DIAGNOSIS, TREATMENT AND PROGNOSIS OF VIRAL HEPATITIS

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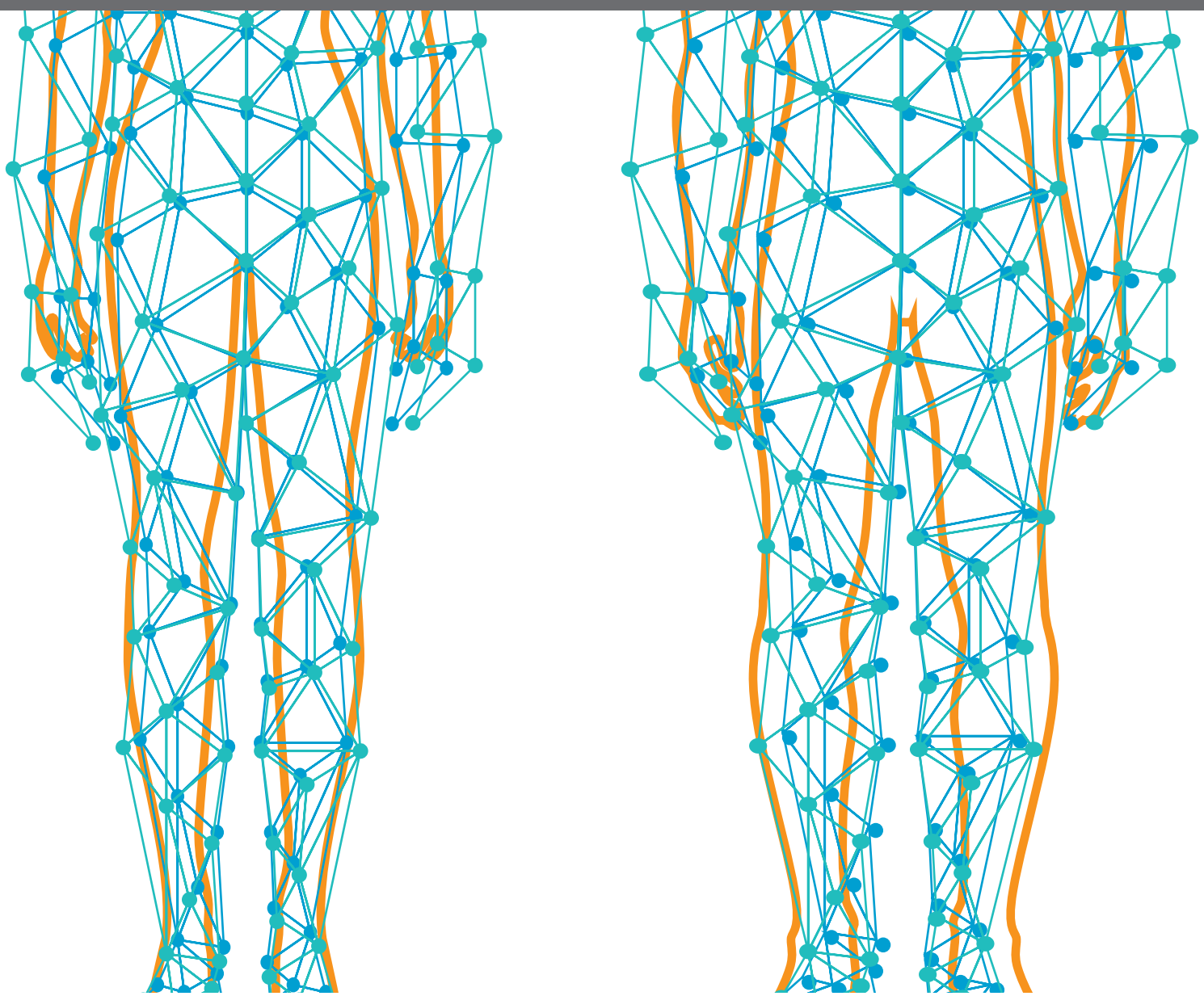

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DIAGNOSIS, TREATMENT AND PROGNOSIS OF VIRAL HEPATITIS

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EDITORIAL
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Editorial: Diagnosis, Treatment, and Prognosis of Viral Hepatitis

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Keywords: viral hepatitis, diagnosis, prognosis, treatment, host immune response

Editorial on the Research Topic

Diagnosis, Treatment, and Prognosis of Viral Hepatitis

Viral hepatitis is an infectious disease mainly manifested by liver lesions caused by various hepatitis viruses, including hepatitis A virus (HAV), hepatitis B virus (HBV), hepatitis C virus (HCV), hepatitis D virus (HDV), and hepatitis E virus (HEV). Viral hepatitis poses a huge threat to human public health (1). In 2010, the 63rd World Health Assembly (WHA) adopted a resolution (WHA63.18) (2) which stated that ∼2 billion people worldwide are infected with HBV, and about 350 million people are chronically infected with hepatitis B. Nowadays, hepatitis C is still not preventable by vaccines, and almost 80% of HCV infections are chronic infections. Given that viral hepatitis is a serious global public health problem, we call on governments, Parties and the population to scale up efforts to prevent, diagnose, and treat viral hepatitis in the control of viral hepatitis.

In recent years, the world has made impressive progress in a number of areas, including innovations in hepatitis treatment and the expansion of HBV vaccine immunization to prevent new HBV infections. However, until 2021, there are still 296 million people with chronic HBV infection, 58 million people with HCV infection. 1.1 million people die from HBV and HCV infection every year (2), and 3 million people are still newly infected with HBV and HCV. Only 30.4 million (10%) patients with chronic hepatitis B were diagnosed, of whom 6.6 million (22%) received antiviral therapy. 15.2 million (21%) people with hepatitis C infection were diagnosed, of which 9.4 million (62%) received antiretroviral therapy (3). The diagnosis of viral hepatitis patients, the evaluation of patient prognosis and the exploration of novel markers of antiviral efficacy are urgent problems to be solved. For a long time in the future, this field would be advanced by leaps and bounds (4).

Therefore, the appearance of this special issue is very timely. This Research Topic aims to collect articles or reviews that provide insights into disease understanding and translational potential for better clinical care. Early diagnosis of hepatitis infection and early assessment of its prognosis are critical for effective treatment and care (5, 6). In this special issue, Zhang et al. evaluated the diagnostic value of adenosine deaminase, α-l-fucosidase, and lactic acid in liver cirrhosis and hepatocellular carcinoma related to hepatitis B. Through the cohort analysis, Ding et al. constructed a novel non-invasive model for the predication of liver fibrosis with chronic hepatitis B. Genetic mutations in TNFSF11 were proved to be associated with the chronicity of hepatitis C by Huang et al.. Guo et al. identified three genes through bioinformatics, and used them to establish immune-related prognostic characteristics for patients with HCV-related cirrhosis. The correlation between protein induced by vitamin K absence or antagonist-II and trend of changes in hepatitis E patients

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was also revealed by Chen et al. Nowadays, the application of exosome and multi-omics technology have strongly promoted the primary screening and early intervention of viral hepatitis. Sun et al. devoted into bile acid metabolism associated with HBV infection through the transcriptome and gut microbiome. Cui et al. reviewed the advances in muti-omics applications in hepatocellular carcinoma. In addition, Zhou H. et al. summarized the role of exosomes in viral hepatitis. Tong et al. also investigated the prognostic value of serum exosomal AHCY expression in liver cirrhosis associated with hepatitis B.

Host manifestations in hepatitis viruses have always been a fundamental issue and focus of controversy in this research field, involving the severe mechanism of viral hepatitis and antiviral efficacy (7, 8). In this special issue, Gong et al. discussed the role of Th22 cells in human viral diseases. Also, Jin et al. focused on the peripheral immune cell exhaustion in patients with chronic hepatitis B. Finally, the therapeutic effect of viral hepatitis has also been evaluated. Cheng et al. concluded the novel treatment choices for HBV and human immunodeficiency virus (HIV) co-infection patients. Zhou J. et al. also summarized the antiviral therapy for chronic HBV infection with persistently normal alanine aminotransferase. Additionally, sirtuins were also considered as the potential therapeutic targets for HBV infection by Kong et al., all of which may provide new insight into treatment choices in viral hepatitis.

This Research Topic aims to collect articles or reviews that provide insights into disease understanding and translational potential for better clinical care. Finally, we hope you enjoy reading this special issue.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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ORIGINAL RESEARCH
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An Immune Signature Robustly Predicts Clinical Deterioration for Hepatitis C Virus-Related Early-Stage Cirrhosis Patients

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Hepatitis C virus (HCV)-related cirrhosis leads to a heavy global burden of disease. Clinical risk stratification in HCV-related compensated cirrhosis remains a major challenge. Here, we aim to develop a signature comprised of immune-related genes to identify patients at high risk of progression and systematically analyze immune infiltration in HCV-related early-stage cirrhosis patients. Bioinformatics analysis was applied to identify immune-related genes and construct a prognostic signature in microarray data set. Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) functional enrichment analyses were conducted with the "clusterProfiler" R package. Besides, the single sample gene set enrichment analysis (ssGSEA) was used to quantify immune-related risk term abundance. The nomogram and calibrate were set up via the integration of the risk score and clinicopathological characteristics to assess the effectiveness of the prognostic signature. Finally, three genes were identified and were adopted to build an immune-related prognostic signature for HCV-related cirrhosis patients. The signature was proved to be an independent risk element for HCV-related cirrhosis patients. In addition, according to the time-dependent receiver operating characteristic (ROC) curves, nomogram, and calibration plot, the prognostic model could precisely forecast the survival rate at the first, fifth, and tenth year. Notably, functional enrichment analyses indicated that cytokine activity, chemokine activity, leukocyte migration and chemotaxis, chemokine signaling pathway and viral protein interaction with cytokine and cytokine receptor were involved in HCV-related cirrhosis progression. Moreover, ssGSEA analyses revealed fierce immune-inflammatory response mechanisms in HCV progress. Generally, our work developed a robust prognostic signature that can accurately predict the overall survival, Child-Pugh class progression, hepatic decompensation, and hepatocellular carcinoma (HCC) for HCV-related early-stage cirrhosis patients. Functional enrichment and further immune infiltration analyses systematically elucidated potential immune response mechanisms.

Keywords: hepatitis C virus, cirrhosis, immune microenvironment, prognosis, prediction

INTRODUCTION

For approximately 1.6% (range: 1.3–2.1%) population with positivity for anti-hepatitis C virus (HCV) antibodies worldwide (1). Removing either spontaneously or as a result of antiviral treatment, the global viraemic prevalence (positive for HCV RNA) is estimated at 1% (range: 0.8–1.14%) individuals with HCV infection in reality (1). Meanwhile, 50–80% of HCV infection patients develop into chronic hepatitis C (1). Chronic HCV infection is the primary cause of liver cirrhosis, especially in the developing world (2). Liver cirrhosis affects hundreds of millions of people worldwide, causing more than one million deaths in 2010 (3). Cirrhosis is the major driver in the development of hepatocellular carcinoma (HCC). Liver cirrhosis develops from ongoing fibrosis injury and eventually leads to liver failure and HCC. HCC incidence in HCV-related cirrhosis was extremely high (up to 7% per year) (2). Mechanically, fibrosis results from the breakdown of the dynamic balance between extracellular matrix deposition and degradation in chronic diseases of the liver and other parenchymal organs (4). Until recent years, treatment for HCV with direct-acting antivirals (DAAs) regimens were associated with moderate success but were challenging to tolerate (5).

In fact, it was uncertain that at what stage cirrhosis becomes irreversible, but irreversibility becomes more likely as extracellular matrix collagen deposition (6). Generally, the prognosis of fibrosis mainly depends on early detection and clinical intervention. It was clear that early diagnosis and timely intervention can prevent or reverse the decompensation process (7). Importantly, patients with early cirrhosis, which is more common than liver cancer, lack a valid clinical prognostic marker.

Thus, we developed a robust immune-related prognostic index for patients with HCV-related early-stage cirrhosis who never developed HCC or cirrhosis complications at the enrolled time. In addition, our work showed the immune microenvironment and immune functionalities.

MATERIALS AND METHODS

Data Collection

Gene expression profiling of liver needle biopsy specimens and clinical information from 216 patients with hepatitis C–related Child–Pugh class A cirrhosis were available at National Center for Biotechnology Information Gene Expression Omnibus (GEO; GSE15654; https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE15654). External validation, GSE54100 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE54100) was performed using archived liver biopsy specimens from 145 patients with HCV-related compensated cirrhosis who had a liver biopsy and were followed at Massachusetts General Hospital. Another validation cohort GSE54099 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE54099) was performed using formalin-fixed, paraffin-embedded (FFPE) tissues sections (10 micron-thick) sliced from FFPE blocks from 90 HCV-related patients. All HCV infection was confirmed by serum HCV antibody and/or RNA. External independent validation dataset GSE54100 ($n = 145$) and GSE54099 ($n = 90$) with common clinicopathological characteristics. Besides, transcriptome profiling and the related clinical materials of HCC patients ($n = 371$) were obtained from The Cancer Genome Atlas (TCGA, https://cancergenome.nih.gov/) database.

Abbreviations: HCV, hepatitis C virus; HCC, hepatocellular carcinoma; DAAs, direct-acting antivirals; GEO, gene expression omnibus; ImmPort, the immunology database and analysis portal; LASSO, the least absolute shrinkage and selection operator; GO, gene ontology; KEGG, Kyoto encyclopedia of genes and genomes; BP, biological process; CC, cellular component; MF, molecular function; ssGSEA, the single sample gene set enrichment analysis; PPI, protein-protein interaction networks; SVR, sustained virological response; ISGs, interferon-stimulated genes.

Identification of Immune-Related Genes Related to the Prognosis of HCV-Related Early-Stage Cirrhosis Patients

Immune-related genes were obtained using the Immunology Database and Analysis Portal (ImmPort) database (https://immport.niaid.nih.gov) (8). Then, the immune-related genes expression matrix was extracted via matching immune genes in the primary GEO expression matrix. Subsequent analysis in the relationship between immune-related genes expression matrix and 216 HCV-related early-stage cirrhosis patients survival information was conducted *via* univariate Cox regression analysis.

Construction and Validation of an Immune-Related Prognostic Signature

Immune-related prognosis genes screened by univariate Cox regression were then analyzed using the least absolute shrinkage and selection operator (LASSO) regression analyses method (9). Then four genes were screened out. Further, a prognostic signature was built by performing multivariate Cox regression analysis using the selected genes. Previous researches (10) were employed for the determination of the risk score for every patient applying the formula below: Risk score $= \text{coef}_{\text{gene1}} * \text{expr}_{\text{gene1}} + \text{coef}_{\text{gene2}} * \text{expr}_{\text{gene2}} + \ldots + \text{coef}_{\text{gene}n} * \text{expr}_{\text{gene}n}$. A linear integration of the expression levels of genes weighted by regression coefficients (coef) was used to assign the risk score. Log transformation of the hazard ratio (HR) from the multivariate Cox regression analysis was employed to calculate the coef value. Patients enrolled in the training group were fallen into the groups of high-risk and low-risk based on the median score as a cutoff value. The Kaplan–Meier (K-M) method and log-rank tests with the "survival" R package were utilized to compare the survival rate between high-risk and low-risk groups based on the median risk score. Besides, the signature's effectiveness was assessed by performing the area under the curve (AUC) value of the time-dependent receiver operating characteristic (ROC) curves using "survivalROC" packages (11).

The Relationship Between the Prognostic Signature and Other Clinical Outcomes

Information of clinical deterioration terms including hepatic decompensation, progression of Child–Pugh class and HCC were extracted from GEO dataset. Patients were divided into high

and low group based on the median risk score. The cumulative incidence of these clinical endings was calculated and drawn using GraphPad Prism (v. 8.0.1). *P*-value and Hazard ratio were also performed.

Independence of the Prognostic Signature From Traditional Clinical Characteristics

Univariate and multivariate Cox regression analyses were explored to determine whether the immune-related prognostic signature was an independent factor compared with clinical characteristics (presence of varices, bilirubin $\geq$ 1.0 mg/dl and platelet $<$ 100,000/mm^3) in HCV early-stage cirrhosis patients.

Construction and Validation of a Predictive Nomogram

The construction of nomogram from clinical factors was made from multivariate regression analysis in the 216-patient cohort. The assessment of the discrimination and calibration of the predictive nomogram was made by applying the concordance index (C-index) and the calibration curve. The construction of nomogram and calibrate was made by the "rms" package (12). Besides, the signature's accuracy was assessed by performing ROC curve using "survivalROC" package (11).

GO and KEGG Pathways Enrichment Analyses

To explore the mechanisms whereby the identified immune-related prognostic signature may influence HCV-infected early-stage fibrosis patient clinical outcomes, Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) functional enrichment analyses were conducted with the "clusterProfiler" R package (13). GO functional enrichment analysis including biological process (BP), cellular component (CC), and molecular function (MF). The top 10 GO terms and the top 20 KEGG pathways were identified as being significant using the "ggplot2" R package (14). The statistical significance threshold of functional enrichment analysis was set at an adjusted p-value < 0.05.

ssGSEA Analysis

To explore immune cell infiltration, immune pathway activity, and functionality in HCV-related compensated cirrhosis patients, the single sample gene set enrichment analysis (ssGSEA) was conducted to built immune-related term enrichment scores. The ssGSEA function in the "gsva" R package was used to quantify immune cell infiltration based on the expression level of immune cell-specific marker genes (15). Scores corresponding to 29 different immune-related terms, including innate and adaptive immune cells, were determined for HCV-related early-stage cirrhosis patients. Type I and type II interferon response family genes, plasmacytoid dendritic cell precursors (pDCs) family genes, immune cell markers, and checkpoint genes expression levels were assessed in different clusters. Subsequent visualize differences analysis in the distributions of immune terms in the low- and high-risk patient groups via the "vioplot" R package (16).

TABLE 1 | Clinical features of 216 hepatitis C–related early-stage cirrhosis patients.

Characteristic at enrollment	*n* (%)
Presence of varices[a]	
YES	52 (26)
NO	159 (74)
Bilirubin $\geq$ 1.0 mg/dl	
YES	108 (50)
NO	108 (50)
Platelet $<$ 100,000/mm^3	216
YES	99 (46)
NO	117 (54)
Clinical outcome	
Death	66 (31)
Hepatic decompensation[b]	71 (34)
Progression of Child–Pugh class	66 (31)
HCC	65 (30)

[a] *The varices information could not be calculated in 4 patients due to missing data.*
[b] *The hepatic decompensation information could not be calculated in five patients due to missing data.*

Statistical Analysis

All statistical analyses were conducted using Rstudio (v.1.4.1106) and GraphPad Prism (v. 8.0.1). Continuous data are given as medians or as means $\pm$ standard deviation (SD). $P < 0.05$ was considered statistically significant.

RESULTS

Identification and comprehensive analysis of immune genes related to prognosis in HCV-related early-stage cirrhosis patients.

To develop a prognostic index, we download genomic data and clinical information from the GEO website. Clinical variates of the enrolled HCV-related early-stage cirrhosis patients were summarized in **Table 1**. Two hundred sixteen patients were enrolled, and 52 patients have esophageal/gastric varices, 108 patients with bilirubin $\geq$ 1.0 mg/dl, and 99 patients with platelet $<$ 100,000/mm^3. After a median follow-up time of 10 years, 66 patients died, 71 patients developed decompensation, 66 patients developed Child-Pugh B or C, and 65 patients developed to HCC. Genomic expression profiling was extracted from GSE15654. A total of 1,250 immune-related genes were matched in the microarray data. Then, we performed univariate Cox regression to explore the relationship between the expression profiles of the 1,250 genes and 216 patients with survival information. The results indicated that 156 of 1,250 genes were significantly associated with the prognosis of HCV-related early-stage cirrhosis patients ($p < 0.05$, **Supplementary Table 1**).

Protein-protein interaction networks (PPI) of the selected 156 genes were analyzed using the website tool STRING (https://string-db.org) (**Figure 1A**). The top 30 genes with enriched functional partners were performed (**Figure 1B**). Next, GO, and KEGG functional enrich analyses were conducted with the "clusterProfiler" R package. Enriched

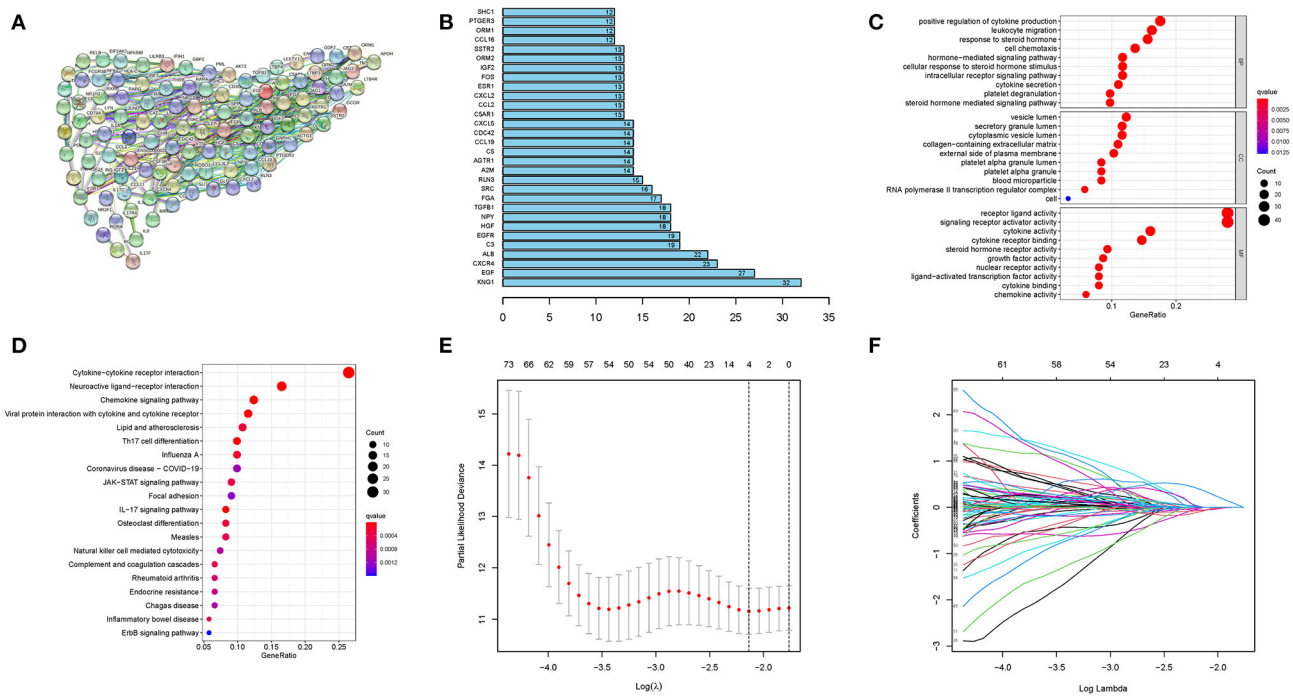

FIGURE 1 | Construction of prognostic model. **(A)** PPI network and **(B)** the top 30 genes with enriched functional partners. **(C)** GO and **(D)** KEGG bubble graph. **(E)** and **(F)** LASSO Cox regression.

biological processes (BP), including cell chemotaxis, leukocyte migration, leukocyte chemotaxis, T cell activation, platelet degranulation, positive regulation of cytokine production, and cytokine secretion (**Figure 1C**). Meanwhile, cytokine activity, cytokine receptor binding, growth factor activity, chemokine activity, and cytokine binding were the regular molecular function (MF) (**Figure 1C**). KEGG analysis showed that cytokine–cytokine receptor interaction, chemokine signaling pathway, IL-17 signaling pathway, and viral protein interaction with cytokine and cytokine receptors were common pathways in the collected genes (**Figure 1D**).

These results indicated vital roles of immune-related terms, including cell chemotaxis, cytokine activity, and inflammation signaling pathway, in the progress of HCV-related cirrhosis.

Establishment and Validation of a Prognostic Signature

Based on the LASSO regression with 10-fold cross-validation, we screened four genes (CXCL2, ENG, GHR, and ORM2) from 156 selected genes with a repetition frequency >900 times in 1,000 substitution samplings (**Figures 1E,F**). Further multivariate Cox regression was applied and finally determined three genes (ENG, GHR, and ORM2) of the four genes to construct a prognostic index (**Table 2**). The prognostic risk score = (1.291 * expression level of ENG) + (−0.568 * expression level of GHR) + (−0.597 * expression level of ORM2). In the signature, the positive coefficient of ENG suggesting that it may be a risk factor because its high expression is related to poor prognosis. However, the high expression of GHR and ORM2 may be protective factors, considering their expression levels were related to longer survival time.

TABLE 2 | Three-gene identified using Cox regression analysis and the LASSO regression method.

Id	Coefficient	Hazard ratio	*P*-value
ENG	1.291	3.635	2.304e−06
GHR	−0.568	0.567	0.021
ORM2	−0.597	0.550	0.024

The above formula was adopted to obtain the risk score of every member in the database, and the median-risk score was used to cluster these patients into different groups as the cutoff value. The group with a higher score was called the high-risk group, and the other group was called the low-risk group. According to the K-M analysis, overall survival (OS) was significantly worse in the high-risk group than in the low-risk group. ($p < 0.001$) (**Figure 2A**). The distribution of immune risk score, survival status, and expression matrix of the three genes for patients with HCV-related early-stage cirrhosis was performed in **Figures 2B–D**. Besides, AUC in the time-dependent ROC curve analysis reached 0.801, 0.767, and 0.775 at 1, 5, and 10 years, respectively (**Figure 2E**), indicating robust specificity and sensitivity of the prognostic signature in predicting survival.

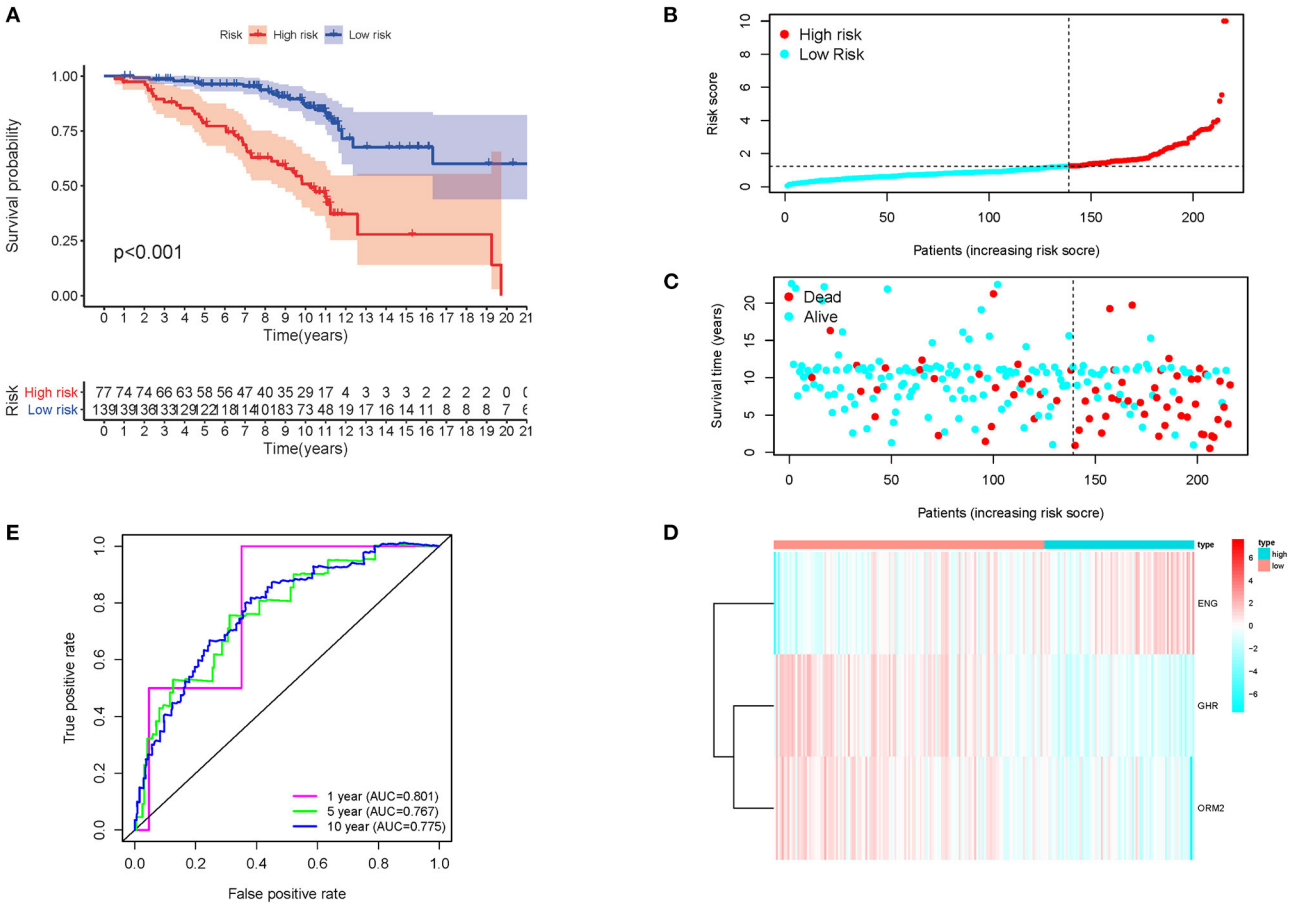

FIGURE 2 | K-M survival analysis, risk score distribution, and time-dependent ROC curves of a prognostic model in the HCV-related early-stage cirrhosis cohort. **(A)** K-M survival curve analysis between high and low-risk groups. The distribution of **(B)** immune risk score, **(C)** survival status, and **(D)** expression matrix of the three genes. **(E)** The time-dependent ROC curve analysis for measuring the predictive performance on OS.

