV diagnosis is anti-HEV IgM/IgG positive. Currently, the most widely used test for diagnosing HEV infection is a serological assay that detects anti-HEV IgM, which is a rapid, cost-effective, and clinically easy-to-use method. Recently, a study reported by Riveiro-Barciela et al. revealed unexpected long-lasting anti-HEV IgM positivity: anti-HEV IgM was detected positively in 56% (Mikrogen) and 24% (Wantai) of patients after a median 34 month follow-up (20). In addition, Abravanel et al. revealed low sensitivity for performance of anti-HEV IgM assay in patients receiving immunosuppressive therapy (21), similar to previous studies (15, 22). In our study, humoral response against HEV was observed since Dec 12nd 2018 (day -126), and anti-HEV IgG titer had a maximum of 2.9 WHO unit per milliliter (Wu/mL). Though our case showed a serological reaction, it suggested that anti-HEV IgM testing has some limitations in diagnosis of HEV infection.

Prevention, management, and treatment of HEV infection in HSCT recipients should be further studied. In Sweden and Europe, 25% to 47.6% of HSCT recipients gradually developed chronic HEV infection and death with ongoing HE occurred in 10% of chronic HEV-infected patients (11, 23). Management of reduced immunosuppressive therapy in HSCT recipients with HEV infection was preferably recommended by some reports (24, 25). Some studies revealed GvHD and death associated with

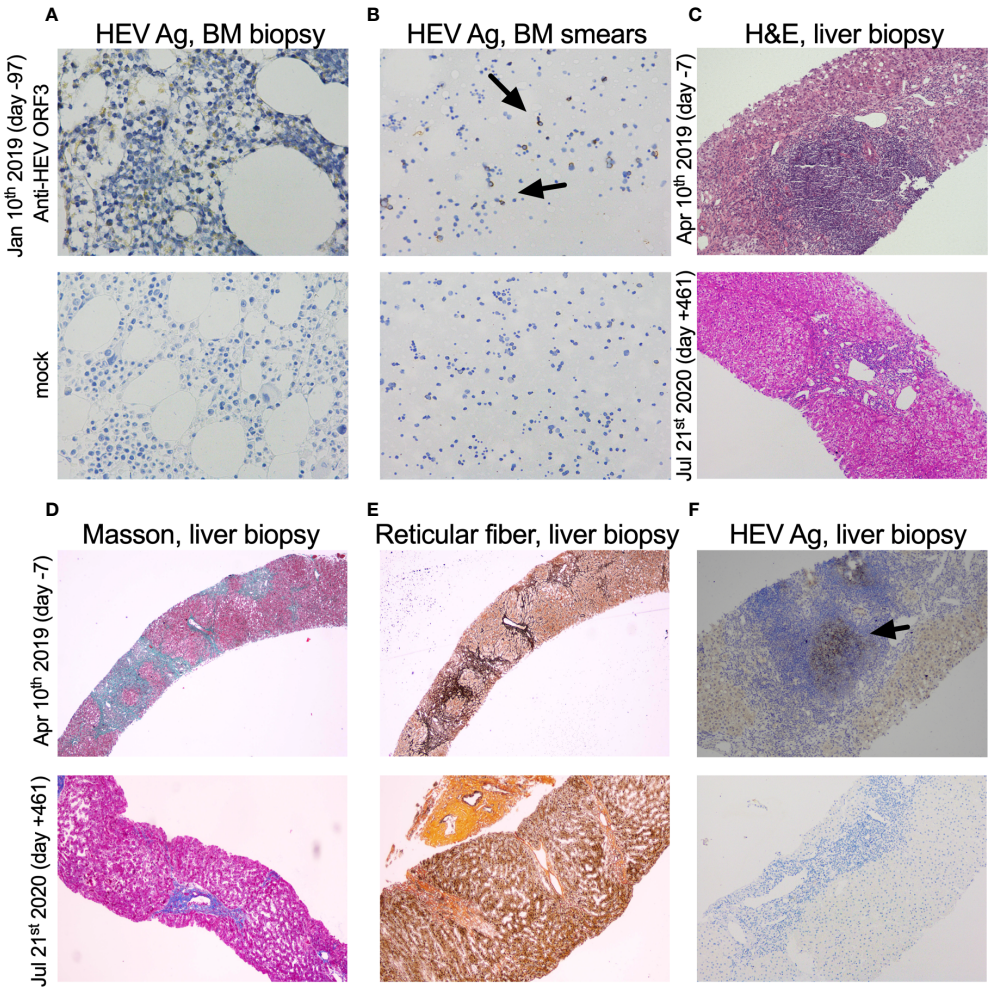

FIGURE 3
Immunohistochemical detection of HEV antigen and pathological analysis of bone marrow and liver biopsy samples. **(A, B)** HEV antigen (HEV Ag) detection of bone marrow (BM) biopsy (×400) and smears (×200). BM biopsy and smears (Jan 10th 2019, day -97) collected from the case were shown. **(C)** H&E of the liver biopsy (×100). **(D, E)** Masson trichrome and reticular fiber staining of liver biopsy (×40). **(F)** HEV Ag detection of liver biopsy (×100). Liver biopsy samples prior to and after oral ribavirin (Apr 17th 2019, day 0) were shown above (Apr 10th 2019, day -7) and below (Jul 21st 2020, day +461), respectively. The time of sample was designated on the left side of each row of pictures. HEV-specific anti-ORF3 1E6 antibodies (Millipore, Temecula, CA) were used in IHC of HEV Ag.

immunosuppressive alleviation (11, 26). Consequently, ribavirin should be considered firstly and early in HSCT recipients with HEV infection instead of immunosuppressive alleviation. Red cell distribution width (RDW), neutrophil to lymphocyte ratio (NLR), and RDW to lymphocyte ratio (RLR) are capable of predicting the development of liver failure during treatment (27).

The first case of chronic HEV infection in patients with hematological diseases was reported by 7 in Canada, in which chronic HEV G3 (subtype 3a) infection was observed in a child with acute lymphoblastic leukemia after HSCT (7). In 2014, Geng et al. reported the first chronic HE case with lymphoblastic leukemia caused by HEV G4 (6). In another two studies, four cases of chronic HE caused by G4 in renal transplant recipients were reported by Sridhar et al. and Wang et al. (28, 29). Future research into G4 chronic HEV in SOT or non-SOT recipients should be considered.

In this study, HEV-G4-induced chronic HE was observed in a male patient with severe AA. Although Mallet et al. reported that patients who were treated with ribavirin for three months cleared HEV and achieved sustained virological response (SVR) rates of ~80% (30), HEV Ag remained positive in specimens at 103 days after treatment in our case. The extended duration (six months) for antiviral therapy was considered. One study emphasized that, although serum and feces may be negative for viral RNA due to inhibition of HEV replication by drug (ribavirin and others), viral Ag can still be detected, suggesting that the virus may enter liver cells under a low replication status (31). The liver biopsy indicated

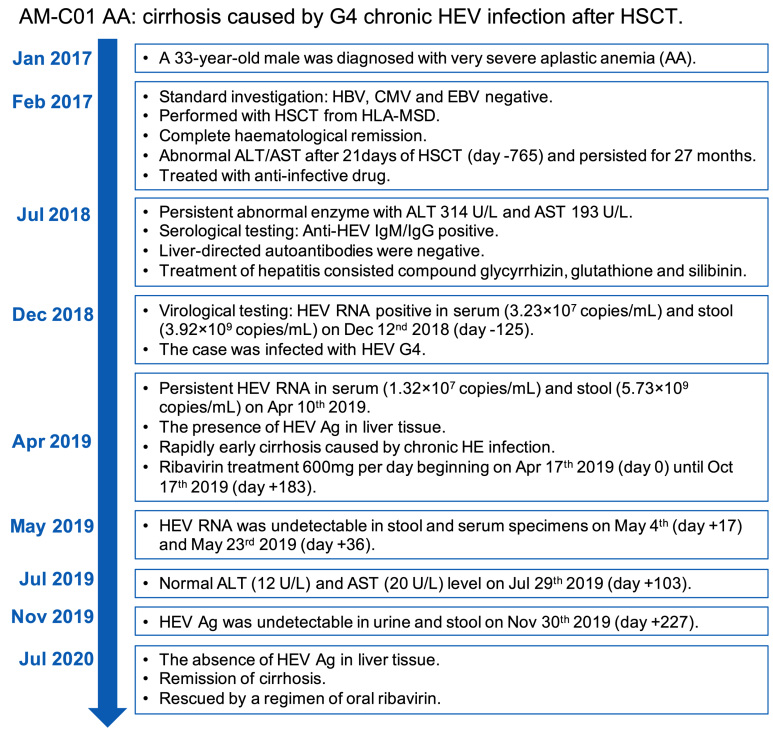

FIGURE 4
Overview of the case's diagnosis, management, and follow-up process and timelines of major clinical events.

fibrosis and cirrhosis in the patient before antiviral treatment. In the early stage of liver cirrhosis, most liver cells and tissue maintain a normal physiological status, and timely treatment may effectively reduce liver inflammation. Indeed, liver function could be partially restored after the removal of pathogenic factors in the patient.

In summary, we report the first case, as far as we know, of G4 HEV-induced chronic infection in a non-SOT recipient. It is also the sixth reported case of chronic HE caused by G4. The study suggests that HEV G4-infected patients may be at risk of developing chronic hepatitis if they are also HSCT recipients.

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 School of Public Health, Xiamen University. The patients/participants provided their written informed consent to participate in this study.

Author contributions

Conceptualization, ZT, ZZ, LY, and NX. Methodology, ZC, JW, LJ, DY, WT, MZ, SW, CL, YW, and TW. Resources, JW, LJ, MZ, YW, TW, ZT, ZZ, LY, and NX. Writing—original draft preparation, ZC and ZT. Writing—review and editing, ZC, GW, CL, and ZT. Funding acquisition, GW, ZT, ZZ, and NX. All authors contributed to the article and approved the submitted version.

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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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Effect of pegylated interferon-α2b add-on therapy on renal function in chronic hepatitis B patients: A real-world experience

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Background and aim: Controversy remains as to pegylated interferon-α (PEG-IFNα) antiviral therapy to renal function in chronic hepatitis B (CHB) patients. The aim of this study was to evaluate the influence of PEG-IFNα2b (Y shape, 40 kD) add-on treatment for renal function in CHB patients who received entecavir therapy.

Methods: This was a retrospective observational study to investigate factors related to renal function in 114 CHB patients who received PEG-IFNα2b add-on therapy to entecavir for 48 weeks. Changes of blood urea nitrogen (BUN), serum creatinine (sCr), and estimated glomerular filtration rate (eGFR), which was calculated with both Chronic Kidney Disease Epidemiology Collaboration and Modification of Diet in Renal Disease (MDRD) formulas, were analyzed by one-way analysis of variance. A linear mixed effects model for repeated measures was used to assess the correlation between baseline information and eGFR changes at 24 and 48 weeks of therapy. The model considered the baseline age, gender, body weight, viral load, hepatitis B surface antigen, BUN, sCr, and treatment strategy as fixed effects and incorporated random effects for individual subjects.

Results: BUN and sCr was decreased, while eGFR was increased at 12 weeks of therapy. Only eGFR maintained at 24 and 48 weeks of therapy. Patients with female gender, age $\geq$ 40 years, and baseline HBsAg level $<$ 250 IU/mL showed significant improvement of renal function with PEG-IFNα2b add-on therapy. The linear mixed effects model revealed that female gender, baseline sCr, and

PEG-IFNα2b add-on were significant positive predictors for eGFR elevation at 24 and 48 weeks of therapy.

Conclusion: In real-world experience, PEG-IFNα2b add-on therapy might be associated with increased eGFR in CHB patients.

KEYWORDS

chronic hepatitis B, renal function, pegylated interferon-α2b, antiviral, mixed linear model

Introduction

Hepatitis B virus (HBV) infection is still a world-wide public health challenge. There is a close relationship between chronic hepatitis B (CHB) and chronic renal disease (Combes et al., 1971). HBV infection is associated with nephropathy, and renal dysfunction is frequently observed in patients with CHB and liver cirrhosis (Ren et al., 2019). HBV-induced impairment of renal function is mainly through deposition of immune complex in the kidney, leading to membranous nephropathy and mesangiocapillary glomerulonephritis (Gupta and Quigg, 2015; Shah and Amarapurkar, 2018). The potential risk factors contributing to renal dysfunction in CHB patients include elder age, co-infection with human immunodeficiency virus (HIV), pre-existing renal failure and comorbidities, hypertension, diabetes mellitus, end-stage liver diseases, and administration of nephrotoxic agents (Mallet et al., 2015; Rodriguez-Novoa et al., 2016; Yang and Choi, 2017; Zhang et al., 2017). Thus, it is pivotal to monitor renal function to avoid potential nephrotoxic effects before and during anti-HBV treatment by determination of blood urea nitrogen (BUN), serum creatinine (sCr), and estimated glomerular filtration rate (eGFR) (Ren et al., 2019).