The Relationship Between the Prognostic Signature and Other Clinical Deterioration

To further expand the application spectrum of the signature, the cumulative incidence of the clinical outcomes, including hepatic decompensation, progression of Child-Pugh class, and HCC were calculated. Surprisingly, a robust separation between the high and low-risk groups was performed. Patients in the high-risk group have a high incidence of poor clinical endings (hepatic decompensation, $p = 0.0313$, HR $= 1.568$; progression of Child-Pugh class, $p = 0.0074$, HR $= 1.544$; HCC, $p = 0.0001$, HR $= 2.421$; **Figures 3A–C**). In addition, time-dependent ROC curves were performed to validate sensitivity and specificity of the index in predicting malignant clinical events at 3, 5, 10 years, respectively (**Figures 3D–F**). These results revealed that the prognostic signature could be used in different clinical outcomes prediction with high efficiency, which benefits clinical applications. The reliable accuracy of the prognostic biomarker could help screen out early-stage cirrhosis patients who benefit from preventive interventions to alleviate cirrhosis complications.

The Relationship Between Risk Score and Clinical Parameters

Moreover, we investigated the differences in the prognostic signature scores in different subgroups of clinical parameters. The results revealed that risk score was significantly different in bilirubin ≥ 1.0 mg/dl ($p = 0.006$; **Figure 4A**) and platelet $< 100{,}000/\text{mm}^3$($p = 0.042$; **Figure 4B**). There was no significant difference in the presence of varices between the two groups ($p = 0.25$; **Figure 4C**). A higher gene score was found in more serious clinical parameters and advanced disease stages.

Association of Prognostic Signature and Immune Infiltration

Immune cell types, immune functions, or pathways were enrolled to assess immune cell infiltration among HCV-related early-stage cirrhosis patients in an integrated fashion via ssGSEA analysis of transcriptome profiling. Immune cell infiltration plays a vital role in HCV infection. Twenty-nine immune items were incorporated into this analysis, and 15 of these immune items differed significantly between the high- and low-risk groups in

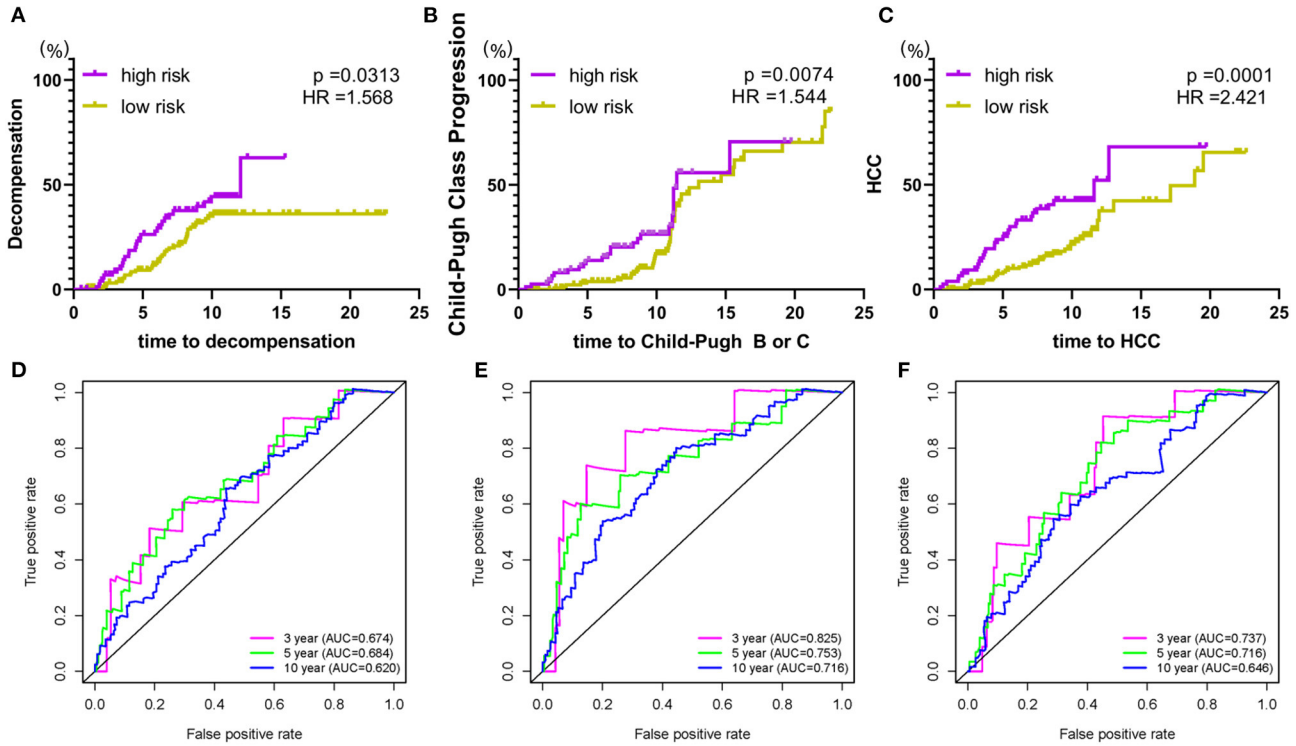

FIGURE 3 | The prognostic signature is associated with poor clinical endings. **(A)** risk score and hepatic decompensation; **(B)** risk score and Child-Pugh class progression; **(C)** risk score and HCC. **(D–F)** The time-dependent ROC curve analysis for verification of the index on different malignant clinical events. Hazard ratios and p-values were given for the difference between the high and low-risk groups.

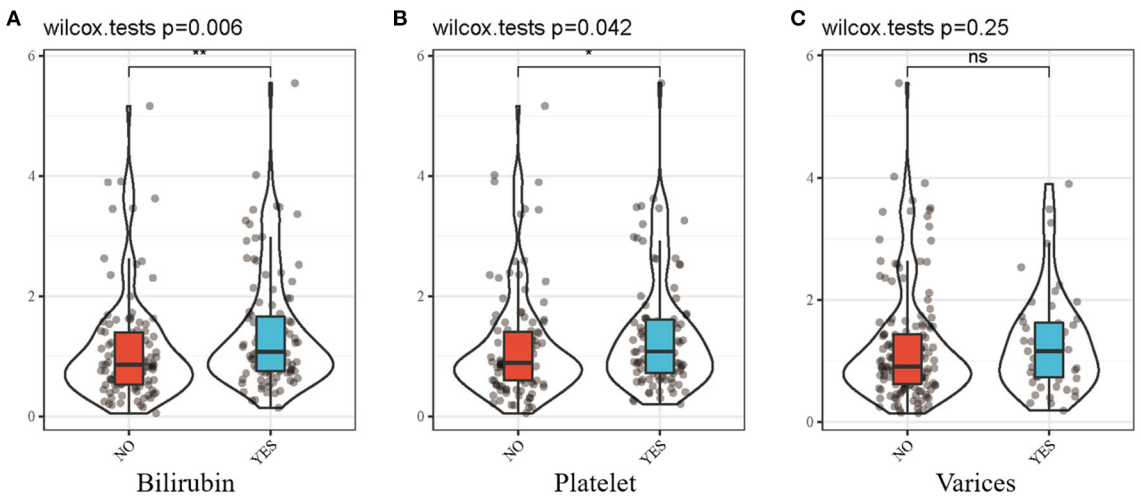

FIGURE 4 | Differential analysis between risk score and clinicopathological features. **(A)** risk score and bilirubin. **(B)** risk score and platelet. **(C)** risk score and varices. Bilirubin, bilirubin ≥ 1.0mg/dl; Platelet, platelet < 100,000/mm^3; Varices, presence of varices. *$p < 0.05$; **$p < 0.01$.

the overall patient cohort (**Figure 5A**). Among these significantly different items, antigen-presenting cell (APC) co-stimulation, CCR, checkpoint, immature dendritic cells (iDCs), macrophages, mast cells, pDCs, and follicular T helper cells (Tfh) were positively related to the risk score. However, the expression of MHC class I, neutrophils, natural killer (NK) cells, Treg, APC co-inhibition, type I interferon (IFN) response, and type II IFN response was opposite.

Then, we explored the further relationship of the expression of pDCs family genes and type I and type II IFN response family genes and risk score (**Figures 5B,C**). All differently expressed type I and type II IFN response family genes in patients with

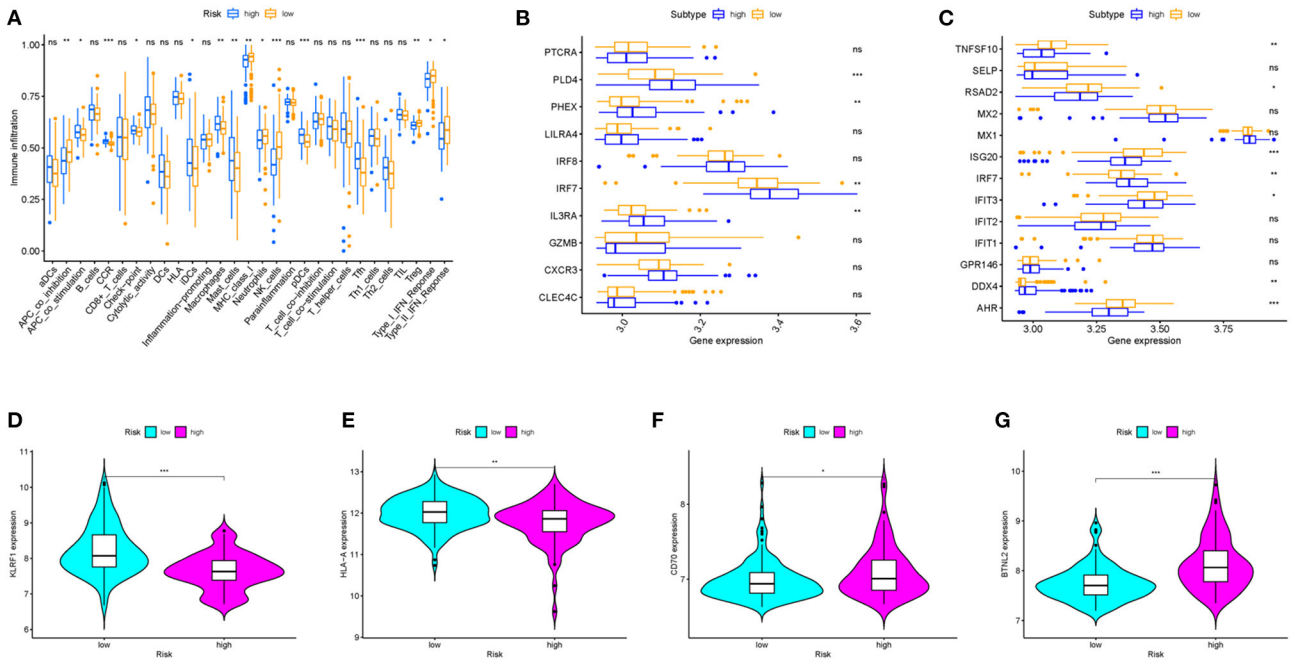

FIGURE 5 | The relationship between prognostic signature, immune cell infiltration, and immune functionality. **(A)** The relative enrichment of 29 immune-related risk terms in high- and low-risk HCV-related early-stage cirrhosis patients. **(B)** pDCs family genes and **(C)** type I and type II IFN response family genes expression profiles in high and low-risk groups. **(D–G)** KLRF1, HLA-A, CD70, and BTNL2 expression levels in different groups.

low-risk scores were higher expressed than patients with high-risk scores except for IRF7 and DDX4. IFN has important antiviral activity and immunomodulatory function in HCV infection and autoimmunity disease (17, 18). IFN upregulates the antiviral and immune regulatory activities of IFN-stimulated genes (ISGs) through binding to its receptor (19, 20). Moreover, patients in the high-risk group were related with a significantly lower expression level of KLRF1, one marker of NK cells (**Figure 5D**). NK cells play an important role in alleviating liver fibrosis through activation of metabotropic glutamate receptor 5 or Siglec-7 expression (21–23). Patients with end-stage cirrhosis often lack NK cells and have a weak response to cytokine stimulation (24). In addition, the expression of HLA-A, one marker of MHC class I cells, and CD70 and BTNL2, members of check-points genes, were also performed (**Figures 5E–G**). These findings served systematic analysis of immune infiltration in HCV-related early-stage cirrhosis.

Nomogram Construction and Validation

To determine whether the predictive prognostic signature was independent of other clinical characteristics, we performed univariate and multivariate Cox regression analyses. Univariate and follow-up multivariate analyses showed that bilirubin $\geq$ 1.0 mg/dl ($p < 0.001$), platelet $<$ 100,000/mm3 ($p < 0.05$) and risk score ($p < 0.001$) were independent elements for the poor prognosis of the HCV-related early-stage cirrhosis cohort (**Table 3**).

Next, a nomogram was built by combining clinical various of varices, bilirubin, platelet and risk score (**Figure 6A**). Each parameter in the nomogram was assigned a specific score. Based on the actual situation of every sample, the score related to every prognostic element to get the total score, which corresponded to the corresponding scale. The survival rates of patients at the first, fifth, and tenth year could be obtained. By measuring the extent of fit between the C-index forecast by the nomogram in the standard curve and the baseline time, the predictive ability of the nomogram model could be evaluated and quantified. The C-index was 0.737 (95% CI:0.671–0.802) for the nomogram, with 1,000 cycles of bootstrapping. Clinical usefulness of the prognostic model was estimated by decision curve analysis (DCA), which was a plot of the "Net Benefit" against "Risk Threshold Probabilities." The higher Net Benefit value, the more patients benefit. It was clear that, compared to clinical parameters, including varices, bilirubin, and platelet, the risk model has a better Net Benefit in a wide risk threshold at 5 years (**Figure 6B**). Moreover, according to the time-dependent ROC curve analysis, the AUC value for the immune-related prognostic signature was 0.767 at 5 years, which was higher than the AUC values for presence of varices (AUC = 0.579), bilirubin

TABLE 3 | Univariate and multivariate Cox regression analyses.

	Univariate analysis			Multivariate analysis		
	HR	**95% CI**	***P***	**HR**	**95% CI**	***P***
Varices	1.946	1.138–3.325	0.015	1.646	0.944–2.872	0.079
Bilirubin	3.295	1.908–5.690	<0.001	2.98	1.667–5.327	<0.001
Platelet	3.168	1.799–5.581	<0.001	1.985	1.095–3.598	0.024
Risk score	1.167	1.097–1.242	<0.001	1.192	1.111–1.278	<0.001

≥ 1.0 mg/dl (AUC = 0.595), and platelet < 100,000/mm3 (AUC = 0.634) (**Figure 6C**). In order to estimate calibration of the nomogram model, we performed calibration curves at the third, fifth and tenth year. Although the performance of the calibration curves at the third year was poor, the performance at the fifth year was better than that at the third year, and with the extension of follow-up time, the calibration curves of the nomogram showed great consistency between the predicted OS rates and actual observations at the tenth year (**Figures 6D–F**). These results indicated that the nomogram model did not perform well in short-time prediction, but show robustly ability in long-time survival prediction. Our work focuses on the prognosis of compensated cirrhosis, which usually has a longer survival time than decompensated cirrhosis and liver cancer.

Besides, distinct expression levels of the three genes in patients with different survival status in training cohort were

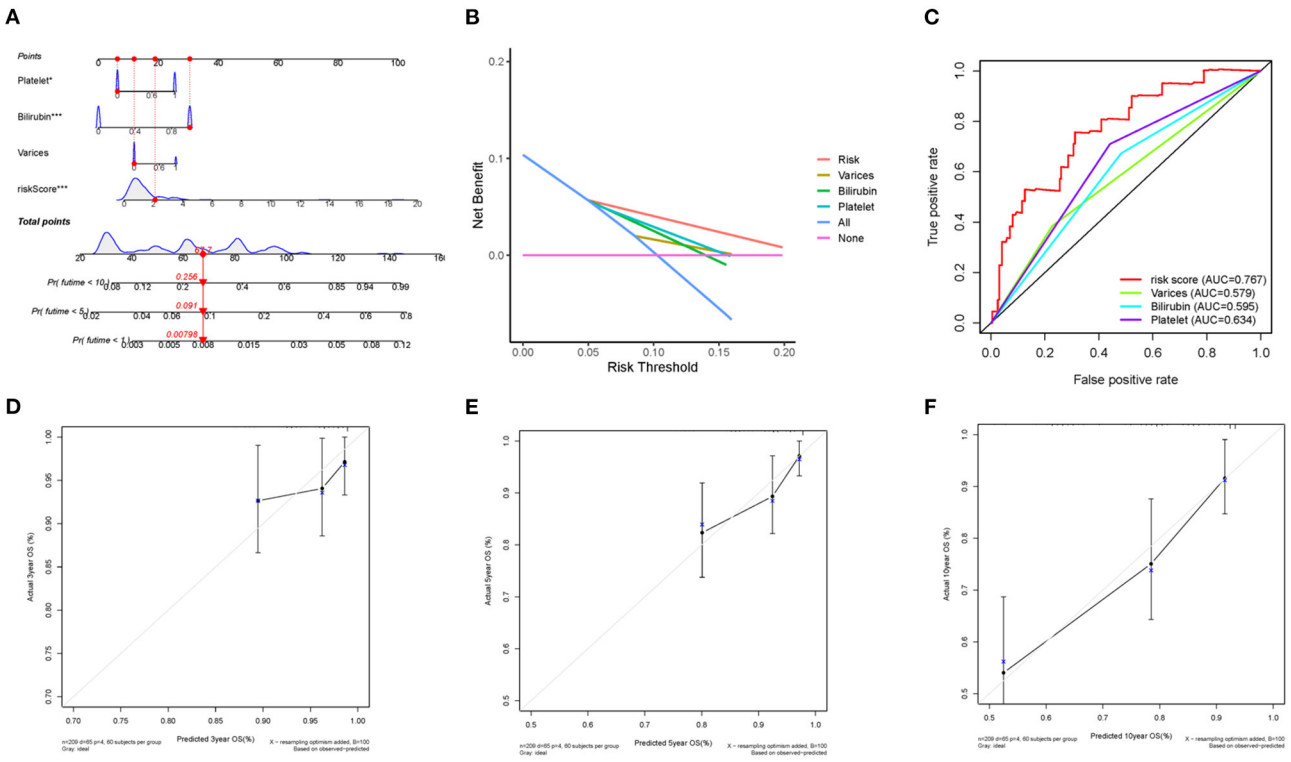

FIGURE 6 | Nomogram construction and validation. **(A)** the predictive nomogram. **(B)** the decision curve analysis and **(C)** ROC curve for the signature and clinical parameters. **(D)** 3-year, **(E)** 5-year and **(F)** 10-year calibration curves for OS.

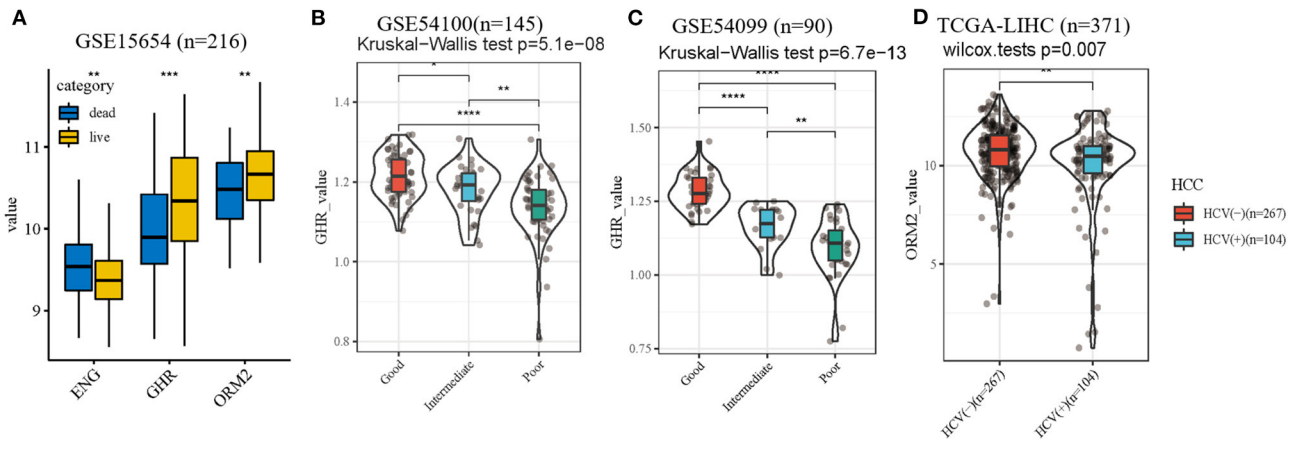

FIGURE 7 | Internal and external datasets verification. **(A)** expression levels of the three genes in patients with different survival status. **(B,C)** the association of GHR value with different clinical outcomes in external independent datasets. **(D)** different ORM2 value of HCC patients with HCV infection or not. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$.

performed (**Figure 7A**). To further validate the accuracy of the prognostic index, external independent datasets were enrolled. The relationship between the expression level of GHR gene and prognostic stratification in GSE54100 ($n = 145$) and GSE54099 ($n = 90$) were performed (**Figures 7B,C**). These results verified GHR was a protective factor in HCV-related cirrhosis development. In addition, due to limited data resources, we analyzed the differential expression of ORM2 in HCC patients with HCV infection or not (**Figure 7D**). HCC patients with HCV infection have a lower expression level of ORM2 compared with non-HCV infection HCC patients.

DISCUSSION

In the absence of highly sensitive and accurate molecular prognostic biomarkers for HCV-related compensated cirrhosis patients, we developed a satisfactory 3-gene signature, which can successfully predict the patients' clinical outcomes. More importantly, this signature can also accurately predict Child-Pugh class progression, hepatic decomposition, and development to HCC, which means the signature was a sensitive measure of the severity of HCV-related cirrhosis and lethality even in compensated patients. Our work revealed the ability of the index in predicting the tendency of liver cirrhosis to worsen. This model revealed a close association between HCC and liver failure state by Child-Pugh class classification progression compared with other HCC prognostic markers (25).

Besides, based on the immune-related prognostic index, our finding further revealed that patients with different risk score stratifications have distinctly different immune microenvironments, which may explain different clinical endings. In the pathogenesis of cirrhosis, the immune system not only plays the role of immune-mediated inflammatory mechanisms, persistent inflammatory stimulation and cell damage promote fibrogenesis through activate hepatic stellate cells and cause immune dysfunction (26). Innate immune cells, including Kupffer cells, mast cells, and resident masters, are the first defense mechanisms against pathogens. However, with the deterioration of cirrhosis, the antibacterial function of circulating neutrophils and monocytes is gradually damaged, end-stage cirrhosis patients were susceptible to bacterial infection (27). Meanwhile, chronic viral infection led to persistent specific immune inflammation stimulation, which aggravating fibrosis and may cause acute compensatory dysfunction and liver failure, both of which are associated with high short-term mortality. Systemic inflammation (elevated steady-state immune cell activation and circulating inflammatory mediators) aggravates hemodynamic derangement and kidney injuries, regularly occurring in patients with acute-on-chronic liver failure (28). An initial systemic inflammatory response (cytokine storm) works as a trigger of the systemic inflammation leading to a compensatory anti-inflammatory response that impairs resistance to infection. Cirrhosis patients with immunodeficiency accompany systemic inflammation were regarded as cirrhosis associated immune dysfunction (CAID) (26). In fact, the mechanism of damage and pathogen-associated molecular patterns activating immune cells and promoting systemic inflammatory response, with ongoing fibrosis progression, is complex, which has not been fully understood.

Direct-acting antivirals (DAAs) has made rapid advance in chronic HCV infections therapy (5, 29). IFN-based therapies, which are no longer recommended, have been replaced by DAA regimens through different viral elimination mechanisms (30). Sustained virological response (SVR) was defined as undetectable serum HCV RNA at least 12 weeks posttreatment (31). SVR after DAA treatment was regarded as closely associated with long-term clinical benefits (31). Thus, almost all stage patients with chronic HCV infection should be treated with DAAs, including patients with decompensated cirrhosis (32–35). Emerging large cohorts further support the results.

DAAs treatment inhibits IFN-λ production and recover exogenous IFN-α reactivity. Besides, it was reported that DAAs downregulated interferon-stimulated genes (ISGs) induced by Hepatitis C virus infection and alleviate HCV-induced extrahepatic symptoms (36).

In addition, there were still some limitations in the research. First, the clinical application of molecular biomarkers based on gene expression has been challenging because of the poor reproducibility of measurements (37). Second, the prognostic model was established relying on the GEO dataset only. Further laboratory experiments and clinical trials are needed to validate the reliability of the results.

CONCLUSION

Conclusively, through a comprehensive analysis of the GEO data set, our work identified an immune-related genes index with accurate and efficient clinical deterioration prediction for HCV-related early-stage cirrhosis patients. Besides, enriched immune function and pathways of prognostic immune genes were exhibited. In addition, we elucidated systematic analysis of the immune microenvironment in cirrhosis patients in different risk groups. However, informatics analysis alone is not sufficient to verify the validity of the indicators and to research possible molecular immune mechanisms of HCV-related compensated cirrhosis. Thus, subsequent lab experiments and follow-up experiments will devote to uncover the mechanism involved in HCV-related cirrhosis.

DATA AVAILABILITY STATEMENT

Publicly available datasets were analyzed in this study. This data can be found at: https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE15654, https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE54100, https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE54099, https://cancergenome.nih.gov/, and https://immport.niaid.nih.gov.

ETHICS STATEMENT

The gene expression profiling and corresponding clinical information in this study were obtained from GEO and TCGA databases and were freely available to the public, which

means this study does not require ethical approval from an Ethics Committee.

AUTHOR CONTRIBUTIONS

CG, YG, and ZW designed the study. CG, CD, JZ, RW, JZ, ZW, BJ, and BM complicated and analyzed data. CG and CD wrote this manuscript. All authors have read and approved the final 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/fmed.2021.716869/full#supplementary-material

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REVIEW
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Role of Th22 Cells in Human Viral Diseases

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Naive $CD4^+$ T cells can differentiate into different cell subsets after receiving antigen stimulation, which secrete corresponding characteristic cytokines and thereby exert biological effects in various diseases. Th22 cells, a novel subset of $CD4^+$ T cells, are different from Th1, Th2, Th17, and Treg cell subsets, which have been discovered in recent years. They can express CCR4, CCR6, and CCR10 molecules and secrete IL-22, IL-13, and TNF-α. They are not able to secrete IL-17, IL-4, and interferon-γ (IFN-γ). IL-22 is considered as a major effector molecule of Th22 cells whose functions and mechanisms of regulating cell differentiation have been constantly improved. In this review, we provide an overview of the origin, differentiation of Th22 cells. Moreover, we also describe the interrelationships between Th22 cells and Th17, Th1, and Th2 cells. Additionally, the role of Th22 cells were discussed in human diseases with virus infection, which will provide novel insight for the prevention and treatment of viral infection in human.

Keywords: naive $CD4^+$ T cells, Th22 cell, IL-22, virus hepatitis, hand, foot, and mouth disease, HIV disease, COVID-19

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INTRODUCTION

T lymphocytes are a significant component of the human immune system and can be further classified as $CD4^+$ T cells and $CD8^+$ T cells. According to functions, surface markers, and secreted effector molecules, $CD4^+$ T cells, also known as T helper (Th) cells were mainly divided into Th1, Th2, Th17, T follicular helper (Tfh) cells, regulatory T (Treg) cells, and other Th cell subsets (1, 2). The different subsets of Th cells subsets play important roles in the development and progression of human autoimmune diseases, infections, and tumors (3, 4). During research to further understand Th cell subsets, new subgroups have been gradually discovered. In the early phases, studies have demonstrated that both Th17 and Th1 could secrete IL-22 (5, 6). Further studies on IL-22 found a group of special cell subsets that can secrete IL-22 and IL-13 instead of IL-17, IL-4, and IFN-γ. These subsets were later confirmed to be $CD4^+$ T cell subsets and independent of Th1, Th2 and Th17 cells. This subset of cells was named Th22 (7). Th22 cells can express CCR4, CCR6, CCR10 and several fibroblast growth factors (FGFs) molecules. They also participate in the homeostatic regulation of skin and pathological processes (7), promote angiogenesis, and accelerate wound healing (8). IL-6 and TNF-α can induce the differentiation of naive $CD4^+$ T cells into Th22 cells, while TGF-β was found to inhibit differentiation (7).

Viral infectious diseases, such as Acquired Immune Deficiency Syndrome (AIDS), viral hepatitis and so on, seriously threaten human health, and become one of the problems that people need to solve. Recent studies have shown Th22 cells may be involved in regulating the pathological

processes of many viral infectious diseases (9–11). The traditional concept of simple division of Th cells is continuously being renovated. This speaks to the diversity of T cell functional subsets and the complexity of immune regulatory functions. As a novel Th cell subset, Th22 cells further expand the understanding of immune regulation (8, 12). The differentiation and role of Th22 cells and their relationships with other T cell subsets are extremely important for recognizing T cell immune response. These findings will also help with understanding the pathogenesis of diseases and exploring more effective targets of disease intervention. This review will focus on the differentiation and regulation of Th22 cells and discuss the research progress for the role of Th22 cells in several common human viral diseases.

THE ORIGIN OF TH22 CELLS

The origin of Th22 cells was derived from a study on IL-22. In 2000, Dunamtier et al. used IL-9 to stimulate mice lymphoma cells. They found that these cells expressed a cytokine closely resembling the secondary structure of IL-10. They named this cytokine Interleukin-10-related T cell-derived inducible factor (IL-TIF) (13). Also in 2000, Gurney et al. identified a new sequence from T cells isolated from humans with 23% of the encoded amino acids being homologous to IL-10, and 87% were similar to IL-TIF (14). This sequence was designated as IL-22. Initially, IL-22 was considered to be a cytokine associated with T helper type 1 cells (Th1) (5, 15). It was later found to be closely related to the expression of IL-17 by IL-17-producing T helper cells (Th17 cells) (6, 16). IL-22 can also be derived from natural killer T cells (17) and lymphoid tissue–inducer cells (LTi cells) (18). In mice, IL-22 is mainly produced by Th17 cells. Retinoid-related orphan receptor-γt (ROR-γt), known as RORC in humans, is a transcription factor that controls the generation of Th17 cells. Retroviruses were used to transfect ROR-γt into T cells of mice, which were endowed with the ability to express IL-17 and IL-22 (19, 20). Human T cells transfected with RORC cannot induce IL-22 expression (21). Another transcriptional regulator of Th17 cells is Aryl hydrocarbon receptor (AHR). AHR can also promote the expression of IL-17 and IL-22 (22).

In 2009, Sara Trifari et al. identified a group of $CD4^+$ memory T cells with the phenotype of $CCR4^+CCR6^+CCR10^+$ in the blood of healthy adults. These T cells produced IL-22 and IL-13, but did not secrete IL-17 and IFN–γ. The expression of IL-22 could be promoted by upregulation of AHR or the transcription factor RORC (23). In 2009, Duhen et al. classified $CD4^+CD45RA^-CD25^-$ memory T cells isolated from peripheral blood of healthy individuals (7). Cells were grouped according to whether they expressed CCR6, and the expressions of IL-17, IL-22, and IFN-γ were analyzed. After polyclonal stimulation, both intracellular and cultured supernatants showed expression of IL-17 and IL-22 was completely restricted to $CCR6^+$ subset cells, while IFN-γ was expressed in both $CCR6^+$ and $CCR6^-$ subsets. Based on the biological function of IL-22, the researchers hypothesized that IL-22- producing cells might have the characteristics of skin-homing T cells, so T cells were separated into four subsets according to the expression of CCR6, CCR4, and CCR10. Further analysis showed that $CCR10^+CCR6^+CCR4^+$ subset cells only expressed IL-22. Neither IL-17 nor IFN-γ was expressed after stimulation with anti-CD3 and anti-CD28. Researchers confirmed the independence of Th22 cells through the following experiments (8). They placed Th22 cell clones derived from patients with psoriasis in an inducible environment of Th1 cells, Th2 cells, Th17 cells, and Treg cells. They found that these cells maintained the ability to secrete IL-22 and did not secrete the characteristic cytokines of other T cell subsets. Thus, it can be determined that Th22 cells are an independent and stable lineage. In 2017, Plank et al. carried out the whole gene chip to analyze the mRNA transcription profile of Th22 and Th17 cells (24). This research further confirmed the differences between Th22 and Th17 cells.

MOLECULAR CHARACTERISTICS AND DIFFERENTIATION OF TH22 CELLS

Th22 cells are a novel subset of $CD4^+$ Th cells that are distinct from Th1, Th2, and Th17 cells (**Figure 1**). Th22 cells are able to secrete IL-22, IL-26, IL-13, TNF-α, and granzyme B (24). They are not able to produce INF-γ, IL-4, or IL-17, and IL-22 is its main effector molecule. Th22 clones begin to release IL-22 at the 6th h and reach a peak at the 12th h. The expression of IL-22 can be maintained at this level for 48 hours. Th22 cells have a $CD3^+CD4^+$ phenotype. In addition, they also express the skin chemokine receptors CCR4, CCR6, and CCR10. CD8 and NK cell markers CD56, NKp44, and NKp46 were found to be negative (8). Human skin typically expresses abundant levels of chemokines. This explains why there are relatively more Th22 cells found in the skin and fewer found in circulation throughout the body.

The differentiation of Th22 cells is regulated by many factors, mainly involving into the cytokine, cellular membrane molecules, and transcription factors. Duhen et al. demonstrated that IL-6 and TNF-α together jointly induced Th22 cell differentiation (7). For this process, IL-6 was crucial for Th22 cell differentiation and stimulation with IL-6 alone resulted in a substantial differentiation of naive $CD4^+$ T cells into Th22 cells. This suggests that IL-6 may be a priming factor for Th22 cell differentiation. When IL-6 and TNF were used in combination, researchers found that the proportion of Th22 cells was higher than when IL-6 was used alone. This suggests that TNF may play a role in promoting Th22 cell differentiation. High doses of TGF-β exerted an inhibitory effect on the differentiation of Th22 cells. Plank et al. optimized the condition of Th22 cell differentiation (24). For intervention, they first combined with four factors: IL-6, IL-23, IL-1β, and 6-formylindolo [3,2-*b*] carbazole (FICZ). This study found that IL-17A was still secreted. In order to inhibit IL-17A secretion, researchers added a TGF-βR inhibitor (galunisertib), which effectively inhibited IL-17A production without affecting the secretion of IL-22. IL-13 and granzyme B levels were also significantly increased under this culture condition. IL-21 and IL-23 can also induce the differentiation of Th22 cells. Yeste et al. found that either IL-21 alone, IL-21 combined with IL-23 or IL-1β could induce Th22

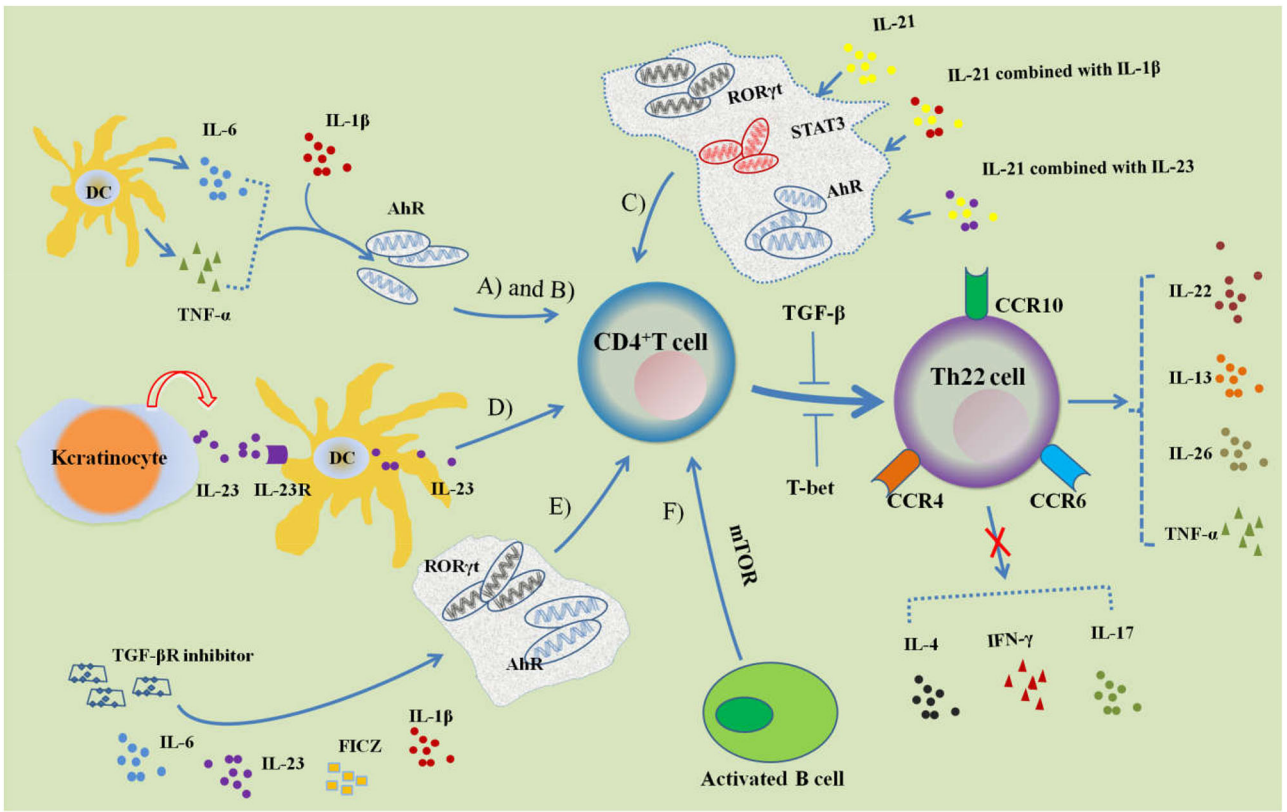

FIGURE 1 | Regulation of Th22 cell differentiation. **(A)** IL-6 and TNF-α induce naive CD4$^+$ T cells to differentiate into Th22 cells characterized with CCR4, CCR6, CCR10 and IL-22 expression, which can be promoted by IL-1 β. AhR is involved in the regulation of differentiation. **(B)** Activated DCs can secrete IL-6 and TNF-α to induce Th22 cell differentiation. **(C)** IL-21, IL-21 combined with IL-23 or IL-1β can induce Th22 cell differentiation and IL-22 expression, while the transcription factors STAT3, RORγt, and AhR participate in its regulation. **(D)** Keratinocytes are stimulated by endogenous TLR4 ligands and secrete IL-23, which combines with IL-23R of DCs; activated DCs further secrete IL-23 and induce CD4$^+$ T cells to differentiate into Th22 cells. **(E)** Combined with IL-6, IL-23, IL-1β, FICZ and TGF-βR inhibitors can promote the differentiation of CD4$^+$ T cells into Th22 cells. **(F)** Activated B cells can induce Th22 cell differentiation by activating mTOR signaling. Both TGF-β and T-bet can inhibit the differentiation of Th22 cells.

cell differentiation and IL-22 expression (25). The transcription factors signal transducer and activator of transcription 3 (STAT3), RORγt, and AhR were involved in their regulation. The endogenous TLR4 ligand stimulates keratinocytes to secrete IL-23 and binds to the IL-23 receptor of skin dendritic cells. This activates the secretion of endogenous IL-23, induces the differentiation of naive T cells into Th22 cells, and releases IL−22 (26). Th22 cells are different from other Th cell subsets for characteristic molecules, and the differentiation of Th22 cells are associated with the important cytokines and transcript factors.

Duhen et al. used conventional DCs (cDCs) and plasmacytoid-like DCs (pDCs) to stimulate naive CD4$^+$ T cells (7). This research revealed that pDCs had a stronger induction effect on Th22 cells than cDCs. The addition of mature pDCs to the culture medium of stimulated differentiated cDCs promoted the differentiation of Th22 cells, while adding mature cDCs to the culture medium of stimulated differentiated pDCs and inhibited the differentiation of Th22 cells. These results indicate that mature pDCs may indirectly drive the differentiation of naive CD4$^+$ T cells into Th22 cells. Further studies found that both cDCs and pDCs released high concentrations of TNF-α and IL-6 after activation, without secreting IL-12, IL-23, and IL-1β. In addition, cDCs were able to produce a small amount of IL-10. This experiment showed that blocking TNF and IL-6 inhibited 70% of Th22 differentiation. These results suggest that DCs may promote Th22 differentiation in both direct and indirect ways. Fujita et al. isolated Langerhans cells (LCs) that were HLA-DR$^+$ and CD207$^+$ (27). They also isolated dermal DCs (HLA-DR^{hi}CD11c$^+$BDCA-1$^+$ cells) from the epidermis and dermis of normal people. This could induce peripheral blood T cells and naive CD4$^+$ T cells to differentiate into Th22 cells, prompting the idea that cutaneous resident DCs may regulate the differentiation of T cells into Th22 and enter the skin. Foreign antigens such as microbes can activate DCs, promote the production of cytokines, and then induce the differentiation of Th22 cells. Díaz-Zúñiga et al. showed that DCs and naive CD4$^+$ T cells isolated from the peripheral blood of healthy people were stimulated by different aggregatibacter actinomycetemcomitans serotypes (28). They found that the levels of TNF-α and IL-6 were significantly increased as well

as the expressions of IL-22 and AhR, which might initiate the polarization of Th22 cells.

In vitro, activated B cells and naive T cells were co-cultured under Th17 cell culture conditions. This revealed that activated B cells could significantly inhibit the production of IL-17 and the expression of RORγt. In contrast, they could stimulate Th22 differentiation and IL-22 production. Further studies *in vivo* demonstrated that treatment with injection of activated B cells in the MRL/lpr lupus mice models reduced anti-dsDNA antibody and protein levels in urine. This also suppressed Th17 cell differentiation and enhanced Th22 cell differentiation (29). The aryl hydrocarbon receptor (AhR) is an endogenous ligand nuclear transcription factor. Activated AhR can significantly promote the differentiation of naive $CD4^+$ T cells into Th22 cells (23). Runt-related transcription factor 3 (RUNX3) is a Runt-domain family transcription factor. Studies have shown that RUNX3 is involved in the differentiation of Th22 cells in patients with psoriasis (30). The number of Th22 cells decreased significantly after RUNX3 levels of $CD4^+$ T cells from psoriasis patients were restricted by RNA interference. Plank *et al.* isolated naive $CD4^+$ T cells from Rorc (γt) knockout mice and found a partial reduction in IL-22 expression under Th22 culture conditions, and naive $CD4^+$ T cells that came from Tbx21 knockouts and were cultured in Th22 conditions exhibited a more than two-fold increase in IL-22 expression (24). It is believed that RORγt is partly involved in the differentiation of Th22 cells, while T-bet inhibits the differentiation of Th22 cells. These findings indicated that Th22 cell differentiation could be initiated by innate immune cells including DCs, LCs and B cells that expressed cytokines and membrane molecules.

A recent study indicated that when $CD4^+$ T cells isolated from peripheral blood of patients with coronary heart disease were transfected with miR-31 mimic, the differentiation of Th22 cells and the expression of transcription-related factor AHR were significantly promoted (31). This also remarkably accelerated IL-22 secretion. Further research showed that miR-31 overexpression increased the differentiation of Th22 cells by inhibiting BTB domain and CNC homolog 2 (Bach2). These findings indicate that microRNAs may also participate in the regulatory differentiation of Th22 cells.

THE RELATIONSHIP BETWEEN TH22 CELLS AND OTHER TH CELLS

Both Th22 cells and Th17 cells are T helper cells that are differentiated from naive $CD4^+$ T cells (**Figure 1**). Th17 cells are a subset of $CD4^+$ T cells that are independent of Th1 and Th2 that was first identified by Park et al. in their research of autoimmune encephalomyelitis and collagen-induced arthritis (3). Cytokines involved in Th17 cell differentiation include TGF-β, IL-6, IL-1β, IL-21, and IL-23 (4, 32). IL-6 and TGF-β are especially important in this process. TGF-β along with IL-6 initiates Th17 cell differentiation through the RORγt signal transduction pathway (19). Th17 cells can specifically secrete cytokines such as IL-17A, IL-17F, IL-21, IL-22, and TNF-α instead of IL-4 or IFN-γ. This allows Th17 cells to exert their biological effects (4).

Both Th22 cells and Th17 cells are activated to play an immunomodulatory role in some diseases. TNF-α is a cytokine secreted by Th22 cells and Th17 cells. Andersen et al. used anti-TNF-α to treat patients with spondyloarthritis (SpA) for 52 weeks of treatment (33). In this time, the number of Th22 cells and Th17 cells increased continuously, while the expression of IL-23 receptor decreased significantly. Two kinds of cell subsets were positively correlated with the Ankylosing Spondylitis Disease Activity Score and the Bath Ankylosing Spondylitis Activity Index. Increased frequencies of Th22 and Th17 cells in peripheral blood may be related to the activity and duration of the autoimmune thyroid disease (12). Th22 cells and Th17 cells also showed a certain correlation in patients with preeclampsia. Studies have found that the percentage of Th22 cells and Th17 cells in the peripheral blood of patients with preeclampsia was significantly increased and there was a positive correlation between Th22 cells and Th17 cells (34).

In some situations, Th22 cells and Th17 cells restrict each other and behave as opposite immune effects. In immune-mediated skin diseases, such as atopic dermatitis, allergic contact dermatitis, and psoriasis, Th22 cells and Th17 cells jointly activated keratinocytes and initiated non-specific immunity to protect the skin against the invasion of pathogens. In the pre-inflammatory state, the roles of the two types of cells are not exactly the same. Th17 mainly relies on the activation of IFN-γ and IL-17 to enhance the expression of adhesion molecules on keratinocyte, which in turn initiates the T cell-mediated cytotoxicity (35, 36). Th22 cells maintain the integrity of the skin by inducing the proliferation and migration of keratinocytes (37).

Th22 cells and Th17 cells may play roles at different stages of the disease and help to regulate each other. $ApoE^{-/-}$ mice were fed the Western diet in order to induce the atherosclerosis model. It was found that the proportions of both Th22 and Th17 cells increased. Levels of Th17 cells began to decrease at the fourth week and almost declined to the initial levels at the 8th week. Th22 remained at a high level throughout observation. After 12 weeks of feeding and treatment with recombinant mouse IL-22, large plaques appeared in the aorta and the aortic root and increased the levels of Th17 cells, DCs, and pSTAT3. Anti- IL-22 monoclonal neutralizing antibody treatment may have the opposite effect. Further studies have revealed that rIL-22 and ox-LDL stimulation can induce the maturation of bone marrow-derived dendritic cells, which further induces Th17 cell proliferation through the IL-6/STAT3 pathway, thereby aggravating the development of atherosclerosis (38).

The differentiation of Th22 cells is different from that of Th17 cells. RORγt (RORC) is the transcription regulator of Th17 cells, and AHR is the main transcription regulator of Th22 cells. A recent study found that medroxyprogesterone acetate (MPA) could inhibit the expression of IFN-γ, IL-22, IL-17A, and RORC in Th17/Th1 cell clones of peripheral blood, but MPA also significantly increased the expression of AHR, T-bet, and IL-22 in Th22 cell clones (39). TGF-β is an important differentiation factor for Th17 cells, but it exerts an inhibitory effect on Th22 cell differentiation. *In vitro*, activated B cells and naive T cells were co-cultured under Th17 cell culture conditions. It was found that activated B cells could significantly

inhibit the production of IL-17 and the expression of RORγt, but were able to stimulate Th22 differentiation and IL-22 production (27).

Th1 cells mainly secrete IL-2, IL-12, IFN-γ, TNF-α, and TNF-β, participate in cellular immunity, activate cytotoxic T lymphocytes and macrophages, mediate organ specific immune response, and eliminate intracellular pathogens. Wolk et al. discovered that IL-22 expression could be increased by inducing Th1 polarization *in vitro* (40). The percentage of Th1 and Th22 cells in untreated immune thrombocytopenia patients was significantly higher than that in healthy controls. IL-22 levels were positively correlated with the proportion of Th1 and Th22 cells. After dexamethasone treatment, the number of Th1 cells and Th22 cells significantly decreased and the level of IL-22 notably declined as well. The polarization of Th1 cells and Th22 cells may contribute to IL-22 expression (41). T-bet is a specific transcription factor for Th1 cell differentiation that mediates the specific expression of IFN-γ in Th1 cells and can inhibit the differentiation of Th2 cells. However, T-bet exerts an inhibitory effect on the differentiation of Th22 cells. Under the condition of Th1 differentiation, 30–50% of Th22 cells can express IFN-γ (24). This indicates that Th22 cells have plasticity to differentiate into Th1 under specific environmental conditions. Th1, Th17, Th22, and Treg cells maintain a balance and jointly regulate the progress of some autoimmune diseases (42). In chronic myelogenous leukemia (CML), Th22, Th17, and Th1 levels were significantly reduced in both the bone marrow and peripheral blood in patients newly diagnosed with CML. These levels were inversely correlated with the percentage of BCR-ABL gene fusion [BCR-ABL (%) IS] (43).

The cytokines secreted by Th2 cells mainly include IL-4, IL-5, IL-6, IL-13, and IL-10. These cytokines can promote the proliferation and differentiation of B cells as well as induce and promote humoral immunity. Cytokines such as IL-6 could promote the differentiation of Th22 cells. In fact, activation of Th2 and Th22 produced differences in disease states. During the acute phase of atopic dermatitis, the bias of Th2 and Th22 cytokines was observed, while Th1 and Th17 cytokines did not show a significant increase (44). Th2 cell activation could be observed in both intrinsic and extrinsic atopic dermatitis (AD). When compared with extrinsic AD, intrinsic AD showed more prominent immune activation, especially on the Th22/Th17 axes (45). In Alopecia areata, Th1 and Th2 cells were activated, but markers of Th17/Th22 cells did not increase significantly (46).

THE ROLES OF TH22 CELLS IN HUMAN VIRAL DISEASES

With continuous research, studies have found that Th22 cells play a regulatory role in the occurrence and development of many diseases (**Table 1**). The cellular and humoral immune responses play an important role in the pathogenesis of viral infectious diseases. In addition to Th1, Th2, Th17, Tfh, and Treg cells, an increasing number of studies have found that Th22, which produces IL-22, is involved in the pathogenesis of multiple viral infectious diseases. Both pathologic and protective roles have been attributed to Th22 in maintaining immunologic homeostasis.

Studies have shown that Th22 cells play a protective role in the process of HIV infection. Kim et al. found that the expression of Th22 cells in the HIV-infected sigmoid colon mucosa was dramatically absent and could be reversed after prolonged antiretroviral therapy (9). Th22 cells expressed the molecules CCR5 and α4β7 for binding to HIV receptor in circulation. The recombinant IL-22 can resist HIV-induced destruction of epithelial cell integrity. In some HIV-resistant individuals (HIV-exposed uninfected individuals), the number of Th22 cells was significantly higher than that of healthy controls and HIV-infected people. Some protein molecules, including IL-22, were involved in the innate host resistance mechanism (47, 48). In HIV-infected children, Th22 cells and mucosal-associated invariant T cells (MAIT) were significantly reduced. There was a clear correlation after successful antiretroviral therapy (ART). Th22 cells in circulation were significantly increased, suggesting that the proliferation of cTh22 could provide immunological advantages for suppressing HIV-1 infection (49). The expression of Th22 and Th17 cells were decreased in the mucosal tissues of HIV infected patients (50). Th17 cells are incompletely restored at normal frequencies in most HIV-infected individuals on antiretroviral therapy,but Th22 cells complete migration through the CCR10-CCL28 axis, which plays a protective role in the mucosa (51). These findings implied that Th22 cells played more important protective role than that of Th17 cells in HIV infection.

Hepatitis B virus infection is the main cause of liver cirrhosis and liver cancer. Th22 cells in the blood of patients with hepatitis B virus infection were found to be increased, along with significantly higher levels of IL-22 (10, 52, 53). The levels of Th22 cells and IL-22 were related to the severity and prognosis of the disease (10, 54). Injection of IL-22 could promote the expression of pro-inflammatory genes in the liver of HBV transgenic mice instead of directly inhibiting virus replication. Transplanting spleen cells from HBV-immunized mice to HBV transgenic mice and neutralizing IL-22 could reduce liver damage in model mice and significantly inhibited the accumulation of antigen-non-specific inflammatory cells to the liver (53). These results indicated that IL-22 has a pro-inflammatory effect during HBV infection. Other research suggests that IL-22 plays a protective role in liver damage. Radaeva et al. detected the increase of IL-22 and IL-22 receptors in the hepatitis model induced by Concanavalin A (ConA). An IL-22 neutralizing antibody could aggravate liver injury, and overexpression of IL-22 was found to activate STAT3 and reduce the apoptosis of hepatocytes (55). In chronic HBV infected patients and animal models, IL-22 was able to promote the proliferation of liver stem/progenitor cells (LPCs) through activation of the STAT3 pathway (54). These reports indicated that Th22 cells and IL-22 played a protective role in HBV infection.

Chronic hepatitis C (CHC) is another common viral hepatitis. Th22 cells and IL-22 are significantly increased in blood and liver of CHC patients, which play an important role in regulating liver immunity (11, 56, 57). IL-22 was involved in hepatofibrosis (HF) and cirrhosis associated with HCV infection. On one hand, IL-22 can activate the innate immunity of liver,

TABLE 1 | The role of Th22 cells in human viral diseases.