HBV-associated renal diseases always improve through inhibition of viral replication mediated by antiviral treatments (Kupin, 2017). Therapeutic approaches for CHB include nucleos(t)ide analogs (NAs) and interferon-α. Six NAs are available for CHB treatment in China, including three nucleoside analogs [lamivudine (LAM), telbivudine (LdT), and entecavir (ETV)] and three nucleotide analogs [adefovir (ADV), tenofovir disoproxil fumarate (TDF), and tenofovir alafenamide (TAF)]. Treatment of CHB irrespective of medication, including LAM, ADV, ETV, and TDF, seems to result in a mild decrease of renal function (Mauss et al., 2011; Udompap et al., 2018), because the primary route of NAs elimination is renal excretion with unchanged drugs (Zhang et al., 2017). Patients receiving ADV and TDF therapy even experience more rapid loss in eGFR (Udompap et al., 2018). In contrast, several studies suggested that TDF is not associated with greater degree of kidney injury compared with other NAs (Trinh et al., 2019; Fischer et al., 2021) and untreated HBV-infected individuals (Wang et al., 2021). LdT has been demonstrated to improve renal function in CHB patients (Gane et al., 2014), in liver transplant recipients for HBV-related cirrhosis (Cholongitas et al., 2015), and in liver transplant recipients with long-term chronic kidney disease (Lee et al., 2017). However, the renal protective effect of LdT is still uncertain for hepatitis B surface antigen (HBsAg)-positive renal transplant recipients (Yang et al., 2020). Importantly, TAF reveals continuous improvement of kidney function in both treatment-naïve CHB (Agarwal et al., 2018) and TDF-experienced patients (Fong et al., 2019; Farag et al., 2021).

Pegylated interferon-α (PEG-IFNα) is also one of the first-line antiviral agents due to higher HBsAg loss, finite therapeutic duration, and absence of drug resistance (Charatcharoenwitthaya et al., 2021), leading to functional cure of CHB (Ning et al., 2019). Combination of PEG-IFNα and NAs therapy may improve the serological response, but remains controversial (Li et al., 2015; Bourliere et al., 2017). However, few studies focus on the safe renal profile of PEG-IFNα therapy in CHB patients. We previously showed that 48-week PEG-IFNα2a treatment revealed a renal protective effect for CHB patients (Zhang et al., 2017). In contrast, Su et al. (2018) showed that PEG-IFNα2b monotherapy or combined with ADV therapy did not cause further renal impairment. Herein, we aimed to assess the change of renal function under PEG-IFNα2b (Y shape, 40 kD) add-on therapy to ETV in CHB patients.

Materials and methods

Institutional review board

The study protocol was approved by Ethics Committee of Tangdu Hospital (Approval No. TDLL-201505-013) and Ethics Committee of the Third People's Hospital of Taiyuan (Approval No. 2021-19). The study was conformed to the guidelines of the Declaration of Helsinki and the principles of Good Clinical Practice. Written consent was obtained from all patients, whose data were anonymized. The data were collected on April and May, 2022. We had access to information that could identify individual enrolled subjects during and after data collection.

TABLE 1 Baseline characteristics of enrolled patients.

Characteristic	Value
Patients enrolled, *n*	114
Male gender, *n* (%)	78 (68.42%)
Body weight (kg)	69.23 ± 13.18
Age, years, mean ± SD	41.49 ± 9.91
<40 years, *n* (%)	48 (42.11%)
≥40 years, *n* (%)	66 (57.89%)
HBV DNA undetectable (<50 IU/mL), *n* (%)	100 (87.72%)
HBV DNA detectable (>50 IU/mL), *n* (%)	14 (12.28%)
HBsAg, IU/mL, median (range)	182.9 (0.13∼1295.37)
HBsAg < 250 IU/mL, *n* (%)	63 (55.26%)
HBsAg > 250 IU/mL, *n* (%)	51 (44.74%)
HBeAg positive, *n* (%)	7 (6.14%)
HBeAg negative, *n* (%)	107 (93.86%)
BUN, mmol/L, mean ± SD	4.57 ± 1.13
sCr, μmol/L, mean ± SD	65.60 ± 12.07
eGFR (CKD-EPI), mL/min/1.73 m^2, mean ± SD	111.0 ± 12.46
eGFR (MDRD), mL/min/1.73 m^2, mean ± SD	116.9 ± 22.22

SD, standard deviation; HBsAg, Hepatitis B surface antigen; HBeAg, Hepatitis B e antigen; BUN, blood urea nitrogen; sCr, serum creatinine; eGFR, estimated glomerular filtration rate; CKD-EPI, Chronic Kidney Disease Epidemiology Collaboration; MDRD, Modification of Diet in Renal Disease.

Study design

The retrospective observational cohort study was conducted. Inclusive criteria: (1) Diagnosis of CHB met the diagnostic standard of Chinese National Program for Prevention and Treatment of Viral Hepatitis; (2) Patients had received ETV therapy for more than 1 year; (3) HBV DNA level < 1,000 IU/mL. (4) HBsAg level < 1,500 IU/mL, because baseline HBsAg less than 1,500 IU/mL was associated with high rate of HBsAg loss (Hu et al., 2018; Ning et al., 2019; Wu et al., 2020). Exclusive criteria: (1) Co-infected with other hepatitis viruses or HIV; (2) Concurrently afflicted by end-stage liver diseases (including decompensated liver cirrhosis, severe hepatitis, liver failure, and hepatocellular carcinoma); (3) Afflicted by autoimmune disorders; (4) Afflicted by alcoholism or drug addiction; (5) Afflicted by solid cancers or leukemia. The enrolled patients received PEG-IFNα2b (Y shape, 40 kD; 180 μg, subcutaneous injection weekly; Xiamen Amoytop Biotech Co., Ltd., Xiamen, Fujian Province, China) add-on therapy to ETV (0.5 mg, orally once daily) for 24∼48 weeks between July 2018 and March 2022 in Tangdu Hospital or the Third People's Hospital of Taiyuan. "Add-on" strategy was defined as addition of PEG-IFN to on-going NAs therapy in virally suppressed patients (Ning et al., 2019). The five observation time point was baseline, 12, 24, 36, and 48 weeks post add-on of PEG-IFNα2b. The blood samples were collected from each enrolled patients at least in three observation time points.

Evaluation of virological index

Serum HBV DNA was quantified by real-time fluorescence quantitative polymerase chain reaction using a commercial HBV DNA detection (Xiamen Amplly, Xiamen, Fujian Province, China) with a detection limit of 50 IU/mL. HBsAg, anti-HBs, hepatitis B e antigen (HBeAg), anti-HBe was quantified using the ARCHITECH HBsAg, anti-HBs, HBeAg, and anti-HBe reagent kit (Abbott GmbH & Co., KG., Wiesbaden, Germany), respectively.

Assessment of renal function

BUN and sCr was measured using an automatic analyzer (Hitachi 7170A, Hitachi Ltd., Tokyo, Japan). The eGFR was estimated using the following formulas based on age and sCr as previously described (Zhang et al., 2017). The Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) calculation for eGFR (mL/min/1.73 m^2) = 141 × min (sCr/κ, 1)$^{\alpha}$ × max (sCr/κ, 1)$^{-1.209}$ × 0.993^{age} × 1.018 (if female). κ is 0.7 for female and 0.9 for male. α is −0.329 for female and −0.411 for male (Levey et al., 2009). The Modification of Diet in Renal Disease (MDRD) calculation for eGFR (mL/min/1.73 m^2) = 186 × sCr$^{-1.154}$ × age$^{-0.203}$ × 0.742 (if female) (Levey et al., 1999).

Statistical analysis

SPSS 23.0 was used for general statistical analysis. Shapiro-Wilk test was used for normal distribution assay. The variables following normal distribution were presented as mean ± standard deviation (SD), and the statistical significance was determined by one-way analysis of variance (ANOVA) followed by Tukey test. The variables following skewed distribution were presented as median (range). To evaluate the association between several variables and eGFR changes over time, a linear mixed effects model for repeated measures was used by SAS 9.4 with MIXED procedure. The model considered the baseline age (in years), gender, body weight, HBV DNA, HBsAg, BUN, sCr, and treatment strategy as fixed effects and incorporated random effects for individual subjects. All *P*-values are two-sided, and type I error was set as 5%.

Results

Characteristics of enrolled patients

A total of 114 CHB patients with PEG-IFNα2b add-on therapy were enrolled in this study. Baseline characteristics for patients were shown in **Table 1**. Fourteen (12.28%) CHB

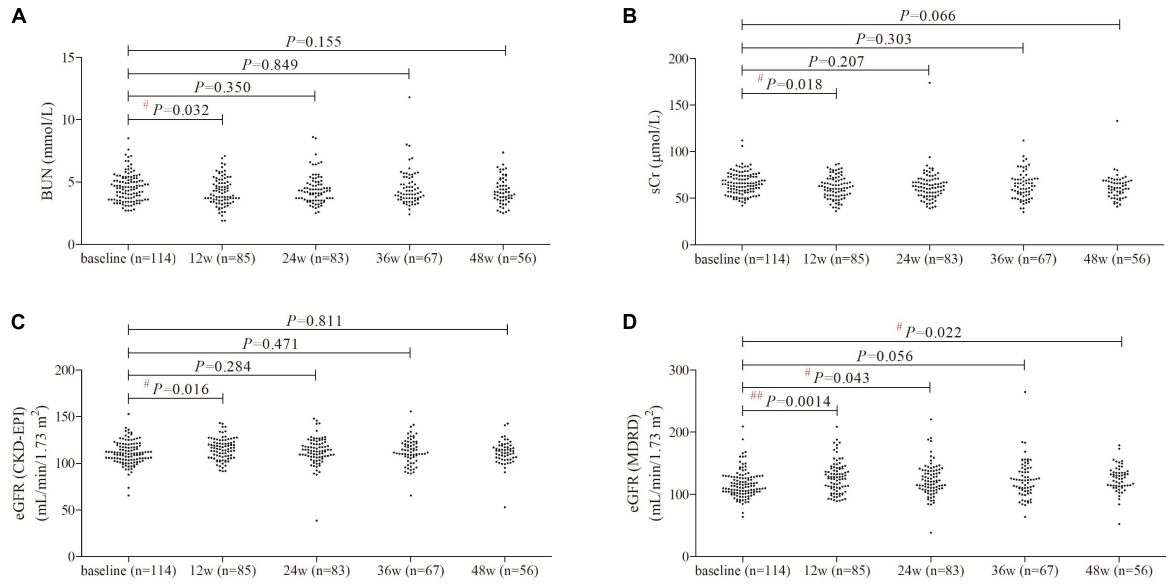

FIGURE 1
Evolution of renal function by PEG-IFNα2b add-on therapy over 48 weeks. **(A)** Change of BUN. **(B)** Changes of sCr. **(C)** Changes of eGFR as calculated by CKD-EPI formula. **(D)** Changes of eGFR as calculated by MDRD formula. Individual level for each value was shown. Statistical analysis was performed using one-way ANOVA followed by Tukey test.

patients had detectable serum HBV DNA with low level viremia [89 (52∼712) IU/mL]. Baseline HBsAg level was 182.9 (0.13∼1295.37) IU/mL. Based on CKD-EPI formula, three patients showed an eGFR less than 90 mL/min/1.73 m^2. Based on MDRD formula, seven patients revealed an eGFR less than 90 mL/min/1.73 m^2. No patients showed a baseline eGFR less than 60 mL/min/1.73 m^2 based on both formulas. All patients received at least 24-week PEG-IFNα2b therapy. Twenty two patients withdrew from PEG-IFNα2b therapy due to poor response (less than 10% of HBsAg down-regulation compared with baseline level) at 24 weeks of treatment, and 36 patients withdrew at 36 weeks of therapy. Fifty six (49.12%) patients completed 48-week PEG-IFNα2b add-on therapy.