Diseases	Th22 cells and IL-22	Associated molecules	Roles	References
AIDS	Th22 cells, IL-22, MAIT ↓	CCR5, CCR10, CCL28	Protect mucous membrane	(44–49)
Hepatitis B	Th22 cells, IL-22 ↑	STAT3, IL-22R	Anti-inflammatory or pro-inflammatory, promote the proliferation of liver stem/progenitor cells (LPCs), inhibit hepatocyte apoptosis.	(50–54)
Hepatitis C	Th22 cells, IL-22 ↑	IL-22BP, IL-22R, Notch signaling proteins	Promote the expression of pro-inflammatory factors, reduce the apoptosis of liver cells, activate hepatic stellate cells (HSCs)	(55–64)
HFMD	Th22 cells, IL-22 ↑	AhR and RORγt	Related to the severity of the disease	(65, 66)
COVID-19	Th22 cells, IL-22 ↓↑	Unclear	Negatively related to the severity of the disease	(67–70)
IAV infection	Th22 cells, IL-22 ↑	AhR and RORγt, IL-22BP, IL-22Ral	Reduce pulmonary inflammation	(71–75)

AIDS, Acquired Immune Deficiency Syndrome; HFMD, hand, foot, and mouth disease; COVID-19, coronavirus disease 2019; IAV, influenza A virus; CCR5, chemokine receptor 5; CCR10, chemokine receptor 10; MAIT, mucosal-associated invariant T cells; STAT3, Signal Transducer and Activator of Transcription 3; AhR, aryl hydrocarbon receptor; IL-22BP, IL-22 binding protein; IL-22Ra1, IL-22 receptor a1; ↑, Increased; ↓, Reduced.

promote the expression of pro-inflammatory factors, promote the proliferation, migration and tissue regeneration of liver cells, and reduce the apoptosis of liver cells (58–60). Hence, it may be an effective target for the treatment of liver fibrosis and HCC. Overexpression of IL-22 binding protein (IL-22BP), which is a competitive inhibitor of IL-22, can aggravate the progression of liver fibrosis and cirrhosis (60). On the other hand, it has been reported that high levels of IL-22 positive cells and IL-22 in intrahepatic and peripheral blood are positively correlated with the progression of liver fibrosis and α- smooth muscle actin (α- SMA) level (61). IL-22 can activate hepatic stellate cells (HSCs) by binding with IL-22R1, which can increase the synthesis of extracellular matrix (ECM) and aggravate HCV associated liver fibrosis (61). IL-22 can promote the proliferation of HSCs *in vitro* and accelerate the progression of liver fibrosis from hepatitis C virus recurrence after orthotopic liver transplantation (HCV-OLT) (62). Notch signaling promotes IL-22 secretion by regulating the expression of AhR. The levels of Th22 cells and Notch signal in peripheral blood of CHC patients were significantly increased. Inhibition of Notch signal could reduce the expression of Th 22 cells, IL-22 and AhR in HCV infected patients (63). The imbalance of Tregs and Th17 cells is a key factor of persistent chronic HCV infection. Th17 mediated immune response could be inhibited when Notch signal is suppressed, and the expression of ROR-γand IL-17/IL-22 was decreased (64). HCV is an RNA virus whose genetic material can be directly integrated into the host genome, which increases the risk of HCC. HCV core protein is critical to drive the transformation of normal hepatocytes into cancer cells. Suppressor of cytokine Signaling 3 (SOCS-3) proteins can cause the dysfunction of IL-22-mediated hepatocyte regeneration, and HCV core protein and SOCS-3 are highly expressed in patients with liver cirrhosis and HCC (58). These findings indicate that Th22 cells and associated molecules play a crucial role in the pathogenesis of HCV infection.

Hand, foot, and mouth disease is an infectious disease caused by enteroviruses. Coxsackievirus A16 (CV-A16) and enterovirus 71 (EV-71) are the most common pathogens that cause this disease. Previous studies found that levels of Th22 cells in the peripheral blood of EV-71 associated severe patients and mild patients were significantly higher than those in healthy controls (65). The levels of IL-22, IL-17A, IL-23, IL-6, TNF-α, AhR, and RORγt were different among mild patients, severe patients, and healthy controls. In convalescent patients, Th22 cells decreased significantly. This research suggests that Th22 cells play an important role in the pathological process of EV-71 infection. IL-22 may have different pathological effects in hand, foot, and mouth disease that is caused by different pathogens. Research has found that hand, foot, and mouth disease patients with encephalitis had higher levels of IL-5, IL-22, and IL-23 (66). In addition, those with EV-71 infection had higher levels of IL-22 than those with CV-A16 infection. These results indicated that Th22 cells and associated molecules were associated with the severity of HFMD caused by CV-A16 or EV-71 infection.

The outbreak of the coronavirus disease 2019 (COVID-19), caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), is a serious threat to human health. The immunological dynamics in SARS-CoV-2 infected patients has been investigated by many researchers. Daniela Fenoglio *et al.* analyzed Th subsets in peripheral blood of 13 patients with severe COVID-19 and 10 healthy controls, and found that the frequencies of Th1 and Th17-1 cell were reduced in COVID-19 patients (67). Another study observed a significantly lower lymphocyte count in COVID-19 patients compared to healthy controls. They found that the percentages of Th1, Th1/Th17, and TFH cells were significantly reduced in both non-ICU hospitalized and ICU hospitalized patients compared to healthy controls. There is some controversy about Th22 cells (68). Daniela Fenoglio *et al.* found that there was no significant difference about Th22 between COVID-19 patients and controls (67). However, Juan Francisco Gutie'rrez-Bautista *et al.* found that Th22 showed the opposite change. Th22 was slightly elevated in non-ICU hospitalized patients and asymptomatic recovered donors, but significantly reduced in ICU hospitalized patients

compared to healthy controls (68). This may be related to the excessive consumption of Th22 in severe patients. In addition, functional assays revealed that the ability to produce IL-22 of peripheral blood mononuclear cell (PBMCs) from critically ill patients was significantly lower than that of health controls (69). However, another study suggested that IL-22 was highly expressed in the infected human bronchial epithelial cell line (16HBE) (70). This suggested IL-22 showed different distribution in peripheral blood and tissues.

Influenza is another common disease that endangers human health. IL-22, the main effector of Th22, plays a crucial role in influenza A virus (IAV) infection. It has been indicated that a higher level of IL-22 expression was detected in the lung tissue during the early stages of IAV infection (71, 72). RORγt and aryl hydrocarbon receptor was crucial in IL-22 synthesis after IAV infection (71). During IAV infection, IL-22 plays protective role in lung injuries.but,IL-22 does not appear to affect pulmonary pathogenesis during lethal IAV infection (71). Interestingly, when human peripheral blood mononuclear leukocytes (PBML) exposed to vaccines against influenza virus, the level of IL-6, IL-1β, TNF-α and IL-22 obviously increased (73). This further suggested that the influenza virus can activate the expression of IL-22. After influenza virus infection, IL-22 may reduce pulmonary inflammation via IL-22Ra1 or the IL-22/IL-22BP axis (74, 75).

Notably, in addition to Th22 cells, IL-22 can also be derived from Th17 cells, natural killer T cells, $\gamma\delta$T cells, and type 3 ILCs. The pathogenesis of each disease is intricate. Various cell subsets regulate each other and collectively participate in the process of the disease. The mechanism of Th22 cells in disease still needs more complete research.

CONCLUSION

The exploration of T cells has made rapid progress over the last 20 years, and newly discovered T cell subsets show a variety of differentiation characteristics. Various cell subsets are able to regulate each other in order to keep the body in a delicate and complex balance. The discovery of Th22 cell subsets further enriches the complex system of immune regulation gridding and illustrates the high plasticity of naive T cells. IL-22 is the main effector of Th22 cells, and AhR is the differentiation transcription factor of Th22 cells that can be promoted by IL-6, TNF-α, AhR agonists, and TGF-β receptor inhibitors. TGF-β and IL-10 can inhibit the differentiation of Th22 cells. The function of Th22 cells extends from the initial involvement in skin inflammation and wound healing to the regulation of pathological processes such as autoimmune diseases, infectious diseases, tumors, hematological diseases, and kidney diseases. Circulating Th22 cells can migrate to pathological tissues through chemokine receptors, express cytokines, and bind to the corresponding receptors. This allows Th22 cells to elicit biological effects. In some diseases, Th22 cells and their effector molecules play a protective role; however, in other diseases, Th22 cells can aggravate the disease progression. The different roles of Th22 cells and associated molecules are closely associated with the types of viral diseases and duration of viral infection. Although the study of Th22 cell has been extended to various systems, the understanding of this precise regulatory mechanism is still very limited. In addition, the regulation of Th22 cell differentiation and downstream pathways are still not completely clear. The relationship with Th17, Th9, Th1, and other cells is not completely understood, along with whether it is involved in the regulation of humoral immunity. Further research should be done to examine the differentiation and regulation mechanism of Th22 cells, explore their interaction with other immune cells, and analyze their mechanism in various diseases. These will help to provide new targets and strategies for diagnosis and treatment of many diseases.

AUTHOR CONTRIBUTIONS

DC and QH designed the study and revised the manuscript. JG, DC, and HZ drafted the manuscript. JG and YL drew the figure and Table. All authors have read and approved the final version of the manuscript.

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Auxiliary Liver Graft Can Be Protected From HBV Infection in HBsAg Positive Blood Circulation

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Auxiliary grafts have a high risk of Hepatitis B virus (HBV) infection in patients with chronic HBV-related diseases. Hepatitis B virus-related auxiliary partial orthotopic liver transplantation (APOLT) cases were reviewed to show the results of current methods to block native-to-graft HBV transmission. Three patients received APOLT for HBV-related liver cirrhosis and a recurrent upper gastrointestinal hemorrhage between April 2015 and January 2017 by the liver transplant team of Beijing Friendship Hospital affiliated with Capital Medical University. All three patients were positive for HBV surface antigen (HBsAg) and had a negative HBV DNA test result before transplantation. After auxiliary transplantations, HBsAg was found to be positive in two patients and negative in one patient. To avoid graft infection of HBV, entecavir-based therapy was employed and the remnant native livers of the recipients were removed 51–878 days after liver transplantation. Then, serum conversions of HBsAg were found in all three cases. For the first time, this case series shows the possibility of blocking the transmission of HBV from a native liver to a graft in auxiliary transplantation by entecavir-based therapy. Among the cases, a left lobe graft was successfully implanted as a replacement of the right lobe of the recipient, which is also discussed.

Keywords: APOLT, entecavir, liver cirrhosis, liver regeneration, hepatitis B recurrence

INTRODUCTION

In auxiliary partial orthotopic liver transplantation (APOLT), a liver graft is implanted while the partial or entire native liver is preserved. Two livers will co-function and support each other. Auxiliary partial orthotopic liver transplantation is commonly used in patients with acute liver failure or metabolic liver diseases (1, 2). Patients who receive APOLT for acute liver failure, a reversible liver disease, may be able to discontinue immunosuppressant treatment after their native liver has regenerated (3). Some inherent liver metabolic defects can be corrected by a small auxiliary graft as a special form of gene therapy (4, 5). Auxiliary partial orthotopic liver transplantation can also be employed when a whole liver graft cannot be acquired or the graft volume is inadequate (6, 7).

Hepatitis B virus (HBV)-related diseases are regarded as a contraindication for APOLT. The HBV-infected native liver is preserved in APOLT, which may potentially relieve HBV and increase the risk of graft infection. Hepatitis B virus recurrence after liver transplantation may progress rapidly and become difficult to control. Several reports showed attempts to carry out auxiliary liver transplantations in patients with HBV-related liver diseases. In the report of Onno, in 1988, four cases received a heterotopic liver transplantation for HBV-related liver cirrhosis, and immunofluorescence stains for the core antigen were positive in all of the grafts within 3 weeks after transplantation (8). One of these patients had clinical signs (recurrence of ascites). In 1991 and 1992, Kate FJ reported chronic hepatitis and cirrhosis in the auxiliary graft after auxiliary liver transplantation for HBV related liver diseases (9, 10). In the auxiliary liver transplantations for fulminant hepatitis B reported by Durand in 2002 (11), three patients who were positive for HBV surface antigen (HBsAg) received an auxiliary liver transplantation. Two patients survived, and one was followed up more than 1 year later. HBV surface antigen was found to be negative in these patients after the hepatitis B immune globulin (HBIG) treatment. However, in the only patient followed for more than 1 year, the HBIG titer was negative, which was most likely a residue of HBV. In 2008, Quaglia reported auxiliary liver transplantations for seronegative liver failure (12). Four cases of HBV infections were included. One of these patients died of HBV recurrence on day 1,505, and the other three patients lived (12). According to the results of these cases, the auxiliary graft conveyed a high risk of HBV infection in patients with chronic HBV-related diseases.

Great improvements in anti-HBV agents have been made. Nucleos(t)ide analogs and HBIG have been used as a combined therapy to prevent HBV recurrence after liver transplantation, which significantly reduces the HBV recurrence rate. In a report by Wang in 2017 (13), four patients with HBV-related liver cirrhosis received APOLT. Nucleos(t)ide analogs were administered after APOLT. After transplantation, all recipients were positive for serum HBsAg and negative for the HBsAg and HBV core antigen (HBcAg) in grafts. All patients lived and showed normal graft function within a mean follow-up period of 21 (13–26) months. However, immunostains for HBsAg and HBcAg at 1, 6, and 12 months after transplantation were not enough to exclude HBV infection in the auxiliary grafts. Whether the nucleos(t)ide analog blocked HBV infection should be further confirmed.

Because of insufficient donor graft volumes, three patients received APOLTs for HBV-related liver cirrhosis in our center. To avoid HBV infection of the auxiliary grafts, we removed the remnant native livers of the recipients 51–878 days after the liver transplantation. Then, serum HBsAg became negative in all three recipients. For the first time, this small case series shows that it is possible to completely block the transmission of HBV from the native liver to a graft in auxiliary transplantation by entecavir monotherapy or in combination with HBIG therapy.

Abbreviations: APOLT, auxiliary partial orthotopic liver transplantation; HBV, Hepatitis B virus; HBsAg, HBV surface antigen; HBIG, Hepatitis B immune globulin; HBcAg, HBV core antigen; GRWR, graft-to-recipient weight ratio; SFSS, small for size syndrome; HBsAb, HBV surface antibody; ICU, intensive care unit; ALT, alanine aminotransferase; AST, aspartate aminotransferase; TBIL, total bilirubin; MRCP, magnetic resonance cholangiopancreatography; PTCD, percutaneous transhepatic cholangiodrainage; HBeAg, HBV e antigen; HBeAb, HBV e antibody; Pre-S1Ag, pre S1 antigen; PV, portal vein.

PATIENTS AND METHODS

Three patients who received APOLTs for HBV-related liver cirrhosis were reviewed; These APOLTs were conducted by the liver transplant team of Beijing Friendship Hospital affiliated with Capital Medical University. This study and the APOLT treatment were approved by the ethics committee of Beijing Friendship Hospital. Informed consent for the operations and this study was obtained. All three patients received APOLT for HBV-related liver cirrhosis and recurrent upper gastrointestinal hemorrhage. Living donor liver transplantation was the only option for these patients due to the shortage of organs (14). However, the left lobe of each donor liver was too small to be transplanted, with a graft-to-recipient weight ratio (GRWR) less than 0.8%. Additionally, the right lobe could not be donated because the volume of the remnant liver was insufficient to ensure the donor's safety because the proportion was <35% (**Table 1**). Thus APOLT was employed to prevent small for size syndrome (SFSS).

The characteristics of the donors and recipients are provided in **Table 2**. Patient A was 55 years old and was admitted for a recurrent upper gastrointestinal hemorrhage. His first upper gastrointestinal hemorrhage was found on January 6th, 2015, and then, splenectomy and disconnection were performed in 2007. Patient C, a 52-year-old man, was admitted with the same diagnosis and no surgical history. Both of these patients received left lobe APOLT by replacing the left lobes of their native livers on April 26th, 2015, and January 11th, 2017, respectively. Patient B was 29 years old. The patient experienced an upper gastrointestinal hemorrhage in 2010. Splenectomy and disconnection were conducted to prevent recurrent hemorrhage. In November 2011, the patient suffered from a severe upper gastrointestinal hemorrhage again. Although endoscopic sclerotherapy was performed, it did not stop recurrent hemorrhage. APOLT was planned. Abdominal adhesions due to previous surgery limited the space of the upper left abdominal cavity. Finally, we implanted the left lobe graft into the upper right abdominal cavity of the recipient after removing the right lobe of his native liver on August 16th, 2016 (**Figure 1**).

Left lobes without the middle hepatic vein were procured as grafts in all of these APOLTs. In patient A, the left hepatic vein, left portal vein and left hepatic artery were anastomosed to their corresponding parts of the recipient. In patient B, the left hepatic vein, left portal vein, and left hepatic artery were anastomosed to the right hepatic vein, right portal vein, and right hepatic artery of the recipient. The left hepatic duct was reconstructed by biliary-enterostomy in patient B. In patient C, the portal vein (PV) of the graft was elongated by a vein conduit taken from the left portal vein of the recipient and anatomized to the recipient's PV by the end to side method. The left hepatic artery of the graft was reconstructed to the left hepatic artery of the recipient. An auxiliary left hepatic artery was also found in patient C.

TABLE 1 | Volumetric assessment of left/right lobe of living donor by computerized tomography.

Donor and patient	Potential graft	Volume of graft (ml)	RLV/WLV (%)	Body weight of recipient (kg)	GRWR (%)	Conclusion
Donor of patient A	Left lobe	396	69.9	68	0.58	Insufficient graft
	Right lobe	921	30.1		1.35	Insufficient remnant liver
Donor of Patient B	Left lobe	448	68.0	76	0.59	Insufficient graft
	Right lobe	952	32.0	76	1.25	Insufficient remnant liver
Donor of Patient C	Left lobe	390	72.7	73	0.53	Insufficient graft
	Right lobe	1,041	27.3	73	1.42	Insufficient remnant liver

RLV, remnant liver volume; WLV, whole liver volume; GRWR, graft recipient's body weight ratio.

TABLE 2 | Characteristics of donors and recipients in auxiliary liver transplantation.

	Patient A	Patient B	Patient C
Recipient			
Age (year)/sex	55/male	29/male	52/male
Cause of liver cirrhosis	Hepatitis B	Hepatitis B	Hepatitis B
ABO blood group	A	B	O
Serum result of HBV	HBsAg(+) HBsAb(–) HBeAg(–) HBeAb(–) HBcAb(+)	HBsAg(+) HBsAb(–) HBeAg(–) HBeAb(+) HBcAb(+)	HBsAg(+) HBsAb(–) HBeAg(–) HBeAb(+) HBcAb(+)
Child grade	6	6	7
MELD score	11	9	11
Platelet count	102×10^9/L	382×10^9/L	45×10^9/L
Albumin (g/L)	33.3	38.0	43.1
Total bilirubin (μmol/L)	22.0	18.87	37.96
INR	1.43	1.18	1.18
Creatinine (lmol/L)	66.0	67.5	73.1
Serum sodium (lmol/L)	138.9	139	140.8
Ascites	No	No	mild
Esophageal and gastric varices	Severe	Severe	Severe
Donor			
Age (year)/sex	45/female	50/male	47/female
Serum result of HBV	HBsAg(–) HBsAb(+) HBeAg(–) HBeAb(–) HBcAb(+)	HBsAg(–) HBsAb(+) HBeAg(–) HBeAb(–) HBcAb(–)	HBsAg(–) HBsAb(+) HBeAg(–) HBeAb(–) HBcAb(–)
Graft			
Type of graft (lobe)	Left lobe (without middle hepatic vein)	Left lobe (without middle hepatic vein)	Left lobe (without middle hepatic vein)
Graft weight during operation (g)	386	408	349
ABO blood group	A	O	O
Cold ischemic time	2 h 5 min	6 h 15 min	4 h 10 min
Warm ischemic time	1 min	1 min	1 min
GW/RW	0.57%	0.54%	0.46%
Duration of operation	9 h 36 min	13 h 50 min	12 h 20 min
Blood loss (ml)	200	3,000	2,400
Post-operative complications	Anatomotic stricture	None	Anatomotic stricture
Follow-up (days)	1,200	566	525

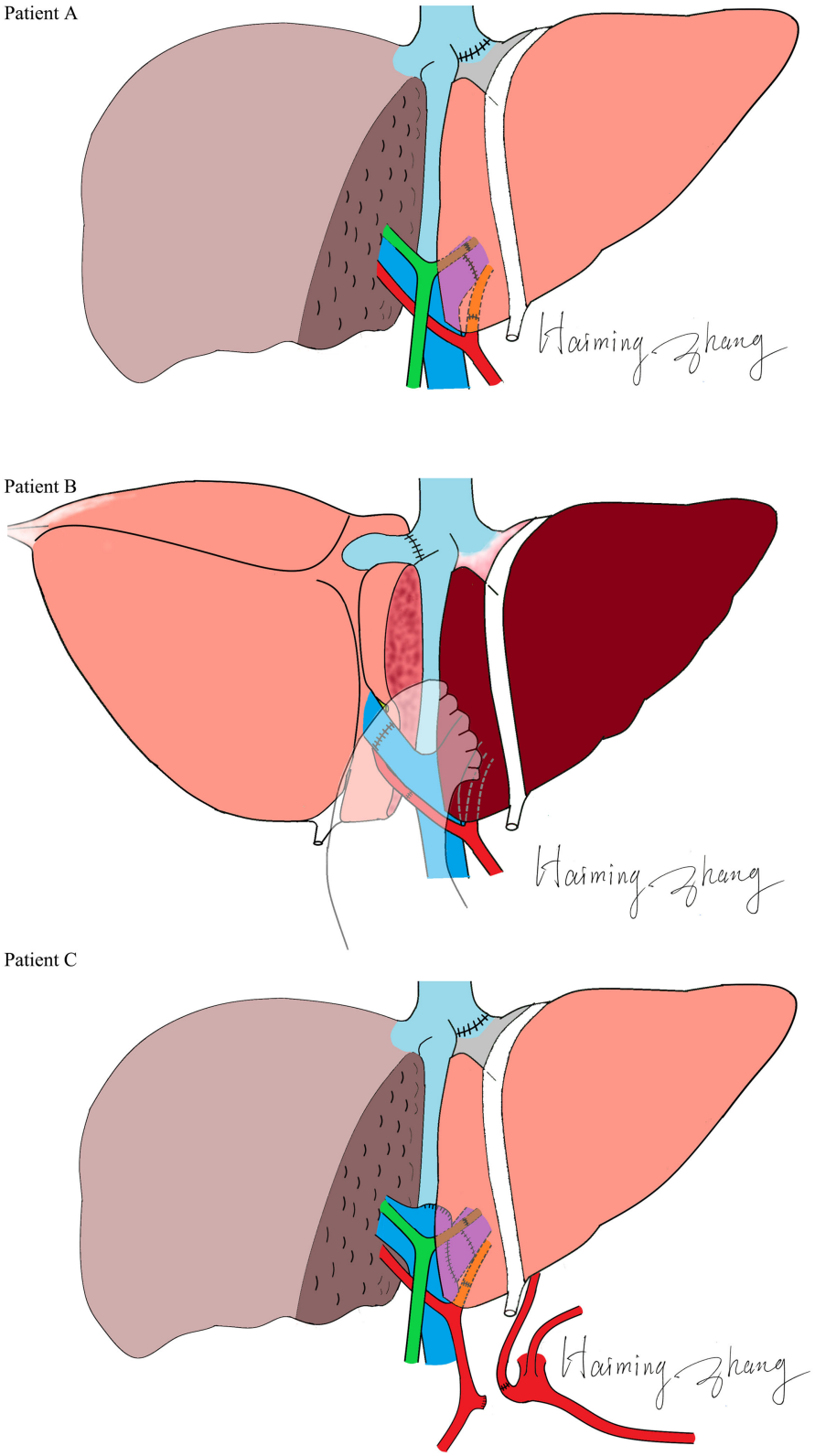

FIGURE 1 | Methods of auxiliary graft reconstructions.

The common hepatic artery of patient C was transected. The proximal end was connected to the auxiliary left hepatic artery of the graft, and the distal end was closed. End to end biliary anastomoses were performed in patients A and C.

After APOLT, the biochemistry results showed normal liver function in all three patients. The volumes of the grafts increased over time, and atrophy was found in the remnant native livers. We removed the remnant native livers at 51–878 days after transplantation to prevent HBV infection (patients A, B, and C at 878, 136, and 51 days, respectively).

Antibiotics, immunosuppressant, and antithrombotic agents were administered. Tacrolimus, mycophenolate mofetil, and steroids were included in the immunosuppression regimen. The target serum level of tacrolimus was 6 and 8 ng/ml. Entecavir was administered continuously since APOLT. Hepatitis B immune globulin was not given to patient A. Patient B and patient C received 4,000 IU HBIG during APOLT and 2,000–4000 IU HBIG once per day within the first week after transplantation. If the patient was positive for the HBV surface antibody (HBsAb), HBIG was given according to the blood titer. Hepatitis B immune globulin therapy was not continued if a patient was persistently positive for HBsAg and negative for HBsAb. In patient B and patient C, 4,000 IU HBIG was also given during the hepatectomy, and then, HBIG was administered based on the blood titer.

Routine blood tests, biochemical markers for liver and kidney functions, and ultrasonography were conducted at the early stages, after transplantation and at each follow-up. Computed tomography (CT) was used to estimate the volumes of the grafts and native livers, if necessary. Liver biopsies were performed during the hepatectomies.

RESULTS

Graft Function and Complications

All three patients recovered from the operations, and their liver functions were normal at the end of the follow-up. The duration of the intensive care unit (ICU) stay was 3, 2 and 2 days for patients A, B, and C, respectively. No serious infections or surgical complications were found. Antibiotics were stopped 5–7 days after transplantation. Patients were discharged 21–28 days after transplantation. Patient A showed elevated levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), and total bilirubin (TBIL) 136 days after transplantation. Magnetic resonance cholangiopancreatography (MRCP) showed a biliary anastomotic stricture, and a percutaneous transhepatic cholangiodrainage (PTCD) was placed. Then, the biochemical parameters returned to normal. However, choledocholithiasis occurred 829 days after transplantation. Choledoenterostomy was performed during the hepatectomy at the 878th day after transplantation. Finally, the biochemical parameters of the liver function became normal. Patient B recovered smoothly; no complications were found during follow-up. A biliary anastomotic stricture was also found in patient C 391 days after transplantation, which was treated successfully by PTCD. No serious infections, acute rejections or chronic rejections were found among these patients. According to the results of ultrasonography, the artery, PV, and hepatic vein of the grafts were patent and the blood flow velocities were within the normal range. The levels of ALT, AST, and TBIL are shown in **Figure 2**.

HBV Markers

All recipients were positive for serum HBsAg before liver transplantation. Patient A and patient B were HBsAg positive after transplantation even after receiving entecavir and HBIG therapy. Patient C showed unstable HBsAg and HBsAb levels after transplantation. All three patients were HBsAg negative and HBsAb and HBV core antibody (HBcAb) positive after hepatectomy (**Figure 3**). Three hundred and twenty-two days after the complete removal of his native liver, patient A was negative for all of the serum markers of HBV, including HBsAg, HBsAb, HBV e antigen (HBeAg), HBV e antibody (HBeAb), HBcAb, and pre S1 antigen (Pre-S1Ag). Patient B and patient C were followed for 566 and 525 days, respectively, and no changes in the serum markers of HBV were found since hepatectomies. All three patients were negative for HBV DNA before and after transplantation, which was tested every 2 weeks after transplantation until discharge after hepatectomy. Biopsies were taken from the grafts during hepatectomy. Immunostaining of donor liver tissues for HBsAg and HBcAg were all negative.

Graft Volume

Computed tomography data of patients could be found at several time points. We calculated volumes of grafts and native livers by the available Computed tomography data and showed them in **Figure 4**. The volumes of the grafts increased over time, especially in the early stages after liver transplantation (**Figure 4**). Since the remnant native livers were cirrhotic, the blood supply to them was very low, which was detected by ultrasonography but could not be quantified. The volume of the remnant native liver continued to reduce as a result of portal blood competition with the graft (**Figure 4**). The trend of the PV blood velocity changes was roughly matched with the total volume changes of grafts. However, the PV blood velocities varied greatly at the early stages after transplantation, and the low density of examinations at the late stages cannot show a clear trend of PV blood velocity changes.