Changes in renal function in response to pegylated interferon-α2b add-on therapy

BUN and sCr level was reduced at 12 weeks of therapy ($P < 0.05$), but there were no significant differences of either BUN or sCr level at 24, 36, or 48 weeks of therapy compared with baseline ($P > 0.05$, Figures 1A,B). Based on CKD-EPI formula, eGFR was increased at 12 weeks of therapy compared with baseline (+4.2 mL/min/1.73 m^2, $P = 0.016$, Figure 1C), but did not remarkably change at 24, 36, or 48 weeks of therapy ($P > 0.05$, Figure 1C). One patient had lowest eGFR CKD-EPI level (38.54 mL/min/1.73 m^2) at 24 weeks of therapy whose baseline level was 65.64 mL/min/1.73 m^2, and eGFR CKD-EPI level was 52.95 mL/min/1.73 m^2 at 48 weeks of therapy. Based on MDRD formula, there was maintenance of eGFR improvement during 48 weeks of PEG-IFNα2b add-on treatment (Figure 1D). eGFR MDRD reached peak level at 12 weeks of therapy (127.9 ± 25.49 mL/min/1.73 m^2, $P = 0.0014$, Figure 1D), and maintained at 125.1 ± 21.00 mL/min/1.73 m^2 at 48 weeks of treatment ($P = 0.022$, Figure 1D). Based on the MDRD formula, two patients had eGFR less than 90 mL/min/1.73 m^2 at 48 weeks of therapy.

Predictors of significant renal function changes in response to pegylated interferon-α2b add-on therapy

BUN level revealed statistical down-regulation at 12 weeks of therapy in male gender, elder patients, and patients with baseline HBsAg less than 250 IU/mL ($P < 0.05$, Figure 2A). sCr level was significantly decreased in female gender at 12 weeks of therapy, and maintained in lower lever at 36 and 48 weeks of therapy in female gender (Figure 2B). However, sCr level was only statistically reduced at 12 weeks of therapy in male gender ($P = 0.028$, Figure 2B) and in patients with age ≥ 40 years ($P = 0.039$, Figure 2B). Based on CKD-EPI formula, eGFR level did not remarkably change over time in either genders ($P > 0.05$, Figure 2C). eGFR CKD-EPI level was increased at 12 weeks of therapy in patients with age ≥ 40 years ($P = 0.012$, Figure 2C) and in patients with baseline HBsAg less than 250 IU/mL ($P = 0.034$, Figure 2C). Importantly, based on MDRD formula,

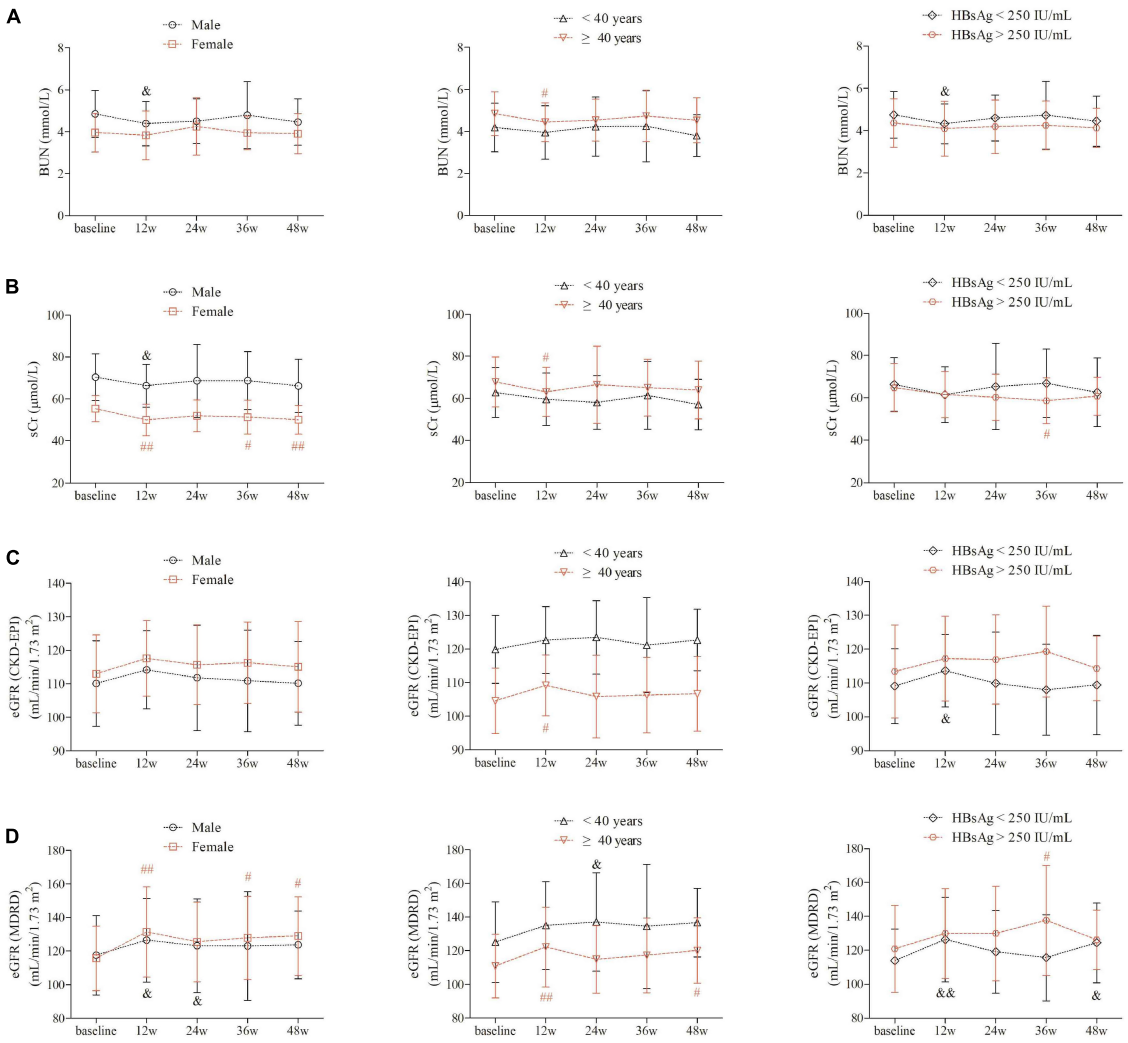

FIGURE 2
Evolution of renal function by PEG-IFNα2b add-on therapy over 48 weeks under different factors. **(A)** Change of BUN between different gender, between different age (<40 years and ≥40 years), and between different baseline HBsAg level (<250 and >250 IU/mL). **(B)** Changes of sCr between different gender, between different age (<40 and ≥ 40 years), and between different baseline HBsAg level (<250 and >250 IU/mL). **(C)** Changes of eGFR as calculated by CKD-EPI formula between different gender, between different age (<40 and ≥40 years), and between different baseline HBsAg level (<250 and >250 IU/mL). **(D)** Changes of eGFR as calculated by MDRD formula between different gender, between different age (<40 and ≥40 years), and between different baseline HBsAg level (<250 and >250 IU/mL). Statistical analysis was performed using one-way ANOVA followed by Tukey test. $^{\&}$ and $^{\#}$indicates $P < 0.05$ compared with baseline. $^{\&\&}$ and $^{\#\#}$indicates $P < 0.01$ compared with baseline.

eGFR was robustly elevated at 12 weeks of therapy, and was maintained in higher level in female gender, patients with age ≥40 years and in patients with baseline HBsAg less than 250 IU/mL ($P < 0.05$, Figure 2D).

Furthermore, we entered all variables as fixed effects and incorporated random effects in the linear mixed model accounting for repeated measures. Previous studies have been demonstrated that ETV therapy was not associated with the either improvement or deterioration of renal function in CHB patients (Park et al., 2017; Zhang et al., 2017; Suzuki et al., 2019), ETV monotherapy in 44 CHB patients who were continuously monitored renal function over 48 weeks was set as reference in this model. Results with CKD-EPI and MDRD equation showed comparable predictor value for eGFR changes at 24 weeks of PEG-IFNα2b add-on therapy. Female gender, baseline detectable HBV DNA, sCr, and PEG-IFNα2b add-on were significant predictors for increase eGFR (Table 2). At 48 weeks of therapy, female gender was also a positive predictor for eGFR MDRD. Female gender also revealed an estimated value of 4.135 for eGFR CKD-EPI, but it failed to achieve statistical difference ($P = 0.075$, Table 3). Baseline sCr and PEG-IFNα2b add-on were observed to be positively affected eGFR values at 48 weeks of

TABLE 2 Predictors for eGFR changes at 24 weeks of therapy.

	eGFR (CKD-EPI)			eGFR (MDRD)		
	Estimate	**Standard error**	***P*-value[a]**	**Estimate**	**Standard error**	***P*-value[a]**
Female gender	3.915	1.690	0.021	11.553	4.740	0.015
Body weight	−0.076	0.056	0.169	−0.074	0.143	0.603
Age	−0.043	0.066	0.516	−0.146	0.178	0.412
HBV DNA > 50 IU/mL (baseline)	3.065	1.132	0.007	8.022	3.208	0.012
HBsAg level > 250 IU/mL (baseline)	−0.002	0.003	0.397	−0.008	0.007	0.253
BUN (baseline)	−0.282	0.549	0.608	−0.574	1.465	0.695
sCr (baseline)	0.218	0.075	0.004	0.418	0.206	0.043
PEG-IFNα2b add-on	3.655	1.532	0.017	9.413	4.032	0.020

[a]Results from the linear mixed effects model for repeated measures.

TABLE 3 Predictors for eGFR changes at 48 weeks of therapy.

	eGFR (CKD-EPI)			eGFR (MDRD)		
	Estimate	**Standard error**	***P*-value[a]**	**Estimate**	**Standard error**	***P*-value[a]**
Female gender	4.135	2.326	0.075	17.090	5.055	0.0007
Body weight	−0.101	0.057	0.077	−0.153	0.115	0.183
Age	0.046	0.072	0.519	0.149	0.187	0.425
HBV DNA > 50 IU/mL (baseline)	2.268	1.311	0.084	2.505	3.294	0.447
HBsAg level > 250 IU/mL (baseline)	−0.000	0.0001	0.688	0.000	0.0001	0.998
BUN (baseline)	0.561	0.711	0.430	1.369	1.647	0.406
sCr (baseline)	0.432	0.091	< 0.0001	1.078	0.207	< 0.0001
PEG-IFNα2b add-on	6.780	1.559	< 0.0001	14.949	3.896	0.0001

[a]Results from the linear mixed effects model for repeated measures.

therapy (all $P < 0.001$, Table 3). However, the changes of eGFR was not remarkably associated with body weight, age, baseline HBsAg level, or baseline BUN ($P > 0.05$, Tables 2, 3).

Discussion

Chronic HBV infection robustly elevated the risk of end-stage renal diseases (Chen et al., 2015). Inhibition of HBV replication by antiviral agents could decrease HBsAg level, leading to the reduction or even clearance of HBV antigenemia and further improvement of renal impairment in patients with hepatitis B related-glomerulonephritis (Liu et al., 2020). However, antiviral therapy might also result in deterioration of kidney injury due to the nephrotoxicity of drugs (Liu and Kao, 2019). Herein, we designed to assess the renal function of CHB patients who received PEG-IFNα2b (Y shape, 40 kD) add-on therapy to ETV. The important finding was that eGFR improved remarkably in response to PEG-IFNα2b combined with ETV treatment over 48 weeks. While ETV therapy showed dispensable effect to renal function (Zhang et al., 2017), PEG-IFNα2b might mainly contribute to the renal protective effect during the combination therapy. Interestingly, female gender was proven to be an important positive predictive factor for eGFR changes during PEG-IFNα2b plus ETV therapy under both one-way ANOVA analyses and linear mixed effects model for repeated measures. Collectively, PEG-IFNα2b add-on therapy might closely associate with renoprotective effect for CHB treatment.

Controversy remained as to the IFNα monotherapy or combination with NAs therapy to renal function. Recombinant IFNα2b improved immune response to hepatitis B vaccination in hemodialysis patients (Miquilena-Colina et al., 2009), indicating the safety profile of IFNα2b to patients with renal dysfunction. Renal allograft recipients who had chronic hepatitis B, C, and D were treated with IFNα three times weekly for 6 months. Renal allograft function remained stable in 31 patients (73.81%) during IFNα therapy, and the antiviral efficiency was only mild to moderate during long-term followed-up (Durlik et al., 1998). IFNα was not recommended for renal allograft recipients with hepatitis virus infection (Durlik et al., 1998). eGFR level was notably lower in patients receiving ADV therapy compared with PEG-IFNα2a treatment in chronic hepatitis B/D co-infection (Mederacke et al., 2012). Importantly, Combination treatment of PEG-IFNα2a plus ADV did not lead to further renal dysfunction (Mederacke et al., 2012),

indicating the potential renal protective effect of PEG-IFNα2a, which might counteract the nephrotoxicity of ADV. The current finding of the renoprotective effect of PEG-IFNα2b (Y shape, 40 kD; 180 μg) add-on therapy was consistent with our previous reports on PEG-IFNα2a (U shape, 40 kD; 180 μg) monotherapy in both treatment-naïve and ETV-experienced CHB patients (Zhang et al., 2017), however, was not in line with the findings of PEG-IFNα2b (line shape, 12 kD; 1.5 μg/kg) monotherapy and combined with ADV treatment in CHB patients, which showed the improvement of renal function at 4 weeks of therapy but steadily declined over 48 weeks (Su et al., 2018). In our opinions, the differential effect of PEG-IFNα2b (Y shape, 40 kD; 180 μg) and PEG-IFNα2b (line shape, 12 kD; 1.5 μg/kg) might mainly due to the different molecule weight of PEG and branched monomethoxy PEG (Monfardini et al., 1995). The branched PEG (Y shape and U shape) demonstrated higher molecule weight than linear PEG (40 kD vs. 12 kD). Moreover, the proteins modified by branched PEG revealed not only elevated *in vitro* activity and proteolytic resistance, but also improved stability toward temperature and pH variations as well as enhanced half-life (Monfardini et al., 1995). Thus, PEG-IFNα2a (U shape, 40 kD) and PEG-IFNα2b (Y shape, 40 kD) could maintain higher concentration in peripheral blood than PEG-IFNα2b (line shape, 12 kD). Both high molecule weight and branched shape might contribute to the significant reduction in renal excretion ratio of drugs, resulting in the potential renoprotective priority of branched PEG modified IFNα. Furthermore, it is generally elucidated that deposition of immune complexes of HBV antigens and host antibodies mediate glomerular injuries. Peripheral $CD4^{+}CXCR5^{+}$ follicular T helper (Tfh) cell frequency was negatively correlated with the value of eGFR (Liu et al., 2014), suggesting that Tfh might contribute to IFN-induced improvement of eGFR. However, the specific mechanisms by which PEG-IFNα2b (Y shape, 40 kD) exert the renoprotective effects remains to be clarified.