DISCUSSION

In these three APOLT recipients, the remnant native livers releasing HBsAg may be potential sources of HBV. However, the liver grafts were not infected, which can be supported by HBsAg conversions after removing native livers. Thus, in these APOLTs, entecavir completely blocked HBV transmission from the HBV-infected native livers to the grafts, even though the two partial livers shared blood circulation for 51 to 878 days. The combination of lamivudine plus HBIG is the most widely used treatment to prevent HBV recurrence after liver transplantation for HBV-related diseases. Lamivudine alone has been shown to be insufficient to completely block the transmission of HBV to the HBV-free graft after liver transplantation (15). New nucleos(t)ide analogs have been used and showed high efficacy in preventing HBV recurrence (16). Entecavir is also used as monotherapy to prevent HBV recurrence (17). However,

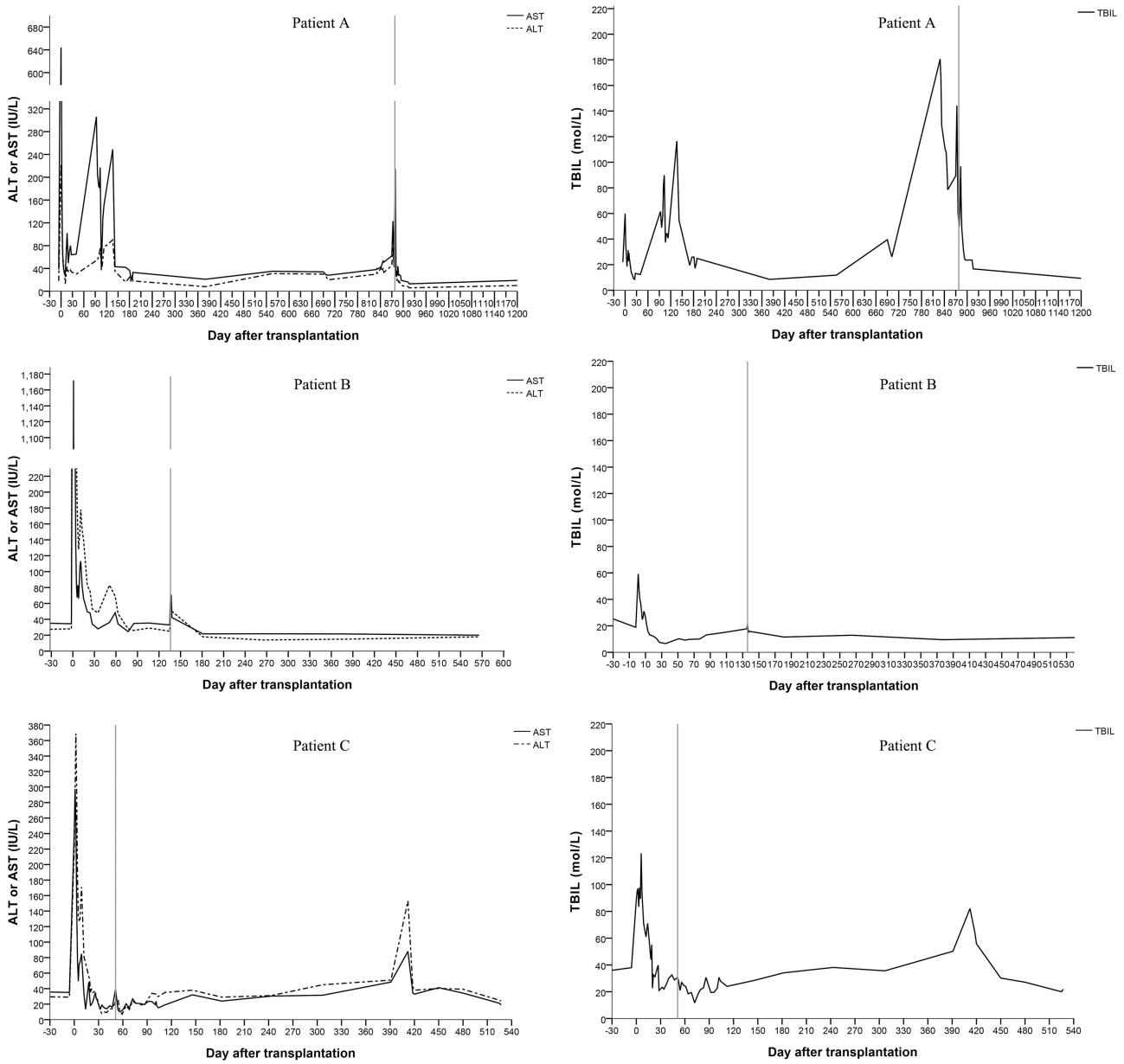

FIGURE 2 | Changes of the serum biochemical parameters for liver function (the vertical line shows the date of the hepatectomy of the remnant native liver).

it is not clear whether HBV transmission can be completely blocked by nucleo(t)ide analogs in HBV-related APOLT. In classic liver transplantation, the HBV infected liver is completely removed. Thus, entecavir can block HBV infection after the serum conversion of HBsAg. In auxiliary liver transplantation, part of the HBV-infected liver continues to release HBsAg. The auxiliary graft seems to inevitably become infected by HBV if entecavir cannot completely prevent viron formation. Liver transplant recipients are immuno-compromised, which also increases the risk of HBV infection. Previous clinical studies (see the introduction) of HBV-related auxiliary liver transplantation showed that auxiliary grafts were normal in function and negative in immunological stains for HBsAg and HBcAg after entecavir administration, which suggested the practical of APOLT for HBV related diseases. Our cases further demonstrated the possibility of preventing graft infection of HBV in auxiliary liver transplantation, especially in patient A whose native liver was removed 878 days after transplantation. By removing the remnant native liver, HBV infection can be cured after APLOT. Keeping grafts free from HBV infection should be emphasized in the process of auxiliary liver transplantation. In the current series, only patients with liver cirrhosis received APOLT. The blood flow resistance in the cirrhotic native liver increased dramatically compared to that in the grafts. Thus, portal blood

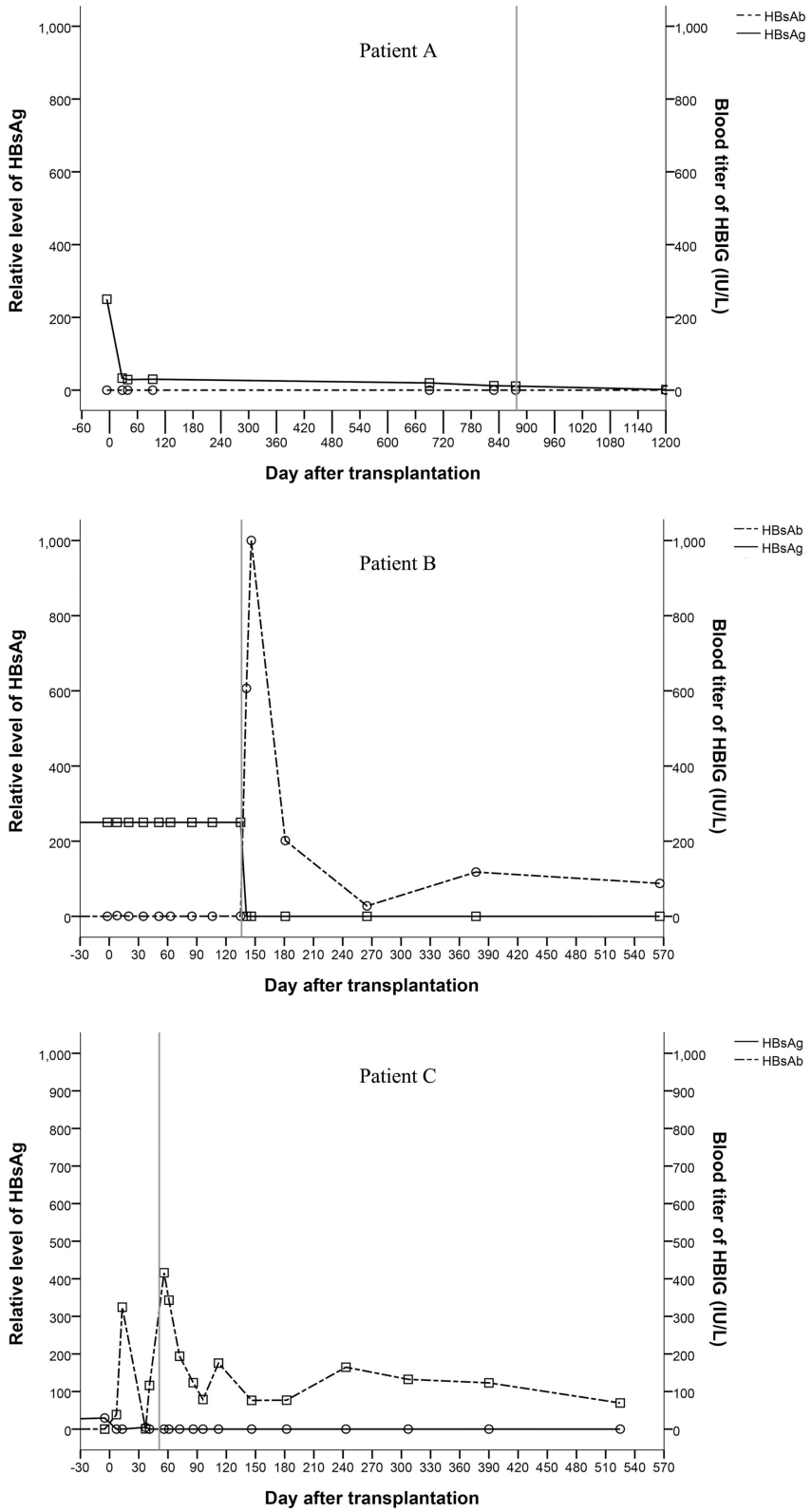

FIGURE 3 | Changes of serum markers for HBV (the vertical line shows the date of the hepatectomy of the remnant native liver).

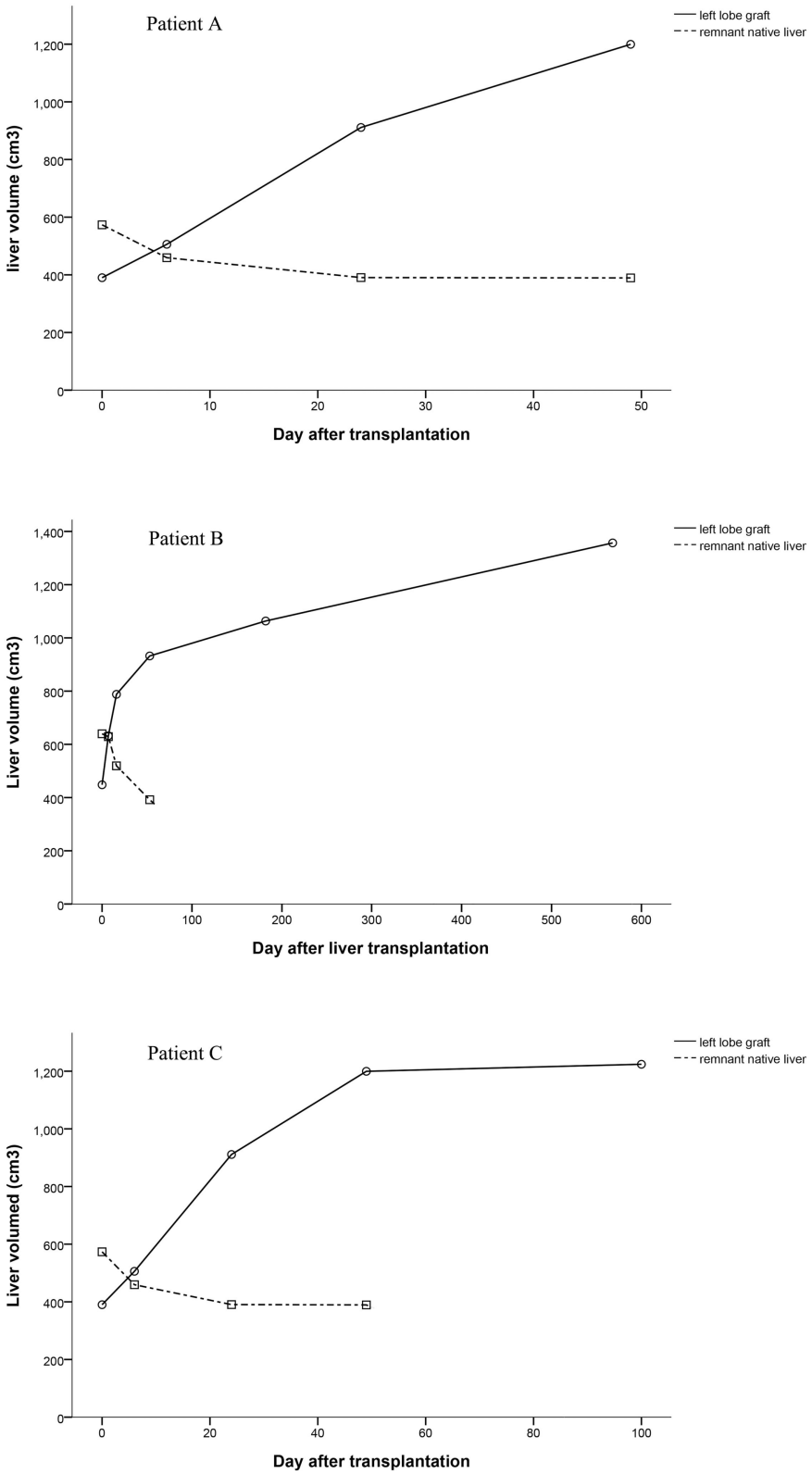

FIGURE 4 | Volume changes of the auxiliary grafts and native liver.

flow of the native livers was too low to be quantified, and most of the portal blood supply went to the grafts. Fast increases in the graft volumes were found, together with atrophy of the native livers (**Figure 4**). In functional respects, the remnant native livers contributed little to the total liver function and could be safety removed at relative early stages. Therefore, we removed the remnant native liver of patient C only 51 days after transplantation to reduce the graft exposure to HBsAg positive circulation. Thus, effective anti-HBV treatment and early removal of the remnant liver are suggested in APOLT for HBV-related liver cirrhosis to reduce the risk of HBV infecting grafts.

A special manner of left lobe graft implantation was used in patient B. The left lobe graft was implanted instead of the right lobe of the recipient. This successful case suggested the practicability of this operation manner, which may be a promising way to settle the space issue in left lobe implantation. Abdominal adhesions resulting from previous surgeries are a common problem, as splenectomies with disconnection are conventional treatments. By reconstructing the normal anatomical structure, a more stable blood flow can be maintained. As reported, orthotopic auxiliary liver transplantation may lead to fewer complications than heterotopic transplantation (18). Therefore, transplant surgeons usually make more effort to dissect adhesions rather than change the position of a graft. However, when we rolled the left lobe over, it was well matched with the right lobe in regard to the portal and hepatic veins. The risks of angulation, compression and twist can also be reduced by precise vessel reconstruction. No vessel graft was used in this case. In an APOLT for non-cirrhotic liver metabolic disease, a reduced left lobe graft blood supply was found (18). The relatively high blood flow resistance due to small volume, immunological, and ischemic injuries may result in a continuing reduction of the blood supply of the left graft, especially when in competition with the non-cirrhotic native liver. Placing the left lobe in the right side may contribute to the balance of the PV blood distribution because of the similar volumes of the donor and remnant native livers. More blood supply to a donor from the right branch of the PV may balance the risk of immune and ischemia injuries. Thus, we believe that this technique may have a special advantage in APOLTs for non-cirrhotic liver diseases.

No complications of the blood vessels were found in any of the recipients. Anastomoses of the PVs were carefully designed in these cases. In patient C, the left PV of the graft was reconstructed by the end to side method and the angulation of end to end anastomosis was avoided. The anastomosis of the PV was pushed to the opposite direction of the graft with the increasing volume of the graft after transplantation. This change should be taken into consideration before PV reconstruction. When the end of the donor's (left or right) PV is lower than the bifurcation of the recipient's PV, the end to side method was suggested. Biliary anastomotic strictures were found in the two cases that received end to end biliary anastomosis. Though an improvement was found after PTCD, choledochojejunostomy seemed to be an optimal way to prevent or treat this problem. All patients recovered and had a normal liver function.

As reported (6, 7, 19) previously, current practice also supports auxiliary liver transplantation as an aid for a SFSS graft. However, in patients with severe liver cirrhosis, the function and PV blood flow of the liver decline to a large degree and a relatively large graft would be needed even for auxiliary liver transplantation. In these patients, hepatic failure, PV hypertension, and blood coagulation disorders may also increase the risk and complications of APOLT. Therefore, only patients with relatively mild cirrhosis and an indication of a gastrointestinal hemorrhage were considered for APOLT in the current series. Right lobe and whole liver grafts would be preferred for patents with more severe cirrhosis.

CONCLUSION

It is possible to block the transmission of HBV between the native liver and graft by a sensitive nucleos(t)ide drug treatment. Auxiliary transplantation of left lobe grafts may be a practical option for recipients at risk for SFSS. Left lobe grafts can also be implanted into the space of the right lobe of the liver safely.

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

AUTHOR CONTRIBUTIONS

L-YS: participated in the research design and the patient management. Z-JZ: planned and performed the operations and participated in operations. JX and YL: participated in the patient management after the operations. WQ: participated in operations. C-DW: contributed to treatments and operations as an expert consultant. H-MZ: participated in data analysis and the writing of the paper. LW: participated in the research design and the surgical process of operations. All authors contributed to the article and approved the submitted version.

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MINI REVIEW
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Antiviral Therapy for Chronic HBV Infection With Persistently Normal Alanine Aminotransferase: Controversy and Consensus

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ALT is one of the most sensitive biochemical indexes to reflect liver injury. It is generally believed that hepatitis B virus (HBV) infected patients with normal ALT levels are in either immune tolerance or low replication stage of the natural history of hepatitis B, and there is no or only mild inflammation in liver tissue, so antiviral therapy is not recommended. However, chronic HBV-infected patients with normal ALT levels are not always in a stable state. A considerable number of patients will develop active hepatitis or occult progress to liver fibrosis, cirrhosis, and hepatocellular carcinoma. Therefore, whether antiviral therapy should be recommended for chronic HBV infection with normal ALT level has been a hot topic in clinical practice. In this paper, the definition of immune tolerance, the relationship between ALT and liver inflammation, and the benefits of antiviral therapy were reviewed, and we hope it will be helpful for clinicians to have a deeper understanding of whether antiviral therapy should be considered for chronic HBV infection with normal ALT.

Keywords: hepatitis B virus infection, normal alanine aminotransferase, immune tolerance, disease progression, antiviral therapy

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INTRODUCTION

Hepatitis B virus (HBV) infection is one of the most common infectious diseases in the world. The persistence and active replication of HBV in liver tissue is the main cause of chronic hepatitis, cirrhosis, and hepatocellular carcinoma(HCC). Timely and effective antiviral treatment is the most effective measure to delay and prevent the progress or aggravation of diseases (1). The occurrence of liver damage and its severity depends on the interaction between HBV and host immune system (2). The host immune response can not only clear the virus but also lead to liver inflammation (2, 3).

In East Asian countries, the main route of chronic HBV infection is mother-to-child transmission or infection during infancy. Due to immune tolerance of the host against HBV, some patients are with normal ALT, positive HBeAg, and high HBV DNA levels (4). At present, for such a group of patients, antiviral therapy is not recommended by guidelines unless the patient has evidence of liver injury (5–8).

ALT is the most direct, sensitive, and economic indicator of liver inflammation, and its elevated level usually indicates the occurrence of liver inflammatory (9). Therefore, in addition to positive HBV DNA level, abnormal ALT is also required if antiviral therapy is considered (6, 7). However, ALT in the normal range does not absolutely indicate no inflammatory in liver. Thus, patients with normal ALT is not always in a stable state, and a considerable number of them will develop active hepatitis or occult progression to liver fibrosis, cirrhosis, and HCC (10, 11). Therefore, whether antiviral therapy should be recommended for patients with normal ALT has been a hot topic at present. This article will review the definition of immune tolerance, relationship between ALT and liver inflammation, and benefits of antiviral therapy, so as to help clinicians to have a deeper understanding of whether antiviral therapy should be considered for chronic HBV infection with normal ALT.

DEFINITION AND CONNOTATION OF IMMUNE TOLERANCE

What Is Immune Tolerance?

Generally, immune tolerance is defined as the specific non-response state of the immune system after receiving specific antigen. The liver has a unique immune regulation function, which can promote the tolerance to HBV, and this may be the main reason of HBV persistence and chronic infection. In 1972, Dudley et al. firstly proposed that HBV persistent infection was related to immune tolerance, and the liver injury was determined by T cell-mediated immune response (12). In 1983, Liaw et al. reported that HBeAg clearance was related to the enhancement of host immune response (13). Based on the above findings, Chu *et al.* firstly divided the natural history of HBV infection into three phases of immune tolerance, immune clearance, and residual integration (14), and then divided it into four phases of immune tolerance, immune clearance, inactive carrier status and reactivation (15). The latter four phases are the current widely used classification of hepatitis B natural history.

However, in recent years, some scholars have challenged the concept of "immune tolerance phase", because HBV-specific T cell immune response and liver injury have been found in patients with chronic HBV infection in the so-called "immune tolerance phase" (16). In addition, the vast majority of newborns and infants inoculated with hepatitis B vaccine can produce antibodies, and the antibody titer is very high. These evidence all suggests that the so-called "immune tolerance phase" actually has an immune response. Therefore, there is no absolute immune tolerance state in the natural history of HBV chronic infection. Although there is no consensus on the above views, these new findings at least suggest that the so-called immune tolerance phase does not mean that HBV will not cause damage to the liver. It has been found that the host immune pressure plays the main role for viral mutation (17, 18). Recently, lilly Y *et al.* analyzed whole HBV genome NGS data from 97 treatment-naïve patients and found that the specific HBV variants associated with disease progression (cirrhosis and HCC) were common in immune tolerance stage. Therefore, they proposed that screening for specific viral variants and early antiviral therapy during this phase may help to reduce the risk of HCC (19). Because of these, more and more clinical experts are worried about the management strategy that patients in the so-called "immune tolerance phase" are just given follow-up but no recommendation of antiviral therapy (20).

How to Define the Immune Tolerance Phase of HBV Infection?

The "immune tolerance phase" of HBV infection has been defined and described in many international CHB management guidelines (5–8). Although these guidelines have some differences in the definition of "immune tolerance phase" of HBV infection, they also have common characteristics, such as positive HBsAg (persistent positive for no less than 6 months), positive HBeAg, high level of HBV-DNA (inconsistent threshold), persistently normal ALT (inconsistent upper limit of normal value), as well as no obvious inflammation, necrosis and fibrosis in liver pathology.

At present, the inconsistent definition of immune tolerance phase in different guidelines is mainly manifested in the level of serum HBV DNA and ALT. Because of the controversy in serum HBV DNA and ALT levels, clinicians must pay attention to following details when judging a chronic HBV infection whether in the immune tolerance phase. Firstly, the guidelines require that patients' serum ALT level is persistently normal, rather than a certain cross-sectional serum ALT within the normal range. Therefore, in clinical practice, we need patients to provide reliable laboratory reports of dynamic serum ALT and comprehensively evaluate various potential factors that may cause ALT level fluctuations. Secondly, on the premise of meeting other conditions, the higher the serum HBV-DNA level, the more likely the patient will be in the immune tolerance period. Because only the very high serum HBV DNA level can accurately indicate the peaceful coexistence of the virus and the host.

Although there is no consensus on the lower limit of serum HBV-DNA in immune tolerance phase, it is agreed that the higher the better. And most clinical experts suggested that serum HBV-DNA in immune tolerance phase should be more than $10^7 \sim 10^8$ IU/mL. Therefore, for those HBeAg-positive HBV patients whose serum HBV DNA level is not high and ALT level is within the normal range, we should be careful to judge whether they are really in the immune tolerance phase. In fact, when defining the immune tolerance phase in various international guidelines, it is mentioned that the evidence of liver histological changes must be available. Therefore, clinicians cannot make a hasty decision of the patient in immune tolerance phase in the absence of pathological results of liver tissue (21).

It is worth noting that although liver biopsy may help clinicians to understand the liver inflammation or fibrosis of patients, and judge whether patients are in the immune tolerance phase. Unfortunately, in the real world, it is impractical to determine whether patients have liver inflammation or fibrosis through regular dynamic liver biopsy. Therefore, clinicians did not take the pathological results of liver tissue as an essential index to judge whether patients are immune tolerance phase in

the actual diagnosis and treatment process of hepatitis B. This is also one of the important reasons why there are different opinions on whether antiviral therapy should be recommended in patients with chronic HBV infection with normal ALT and high HBV DNA level. Therefore, there is an urgent need for new laboratory indicators or convenient tools to help clinicians better evaluate the changes of liver histology. However, in the absence of liver biopsy, clinicians should not make a hasty judgment of the immune tolerance phase at present.

Clinical Outcome of HBV Infection in Immune Tolerance Phase

Previous studies have shown that patients in immune tolerance phase have no inflammation in the liver, and their serum ALT level is in the normal range, so they do not need antiviral therapy, and the risk of HCC is relatively low (22, 23). However, some studies have reported that a large proportion of chronic HBV infected patients with normal ALT level and high HBV DNA level have potential liver inflammation (24–26), and the risk of cirrhosis and HCC in these patients is significantly increased in the future.

The limitation of immune partition of natural history of hepatitis B may be an important reason for this embarrassing reality of hepatic histological abnormalities in immune tolerance phase (20). Some scholars recommend no antiviral therapy is based on correct diagnosis of "real immune tolerance" patients. Only paying attention to or emphasizing positive HBeAg, normal ALT and high level HBV-DNA will expand the range of patients in "real immune tolerance" phrase. In fact, there are so-called "gray areas" in both the current immune tolerance phase and inactive phase (27). Patients with chronic HBV infection who are older and/or whose serum HBV DNA level is not particularly high are often not really immune tolerant (21, 27). The development and deterioration of disease observed at present may come from this "gray area" patients in the immune tolerance phase. Thus, patients with normal ALT in the "gray areas" should be paid more attention.

DOES ALT IN NORMAL RANGE MEAN HEALTHY LIVER?

The Function of ALT and the Origin of Its Normal Range

Serum ALT is a kind of intracellular functional enzyme catalyzing amino transfer reaction, which is mainly distributed in the liver. It is one of the most sensitive indicators to measure liver function and reflect liver damage (9). In normal condition, as long as a small amount of ALT is released into the blood, the activity and activity of the enzyme in the serum can be significantly increased. The concentration of ALT in hepatocytes was 1,000∼3,000 times higher than that in serum. As long as 1% of hepatocytes are necrotic and the activity of enzymes in blood is doubled, thus serum ALT is a sensitive marker of acute hepatocyte injury (9).

Currently, the internationally accepted "normal" serum ALT level is generally less than 40 U/L. After years of observation on a large number of blood donors in 2002, some scholars proposed to modify the normal range of serum ALT, and the reference range of serum ALT should be adjusted to 0 ∼ 30 U/L for men and 0∼19 U/L for women, excluding various interference factors such as overweight, drugs, alcohol and virus infection (28). In 2006, experts in the field of liver disease from the United States clearly suggested that the reference range of ALT should be adjusted to 0∼30U/ L for men and 0∼19 U/L for women (29). In 2018, the updated AASLD guideline suggested that the reference level of serum ALT should be adjusted to <35U/L for men and < 25U/ L for women (7). According to the EASL guidelines in 2017, the upper limit of normal serum ALT was lowered by 10 U/L, and serum ALT is considered normal only when its level is lower than 30 U/L (for both men and women) (6).

In fact, serum ALT detection equipment and reagents are different in different countries and regions, thus the results of normal range are not the same (30). The standardization of ALT normal range needs to be solved urgently. And the "individualized ALT reference level" may be also a new direction in the future.