We then investigated the potential indicators associated with eGFR changes over time. Our previous study revealed that age and baseline BUN were significant negative predictors for eGFR changes in CHB patients receiving either PEG-IFNα2a or NAs therapy (Zhang et al., 2017). Su et al. (2018) showed that age, baseline HBV DNA, and ADV-containing therapy are important predictable factors for eGFR decrease in patients with PEG-IFNα2b therapy, which was similar to the findings by Mederacke et al. (2012) in chronic hepatitis B/D co-infection. Herein, we analyzed the potential predictors for eGFR changes in response to PEG-IFNα2b (Y shape, 40 kD) add-on therapy to ETV *via* two different statistical methods. On the one hand, we compared the changes renal function over time between different genders, ages, and baseline HBsAg level using one-way ANOVA. We found that patients with female gender, age $\geq$ 40 years, and baseline HBsAg level $<$ 250 IU/mL showed significant improvement of renal function with PEG-IFNα2b add-on therapy. However, we did not involve baseline HBV DNA level as one of the potential factors due to the limited cases with detectable HBV DNA (n = 14). On the other hand, we entered all baseline variables as fixed effects and incorporated random effects in a linear mixed model accounting for repeated measures. The results showed that female gender, baseline sCr, and PEG-IFNα2b add-on were significant positive predictors for eGFR elevation at 24 and 48 weeks of therapy. Although detectable HBV DNA was the positive indicator for eGFR changes at 24 weeks, it failed to achieve statistical significance at 48 weeks of therapy. Collectively, female gender might be the most important positive predictor for renoprotection during PEG-IFNα2b (Y shape, 40 kD) add-on therapy.

There were several limitations of the present study. Firstly, this was a retrospective analysis of renal function in a real-world setting. The followed-up data were not complete in several patients. We collected the data in at least three observation time points to reduce errors and bias. The large scale prospective study with longer observational time (both during and post treatment) are needed to confirm the current findings. Secondly, NAs might be harmful to both glomerular and tubular cells in the kidney (Kayaaslan and Guner, 2017; Wong et al., 2018). The mechanisms and target cells of PEG-IFNα2b (Y shape, 40 kD) for renoprotective effect were still unclear.

Conclusion

In summary, our present results provided the evidence that PEG-IFNα2b (Y shape, 40 kD) add-on treatment might contribute to the increased eGFR for CHB patients in real-world experience. The mechanisms underlying the beneficial effects remained to be further clarified.

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 Ethics Committee of Tangdu Hospital and Ethics Committee of the Third People's Hospital of Taiyuan. The patients/participants provided their written informed consent to participate in this study.

Author contributions

YZ and YG contributed to study concept, design, and manuscript revision. M-JP, X-QG, JC, WK, and X-FY

contributed to data acquisition. M-JP, W-LZ, YG, and YZ contributed to data analysis. M-JP, X-QG, W-LZ, and JC contributed to manuscript drafting. All authors approved the final version of the manuscript.

Conflict of interest

Author JC was employed by the Xiamen Amoytop Biotech Co., Ltd.

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.

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Micro-PET imaging of hepatitis C virus NS3/4A protease activity using a protease-activatable retention probe

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Hepatitis C virus (HCV) NS3/4A protease is an attractive target for direct-acting antiviral agents. Real-time tracking of the NS3/4A protease distribution and activity is useful for clinical diagnosis and disease management. However, no approach has been developed that can systemically detect NS3/4A protease activity or distribution. We designed a protease-activatable retention probe for tracking HCV NS3/4A protease activity *via* positron emission topography (PET) imaging. A cell-penetrating probe was designed that consisted of a cell-penetrating Tat peptide, HCV NS3/4A protease substrate, and a hydrophilic domain. The probe was labeled by fluorescein isothiocyanate (FITC) and ^{124}I in the hydrophilic domain to form a TAT-ΔNS3/4A-^{124}I-FITC probe. Upon cleavage at NS3/4A substrate, the non-penetrating hydrophilic domain is released and accumulated in the cytoplasm allowing PET or optical imaging. The TAT-ΔNS3/4A-FITC probe selectively accumulated in NS3/4A-expressing HCC36 (NS3/4A-HCC36) cells/tumors and HCV-infected HCC36 cells. PET imaging showed that the TAT-ΔNS3/4A-^{124}I-FITC probe selectively accumulated in the NS3/4A-HCC36 xenograft tumors and liver-implanted NS3/4A-HCC36 tumors, but not in the control HCC36 tumors.

The TAT-ΔNS3/4A-^{124}I-FITC probe can be used to represent NS3/4 protease activity and distribution *via* a clinical PET imaging system allowing. This strategy may be extended to detect any cellular protease activity for optimization the protease-based therapies.

KEYWORDS

cellular protease activity, HCV NS3/4A serine protease, protease-activated retention peptide, micro-positron emission tomography, TAT-ΔNS3/4A-^{124}I-FITC probe

Introduction

The World Health Organization (WHO) estimates that worldwide, at least 170 million people (3% of the world population) are infected with hepatitis C virus (HCV) (Wasley and Alter, 2000; Kondou, 2022). HCV infection often leads to chronic infection and is associated with high risk of the development of liver cirrhosis and hepatocellular carcinoma (Jahan et al., 2012; Fu et al., 2018; Glitscher et al., 2022; Yoo et al., 2022). For example, HCV NS3/4A protease highly expressed in HCV-infected patients is required for viral replication (Tomei et al., 1993) and virus particle assembly (Phan et al., 2011). The NS3/4A protease activity has been reported that is highly associated with the development of liver cirrhosis and hepatocellular carcinoma (McGivern and Lemon, 2009; Presser et al., 2013; Sakata et al., 2013). Sakata and colleagues indicated that the HCV NS3/4A protease enhances liver fibrosis *via* binding to and activating TGF-β type I receptor in HCV-infected chimeric mice (Laurent et al., 2013). Given their relevance, NS3/4A proteases is attractive targets for the design of antiviral drugs (Cai et al., 2020). Currently, the boceprevir and telaprevir are the protease inhibitors and that are approved by FDA for the treatment of HCV genotype 1 infected patients (Laurent et al., 2013; Deeks, 2014). Furthermore, many clinical studies have reported that blocking the NS3/4A protease activity using a protease inhibitor significantly reduces the virus load in patients (Lamarre et al., 2003; Salam and Akimitsu, 2013; Hayashi et al., 2014), indicating that the HCV NS3/4A protease activity may be a useful marker to predict HCV viral activity and disease progression (Pan et al., 2019; Kim et al., 2021). Therefore, the technology to tracking the NS3 protease activity *in vivo* would provide a powerful tool to design the personalized protease inhibitor-based therapies and to monitor the development of liver cirrhosis and hepatocellular carcinoma (Hasegawa et al., 2015).

However, the current approaches to detect HCV NS3/4A protease activity *in vivo* were not sufficient. For example, several cell-based systems for monitoring NS3/4A activity have been reported (Lee et al., 2003; Boonstra et al., 2009). However, these cell-based systems cannot assess the efficacy, toxicity, and bioavailability of HCV NS3/4A protease inhibitors *in vivo*. To overcome this problem, Wang and colleagues developed a stable Huh7-[ANLuc(NS5A/B)BCLuc] cell line that can report the NS3/4A serine protease activity *via* bioluminescence imaging in living animals (Wang et al., 2010). However, the low tissue penetration of the luminescent signal limits these reporters to studies of small animals. Currently, the chimpanzee is still the animal of choice for the study of antiviral therapy against HCV (Abe et al., 1992), meaning that a higher sensitivity and more penetrative imaging probe is necessary. To overcome these challenges, we developed a PET probe that consisted of a cell-penetrating Tat peptide (GRKKRRQRRRPQ) (Vives et al., 1997), HCV NS3/4A protease substrate (DEDEDEDEMEECASHL) (Lee et al., 2003), and the hydrophilic domain (KKKYK). The probe was labeled by FITC and ^{124}I in the hydrophilic domain to generate the cell-permeable probe (TAT-ΔNS3/4A-^{124}I-FITC). Upon cleavage at NS3/4A substrate, the non-penetrating hydrophilic domain is released and accumulated in the cytoplasm and can be visualized by PET or optical imaging (**Figure 1**). We first examined whether TAT-ΔNS3/4A-FITC could selectively accumulate at sites with protease activity, including protease NS3/4A-expressing HCC36 (NS3/4A-HCC36) cells, HCV-infected HCC36 cells, and NS3/4A-containing HCC36 tumors. We also examined whether the TAT-ΔNS3/4A-^{124}I-FITC probe could be selectively retained in NS3/4A-HCC36 tumors in xenograft mice by micro-PET imaging. The radioactivity in selected tissues was then examined to quantify the probe biodistribution. Finally, we evaluated whether this probe could accumulate in NS3/4A-HCC36 liver-implanted mice through micro-PET imaging of mice. The successful development of a clinically available PET probe to represent NS3/4A activity would provide a valuable tool for real-time tracking of protease activity and distribution.

Materials and methods

Cells and animals

Human hepatocellular carcinoma cells HCC36 (American Type Culture Collection, Manassas, VA, United States) were

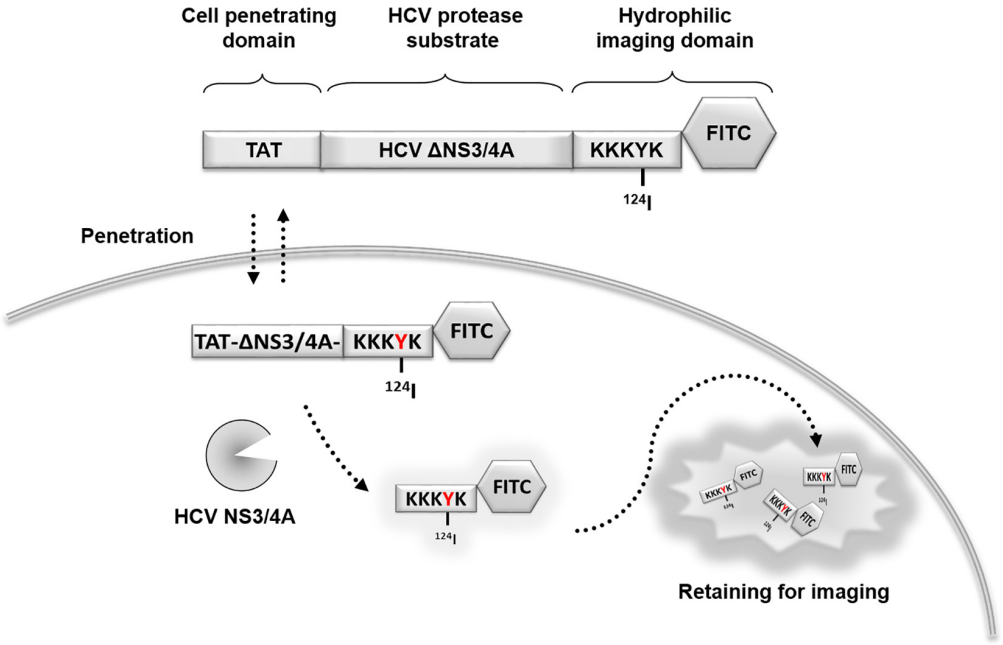

FIGURE 1
Schematic representation of the protease-activated retention micro-PET probe. Hepatitis C virus (HCV) tracking probe was designed using HCV NS3/4A serine protease as a marker of viral activity. A cell-penetrating probe was designed that consisted of a cell-penetrating Tat peptide, a HCV NS3/4A protease substrate, and a hydrophilic domain. For imaging, the probe was labeled by fluorescein FITC and ^{124}I in the hydrophilic domain to form a TAT-ΔNS3/4A-^{124}I-FITC probe. Upon cleavage at NS3/4A substrate, the non-penetrating hydrophilic domain is released and accumulated in the cytoplasm allowing visualization using PET or optical imaging. NS, non-structural protein; TAT, transactivator of transcription of human immunodeficiency virus; FITC, fluorescein isothiocyanate; HCV, hepatitis C virus; PET, positron emission tomography.

cultured in Dulbecco's Minimal Essential Medium (Sigma-Aldrich, Burlington, MA, United States) supplemented with 10% heat-inactivated bovine calf serum, 100 units/ml penicillin, and 100 μg/ml streptomycin (Sigma-Aldrich, Burlington, MA, United States) at 37°C in a humidified atmosphere of 5% CO2. NS3/4A-HCC36 cell lines by transfection of recombinant lentivirus consist package of plasmid: pCMVΔR8.91 (RNAi core, Academia Sinica, Taipei, Taiwan) and pMD.G (RNAi core, Academia Sinica, Taipei, Taiwan) and target plasmid: pLKO_AS3 NS3/4A, respectively; after selection by 1, 3, and 5 μg/ml puromycin dihydrochloride (P8833, Sigma-Aldrich, Burlington, MA, United States), the selection medium was replaced every 2 days for 1 week. Female BALB/c nude mice (6–8 weeks old) were obtained from the National Laboratory Animal Center, Taipei, Taiwan. All animal experiments were performed in accordance with institutional guidelines and approved by the Animal Care and Use Committee of Kaohsiung Medical University.