Relationship Between ALT and Liver Injury

The elevation of serum ALT level in different degrees indicates that there is inflammatory reaction in liver tissue. In the recovery period of the liver disease, with the gradual disappearance of inflammation, ALT levels will gradually return to normal. CHB is a chronic infectious disease which is mediated by HBV and infiltrated by a variety of inflammatory cells in the liver. For example, CTL induces apoptosis of target cells by secreting perforin and expressing FasL; Neutrophils and monocytes in the liver produce reactive oxygen species, carbon monoxide, and reactive nitrogen species, which mediate the injury of infected hepatocytes and the formation of local inflammatory injury and reaction with monocytes/macrophages infiltration. Generally, ALT in peripheral blood of patients with HBV infection leading to liver inflammatory injury will increase in varying degrees, but there are also some patients whose ALT in peripheral blood is still in the normal range or only slightly increased even when liver histology has shown more significant inflammation or fibrosis.

According to the histopathological analysis of 346 patients with chronic HBV infection (including 88 patients with ALT $\geq$ 2×up limit of normal [ULN] and 258 patients with ALT < 2×ULN), 48.8% of them had obvious hepatic tissue inflammation (G $\geq$ 2). Among ALT $\geq$ 2×ULN group, 68.2% of patients had obvious liver inflammation, while 42.2% of patients in ALT < 2×ULN group had obvious liver inflammation. We previously analyzed of the pathological results of 228 CHB patients with ALT < 2×ULN and found that 49.2% of patients had significant inflammation (G $\geq$ 2) and 36.4% of patients had significant fibrosis or cirrhosis (S $\geq$ 2) (26).

It can be seen that serum ALT level and liver inflammation degree are not always parallel, and low level of ALT does not necessarily mean that there is no inflammation or mild inflammation in liver tissue. In fact, chronic HBV infections with normal or slightly elevated ALT levels may have entered the immune clearance period. After hepatocyte injury, ALT released into the blood will soon lose its activity and not remain at a high level. Therefore, it is necessary to dynamically monitor the serum

ALT level to evaluate the liver inflammation. We can't judge whether there is inflammation in the liver simply according to whether the serum ALT level is normal or not.

CHB With Normal ALT Still Had Obvious Liver Histological Abnormalities

We previously analyzed the pathological results of 141 CHB patients with normal ALT and found that 47.5% of the patients had significant inflammation and 33.3% had significant fibrosis or cirrhosis (26). Among HBeAg-positive patients with persistently normal ALT, the proportion of patients with significant liver inflammation or fibrosis was 27.8∼49.4% (31–34). Recently, Prof. Zhuang and his colleagues also reported that 53.2% of HBeAg-negative patients with normal ALT have obvious liver fibrosis (35). Among them, 44.6% of patients with serum ALT >20 U/L had obvious hepatic necrotizing inflammation and 61.0% had obvious hepatic fibrosis; while only 26.5% of patients with ALT ≤20 U/L had obvious hepatic necrotizing inflammation and 41.7% had obvious hepatic fibrosis. These results confirmed that a considerable number of HBeAg-negative CHB patients with normal ALT had obvious liver histopathological changes, and even 46.2% of the patients with low HBV DNA level (<2,000 IU/mL) had obvious liver fibrosis. A long-term follow-up study of 1,965 untreated inactive carrier HBeAg-negative patients in Taiwan found that during an average follow-up of 11.5 years, 16% progressed to reactivation and 3% developed cirrhosis (36). Therefore, patients with normal ALT in inactive carrier status may also have significant liver pathological changes and high risk of liver cancer or death (10, 36, 37).

In some patients with chronic HBV infection, liver inflammation is not serious, ALT level is normal, but liver fibrosis is very obvious. Clinical evidences have shown that serum ALT level is in the normal range for a long time but still could occult progression to cirrhosis, or even HCC (11). Therefore, although serum ALT is a sensitive indicator of liver inflammation, it cannot reflect all the pathological changes of liver tissue, especially the degree of liver fibrosis and its progression. For patients with obvious liver histological changes but low serum level of ALT, the higher ULN of ALT may be a possible reason for this phenomenon.

BENEFITS OF ANTIVIRAL THERAPY IN PATIENTS WITH NORMAL ALT

It has long been recognized that timely and effective antiviral treatment can significantly reduce the risk of end-stage liver disease and related death events in CHB patients. In the past, patients with normal ALT level in the clinical immune tolerance period have not advocated antiviral therapy, because they are worried that after antiviral therapy, not only the viral DNA is not effectively suppressed, but it may induce HBV drug resistance mutations (5–8). In addition, many experts believe that for HBV-infected people with normal ALT in the immune tolerance phase, the virus and the host are in a state of mutual balance, and the virus generally will not cause obvious damage to the host liver. Moreover, whether direct antiviral therapy in patients with immune tolerance will affect the spontaneous immune clearance remains unknown, and the economic burden of patients also increase. Therefore, they infer that these patients may not benefit significantly from antiviral treatment. However, by establishing Markov model, Kim *et al.* found that compared with delaying antiviral treatment to active hepatitis stage, the former can reduce the risk of cirrhosis and HCC (38). Whether or not the patients with immune tolerance are supported to receive antiviral therapy at this stage; and no matter what stage of the natural history of CHB patients, antiviral therapy can delay the progress of the disease, improve the survival time and quality of life.

In recent years, some scholars have carried out in-depth analysis on the relationship between HBV DNA level and the risk of cirrhosis and HCC. Patients with extremely high HBV DNA level may be really in immune tolerance phase. For those patients with normal ALT, it is reasonable not to recommend antiviral therapy. On the contrary, for the patients whose HBV DNA level is not very high but ALT level is normal, antiviral therapy should be considered (27). In addition, due to the limitations of previous detection techniques, HBV DNA in peripheral blood cannot be accurately quantified. However, with the increase of sensitivity of detection techniques, the persistent low-level viremia (LLV) and the risk of disease progression are also concerned (39, 40). It is believed that LLV cannot only increase the risk of drug resistance but also increase the risk of cirrhosis and HCC in the future. In fact, the LLV patients include three groups, namely, naïve patients with low viral load (HBeAg negative chronic HBV infection with normal ALT), nucleos(t)ide analogs-treated patients with low viral load (receiving antiviral treatment, but the HBV DNA in peripheral blood is still detectable) and the patients with low HBV DNA level after drug withdrawal. For these HBV-infected patients with normal ALT, effective antiviral therapy should not be ignored, although the relevant clinical evidence is not sufficient.

SUMMARY

In general, ALT has some limitations as an indication for initiating antiviral therapy and not all patients in so-called immune tolerance stage do not need treatment. Accurate evaluation of "real immune tolerance" is the key to the decision of treatment or not.

For patients in "real immune tolerance" period (positive HBeAg, normal ALT,HBV-DNA > 10^7-10^8 IU/ml, age <30 years, no or mild inflammation/fibrosis in the liver) and without extrahepatic diseases, antiviral treatment can be postponed for the low risk of liver disease progression, and the most important intervention are comprehensive professional follow-up and monitoring. Once the patient enters the immune clearance period, treatment must be initiated in time. For patients who cannot be clearly diagnosed, threatment can be provided with good communication. During the process of treatment, here are some recommendations for patients and physicians: (1) the first-line NAs (ETV,TDF, TAF) are the best choice; (2) instructions on medication use should be given to avoid patients' arbitrary

discontinuation; (3) Serum HBV-DNA should be monitored for all patients on antiviral therapy, and prompt genotypic resistance testing and remedial treatment measures should be taken as early as possible, once increase of HBV-DNA level detected; (4) for eligible patients, combination or sequential PEG-IFN therapy should be provided to improve the clinical cure rate.

It is worth noting that when deciding to treat or not, the patient is the final decision maker while the physician is the advisor. The coexistence of risk and opportunity makes the occurrence of any small probability event a 100% fact for the patient. Physicians should objectively inform patients about the status quo, the pros and cons of antiviral therapy, and leave the decision to patients. Finally, controversies stem from the lack of high-quality information. It is more important for medical professionals to devote efforts to perform high-quality clinical and basic research, revise more reliable diagnostic strategies for immune tolerance phrase, and accelerate development of new drugs to relieve the dilemma of drug resistance and cure CHB eventually.

AUTHOR CONTRIBUTIONS

JZ, F-DW, M-LW, Y-CT, D-BW, Y-JS, G-BX, X-BC, and XC conceived this review and collected the literature, E-QC conducted the study supervision and revised the manuscript. All authors contributed to the article and approved the submitted version.

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SYSTEMATIC REVIEW
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Efficacy and Safety of Treatments for Patients With Portal Hypertension and Cirrhosis: A Systematic Review and Bayesian Network Meta-Analysis

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Background and Aims: Viral hepatitis are one of the main causes of liver cirrhosis. The treatment of portal hypertension caused by liver cirrhosis is difficult and diverse, and the therapeutic effect is unknown. Bayesian network meta-analysis was performed to compare the efficacy and safety of treatments for patients with portal hypertension and cirrhosis, including a transjugular intrahepatic portosystemic shunt (TIPS), endoscopic therapy, surgical therapy and medications.

Methods: Eligible articles were searched for in PubMed, Embase, Cochrane Library and Web of Science databases from their inception until June 2020. Using the "gemtc-0.8.4" package in R v.3.6.3 software and the Just Another Gibbs Sampler v.4.2.0 program, network meta-analysis was performed using a random effects model within a Bayesian framework. The odds ratios for all-cause rebleeding, bleeding-related mortality, overall survival (OS), treatment failure and hepatic encephalopathy were determined within the Bayesian framework.

Results: Forty randomized controlled trials were identified, including 4,006 adult patients and nine treatment strategies. Our results showed that distal splenorenal shunt and TIPS provided the best control of hemorrhage. Endoscopic variceal ligation with medication resulted in the highest OS rate. Medication alone resulted in poor OS and treatment failure.

Conclusions: We performed a systematic comparison of diverse treatments for cirrhotic patients with portal hypertension. Our meta-analysis indicated that a TIPS and distal splenorenal shunt resulted in lower rates of rebleeding than did other therapies. Furthermore, drugs are more suitable for combination therapy than monotherapy.

Keywords: endoscopic therapy, transjugular intrahepatic portosystemic shunt, portal hypertension, liver cirrhosis, network meta-analysis, all-cause rebleeding

INTRODUCTION

Esophageal and gastric variceal hemorrhage is a common and life-threatening complication for patients with cirrhosis and portal hypertension (1). In approximately one-third of patients with cirrhosis, hemorrhage may cause early mortality (2). After the first variceal bleeding is addressed, the incidence of rebleeding within 1–2 years is 60–70%, and the mortality rate can be as high as ∼20–33% (3, 4). Therefore, monitoring or choosing additional appropriate treatment of hemostasis for the first variceal bleeding may help improve quality of life and prognosis.

Over the past 20 years, various effective treatments for portal hypertension have been developed. Non-selective beta-blockers (β-blockers) remain the cornerstone of bleeding and have been used for more than 30 years (5). In addition, somatostatin and terlipressin are potent splanchnic vasoconstrictors (6). These agents significantly decrease both the hepatic venous pressure gradient and portal-collateral (azygos) blood flow and are used to reduce the risk of bleeding (7). Endoscopic therapy (ET), interventional therapy and surgery are also often used to control hemorrhage. ET involves mainly endoscopic injection sclerotherapy (EIS) and endoscopic variceal ligation (EVL). However, EVL has been shown to result in lower rebleeding, mortality and complication rates compared with EIS (8–10). Transjugular intrahepatic portosystemic shunt (TIPS) is a metal stent that connects the hepatic vein and intrahepatic portal vein to effectively decrease portal pressure and prevent ascites aggravation and rebleeding (11). It is ideal to maintain the portal pressure gradient of the portal and inferior vena cava between 10 and 12 mm Hg (5). Some studies have indicated that the primary unassisted patency rates of polytetrafluorethylene-covered stents are similar to those of surgical shunting (10).

Additionally, several randomized controlled trials (RCTs) have shown that combination therapy may be superior to monotherapy in terms of rebleeding, survival and complication rates (12). Argonz et al. reported increased recurrence of bleeding in the EVL group compared with the EVL plus EIS group (31.7 vs. 23%) (13). Similarly, a meta-analysis found that EVL plus nadolol or sucralfate decreased the risk of rebleeding compared with EVL alone. However, Puente et al. noted that a reduction in rebleeding did not improve survival (14).

To use existing study-level data to assess the relative effectiveness of active interventions in cirrhotic patients with a history of hemorrhage, we performed a network meta-analysis of RCTs that included rebleeding and mortality as outcomes. The purpose of this meta-analysis is to provide guidance for clinical policymakers regarding the safety and efficacy of TIPS, EVL, EIS, medication and combinations of these treatments in terms of the 1-, 2-, and 3-year rebleeding and overall survival (OS) rates, treatment failure, bleeding-related mortality, and HE.

Abbreviations: JAGS, Just Another Gibbs Sampler; MCMC, Markov Chain Monte Carlo chain; PSRF, Potential Scale Reduction Factor; CI, Confidence interval; OS, Overall survival; RR, Odds ratio; RCT, Randomized controlled trial; PRISMA-P, Preferred Reporting Items for Systematic Review and Meta-Analysis Protocols; EIS, endoscopic sclerotherapy; EVL, endoscopic variceal ligation; ETA, endoscopic tissue adhesion; ISMN, isosorbide mononitrate; TIPS, transjugular intrahepatic portosystemic shunt; DSRS, distal splenorenal shunt.

MATERIALS AND METHODS

Search Strategy

Two researchers independently screened the titles and abstracts of the articles in terms of the selection criteria. The literature search was performed in various electronic databases (PubMed, Web of Science, MEDLINE, Embase and Cochrane Library) from their inception to June 2020. A combination of free-text terms and medical subject heading terms were used for the subject search, as follows: "liver cirrhosis," "variceal hemorrhage," "variceal rebleeding," "transjugular intrahepatic portosystemic shunt," "balloon-occluded retrograde transvenous obliteration," "endoscopic therapy," "beta-blocker," and "surgery." The article type was restricted to randomized controlled trials.

Study Selection

Studies were considered eligible for inclusion if they fulfilled the following criteria: (1) focused on treatments for patients with cirrhosis and portal hypertension; (2) compared at least two factors among TIPS, EVL, EIS, medication, or combination therapies; and (3) included rebleeding as a primary endpoint. The exclusion criteria were as follows: (1) not written in English; (2) non-clinical article, such as a case report, letter, basic research study or systematic review; (3) lack of sufficient or qualified data; (4) published before 2,000 or included <20 participants per group.

Data Extraction and Quality Assessment

Two researchers independently extracted the data, and a third researcher was consulted to reach a majority decision when needed. The following information was summarized. (1) The authors' names, year of publication, treatment group, country of study, number of patients and follow-up time. (2) Clinical outcomes including all-cause rebleeding; 1-, 2-, and 3-year rebleeding rates; treatment failure; bleeding-related mortality; 1-, 2-, and 3-year OS rates; and HE. Otherwise, treatment failure was defined as the occurrence of two or more episodes of recurrent bleeding or switching to an alternative treatment. This meta-analysis was conducted in accordance with the guidelines of the Preferred Reporting Items for Systematic Review and Meta-Analysis Protocols 2015 statement (15). The methodological quality of the RCTs was evaluated using the Cochrane Collaboration tool (16).

Statistical Analysis

Using the "gemtc" package in R 3.6.3 software, the Markov chain Monte Carlo method was applied to perform Bayesian network meta-analysis (17). This method combines both direct and indirect evidence for any given pair of management strategies and a particular endpoint. The mtc.run function was applied to generate samples, and we set 5,000 simulations for each chain as the "burn-in" period, yielding 20,000 iterations to obtain the odds ratios (ORs) for the model parameters based on three Markov chains. Rank probabilities were calculated to obtain the hierarchical position of each treatment, and a plot of rank probabilities was created using the "gemtc" package (18). Brooks–Gelman–Rubin plots, trace plots and density plots were used to assess model convergence (19).

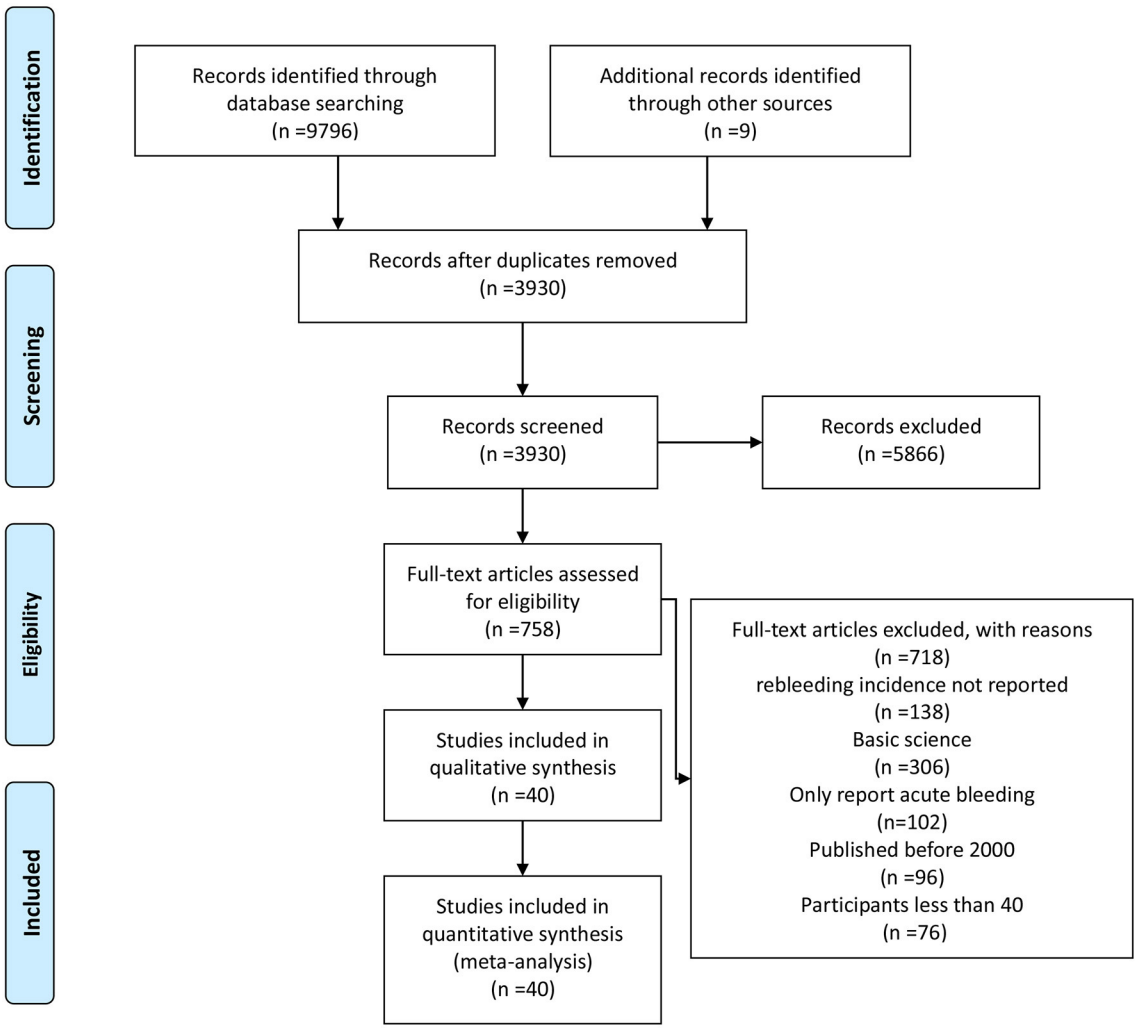

FIGURE 1 | Flow chart of the study selection.

The mtc.anohe command of the "gemtc" package was used to evaluate global heterogeneity. To ensure reliability, a sensitivity analysis was performed by removal of each trial. Begg's test and Egger's test were applied using a $P < 0.1$ threshold of significance for testing publication bias.

RESULTS

Eligible Studies and Characteristics

The literature search generated 9,805 relevant clinical records. After screening titles and abstracts, removing duplicates and assessing eligibility, 5,866 articles were excluded; the remaining articles were subjected to full text review. Finally, 40 RCTs including a total of 4,006 patients met the inclusion criteria and were selected for the meta-analysis. A flow chart of the detailed screening process can be found in **Figure 1**.

Study Characteristics and Quality

The basic characteristics of the selected studies are summarized in **Table 1**. In total, 4,006 patients were enrolled in nine different treatment strategies. The 40 studies included 12 from China, five from India, one from Japan, one from Pakistan, one from Mexico, two from Argentina, one from Canada, one from the USA, one Croatia, one Brazil, one from Italy, four from Egypt, three from Germany, four from Spain, and two from Greece. Thus, 19 studies were performed in Asia, 11 in Europe, six in America, and four in Africa. Seventeen of the studies had a mean follow up time of >2 years. Medications included propranolol, nadolol, octreotide, terlipressin, and isosorbide-5-mononitrate; ET included EIS, EVL, and endoscopic tissue adhesive (ETA) injection. There were two major types of surgical shunts: portacaval and distal splenorenal shunts. All studies were two-arm trials, except for Harras consisting of three arms: EIS, EVL, and EIS + EVL. Detailed results of the bias assessment are shown in **Supplementary Table 1**.

TABLE 1 | Study characteristics.

References	Country	Sarin classification	Etiology of cirrhosis, alcohol/ Hepatitis/others	Male/female	Treatment	Number of patients	Child-Pugh class (A/B/C)	Child-Pugh score	Follow-up time mean (range or SD)
Argonz et al. (13)	Argentina	G2/G3: 27,14	20/12/9	32/9	EVL	41	14/23/4	NA	337 ± 43.4 days
		G2/G3: 22,17	24/6/9	30/9	EVL+EIS	39	11/26/2	NA	386 ± 40.1 days
Hou et al. (9)	China	F3/F2+F: 151,19	13/44/13	57/13	EIS	70	17/34/19	8 ± 1.9	63.4 ± 11.6 months
		F3/ F2+F1: 57,14	11/41/19	56/15	EVL	71	20/26/25	8.4 ± 2.4	60.1 ± 17.5 months
Lo et al. (20)	China	F2/F3: 27,35	20/41/1	49/13	EVL	62	12/28/22	NA	21 months
		F2/F3: 24,36	17/41/2	45/15	EVL+ Drug (Nadolol)	60	13/30/19	NA	21 months
Orozco et al. (21)	Mexico	NA	NA	NA	Drug (Nadolol)	40	22/14/4	NA	45 months
		NA	NA	NA	EIS	46	21/17/8	NA	45 months
Villanueva et al. (22)	Spain	G1/G2/G3: 1,49,22	30/26/13	47/25	EVL	72	11/43/18	8.4 ± 1.9	21 months
		G1/G2/G3: 2,41,29	33/24/10	43/29	Drug (Nadolol+ ISMN)	72	19/39/14	7.9 ± 1.9	21 months
Pomier-Layrargues et al. (23)	Canada	NA	24/5/10	27/12	EVL	39	NA	9.8 ± 1.6	48.5 months
		NA	25/4/12	29/12	TIPS	41	NA	9.6 ± 1.6	22.6 months
Hou et al. (24)	China	NA	NA	31/16	EVL	47	14/17/16	8.2±2.3	11.6 ± 6 months
		NA	NA	36/11	EVL+EIS	47	11/23/13	8 ± 2.1	11.1 ± 5.9 months
Cheng et al. (25)	China	F1/F2/F3: 8,21,13	NA	30/12	EVL	42	14/17/11	NA	NA
		F1/F2/F3: 11,23,10	NA	29/15	EIS+EVL	44	18/14/12	NA	NA
Narahara et al. (26)	Japan	NA	9/24/5	32/6	TIPS	38	NA	6.8 ± 0.3	1,116 ± 92 days
		NA	17/20/3	30/10	EIS	40	NA	7.4 ± 0.3	1,047 ± 102 days
Sauer et al. (27)	Germany	MG 2.4: 43	29/9/5	27/16	TIPS	43	15/16/12	7.9 ± 2.1	4.1 ± 0.26 months
		MG 2.6: 42	24/12/6	23/19	EVL	42	10/19/13	8.2 ± 2.0	3.6 ± 0.25 months
Gülberg et al. (28)	Germany	NA	22/3/3	20/8	TIPS	28	11/15/2	NA	21.6 months
		NA	23/3/0	19/7	EVL	26	10/12/4	NA	24.0 months
Escorsell et al. (29)	Spain	NA	25/NA/19	35/9	Drug (Propranolol+ ISMN)	44	0/28/16	6.3 ± 1.3	15.4 ± 10.3 months
		NA	24/NA/23	33/14	TIPS	47	0/30/17	7.0 ± 1.4	14.4 ± 9.6 months
Viazis et al. (10)	Greece	S/M /L: 9,17,10	15/15/6	21/15	EVL	36	4/18/14	NA	49.6±9.7 days
		S/M/L: 10,15,12	13/17/7	20/17	EIS	37	6/16/15	NA	58.6 ± 10.4 days
Avgerinos et al. (8)	Greece	G1/G2/G3: 3,13,9	17/8/0	19/6	EVL	25	6/8/11	9.4 ± 2.8	42 days
		G1/G2/G3: 3,12,10	15/7/3	20/5	EIS	25	7/6/12	9.2 ± 2.96	42 days

(Continued)

TABLE 1 | Continued

References	Country	Sarin classification	Etiology of cirrhosis, alcohol/ Hepatitis/others	Male/female	Treatment	Number of patients	Child-Pugh class (A/B/C)	Child-Pugh score	Follow-up time mean (range or SD)
Schepke et al. (30)	Germany	NA	40/22/13	50/25	EVL	75	34/31/10	7.3 ± 1.8	34.4 ± 18.9 months
		NA	38/25/14	54/23	Drug (Propranolol)	77	37/31/9	7.0 ± 1.9	34.4 ± 18.9 months
Peña et al. (31)	Spain	II/III/IV: 7,17,13	26/8/3	27/10	EVL	37	6/20/11	NA	15 ± 8 months
		II/III/IV: 10,26,7	27/12/4	33/10	EVL+ Drug (Nadolol)	43	6/25/12	NA	17.5 ± 7.8 months
Sarin et al. (32)	India	NA	18/25/10	51/20	EVL	71	35/26/10	6.9 ± 2.0	12.4 ± 9.7 months
		NA	15/23/8	45/21	Drug (β-blocker+ ISMN)	66	27/28/11	7.2 ± 1.9	11.1 ± 7.9 months
Shah et al. (33)	Pakistan	NA	2/49/NA	32/19	EIS +Drug (Octreotide)	51	9/31/11	NA	24 months
		NA	2/52/NA	36/18	EIS	54	7/33/14	NA	24 months
Zargar et al. (34)	India	G2/3/4: 1,8,27	NA	22/14	EIS	36	NA	NA	NA
		G2/3/4: 2,6,29	NA	24/13	EVL	37	NA	NA	NA
Chen et al. (35)	China	F1/F2/F3: 5,38,19	24/30/8	43/19	EVL	62	13/31/18	8.3 ± 2.0	NA
		F1/F2/F3: 2,41,20	29/29/5	52/11	SMT	63	18/27/18	8.3 ± 2.3	NA
Santambrogio et al. (36)	Italy	NA	14/NA/NA	27/13	DSRS	40	19/21/ NA	NA	109.0 ± 58 months
		NA	26/NA/NA	33/7	EIS	40	11/29/ NA	NA	87.0 ± 61 months
Romero et al. (37)	Argentina	NA	30/8/19	37/20	Drug (Nadolol+ ISMN)	57	40/44/16	7.0 ± 1.9	11.5 months
		NA	24/7/21	35/17	EVL	52	32/58/10	7.0 ± 1.6	12 months
Henderson et al. (38)	America	NA	NA	42/31	DSRS	73	41/32/NA	6.4 ± 1.1	45 months
		NA	NA	44/23	TIPS	67	39/28/NA	6.3 ± 1.0	45 months
Tan et al. (39)	China	G1/G2/IGV1: 11,5,5	3/32/13	34/14	EVL	48	12/25/11	8.10 ± 2.22	610.6 ± 603.04 days
		G1/G2/IGV1: 7,3,1	2/31/16	35/14	ETA	49	13/26/10	7.96 ± 1.86	680.7 ± 710.54 days
Lo et al. (40)	China	G1/G2: 19,14	NA	25/10	TIPS	35	9/20/6	7.8 ± 1.8	33 months
		G1/G2: 17,19	NA	28/9	ETA	37	12/19/6	7.6 ± 1.7	32 months
Morales et al. (41)	Brazil	NA	9/17/14	27/13	EIS+ Drug (Octreotide)	40	4/12/24	6.8 ± 0.3	14 months
		NA	2/11/15	18/10	EIS	28	7/11/10	NA	14 months
Amin et al. (42)	Egypt	NA	NA/68/NA	53/22	EVL	75	20/40/15	NA	NA
		NA	NA/65/NA	55/20	ETA	75	15/32/28	NA	NA
Lo et al. (43)	China	NA	16/32/12	46/14	EVL	60	13/35/12	8.0 ± 1.5	82 months

(Continued)

TABLE 1 | Continued

References	Country	Sarin classification	Etiology of cirrhosis, alcohol/ Hepatitis/others	Male/female	Treatment	Number of patients	Child-Pugh class (A/B/C)	Child-Pugh score	Follow-up time mean (range or SD)
		NA	22/32/7	47/14	Drug (Nadolol+ ISMN)	61	13/35/13	7.8 ± 1.6	81 months
Kumar et al. (44)	India	MG 3.1: 88	33/13/30	75/13	EVL+ Drug (Propranolol+ ISMN)	88	35/31/10	7.3 ± 2.0	15 ± 12 months
		MG 3.2: 89	30/20/25	78/11	EVL	89	26/34/15	7.8 ± 2.1	15 ± 11 months
Lo et al. (12)	China	NA	17/25/4	41/5	Drug (Terlipressin)	46	14/25/7	7.7 ± 1.8	42 days
		NA	15/28/4	36/11	EVL+ Drug (Terlipressin)	47	13/20/14	8.1 ± 1.8	42 days
García-Pagán et al. (45)	Spain	NA	42/18/18	53/25	Drug (β-blocker+ ISMN)	78	18/42/18	8.1 ± 1.8	15 months
		NA	39/25/16	65/15	EVL+ Drug (β-blocker+ ISMN)	80	16/46/18	8.2 ± 1.8	15 months
Sarin et al. (46)	India	II/III/IV: 12,20,19	NA	38/13	EVL	51	NA	5 (5–9)	23 months
		II/III/IV: 17,19,14	NA	32/18	Drug (Propranolol)	50	NA	5 (5–7)	23 months
Mishra et al. (47)	India	NA	12/NA/NA	19/14	ETA	33	4/12/17	9 (6–12)	26 months
		NA	11/NA/NA	26/8	Drug (β-blocker)	34	5/13/16	9 (6–12)	26 months
Harras et al. (48)	Egypt	G1/G2/G3: 0,2,48	NA	NA	EIS	50	16/29/5	NA	17.8 ± 4.85 months
		G1/G2/G3: 0,0,50	NA	NA	EVL	50	14/32/4	NA	17.8 ± 4.85 months
		G1/G2/G3: 0,1,49	NA	NA	EIS+EVL	50	12/36/2	NA	17.8 ± 4.85 months
Ljubicić et al. (49)	Croatia	NA	NA	16/6	ETA	22	4/9/9	NA	60 months
		NA	NA	15/6	EVL	21	8/9/4	NA	60 months
Kong et al. (50)	China	f2/f3: 10,10	2/10/6	14/6	EVL	20	9/11/NA	NA	48 months
		f2/f3: 9,11	1/10/6	9/9	EVL+EIS	18	8/10/NA	NA	48 months
Ali et al. (51)	China	G2/G3: 11,53	4/37/23	51/13	EIS	64	20/33/11	NA	24 months
		G2/G3: 8,52	3/39/18	45/15	EVL	60	22/29/9	NA	24 months
Lv et al. (52)	China	NA	1/21/2	13/11	TIPS	24	9/13/2	7 (6–8)	30 months
		NA	0/22/3	16/9	EVL+ Drug (Terlipressin or Somatostatin)	25	10/14/1	7 (6–8)	30 months
Mansour et al. (53)	Egypt	G1/G2: 49,11	NA/56/4	34/26	EVL	60	8/20/32	NA	12 months
		G1/G2: 45,15	NA/60/0	44/16	EVL+EIS	60	14/22/24	NA	12 months
Elsebaey et al. (54)	Egypt	F1/F2/F3: 2,22,32	NA	42/14	EIS	56	13/23/20	NA	20 months
		F1/F2/F3: 4,24,29	NA	39/18	ETA	57	15/24/18	NA	20 months

G, Grade; Grade 1, visible only during 1 phase of respiration or on performance of valsalva maneuver; Grade 2, visible during both phases of respiration; Grade 3, 3–6 mm; Grade 4, >6 mm; S, small: V arix is flush with the wall of the oesphagus; M, medium: Protrusion of varix no further than halfway to the center; L, large: protrusion more than half way to the center of the lumen; F, varicies; F1, straight, small-caliber varices; F2, moderately enlarged, beady varices; F3, markedly enlarged, nodular or tumor-shaped varices. DSRS, distal splenorenal shunt; TIPS, transjugular intrahepatic portosystemic shunt; EIS, endoscopic injection sclerotherapy; EVL, endoscopic variceal ligation; ETA, endoscopic tissue adhesion; ISMN, isosorbide mononitrate; MG, mean grade; NA, not available.

Network Structure Diagrams

Nine different therapeutic strategies were included among the trials: EVL, TIPS, distal splenorenal shunt (DSRS), medication, EVL + EIS, EIS, EVL + medication, ETA, and EIS + medication. Network structure diagrams were applied to depict the direct associations among the treatment strategies. The thickness of the lines is proportional to the number of comparisons, and the diameter of the circles is proportional to the number of treatments included in the meta-analysis. All diagrams are presented in **Figure 2**.

Brooks–Gelman–Rubin Diagnostic Plot, Density Plot, and Trace Plot

Brooks–Gelman–Rubin diagnostic plots, trace plots and density plots were obtained to assess the convergence of our model. As suggested by Brooks and Gelman (55), the model was considered to be well-fitted if the curves of the plots were consistent and stable, and if the potential scale reduction factor was close to 1.0. For trace plots, each Markov Chain Monte Carlo chain achieved stable fusion from the beginning, and the overlapping area accounted for the majority of chain fluctuation in the subsequent calculations. The fluctuation of single chains could not be recognized by the naked eye, and therefore the degree of convergence was considered satisfactory, as shown in **Supplementary Figure 1**. In the density diagram, the bandwidth tended to be zero and stable, and a smooth curve that conformed to the normal distribution indicated that the model had good convergence (**Supplementary Figure 1**). Furthermore, the potential scale reduction factor for each analysis was close to 1.0 in the Brooks–Gelman–Rubin diagnostic plot, as shown in **Supplementary Figure 2** and **Supplementary Table 2**.

All-Cause Rebleeding

Forty studies, including 4,006 patients with nine therapeutic schedules, reported all-cause rebleeding. There were many significant differences among the therapeutic schedules, such as TIPS vs. EVL (OR 0.31, 95% CI 0.15–0.66), medication vs. TIPS (OR 5.4, 95% CI 2.3–13.0), EIS vs. TIPS (OR 4.1, 95% CI 1.7–10.), medication vs. DSRS (OR 1.3, 95% CI 0.30–5.6), EIS vs. DSRS (OR 5.3, 95% CI 1.3–23.), medication vs. EVL + EIS (OR 2.9, 95% CI 1.1–8), EVL + medication vs. medication alone (OR 0.36, 95% CI 0.15–0.85), ETA vs. medication (OR 0.26, 95% CI 0.10–0.66), and ETA vs. EIS (OR 0.34, 95% CI 0.13–0.88) (**Figure 3**). Subgroup analyses of rebleeding occurring at 1, 2, and 3 years are summarized in **Supplementary Figures 3–5**. Compared with TIPS, EVL (OR 6.1, 95% CI 2.2–18.0; OR 6.6, 95% CI 2.5–18.0; and OR 5.0, 95% CI 0.9–28.0 at 1, 2, and 3 years, respectively) and medication (OR 4.9, 95% CI 1.7–17.0; OR 7.9, 95% CI 2.9–23.0; and OR 11, 95% CI 1.0–120.0 at 1, 2, and 3 years, respectively) had higher rates of rebleeding in all years. In descending order, all-cause rebleeding was best controlled by DSRS, TIPS, EIS + medication, ETA, EVL + medication, EVL + EIS, EVL, EIS, and medication alone (**Figure 4**). In order of decreasing efficacy, rebleeding at 1, 2, and 3 years were best controlled by TIPS, ETA, EVL + medication/EIS, EVL, and medication alone (**Supplementary Figure 6**).

Bleeding-Related Mortality

Twenty-one articles including seven different treatments (EVL, TIPS, medication, EVL + EIS, EIS, EVL + medication, and ETA) were used in the analysis of bleeding-related mortality. No significant results were identified regarding the previously discussed treatments. Our results showed that EVL (OR 5.0, 95% CI 0.6–100.0), TIPS (OR 2.6, 95% CI 0.2–51.0), medication alone (OR 5.0, 95% CI 0.5–100.0), EIS (OR 5.4, 95% CI 0.5–130.0), EVL + medication (OR 2.4, 95% CI 0.2–54.0), and ETA (OR 2.3, 95% CI 0.2–50.0) were associated with a relatively high rate of rebleeding compared with EVL + EIS (**Supplementary Figure 7**). As indicated in the cumulative ranking, EVL + EIS ranked most favorably among the treatments (**Supplementary Figure 8**).

Overall Survival (OS)

Twenty-one of the selected studies assessed the 1-year OS rate in a total of 2,163 patients with cirrhosis. Eleven of the studies assessed 2- and 3-year OS rates in a total of 967 cirrhosis patients. Our results showed that TIPS (OR 1.1, 95% CI 0.5–2.7), DSRS (OR, 0.9 95% CI 0.2–5.9), medication (OR 0.6, 95% CI 0.3–1.3), EIS (OR 0.7, 95% CI 0.2–2.5), EVL + medication (OR 1.5, 95% CI 0.5–5.1), and ETA (OR 1.2, 95% CI 0.4–3.9) did not differ significantly from EVL in terms of 1-year OS (**Figure 5**). There was also no significant difference among the other treatment methods (**Figure 5**). EVL, TIPS, DSRS, medication, EIS and ETA offered no significant benefit in terms of the 2- or 3-year OS rate (**Supplementary Figures 9, 10**). Rank probability analysis showed that medication, EIS, DSRS, TIPS, EVL, ETA, and EVL + medication ranked from worst to best in terms of 1-year OS, and that medication, TIPS, EVL, ETA, DSRS, and EIS ranked from worst to best in terms of 3-year OS (**Figure 6** and **Supplementary Figures 11, 12**).

Treatment Failure

The incidence of treatment failure was examined in nine direct comparisons and just four different treatments among 1,099 patients. The incidence of treatment failure is shown in **Supplementary Figure 13**. Compared with EVL, medication (OR 1.3, 95% CI 0.4–5.7), EVL + medication (OR 0.3, 95% CI 0.0–2.4), and ETA (OR 1.2, 95% CI 0.1–13.0) were not significantly associated with treatment failure. When other treatments were compared, the results were similar. We created a rank probability plot, which showed that EVL + medication had the lowest rate of treatment failure (**Supplementary Figure 14**).

Hepatic Encephalopathy

A total of 1,956 patients experienced hepatic encephalopathy in trials that included 19 direct comparisons and seven different treatments. As shown in **Figure 7**, DSRS and TIPS were associated with a significantly higher incidence of hepatic encephalopathy compared with EVL, medication, EIS, EVL + medication, and ETA. The rank probability analysis confirmed this finding (**Supplementary Figure 15**).

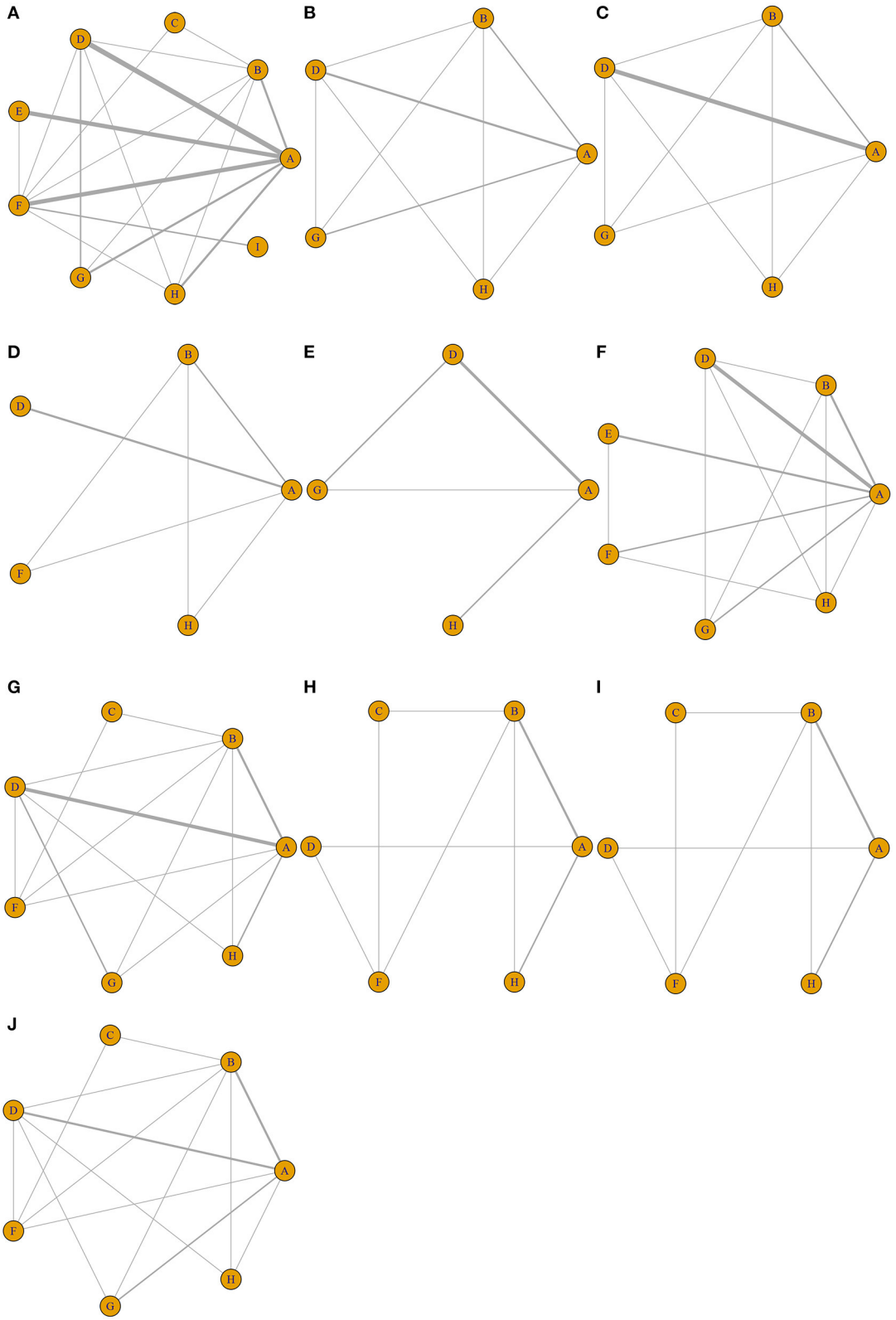

FIGURE 2 | Network structure diagrams. As shown in the figure, the thickness of the lines is proportional to the number of comparisons, and the diameter of the circles is proportional to the number of treatments included in the meta-analysis. **(A)** All-cause rebleeding. Rebleeding at **(B)** 1 year, **(C)** 2 years, and **(D)** 3 years. **(E)** Treatment failure. **(F)** Bleeding-related mortality. OS at **(G)** 1 year, **(H)** 2 years, and **(I)** 3 years. **(J)** Hepatic encephalopathy.

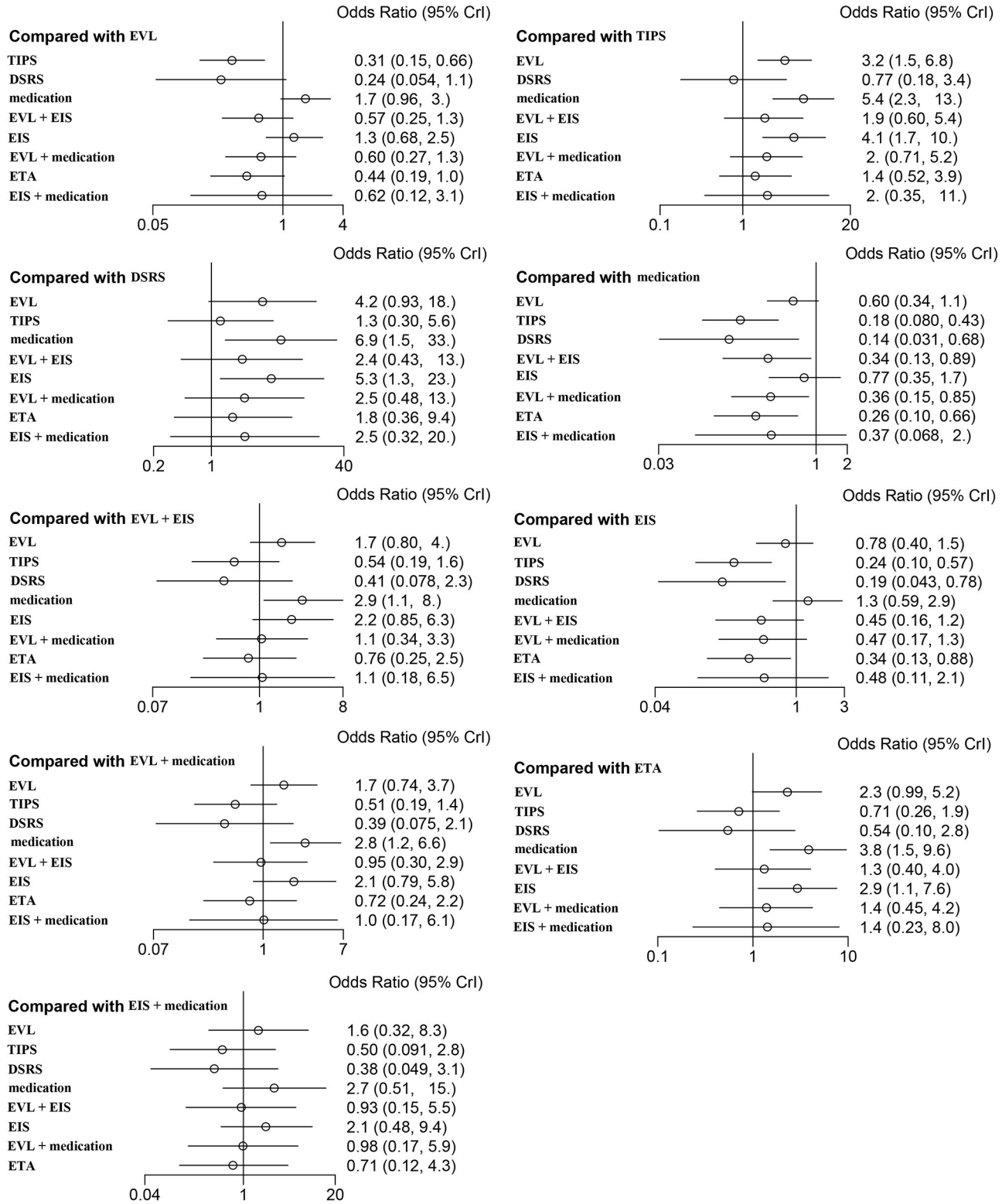

FIGURE 3 | Forest plot of the odds ratios for all-cause rebleeding based on different pairwise comparisons. EVL, endoscopic variceal ligation; TIPS, transjugular intrahepatic portosystemic shunt; DSRS, distal splenorenal shunt; EIS, endoscopic injection sclerotherapy; ETA, endoscopic tissue.

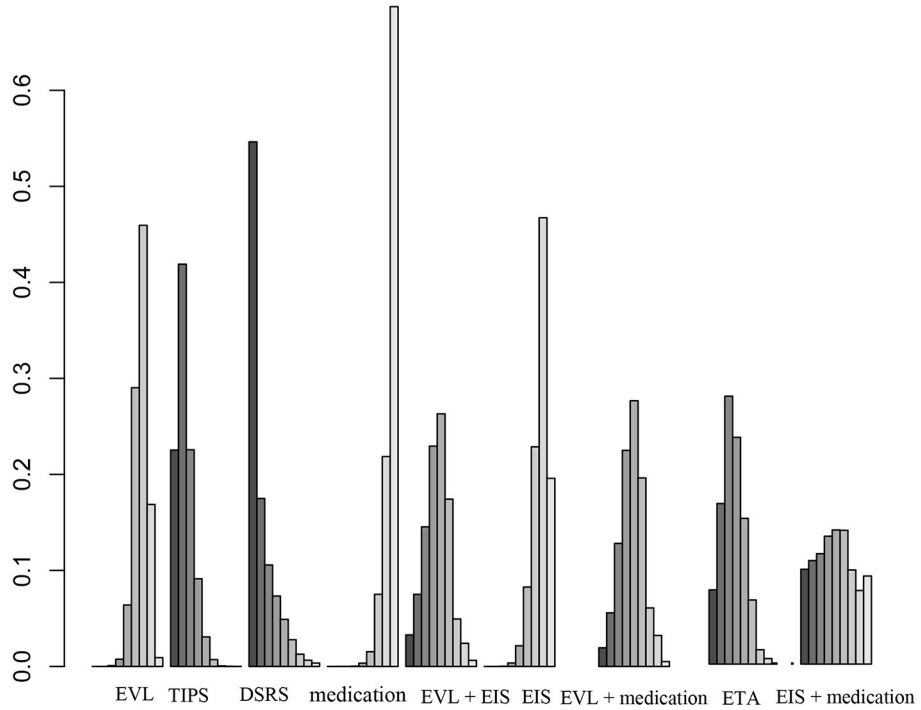

FIGURE 4 | Ranking of therapies based on all-cause rebleeding. EVL, endoscopic variceal ligation; TIPS, transjugular intrahepatic portosystemic shunt; DSRS, distal splenorenal shunt; EIS, endoscopic injection sclerotherapy; ETA, endoscopic tissue.

Sensitivity Analysis and Publication Bias

Sensitivity analysis was performed by excluding several studies. The results were consistent with those of the primary meta-analysis. Begg's and Egger's tests showed that no clear publication bias existed ($P > 0.1$).

DISCUSSION

Abundant research has shown that variceal bleeding and rebleeding are among the most serious complications of portal hypertension in patients with cirrhosis because of a severe impact on prognosis (11, 56). Treating patients based on their individual risk of portal hypertension-related bleeding undoubtedly affects the prognosis. ET, a classic treatment that has been routinely used for 30–40 years, plays a pivotal role in the management of variceal bleeding and rebleeding (57). However, ET is effective for only a short time because portal pressure and blood flow remain unchanged, and varices frequently recur (in ∼50% of cases within 2 years). β-blockers, such as propranolol, timolol, nadolol and carvedilol, decrease cardiac output by β1 adrenergic receptors and reduce splanchnic blood flow by β2 receptors. None of these medications are clearly more effective than others; their usage is driven by doctors' recommendations and patient compliance (58, 59). It has been reported that combination therapy with β-blockers and EVL is significantly more effective than either EVL or medication alone in preventing recurrent hemorrhage (46, 60). In addition, adding low-dose isosorbide-5-mononitrate to β-blockers has been shown to provide a greater portal pressure-reducing effect than β-blockers alone (37, 61). As shown in our network diagram, many studies have directly compared the above treatments, but several treatments have not been compared directly (e.g., EIS + medication vs. DSRS, EIS + medication vs. TIPS, etc.). We conducted our network meta-analysis to address these gaps and provide further guidance for clinical practice.

Our network meta-analysis included 40 RCTs that were conducted within the past 20 years and compared rates of rebleeding, treatment failure, OS and HE due to variceal hemorrhage. A total of 4,006 patients were treated with nine therapeutic methods, including vasoactive medications, DSRS, EVL, EIS, ETA, and combination therapies.

We found that TIPS and DSRS were associated with a lower likelihood of variceal rebleeding compared with ET or medication, either alone or combined. However, TIPS and DSRS were also associated with a higher rate of HE, which is consistent with the American Association for the Study of Liver Diseases practice guidelines (62). Patients experienced a peak incidence of ascites early after DSRS placement (∼10% within the 1st month) (38, 63). Conversely, there was a high rate of ascites in the TIPS group at later follow-up time points. TIPS is preferred over DSRS for patients with poor liver function after ineffective conservative therapy and ET. Otherwise, both DSRS and TIPS appear to offer equivalent outcomes (59). According to the AALSD and Baveno guidelines, although TIPS and DSRS are

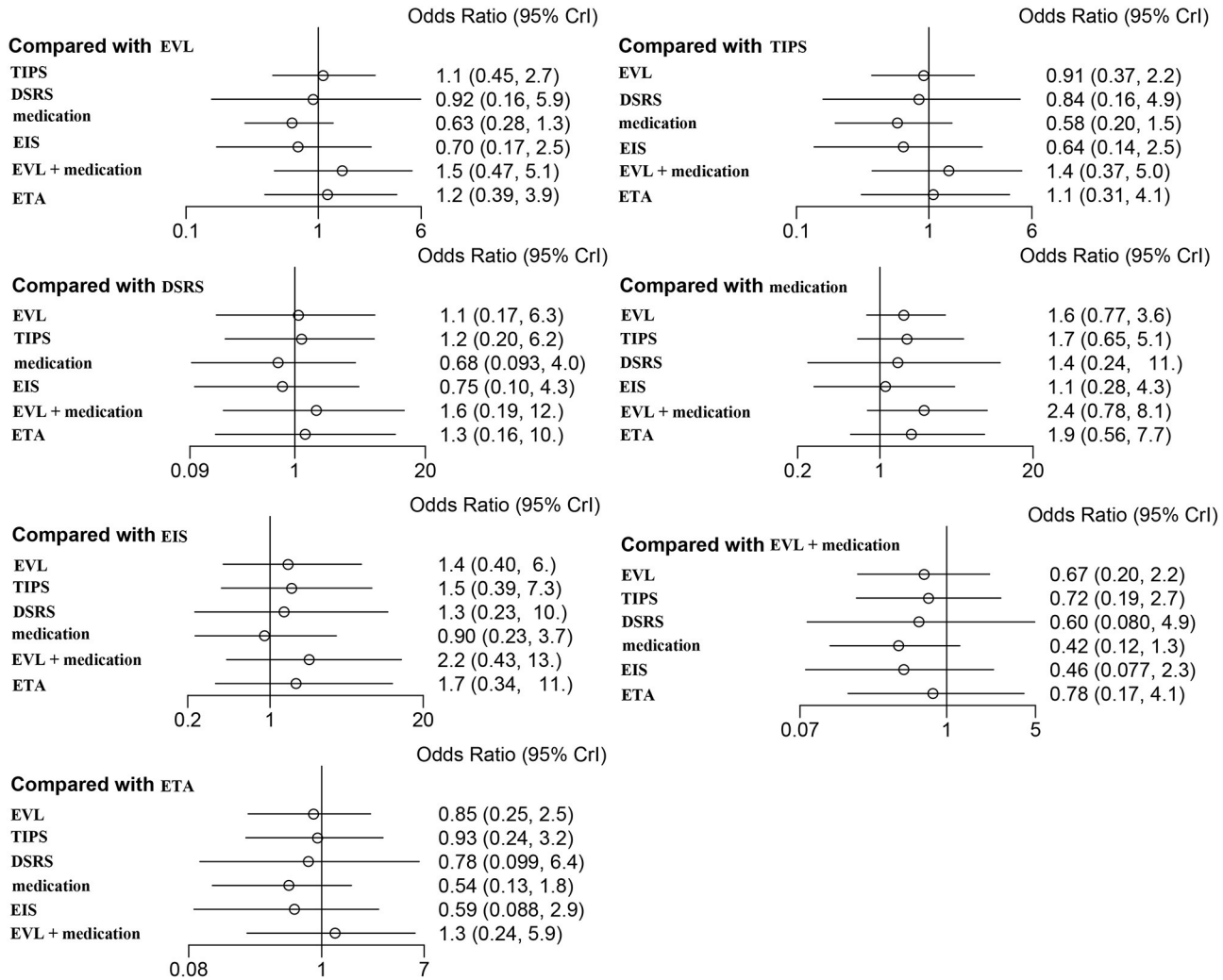

FIGURE 5 | Forest plot of the odds ratios for 1-year OS based on different pairwise comparisons. EVL, endoscopic variceal ligation; TIPS, transjugular intrahepatic portosystemic shunt; DSRS, distal splenorenal shunt; EIS, endoscopic injection sclerotherapy; ETA, endoscopic tissue.

effective in controlling rebleeding, we need to discuss treatment indications the issue of critical liver reserve. TIPS is only recommended for early intervention (72 h) and is not suitable for serious decompensated cirrhosis patients, such as multi-organ failure, abnormal coagulation function, etc. Besides, it should be mentioned that our study did not distinguish between bare and covered stents, which may underestimate the effectiveness of TIPS.