Synthesis of TAT-ΔNS3/4A-FITC

TAT-ΔNS3/4A-FITC (GRKKRRQRRRDEDEDEDEMEECASHLKKKYK-FITC) and scrambled sequence probe (GRKKRRQRRR-ECEEEEESMDDDAH-LKKKYK) were synthesized by Kelowna International Scientific (Taipei, Taiwan) and purified to > 95% purity by high-performance liquid chromatography [Merck Hibar C18 column, 4 × 250 mm; eluted at 1 mL/min with a gradient starting from 95% solvent A (0.1% trifluoroacetic acid in water) and 5% solvent B (0.1% trifluoroacetic acid in MeCN) to 5% solvent A and 95% solvent B at 30 min].

Specific retention of TAT-ΔNS3/4A-FITC in NS3/4A-expressing cells

HCC36 or NS3/4A-HCC36 cells (1×10^5) were seeded with DMEM containing 10% serum in a 24-well plate at 37°C in a CO_2 incubator overnight. The cells were incubated with 10 μM of TAT-ΔNS3/4A-FITC in the presence or absence of 2 μM telaprevir (purchased from Legend Star International, Taiwan) at 37°C for 1 h. After probe staining, the cells were washed using DMEM containing 10% serum per 20 min until the end of the experiment. After 8 h washing, the fluorescence of viable cells was observed under a fluorescence microscope at the indicated times (Axiovert 200, Carl Zeiss MicroImaging, GmbH, Germany).

Specific retention of TAT-ΔNS3/4A-FITC in hepatitis C virus-infected cells

Infectious HCV particles were generated as previously described (Hayashi et al., 2014). HCC36 cells (1×10^5) were seeded in DMEM containing 10% serum in a 24-well plate at 37°C in a CO_2 incubator overnight. The cells were infected with JFH-1 and incubated with or without 2 μM telaprevir. After 5 days, the parental HCC36 and JFH-1-infected cells were stained with 10 μM of TAT-ΔNS3/4A-FITCat 37°C for 1 h. After probe staining, the cells were washed using DMEM containing 10% serum per 20 min until the end of the experiment. After 8 h washing, the fluorescence of viable cells was observed under a fluorescence microscope at the indicated times (Axiovert 200, Carl Zeiss MicroImaging, GmbH, Germany).

Histological analysis of the fluorescence intensity and protease activity in tumors

BALB/c nude mice (n = 3) bearing established HCC36 and NS3/4A-HCC36 (100–200 mm^3) tumors in their right and left hind legs, respectively, were intravenously injected with 500 μM (in 100 μL) TAT-ΔNS3/4A-FITC and sacrificed 4 h later. Tumors were excised and embedded in OCT compound (Tissue-Tek, CA) in liquid nitrogen. The adjacent tumor sections either directly detect the FITC-fluorescent intensity to observe the accumulation of TAT-ΔNS3/4A-FITC probe or stained with a 520 HCV protease Assay Kit (AnaSpec) to visualize NS3/4A activity. The sections were examined on an upright BX4 microscope (Olympus, Melville, NY, USA) or viewed under phase contrast or fluorescence fields on an inverted Axiovert 200 microscope (Carl Zeiss Microimaging, Thornwood, NY, USA).

Synthesis and purification of TAT-ΔNS3/4A-^{124}I-FITC

^{124}I was purchased from IBA Molecular, VA, USA (2.6 TBq/mL, n.c.a.). Radio-iodination (nucleophilic aromatic addition) was performed by adding $Na^{124}I$ (2.6 TBq/mL, no carrier added) into TAT-ΔNS3/4A-FITC (0.5 mg, MW = 4339.8) with hydrogen peroxide solution (H_2O_2: HCl: H_2O = 16:16:68) as an oxidant under condition. The solution was reacted at room temperature for 10 min with vigorous vortexing, followed by adding 2 mM sodium thiosulfate solution and saturated sodium hydrogen carbonate to neutralize the solution. TAT-ΔNS3/4A-^{124}I-FITC was purified by C-18 sep-pak (Waters, Milford). The radiochemical purity of TAT-ΔNS3/4A-^{124}I-FITC was determined using HPLC (Delta 600, Waters). For HPLC analysis, a reverse phase column (Purospher STAR RP-18e, 10 × 250 mm, MERCK, Darmstadt, Germany) was used and eluted with acetonitrile (ACN)/0.1% TFA in water (3/97, v/v) at a flow rate of 4 mL/min.

Specificity and serum half-life of TAT-ΔNS3/4A-^{124}I-FITC

HCC36 or NS3/4A-HCC36 (1×10^5) cells were seeded with DMEM containing 10% serum in a 24-well plate at 37°C in a CO_2 incubator overnight. The cells were incubated with 37 kBq of TAT-ΔNS3/4A-^{124}I-FITC in the presence or absence of 2 μM telaprevir (Vertex, Cambridge MA) at 37°C for 1 h. After probe staining, the cells were washed using DMEM containing 10% serum per 20 min until the end of the experiment. At different time points, the cells were collected by treatment with trypsin. The radioactivity of the cells was then measured using a gamma-counter. The CPM was normalized by protein concentration.

BALB/c nude mice (n = 3) were intravenously injected with 3,700 kBq TAT-ΔNS3/4A-^{124}I-FITC, and blood samples were periodically removed from the tail vein of the mice. The blood was weighed on an analytical balance and assayed for radioactivity in a multichannel gamma-counter. The initial and terminal half-life of the probe was estimated by fitting the data to a two-phase exponential decay model with Prism 4 software (GraphPad Software, San Diego, CA, USA).

Micro-PET imaging of NS3/4A activity *in vivo*

BALB/c nude mice (n = 3) bearing established HCC36 and NS3/4A-HCC36 (100–200 mm^3) in their right and left hind leg, respectively, were anesthetized by halothane vapor with a vaporizer system. The mice were feed the Lugol's solution and then intravenously injected with 3,700 kBq (in 100 μL) TAT-ΔNS3/4A-^{124}I-FITC. PET imaging was sequentially performed at 2, 4, and 6 h. To test the specificity of TAT-ΔNS3/4A-^{124}I-FITC, telaprevir (20 mg/kg/day for 3 days) or control vehicle was intraperitoneally injected into mice (n = 3) 3 days prior to TAT-ΔNS3/4A-^{124}I-FITC.

The orthotopic liver implantation model was generated as previously described (Kim et al., 2009). The NS3/4A-HCC36 tumors or HCC36 tumors from the ectopic tumors were harvested and transplanted into the left liver lobe of SCID mice (n = 3). After 2 weeks, the mice were feed the Lugol's solution, and then, the 3,700 kBq of TAT-ΔNS3/4A-^{124}I-FITC was intravenously injected.

The mice were positioned in a micro-PET scanner (R4; Concorde Microsystems, Knoxville, Tenn) with their long axis

parallel to the transaxial plane of the scanner. The scanner has a computer-controlled bed with a 10.8-cm transaxial and 8-cm axial field of view. It has no septa and operates exclusively in a three-dimensional list mode. All raw data were first sorted into three-dimensional sinograms, followed by Fourier rebinning and ordered-subsets expectation maximization image reconstruction. Fully three-dimensional list-mode data were collected by using an energy window of 350–750 keV and a time window of 6 nsec. Image pixel size was 0.85 mm transaxially, with a 1.21-mm section thickness. The region of interest was analyzed with ASIPro VM version 5.0 (Concorde Microsystems, Knoxville, TN, USA) analysis software.

Biodistribution of TAT-ΔNS3/4A-^{124}I-FITC *in vivo*

In xenograft model, BALB/c nude mice bearing established NS3/4A-HCC36 and HCC36 tumors (n = 3) were intravenously injected with 3,700 kBq (in 100 μL) TAT-ΔNS3/4A-^{124}I-FITC. In orthotopic model, SCID mice (n = 3) with NS3/4A-HCC36 or HCC36 tumors implanted in the liver were intravenously also injected with 3,700 kBq (in 100 μL) TAT-ΔNS3/4A-^{124}I-FITC. Animals were killed after anesthesia with pentobarbital (65 mg/kg) at 2, 4, and 6 h. Radioactivity in isolated tumors and tissues was measured with a multichannel gamma-counter. The biodistribution of the probe was expressed as percentage injected dose per gram of tissue (%ID/g).

Statistical analysis

Statistical significance of differences between mean values was estimated with InStat software (version 3.0; GraphPad software) using the independent Student's t-test for unequal variances. P-values of less than 0.05 were considered statistically significant.

Results

Generation of NS3/4A-expressing cells

To setup NS3/4A stably expressing cells to visualize NS3/4A protease activity *in vitro* and *in vivo*, we constructed the NS3/4A gene (HCV JFH-1 2a strain) into a lentiviral vector, pLKO_AS3 NS3/4A, and the infected cells were selected *via* puromycin to directly express HCV NS3/4A protease in the human hepatocellular carcinoma HCC36 cells (NS3/4A-HCC36). The expression of NS3/4A protease was confirmed by Western blot using anti-NS3 antibodies (ab13830, Abcam UK). As shown in **Supplementary Figure 1A**, NS3/4A protease could be detected in the NS3/4A-HCC36 cells and HCV replicon-containing cell line (AVA5) (Lee et al., 2004) but not in the control HCC36 cells. In addition, these cells were transfected with the NS3 response reporter vector pEG-(DEΔ4AB)-SEAP, containing the NS4A/B junction between egfp and seap, to detect the NS3/4A activity (Lee et al., 2003). Upon NS3/4A cleavage, the secreted embryonic alkaline phosphatase (SEAP) is secreted. The activity of NS3/4A protease can be quantitatively indicated by measuring the SEAP activity in the cell culture medium. Strong SEAP activity could be detected in the NS3/4A-HCC36 cells and HCV replicon-containing cell line (AVA5) but not in the control HCC36 cells (**Supplementary Figure 1B**), indicating strong NS3/4A activity in the NS3/4A-HCC36 cells. These results indicated the NS3/4A protease is stable and functionally expressed in NS3/4A-HCC36 cells.

Specific retention of TAT-ΔNS3/4A-FITC in NS3/4A-HCC36-and hepatitis C virus-infected cells

To evaluate whether this protease-activated retention probe (TAT-ΔNS3/4A-FITC) could specifically accumulate and be retained in the NS3/4A-expressing cells, we incubated the HCC36 cells, NS3/4A-HCC36 cells, or HCV (JFH-1)-infected HCC36 cells with for 1 h. After culturing for 8 h, the accumulation of the fluorescence signal was observed using a fluorescence microscope. **Figure 2** shows that strong fluorescence accumulated in each cell after probe staining. After culturing for 8 h, the strong fluorescence only accumulated in NS3/4A-HCC36 cells and HCV-infected HCC36 cells but not in control HCC36 cells. We also compared the NS3/4A protease activity between NS3/4A-HCC36 cells and JFH-1-infected HCC36 cells. The relative protease activity of HCC36 cells, NS3/4A-HCC36 cells, and JFH-1-infected HCC36 cells was 1.27 ± 0.18, 7.09 ± 0.29, and 5.64 ± 0.22, respectively (**Supplementary Figure 2**). These results indicated that the TAT-ΔNS3/4A-FITC probe selectively accumulated and retained the fluorescence signal in the NS3/4A-expressing cells or virus-infected cells.

To confirm that the accumulation of TAT-ΔNS3/4A-FITC is dependent on protease activity, we incubated the HCC36 cells, NS3/4A-HCC36 cells, or HCV (JFH-1)-infected HCC36 cells with the probe in the presence of NS3/4A inhibitor (Telaprevir, VX-950) (Salam and Akimitsu, 2013). After culturing for 8 h, the accumulation of fluorescence signals was observed under a fluorescence microscope. The fluorescence of the probe did not accumulate in the NS3/4A-HCC36 cells or HCV (JFH-1)-infected HCC36 cells after inhibition, indicating that accumulation of TAT-ΔNS3/4A-FITC probe is dependent on protease activity (**Figure 2**).

We also used the WT probe (GRKKRRQRRR-DEDEDEDEMEECASH-LKKKYK-FITC) and the scrambled

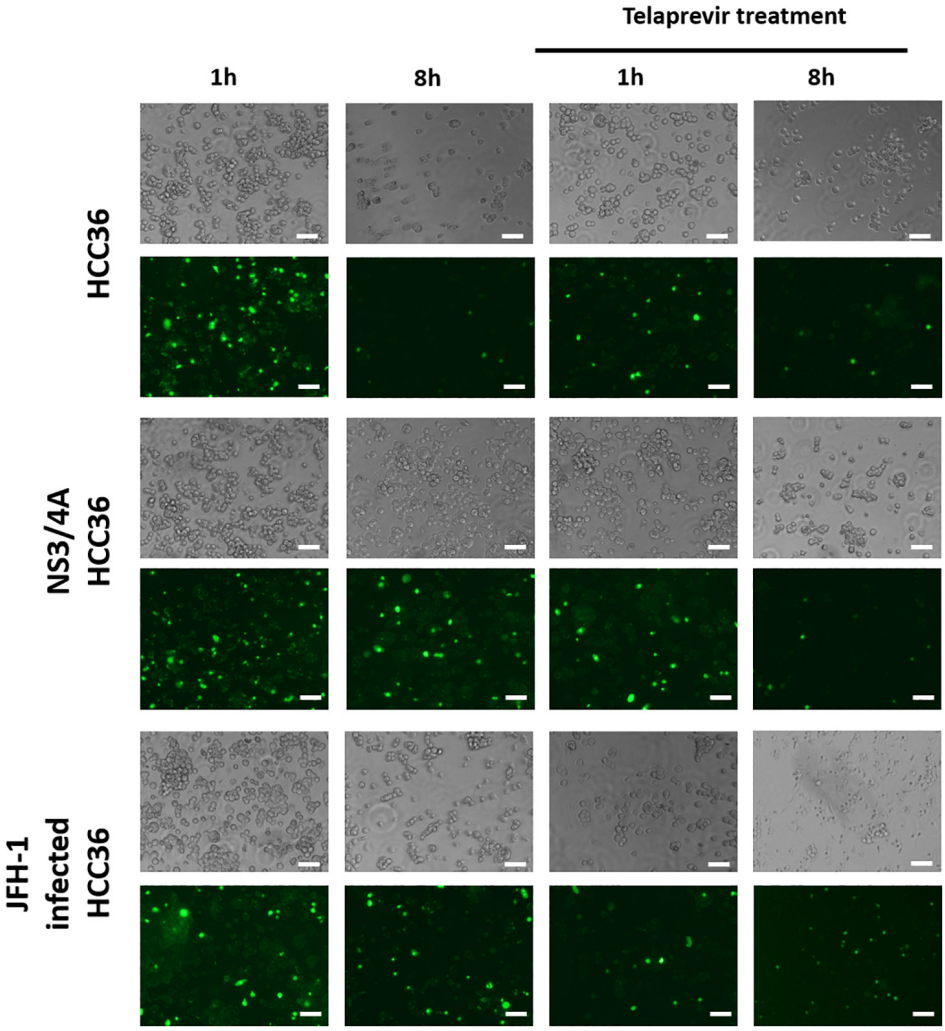

FIGURE 2
Specific retention of TAT-ΔNS3/4A-FITC in NS3/4A-HCC36- and HCV-infected cells. HCC36 cells, NS3/4A-HCC36 cells, or JFH-1-infected HCC36 cells were incubated with 10 μM TAT-ΔNS3/4A-FITC in the absence or presence of 2 μM telaprevir (NS3/4A protease inhibitor) at 37°C for 1 h. The cells were washed with DMEM containing 10% serum three times per hour. After culturing for 1 or 8 h, the phase contrast and fluorescence of viable cells were observed under a fluorescence microscope. Scale bar: 100 μm.

sequence probe (GRKKRRQRRR-ECEEEEESMDDDAH-LKKKYK) that could not be activated by protease as a control group to test the specific retention of probe *via* flow cytometry. HCC36 cells, NS3/4A-HCC36 cells, or JFH-1-infected HCC36 cells were incubated with 10 μM TAT-ΔNS3/4A-FITC or 10 μM TAT-scrambled NS3/4A-FITC in the absence or presence of 2 μM telaprevir (NS3/4A protease inhibitor) at 37oC for 1 h. The cells were washed with DMEM containing 10% serum three times per hour. After culturing for 1 or 8 h, the fluorescence of viable cells was observed *via* flow cytometry. The **Supplementary Figure 5** shows that strong fluorescence of WT probe accumulated in each cell after 1 h probe staining. After culturing for 8 h, the strong fluorescence of WT probe only accumulated in NS3/4A-HCC36 cells and HCV-infected HCC36 cells but not in control HCC36 cells. In addition, the strong fluorescence of WT probe (8 h) could block *via* the 2 μM telaprevir (NS3/4A protease inhibitor). The fluorescence of scrambled sequence probe cannot be accumulated in each cell after 8-h probe staining. These results infected the accumulation of TAT-ΔNS3/4A-FITC probe is dependent on protease activity.

In vivo accumulation of TAT-ΔNS3/4A-FITC in NS3/4A-expressing tumors

To examine whether the TAT-ΔNS3/4A-FITC probes can specifically detect NS3/4A activity *in vivo*, NS3/4A-HCC36 and HCC36 tumors were frozen sectioned at 4 h after the

injection of TAT-ΔNS3/4A-FITC probes in tumor-bearing mice. The adjacent tumor sections either directly detect the FITC-fluorescent intensity to observe the accumulation of TAT-ΔNS3/4A-FITC probe or stained with NS3/4A substrate to visualize NS3/4A activity. FITC-derived fluorescence was accumulated in the NS3/4A-HCC3 tumors, but not the control HCC36 tumors, which was matched with red fluorescence for NS3/4A activity (**Figure 3**). These results indicated that the retention of AT-ΔNS3/4A-FITC probe correlated with NS3/4A activity *in vivo*.

Specificity and half-life of TAT-ΔNS3/4A-^{124}I-FITC

We labeled TAT-ΔNS3/4A-FITC with ^{124}I to generate a TAT-ΔNS3/4A-^{124}I-FITC probe. A representative analytical HPLC chromatogram of the TAT-ΔNS3/4A-^{124}I-FITC probe was shown in **Supplementary Figure 3**. The retention time of ^{124}I and TAT-ΔNS3/4A-^{124}I-FITC was ∼4.7–5 and 6.7 min, respectively. The crude TAT-ΔNS3/4A-^{124}I-FITC was purified by using a C-18 sep-pak (Waters, Milford) separation unit. After purification, the radiochemical purity (RCP) of TAT-ΔNS3/4A-^{124}I-FITC was higher than 95% (**Supplementary Figure 3C**) (radio-peak, 6.7 min). The radiochemistry yield is about 25–55%.

To evaluate whether TAT-ΔNS3/4A-^{124}I-FITC was selectivity retained in the NS3/4A-expressing cells, the HCC36 cells or NS3/4A-HCC36 cells were incubated with 37 kBq of TAT-ΔNS3/4A-^{124}I-FITC probe at 37°C for 1 h in the presence or absence of the NS3/4A inhibitor telaprevir. After culturing for 0, 4, 6, and 8 h, the radioactivity retained in the cells was measured using a gamma-counter. A high level of radioactivity was detected in NS3/4A-HCC36 cells but not in control HCC36 cells, indicating TAT-ΔNS3/4A-^{124}I-FITC was selectivity retained in the NS3/4A-expressing cells (**Figure 4A**). After inhibitor treatment, radioactivity did not accumulate in the NS3/4A-HCC36 cells, indicating that accumulation of the TAT-ΔNS3/4A-^{124}I-FITC probe is dependent on the protease activity.

To evaluate the pharmacokinetics of TAT-ΔNS3/4A-^{124}I-FITC in circulation, BALB/c mice were intravenously injected with TAT-ΔNS3/4A-^{124}I-FITC and the radioactivity in serum samples collected at the indicated times was measured using a gamma-counter. The TAT-ΔNS3/4A-^{124}I-FITC was rapidly eliminated from the blood following one-phase exponential decay kinetics with a half-life of 2.55 min (**Figure 4B**). Radioactivity in the blood was as low as 2.6 ± 0.1% ID/g at 4 h after injection, indicating that TAT-ΔNS3/4A-^{124}I-FITC was rapidly cleared from circulation.

Micro-PET imaging and biodistribution of TAT-ΔNS3/4A-^{124}I-FITC *in vivo*

To evaluate whether TAT-ΔNS3/4A-^{124}I-FITC can be used to visualize NS3/4A activity *in vivo*, the NS3/4A-HCC36 or HCC36 tumor-bearing mice were intravenously injected with TAT-ΔNS3/4A-^{124}I-FITC and imaged at 2, 4, and 6 h. Stronger radio-signals were detected in the NS3/4A-HCC36 tumors than in control HCC36 tumors (**Figure 5A**). Serial imaging analysis indicated that the highest image intensity occurred at 4 h after injection of TAT-ΔNS3/4A-^{124}I-FITC. The radioactivity in the region of interest (ROI) was 1. 67-, 4. 42-, and 2.53-fold higher in NS3/4A-HCC36 tumors than in HCC36 tumors at 2, 4, and 6 h, respectively, suggesting that the TAT-ΔNS3/4A-^{124}I-FITC was accumulated and retained in NS3/4A-HCC36 tumors. After inhibitor treatment, radioactivity did not accumulate in the NS3/4A-HCC36 tumors and the radioactivity was at a similar level in the NS3/4A-HCC36 and HCC36 tumors (**Figure 5B**). ROI in NS3/4A-HCC36 tumors was only 1. 13-, 0. 96-, and 1.31-fold greater than in HCC36 tumors at 2, 4, and 6 h, respectively. These results indicate that TAT-ΔNS3/4A-^{124}I-FITC can be used to accurately represent NS3/4A protease activity *in vivo* using micro-PET.

To investigate the biodistribution of TAT-ΔNS3/4A-^{124}I-FITC *in vivo*, the NS3/4A-HCC36 or HCC36 tumor-bearing mice were intravenously injected with TAT-ΔNS3/4A-^{124}I-FITC and then examined by measuring the radioactivity of the probe in organs at different time points after probe injection. Higher levels of radioactivity were detected in NS3/4A-HCC36 tumors than in control HCC36 tumors 2, 4, and 6 h after probe injection (**Figure 6**). The accumulation of radioactivity in NS3/4A-HCC36 tumors was 1. 6-, 3. 4-, and 2.7-fold higher than in HCC36 tumors at 2, 4, and 6 h, respectively. This result was similar to the micro-PET analysis. Notably, we also noted high levels of radioactivity were observed in the stomach and kidneys. However, the long-term bio-destruction results (**Supplementary Figure 4**) showed that the accumulation of probe in kidney would quickly reduce at 12 and 24 h, implying that the elimination of the radiolabeled probes was through the urinary system.

Micro-PET imaging of NS3/4A activity in an orthotopic liver implantation tumor model

To further assess whether the TAT-ΔNS3/4A-^{124}I-FITC can detect NS3/4A activity in deep liver tissue, we generated an orthotopic implantation tumor model in mice *via* surgery (Kim et al., 2009). The mice transplanted with NS3/4A-HCC36 tumors or control HCC36 tumors were intravenously injected with 3,700 kBq of TAT-ΔNS3/4A-^{124}I-FITC and

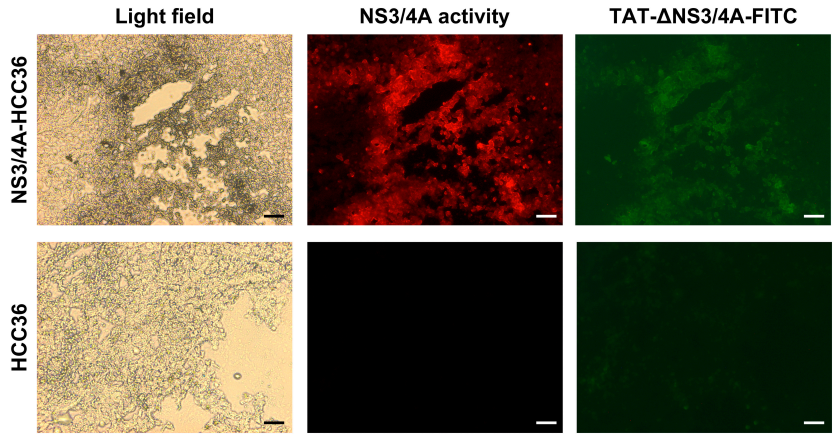

FIGURE 3
Specific retention of TAT-ΔNS3/4A-FITC in NS3/4A-expressing tumors *in vivo*. Mice bearing established NS3/4A-HCC36 and HCC36 tumors were injected with 500 μM TAT-NS3/4A-FITC and sacrificed after 4 h. Sections of NS3/4A-HCC36 (upper panels) and HCC36 (lower panels) tumors were stained with 520 HCV protease Assay Kit (AnaSpec) to detect NS3/4A activity in tumor sections. FITC-derived fluorescence (green) and NS3/4A activity (red) were observed under a fluorescence microscope. Scale bar: 1 mm.

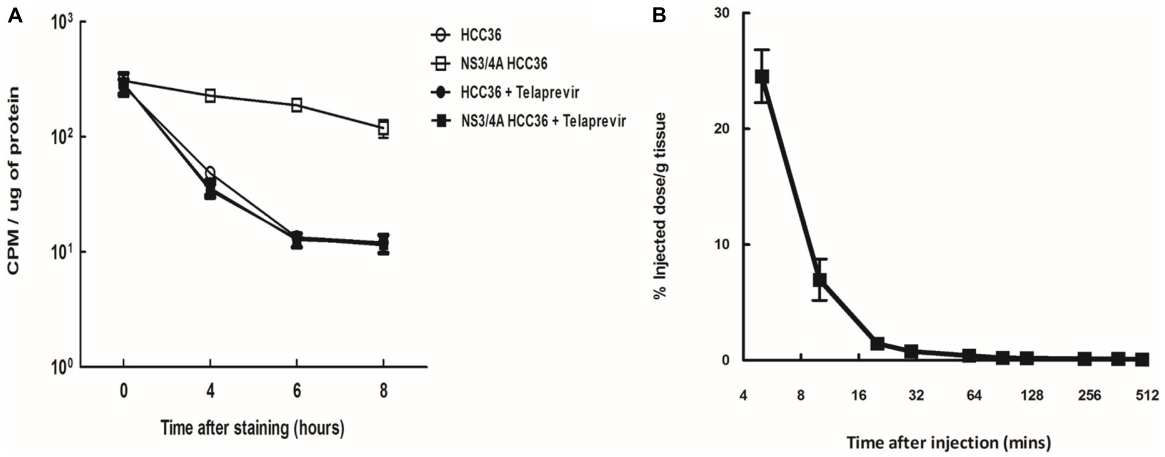

FIGURE 4
Specificity and half-life of TAT-ΔNS3/4A-^{124}I-FITC. **(A)** NS3/4A-HCC36 and HCC36 cells were incubated with 37 kBq of TAT-ΔNS3/4A-^{124}I-FITC in the presence or absence of 2 μM Telaprevir at 37°C for 1 h. The cells were washed with DMEM containing 10% serum three times per hour. The cells were collected by treatment with trypsin at the indicated times. The radioactivity of the cells was then measured with a gamma-counter. The CPM was normalized by protein concentration. **(B)** Kinetics of the TAT-ΔNS3/4A-^{124}I-FITC *in vivo*, BALB/c mice were intravenously injected with TAT-ΔNS3/4A-^{124}I-FITC, and the radioactivity in serum samples collected at the indicated times was measured using a gamma-counter. $t_{1/2}$ = 2.55 min. Error bar: standard error of triplicate determinations.

imaged *via* micro-PET. Radioactivity accumulated in NS3/4A-HCC36 tumors but not in control tumors 4 h after probe injection (Figure 7A). The ROI in NS3/4A-HCC36 tumors was 4.75-fold higher than in HCC36 tumors. In addition, the radioactivity of implanted NS3/4A-HCC36 tumors and HCC36 tumors was measured using a gamma-counter at 2, 4, and 6 h after probe injection. More radioactivity was detected in the implanted NS3/4A-HCC36 tumors than in control HCC36 tumors (Figure 7B). The uptake of radioactivity in implanted NS3/4A-HCC36 tumors was 1. 8-, 4. 7-, and 3.1-fold higher than in implanted HCC36 tumors at 2, 4, and 6 h, respectively, after probe injection. This result was similar to the micro-PET analysis. These results indicate that TAT-ΔNS3/4A-^{124}I-FITC visualized by micro-PET imaging can be used to represent the NS3/4A activity in deep liver tissue.

Discussion

We successfully developed a protease-activated retention peptide that allows real-time imaging of

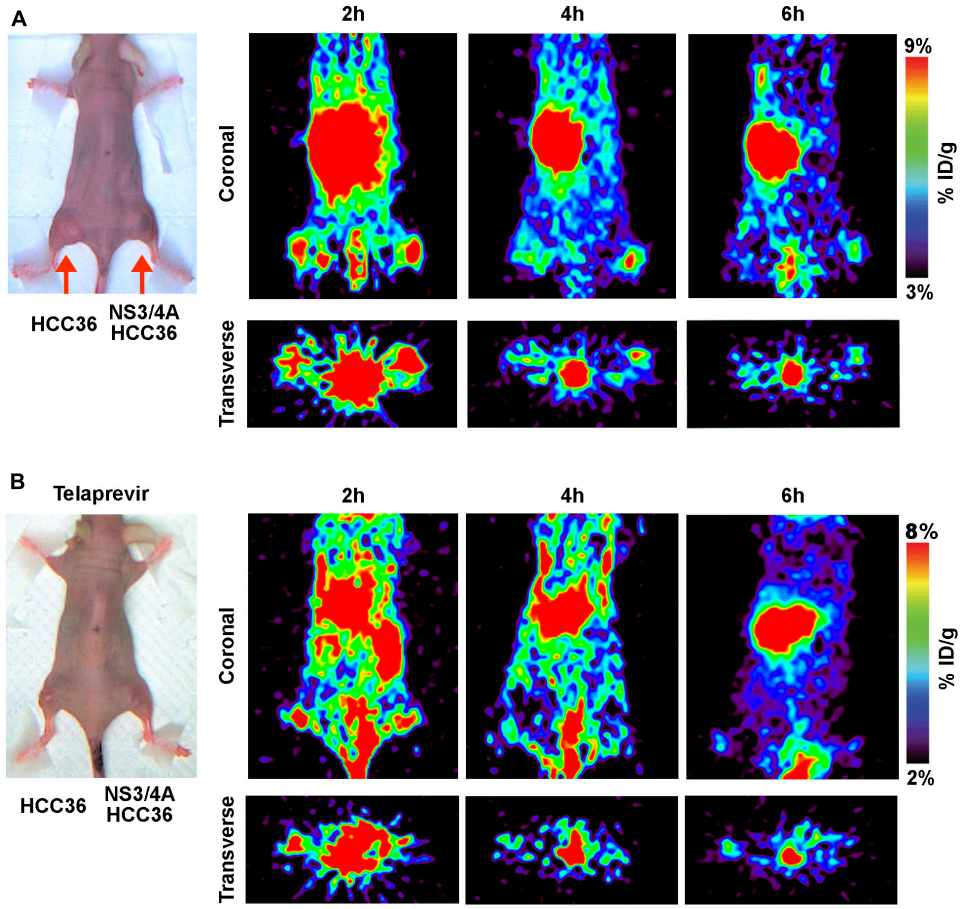

FIGURE 5
Micro-PET imaging of NS3/4A activity *in vivo*. **(A)** Mice bearing established NS3/4A-HCC36 (right hind leg) and HCC36 (left hind leg) tumors were injected with 3,700 kBq of TAT-ΔNS3/4A-^{124}I-FITC. Coronal and transverse images were acquired at 2, 4, and 6 h after injection of the probe. **(B)** Mice bearing established NS3/4A-HCC36 (right hind leg) and HCC36 (left hind leg) tumors were intraperitoneally injected with telaprevir (20 mg/kg/day for 3 days) before intravenous injection of TAT-ΔNS3/4A-^{124}I-FITC (3,700 kBq). Coronal images of tumor sections were acquired at 2, 4, and 6 h after injection of the probe.

HCV NS3/4A protease activity by micro-PET. The *in vitro* and *in vivo* results demonstrated that TAT-ΔNS3/4A-FITC specifically accumulated in the NS3/4A protease-expressing area. Furthermore, PET imaging results showed that the NS3/4A protease activity can be systemically detected in xenograft mice or in orthotopic liver-implanted mice. These results indicate that TAT-ΔNS3/4A-^{124}I-FITC may provide a probe that allows visualization of NS3/4 activity by PET technology to systemically track protease activity and distribution *in vivo*.

The technology to tracking the NS3 protease activity *in vivo* would provide a powerful tool to design the personalized protease inhibitor-based therapies and to monitor the development of liver cirrhosis and hepatocellular carcinoma. Currently, HCV is commonly diagnosed by reverse transcription-PCR (RT-PCR) or quantitative PCR to detect the HCV RNA in patient blood. However, the viral distribution and protease activity cannot be detected by RT-PCR or anti-HCV ELISA in patients. Liver-biopsy is, therefore, currently the best approach to detect the viral distribution (Carreno et al., 2004; Castillo et al., 2004) or viral protease activity in liver (Chao et al., 2011). However, liver biopsy is the partial and invasive method that only detects the infection in liver and may increase the risk of liver damage. The HCV also can infect the extra-hepatic tissue and can increase the incidence of several diseases, including autoimmune disorders (Yang et al., 2014), diabetes (Arase et al., 2009), nervous disorders (Mariotto et al., 2014), and chronic kidney disease (Sumida et al., 2010), indicating the systemically image system is the suitable approach to detect the distribution of NS3/4A protease activity. Here, our results show that our probe could be used to specifically detect the protease activity in NS3/4A protease-expressing cells/tumors and in liver-implanted tumors *via* a PET imaging system. These results indicated that TAT-ΔNS3/4A-^{124}I-FITC may

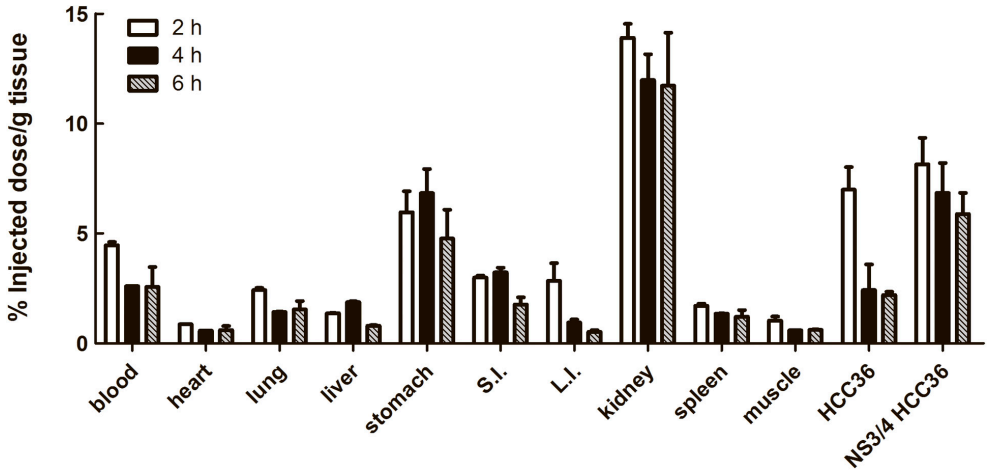

FIGURE 6
Biodistribution of TAT-ΔNS3/4A-^{124}I-FITC in xenograft mice. Mice bearing established NS3/4A-HCC36 and HCC36 tumors were injected with 3,700 kBq of TAT-ΔNS3/4A-^{124}I-FITC. Selected organs and tumors were removed from the mice after 2 (white column), 4 (black column), and 6 (gray column) h. The radioactivity of individual organs was measured using a gamma-counter and normalized for sample weights. The biodistribution of TAT-ΔNS3/4A-^{124}I-FITC in selected organs was expressed as percentage injected dose/g tissue. Data represent mean ± SEM.

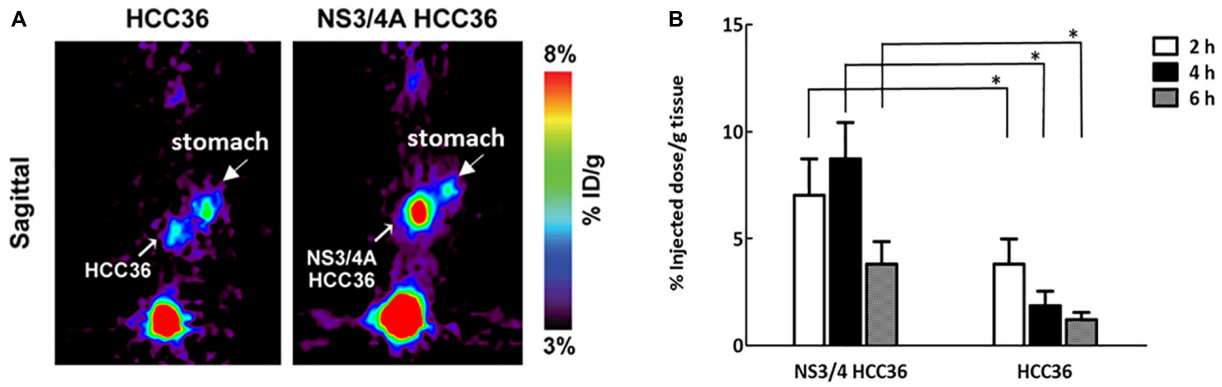

FIGURE 7
Micro-PET Imaging of NS3/4A activity in orthotopic liver implantation model. The NS3/4A-HCC36 tumors or HCC36 tumors from the ectopic tumors were harvested and transplanted into the left liver lobe of SCID mice (n = 3). After 2 weeks, the 3,700 kBq of TAT-ΔNS3/4A-^{124}I-FITC was intravenously injected. **(A)** PET imaging was performed at 4 h after injection of the probe. **(B)** The accumulation of radioactivity in NS3/4A-HCC36 tumors and HCC36 tumors was measured using a gamma-counter at 2 (white column), 4 (black column), and 6 (gray column) h after probe injection. Data represent mean ± SEM. Student's t-test analysis of data. Statistical analysis was compared NS3/4A-HCC36 with HCC36. $P < 0.05$ was considered statistically significant.

provide a clinically available PET probe to represent NS3/4A activity to systemically track protease activity and distribution *in vivo*.

Development of a clinically available imaging probe to detect cellular protease activity has potential applications in many diseases. Overexpression of cellular proteases has been reported to play an important role in many diseases, including various viral infections (Carreno et al., 2004; Castillo et al., 2004; Lee et al., 2004), Alzheimer's disease (Lescar et al., 2008), and cancers (Brik and Wong, 2003; Vermehren and Sarrazin, 2011). Various imaging strategies aiming to evaluate protease activity have been developed. For example, a quenched near-infrared fluorescence (NIRF) probe was developed to track human immunodeficiency virus specific protease (HIV PR) activity *in vivo* (Ta et al., 2013). Various protease-activation bioluminogenic probes have been designed using a similar strategy to image the furin activity in breast tumors (Dragulescu-Andrasi et al., 2009) or to detect caspase 3 activity in gliomas (Shah et al., 2005). However, the shallow penetration of the bioluminogenic imaging still limits its use in the clinic (Cheng et al., 2012; Chuang et al., 2012). Here, we successfully developed a clinically available PET probe to image NS3/4A activity. The protease substrate of this probe can be

changed to allow imaging of other cellular proteases relevant to different diseases. Furthermore, the hydrophilic domain can be easily conjugated to a variety of contrast agents, such as ^{124}I for PET (Pentlow et al., 1996), ^{111}In-DOTA for SPECT, or Gd-DOTA for MRI, giving flexibility in choice of imaging system.

Development of a quickly metabolic and low toxicity probe is very important. The quickly metabolic probe usually be considered has the low toxicity and low side effects. Theoretically, peptide-based probe is metabolized briefly into ammonium and carbon backbone, eventually enter urea cycle and TCA cycle. For example, Chuang and colleagues developed a PEG-peptide-^{18}F-TMR probe to image the *in vivo* MMP protease activity *in vivo* by PET (Park et al., 2012). They also reported this based probe was quickly the elimination of the radiolabeled probes in urine. Our long-term bio-destruction results (**Supplementary Figure 4**) also indicated that the non-specific accumulation of probe in kidney and in stomach at 4 h, and the accumulation of probe in kidney and in stomach would quickly reduce at 12 and 24 h. The result also indicated the radioactivity of probe did not accumulate in the all of tissues at 24 h. These results implied that this probe will be rapidly metabolized and eliminated out of the body through the urinary system. In addition, many studies have been reported that the deiodination of probe would usually happen *in vivo* and that would cause the non-specific accumulation in stomach (Koehler et al., 2010; Oh and Ahn, 2012). We considered that was the reason why our probe would non-specifically accumulate in stomach. However, the result also indicated the radioactivity of probe did not accumulate in the all of tissues at 24 h, implying that this probe will be rapidly metabolized and eliminated out of the body through the urinary system. We also have detected the *in vitro* toxicity of our probe, and we treated the human embryonic kidney cell (HEK293) with different concentrations (0.1–2,000 ug/ml) of probe. The results (**Supplementary Figure 5**) indicated the different concentrations (0.1–2,000 ug/ml) of probe could not inhibit the growth of HEK293 as compared with CPT-11(anti-cancer drug), indicating this probe has the very low toxicity. Therefore, we considered that our probe is quickly metabolic probe, and low toxicity might be more suitable for clinical imaging.

The strategy may be extended to image other cellular proteases in different diseases *via* multimodality system in clinic. Therefore, we hypothesized that the mechanism of cellular clearance of the probe was through (1) directly interaction with inner member and (2) the exocytosis of the TAT peptide contained endosomes. For example, Dr. Rayne has reported the cellular Tat could bind to the phosphatidylinositol-(4,5)-bisphosphate in the inner member, resulting in Tat insertion into the plasma membrane, and then enables efficiently secretion of HIV1 from cytoplasm into medium (Rayne et al., 2010), suggesting the cellular Tat would interact with membrane and exclude from the cells. In addition, several studies have shown the uptake mechanism of arginine rich CPPs, such as TAT peptide or R9 peptide, was dependent on the endocytotic pathways (Rubin et al., 1992; Richard et al., 2003, 2005). After internalization, the TAT peptide would have trapped in endosomes of cells (Ross and Murphy, 2004; Debaisieux et al., 2012) and might slowly diffuse into cytoplasm. Therefore, we considered that the cellular TAT peptide in the endosomes might be excluded from cell *via* exocytosis, such as the mechanism of TAT-derived HIV to cross the BBB and to reach the brain (Banks et al., 2005; Mahajan et al., 2008; Cooper et al., 2012). Therefore, we hypothesized that the mechanism of cellular clearance of the probe was through (1) directly interaction with inner member and (2) the exocytosis of the TAT peptide contained endosomes.

This NS3/4A-activatable PET probe could real-time imaging of NS3/4A protease activity *in vivo*. This strategy would provide several advantages: (1) The probe has high penetrability for imaging of NS3/4A activity in deep tissues; (2) the possibility of changing the substrate potentially allows the generation of a wide range of probes for other cellular proteases; (3) this is a multimodality system that can be easily conjugated with a variety of contrast agents; (4) this probe provides a convenient platform for the development of protease inhibitors in human or big animal studies. Based on these advantages, the TAT-ΔNS3/4A-^{124}I-FITC probe potentially provides a clinical available approach to determine the protease distribution and optimize protease inhibitor-based therapy.

Data availability statement

The original contributions presented in this study are included in the article/**Supplementary material**, further inquiries can be directed to the corresponding authors.

Ethics statement

This animal study was reviewed and approved by Affidavit of Approval of Animal Use Protocol Kaohsiung Medical University, Center for laboratory animals, Kaohsiung Medical University.

Author contributions

M-YH and J-CL: conception and design. C-HC, T-LC, Yi-JH, J-CL, H-EW, W-CC, C-CK, and M-YH:

development of methodology. C-HC, J-CL, W-CC, H-EW, T-LC, Y-CL, Yi-JH, Y-CH, W-WL, Ya-JH, C-CK, K-CH, and M-YH: acquisition of data. C-HC, J-CL, Yi-JH, and M-YH: analysis and interpretation of data. C-HC, M-YH, Yi-JH, H-EW, J-CL, and T-LC: writing, review, and/or revision of the manuscript. H-EW, T-LC, and J-CL: administrative, technical, or material support. C-HC, J-CL, and M-YH: study supervision. All authors contributed to the article and approved the submitted version.

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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/fmicb.2022.896588/full#supplementary-material

SUPPLEMENTARY FIGURE 1
Characterization of the expression and function of NS3/4A-expressing cells. We constructed the NS3/4A gene into a lentiviral vector, pLKO_AS3 NS3/4A, to directly express the HCV NS3/4A protease in human hepatocellular carcinoma HCC36 cells. **(A)** HCC36, NS3/4A-HCC36, and HCV replicon-containing cells (AVA5) were harvested to detect the expression HCV NS3. Western blotting was performed using anti-HCV NS3 (ab13830, 1:1000, Abcam) and anti-GAPDH antibodies (GTX100118, 1:1000, GeneTex). GAPDH was used as a loading control. **(B)** HCC36, NS3/4A-HCC36, and AVA5 were transfected with NS3 response reporter vector Peg (DEΔ4AB) SEAP, containing the NS4A/B junction between *egfp* and *seap*. After 3 days, total cell supernatant was analyzed for SEAP activity according to the manufacturer's instructions. Each value represents the mean fold ± SD of triplicate experiments after normalization of luciferase activities. The error bars denote SD of the mean.

SUPPLEMENTARY FIGURE 2
Characterization of the function of NS3/4A in virus-infected cells. HCC36, NS3/4A-HCC36, and JHF-1-infected cells were transfected with NS3 response reporter vector pEG (DEΔ4AB) SEAP, containing the NS4A/B junction between egfp and seap. After 3 days, total cell supernatant was analyzed for SEAP activity according to the manufacturer's instructions. Each value represents the mean fold ± SD of triplicate experiments after normalization of luciferase activities. The error bars denote SD of the mean.

SUPPLEMENTARY FIGURE 3
HPLC chromatograms of **(A)** $Na^{124}I$ solution, **(B)** crude product of TAT-ΔNS3/4A-^{124}I-FITC, and **(C)** final product TAT-ΔNS3/4A-^{124}I-FITC: radio-peak of ^{124}I and TAT-ΔNS3/4A-^{124}I-FITC. The retention time of ^{124}I and TAT-ΔNS3/4A-^{124}I-FITC was ∼4.7–5 and 6.7 min, respectively.

SUPPLEMENTARY FIGURE 4
Long-term biodistribution of TAT-ΔNS3/4A-^{124}I-FITC in xenograft mice. Mice were injected with 3,700 kBq of TAT-ΔNS3/4A-^{124}I-FITC. Selected organs and tumors were removed from the mice after 4, 12, and 24 h. The radioactivity of individual organs was measured using a gamma-counter and normalized for sample weights. The biodistribution of TAT-ΔNS3/4A-^{124}I-FITC in selected organs was expressed as percentage injected dose/g tissue. Data represent mean ± SEM.

SUPPLEMENTARY FIGURE 5
To evaluate the specific retention of probe *via* flow cytometry, we used the WT probe (GRKKRRQRRR-DEDEDEDEMEECASH-LKKKYK-FITC) and the scrambled sequence probe (GRKKRRQRRR-ECEEEEESMDD DAH-LKKKYK) that could not be activated by protease as a control group to test the specific retention of probe *via* flow cytometry. HCC36 cells, NS3/4A-HCC36 cells, or JFH-1-infected HCC36 cells were incubated with 10 μM TAT-ΔNS3/4A-FITC or 10 μM TAT-scrambled NS3/4A-FITC in the absence or presence of 2 μM telaprevir (NS3/4A protease inhibitor) at 37°C for 1 h. The cells were washed with DMEM containing 10% serum three times per hour. After culturing for 1 or 8 h, the fluorescence of viable cells was observed *via* flow cytometry.

SUPPLEMENTARY FIGURE 6
In vitro cytotoxicity of TAT-ΔNS3/4A-FITC. HEK293 cells were incubated with different concentrations (0.1–2,000 ug/ml) of TAT-ΔNS3/4A-FITC or CPT-11. After incubation for 72 h, cell viability was measured *via* the rate of cellular ATP synthesis to detect the cytotoxicity of TAT-ΔNS3/4A-FITC for HEK293 cells. CPT-11: irinotecan hydrochloride.

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