EIS has been supplanted by EVL as the main therapeutic strategy because of the growing evidence that EVL has lower complication rates (51, 64). EIS is associated with severe complications such as transient dysphagia, retrosternal chest discomfort, low-grade fever and esophageal ulceration. Complications occur in up to 40% of patients. ETA employs N-butyl-cyanoacrylate, a strong tissue adhesive used for hemostasis that causes endothelial fibrosis and venous obturation. ETA is associated with a rebleeding risk of 20–25% when endoscopic tissue adhesion achieves hemostasis (65). Our results showed that rebleeding was more frequent after ET than after TIPS or DSRS. However, patients had significantly lower rates of HE after ETA. According to the 2015 United Kingdom guidelines and the 2017 American Association for the Study of Liver Diseases guidelines, combined therapies are favored over EIS, EVL or ETA alone (66, 67). Similarly, based on our network meta-analysis, EVL + medication resulted in a higher 1-year OS rate compared with EVL, EIS or ETA alone and a lower treatment failure rate compared with EVL or ETA alone. EVL + EIS was superior to EVL or EIS alone in terms of bleeding-related mortality.

β-blockers such as propranolol and nadolol have been used for more than 30 years (5). Terlipressin and somatostatin are potent splanchnic vasoconstrictors that also have systemic circulatory effects; they increase arterial pressure and systemic vascular resistance, inhibit glucagon and other vasoactive peptides, and facilitate adrenergic vasoconstriction (68, 69). Our meta-analysis

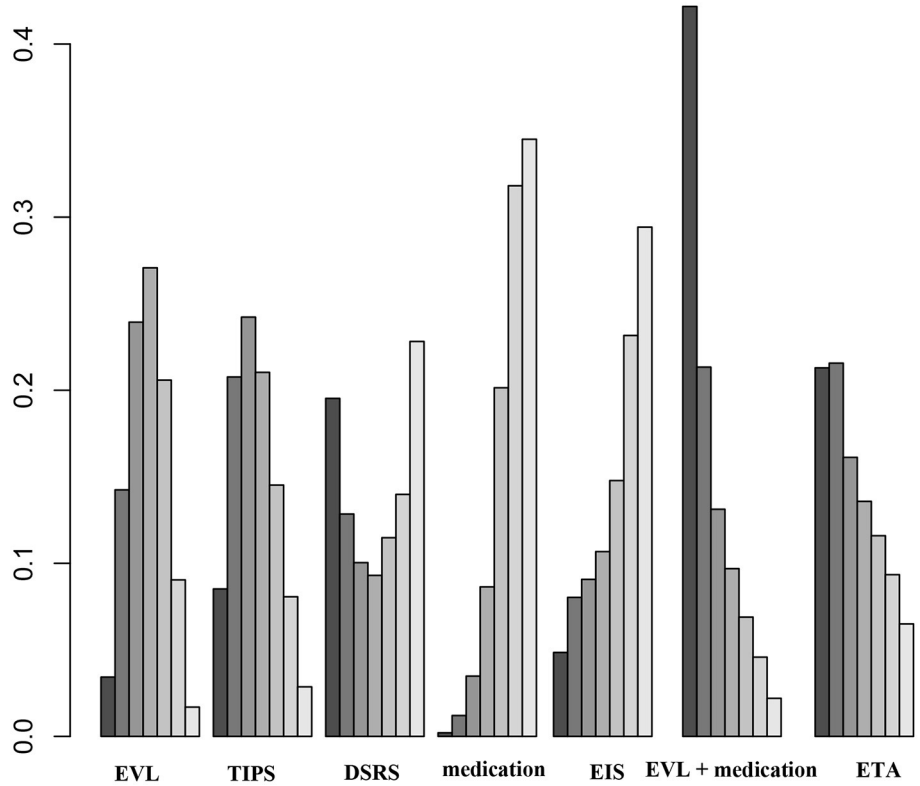

FIGURE 6 | Ranking of 1-year OS among the different therapies. EVL, endoscopic variceal ligation; TIPS, transjugular intrahepatic portosystemic shunt; DSRS, distal splenorenal shunt; EIS, endoscopic injection sclerotherapy; ETA, endoscopic tissue.

showed that simple conservative treatment offers little benefit. β-blockers, terlipressin and somatostatin drugs do not offer greater benefits compared with endoscopy or interventional therapy (29). However, Patch et al. (70) and Saran et al. (46) reported that propranolol is as effective as EVL in preventing variceal rebleeding within a median follow-up period of 1–2 years. This discrepancy may be attributed to the relatively small sample size in these studies; there is a clear difference in efficacy between medications alone and ET + medication (12, 45).

To the best of our knowledge, this is the first study to comprehensively analyze the safety and efficacy of various treatments for patients with portal hypertension and cirrhosis in terms of bleeding-related complications. Our study has several advantages. First, the data were extracted from 40 high-quality randomized controlled trials that involved over 4,000 patients in 13 countries. Second, multiple endpoints were observed, including 1-, 2-, and 3-year rebleeding rates, treatment failure, bleeding-related mortality, 1-, 2-, and 3-year OS rates, and HE.

Our study also had several limitations. First, some of the included subgroups were too small to evaluate effectively. Thus, several subgroup analyses (e.g., balloon-occluded retrograde transvenous obliteration, EVL + EIS + β-blocker, bare stent, covered stents etc.) were not performed. Second, patient characteristics that may have resulted in unavoidable methodological heterogeneity, such as Child–Pugh class, age and sex, varied among individual studies and could not be further addressed by subgroup or sensitivity analyses. Third, many different medications were used, including isosorbide mononitrate, somatostatin, octreotide, terlipressin and β-blockers, and there may have been differences in dosage among the studies.

According to our meta-analysis, TIPS and DSRS were superior to other therapies in terms of short-term and long-term bleeding control. However, these therapies may increase the risk of HE. There was no significant difference among the groups in the 1- or 3-year OS rate. Based on the complexity of the network meta-analysis model, the results of the meta-analysis are closely related to the model parameters, including the initial values and number of iterations. Therefore, the results of this meta-analysis should be interpreted with caution. Prospective RCTs are required to provide more data on TIPS, balloon-occluded retrograde transvenous obliteration and combination therapy.

CONCLUSIONS

In conclusion, TIPS and DSRS should be given priority in patients with portal hypertension and cirrhosis to control

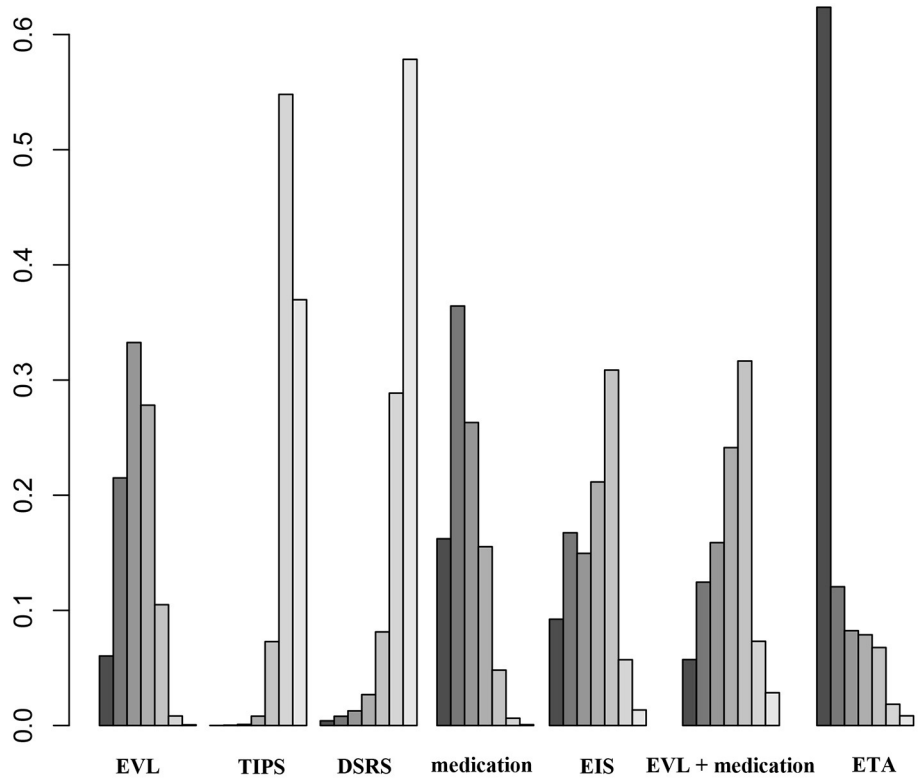

FIGURE 7 | Ranking of the odds ratios for HE based on different pairwise comparisons. EVL, endoscopic variceal ligation; TIPS, transjugular intrahepatic portosystemic shunt; DSRS, distal splenorenal shunt; EIS, endoscopic injection sclerotherapy; ETA, endoscopic tissue.

rebleeding, which may not improve survival. ET together with medication may improve survival. Furthermore, medications should be used in combination with ET or other treatments rather than as the sole therapeutic intervention.

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.

AUTHOR CONTRIBUTIONS

QY: conception and design and data collection. QY, WC, CY, and JY: data analysis, interpretation, and drafting the manuscript. TJ and HC: reviewed data analysis, interpretation, writing the manuscript, read, and approved the final manuscript. 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/fmed.2021.712918/full#supplementary-material

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BRIEF RESEARCH REPORT
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Rat Hepatitis E Virus: Presence in Humans in South-Western France?

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Background: Hepatitis E Virus (HEV) is one of the most common causes of hepatitis worldwide, and South-Western France is a high HEV seroprevalence area. While most cases of HEV infection are associated with the species Orthohepevirus-A, several studies have reported a few cases of HEV infections due to Orthohepevirus-C (HEV-C) that usually infects rats. Most of these human cases have occurred in immunocompromised patients. We have screened for the presence of HEV-C in our region.

Methods and Results: We tested 224 sera, mostly from immunocompromised patients, for HEV-C RNA using an in-house real time RT-PCR. Liver function tests gave elevated results in 63% of patients: mean ALT was 159 IU/L (normal < 40 IU/L). Anti-HEV IgG (49%) and anti-HEV IgM (9.4%) were frequently present but none of the samples tested positive for HEV-C RNA.

Conclusion: HEV-C does not circulate in the human population of South-Western France, despite the high seroprevalence of anti-HEV IgG.

Keywords: hepatitis E virus, rat hepatitis E virus, immunocompromised, South-Western France, chronic hepatitis, hepatitis E, zoonosis, rodent

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INTRODUCTION

Hepatitis E virus (HEV) is a major cause of epidemic waterborne and sporadic hepatitis worldwide (1). HEVs belong to the family Hepeviridae, which contains two genera: Orthohepevirus and Piscihepevirus (1). Genus Orthohepevirus comprises four species: Orthohepevirus A–D. The viruses that make up the species Orthohepevirus A are assigned to one of at least eight distinct genotypes (HEV-1 to HEV-8); the four major ones are the main causes of infection in human (1).

Hepatitis E produces a benign infection in most immunocompetent people, with a wide range of symptoms, including fever, nausea, anorexia, asthenia, vomiting, abdominal pain, and icterus. Liver function tests are often abnormal, with signs of hepatic cytolysis (increased transaminases), cholestasis, and sometimes even liver failure. Severe acute or fulminant hepatitis is rare, usually occurring in patients with underlying chronic liver disease and pregnant women with an HEV-1 or HEV-2 infection (2). The virus can also produce extra-hepatic symptoms like neurological disorders, renal failure, pancreatitis, and hematological disorders (1). HEV replication can persist for over 3–6 months in adults or children on immunosuppressive therapy following transplantation, those on chemotherapy or immunotherapy, and persons with a concomitant HIV

infection. Such infections can lead to chronic hepatitis with progressive liver fibrosis and cirrhosis. The majority of immunosuppressed patients are asymptomatic and present with persistent, mildly abnormal liver function tests (1).

In developed countries, HEV is a zoonosis with pigs being the main reservoir (1). Direct and indirect contact with infected animals or consumption of contaminated food products are the main transmission routes of HEV. Rabbit HEV, which is closely related to HEV3, circulates in rabbits in China, the United States and Europe (3, 4). Other food products are also possible sources of HEV, as HEV3 RNA was detected in red fruits, strawberries, green leaves and spices (1). Furthermore, one case of transmission of HEV7 to an immunocompromised individual through consumption of camel meat and milk has been described (5). HEV RNA was recently identified in goat and sheep milk and could represent a source of infection to consumers (6). Parenteral transmission by blood transfusion is also a potential mode of contamination (1).

An HEV of rat origin has recently been shown to cause zoonotic infections and symptomatic disease (hepatitis) in humans (7). This rat HEV is a genetically distant relative of other mammalian HEVs; it is about 55–60% sequence homologous to HEV genotypes 1–4, and is assigned to the genus Orthohepevirus C (7). In Europe, studies conducted on Norway rats and Black rats, reported the detection of HEV-C RNA with prevalence ranging between 0.3 and 27.2% (8–15); HEV-C RNA was also frequently detected in Asia with prevalence ranging between 0.7 and 26.3% in rats (16–21), 1.9% in musk shrew (22) and between 1.5 and 7.8% in 2 US studies in rats (23, 24). The virus isolated in musk shrew could infect nude mice (25). The first human case identified was a previously healthy Canadian man (26) and there have been a few recent (2017–2020) confirmed cases in Hong Kong (27–29). In the largest epidemiological studies performed in Honk-Hong, HEV-C1 RNA was detected in 6/2,201 (0.27%) patients with hepatitis and 1/659 (0.15%) immunocompromised persons (28). The eight HEV-C1 infections identified included 3 with acute hepatitis, four with persistent hepatitis, and one with a subclinical infection but no hepatitis. Significantly, seven of the eight patients were immunosuppressed (28). The source of transmission from rat to these human cases is still unknown. None of these patients had a history of rat meat consumption, and the practice is uncommon in Hong Kong. Indeed, almost all of them even denied rat infestation in their domestic premises. The adulteration of food products or natural HEV-C infection of pigs may be a possibility (28). Another relevant sanitary aspect of rat as HEV carrier is the possibility of interspecies transmission of HEV-3 between pigs, wild boar, humans and rats. Three studies reported the detection of HEV-3 in rats, in the USA, Japan and Belgium (10, 23, 30). Rats are synanthropic mammals and their ubiquitous presence in urban environments and in rural areas where pigs are farmed may explain the interspecies transmission.

As the seroprevalence of anti-HEV IgG is high among blood donors in our area (52.5%) (31), and HEV-A infections are frequent among immunocompromised patients (32), while cirrhotic patients are at high risk of hepatic decompensation (33) these populations in our area are regularly tested for HEV-A. This prompted us to assess the risk of an HEV-C infection.

Abbreviations: ALT, alanine aminotransferase; HEV, hepatitis E virus; RNA, ribonucleic acid.

PATIENTS AND METHOD

Patients Samples

We investigated prospectively 224 patients who tested negative for HEV-A RNA. They were from a group of individuals at risk of developing a chronic HEV infection, for whom physicians had prescribed regular HEV-A RNA tests. The majority of them were immunocompromised (organ transplant recipients, hematological malignancies, bone marrow transplant recipients and solid cancer). All sera were collected between December 2019 and May 2020 and all tested negative for HEV-A RNA. According to French law (Loi Jardé), because this is an anonymous study without additional blood sampling, institutional review board approval was not required.

Serological Assays

We used commercial ELISA kits (Wantai, Beijing, China) for the serological tests for HEV-IgM and HEV-IgG as recommended by the manufacturer. The capture antigen in these kits is an ORF2 fragment of HEV-A genotype 1. We used the Wantai kits since they cross-react with both anti-HEV-A and anti-HEV-C antibodies (34). Sample were considered as positive if the signal to cut-off ratio (S/Co) was >1.

HEV-C RNA Detection

RNA was extracted using QIAamp Viral RNA Mini kit (Qiagen, Courtabeuf, France). We diagnosed HEV-C infections using an in-house qRT-PCR assay based on HEV-C sequences available in Genbank.

TABLE 1 | Clinical and demographic features of patients.

Mean age (year)	53 ± 17
Gender (Male/Female)	133/91
Underlying pathologies	
Bone marrow transplant recipients	85
Organ transplant recipients	67
Hematological malignancies	48
Solid cancer	5
Cirrhosis	4
Corticosteroids treatment	2
Others	13
ALT (IU/L)	159 ± 378
AST (IU/L)	162 ± 753
Bilirubine (μmol/L)	239 ± 45
GGT (IU/L)	20 ± 488
IgG VHE+	110/224
IgM VHE +	21/224
Total (*n*)	224

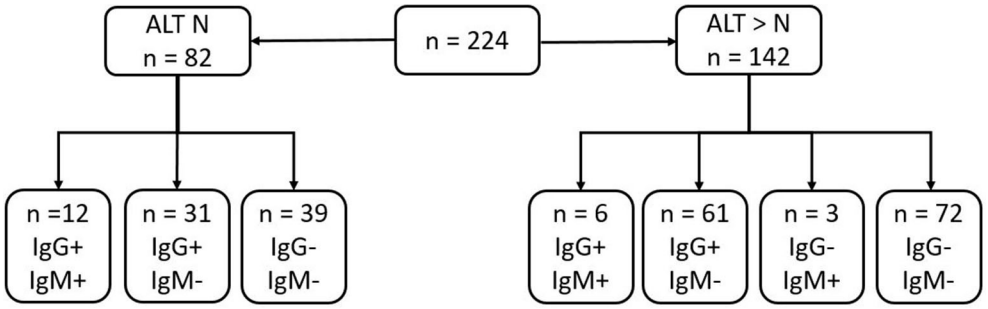

FIGURE 1 | Summary of serological results of samples depending on ALT results (N, normal value: *N* < 35 U/L in our lab).

Reverse transcription and amplification were performed using the SuperScriptIII Platinum One-Step qRT-PCR kit (Invitrogen). The forward primer was 5′-CCACGGGGTTAATACTGC-3′, the reverse primer was 5′-CGGATGCGACCAAGAAACAG-3' and the probe was 5'-6FAM-CGGCTACCGCCTTTGCTAATGC-BBQ-3'. Each 50 μL mix contained: RNA (15 μL), SuperScript III RT/Platinum TaqMix (1 μL), buffer containing 0.4 mM of each dNTP and 6 mM MgSO4, 0.4 μM forward and reverse primers, 0.2 μM probe, and 1 μL RNAse-out. The RT-PCR cycle was: 50°C/25, 95°C/10 min, 95°C/20, and 56°C/45 s. HEV RNA was detected on a Light Cycler 480 Real-Time PCR System (Roche).

The rat HEV-RNA positive control was extracted from a PLC/PRF/5 cell culture supernatant containing rat HEV strain 63 (kindly provided by Prof. Reimar Johne).

RESULTS

The 224 samples analyzed included 207 from immunocompromised patients. Of these, 67 were solid organ recipients, 48 suffered from hematological malignancies, 85 were bone marrow transplant recipients, 5 had solid cancers, 2 were on corticosteroids and 17 were immunocompetent with abnormal liver function tests (including 4 cirrhotic patients). Over half the patients (142/224; 63%) had elevated liver function tests (mean ALT: 159 IU/L) (**Table 1**).

Summary of serological results are presented in **Figure 1**. Anti-HEV IgG was present in 49% of the sera and anti-HEV IgM in 9.4%. The ratios of the HEV IgG and IgM are presented in **Figure 2**. The sera from 14.6 % (12/52) of the patients with normal liver function tests contained anti-HEV IgM, as did the sera from 6.3% (9/142) of those with an elevated ALT.

All samples tested negative for HEV-C RNA.

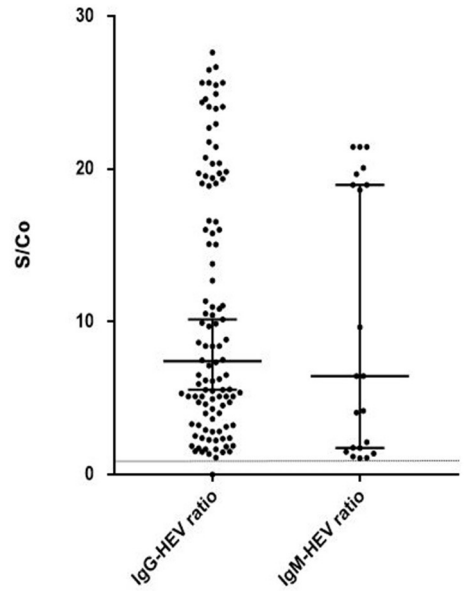

FIGURE 2 | HEV IgG and IgM ratio (S/Co) in positive samples (S/Co:signal-to-cut-off ratio). Limit of positivity is plotted on the vertical axis with the dotted line.

DISCUSSION

Our investigation of individuals, mainly immunosuppressed, who were highly susceptible to viral infections found no HEV-C infections, even in the patients with anti-HEV antibodies or transaminase elevation.

Most of the patients we tested were immunosuppressed and had been referred by their physician for an HEV diagnosis as this population is at risk of persistent HEV-A infection or HEV-C1 infection, as recently reported by Sridhar et al. (28). They found that among the 8 cases of HEV-C infection included 7 were immunocompromised and 3 of them developed a chronic infection. Moreover, prior exposure to HEV-A does not protect against an HEV-C1 infection (34).

Sridhar et al. recently investigated the divergence between HEV-C1 and HEV-A, the usual cause of human hepatitis (34) because genomic divergence reduces the capacity of existing tests to diagnose HEV-C. Human HEV-based antigen and antibody assays, even molecular assays, may not diagnose HEV-C1 infections. We therefore developed an in-house RT-PCR assay to look for HEV-C RNA and tested the samples for anti-HEV-C antibodies using the Wantai IgM and IgG kits, which cross-react with both anti-HEV-A and anti-HEV-C antibodies (34).

There were 21 HEV-IgM positive patients, none of whom had any virus detected, either HEV-A or HEV-C, by PCR. They probably had a HEV-A infection in the past, as HEV-IgM is a persistent marker that can be detected up to 3 years after acute hepatitis (35). Similarly, HEV-IgG can persist for a long period after an HEV-A infection, up to 14 years (1). Additionally, 142/224 (63.4%) of our patients had abnormal aminotransferase activities, but they tested negative for HEV-RNA. Only one of them tested positive for HCV RNA. Our patients may have several causes of transaminases elevation: drug induced liver injury, graft-vs. diseases cirrhosis.

Despite the high HEV-A seroprevalence in South-Western France, HEV-C does not seem to circulate in the human population. However, we focused our study in immunocompromised patients and other groups of patients should be investigated. The epidemiology of HEV-A infections is known to vary greatly from one geographic area to another. The route of transmission between rats and humans in Hong Kong, where the majority of the HEV-C infections have been reported, is still elusive. Certain populations are known to be more at risk of infection, depending on immunocompromised factors and/or life style. One study found a few forestry workers in Germany tested positive for anti-HEV-C antibodies (36). Thus, serological and molecular assays that discriminate between HEV-A and HEV-C would be valuable for determining the real distribution of rat HEV worldwide. Immunoblot assays could help to distinguish the serological profiles of HEV infections (34).

Sridhar et al. reported that HEV-C infections accounted for 8% of all genotyped hepatitis E cases in Hong Kong (28, 34). We therefore expected to find at least some cases of HEV-C1 among the limited number of patients tested, as anti-HEV antibodies are prevalent in our region, particularly in the population investigated. We have designed our RT-PCR assay to detect all HEV-C variants according to the sequences available in Genbank. We used a culture supernatant of a rat HEV to verify the ability of our test to detect this specific species, but we cannot rule out the possibility that our PCR have missed a divergent strain.

In conclusion, we found no case of rat HEV infection between December 2019 and May 2020 despite the high seroprevalence of HEV in our region. A larger study, testing patients with occupational exposure and more immunocompromised patients, using discriminatory serological assays such as immunoblots, could help reveal the presence of HEV-C in South-Western France. Specific molecular assays would also enable us to confirm human cases of HEV-C if serological tests were positive.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

Ethical review and approval was not required for the study on human participants in accordance with the local legislation and institutional requirements. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

JI, DP, and FA: drafting and refining the manuscript. SL: critical reading of the manuscript. DP, FA, JP, ST, and NK collected the data. ID performed the analysis. 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/fmed.2021.726363/full#supplementary-material

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The Applicability of ADA, AFU, and LAC in the Early Diagnosis and Disease Risk Assessment of Hepatitis B-Associated Liver Cirrhosis and Hepatocellular Carcinoma

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Objective: This study aimed to evaluate the applicability of adenosine deaminase (ADA), α-l-fucosidase (AFU), lactic acid (LAC), and their combined detection in the early diagnosis of chronic hepatitis B (CHB), liver cirrhosis (LC), and hepatocellular carcinoma (HCC).

Methods: A retrospective analysis of hepatitis B-positive liver disease patients admitted between 2015 and 2020 was conducted. The receiver operating characteristic (ROC) curve was used to determine the diagnostic value of each indicator in LC and HCC, and binary logistic regression analysis was performed to determine the factors and risks related to the occurrence of the two conditions.

Results: The levels of ADA, AFU, and LAC were significantly increased in patients with CHB, LC, and HCC ($p < 0.05$). The ROC curve showed that the sensitivity and specificity of ADA, AFU, LAC, and their combined detection in the CHB and LC groups as well as in the LC and HCC groups reflected different degrees of clinical value. In the CHB and LC groups, the adjusted odds ratio (OR) values of ADA, AFU, and LAC among patients in the high-level group were 3.218, 1.859, and 11.474, respectively, when the median was considered the cutoff point. When quartiles were considered the cutoff point, the OR risk values of the adjusted levels of ADA, AFU, and LAC were higher than those of the lowest-level group (Q1) ($p < 0.05$). In the LC and HCC groups, the adjusted OR values of ADA, AFU, and LAC among patients in the high-level group were 0.967, 2.365, and 38.368, respectively. When quartiles were considered the cutoff point, the OR risk values of AFU and LAC levels were higher than those of the lowest-level group (Q1) ($p < 0.05$).

Conclusion: ADA, AFU, and LAC demonstrated good value in the early diagnosis of LC and HCC. The combined detection of ADA+AFU+LAC is more effective than single detection for the early diagnosis of the two conditions. ADA, AFU, and LAC can serve as risk predictors of LC, while AFU and LAC can be considered early risk predictors of HCC.

Keywords: ADA, AFU, LAC, hepatitis, liver cirrhosis, hepatocellular carcinoma, diagnostic value, risk assessment

INTRODUCTION

Hepatitis B virus (HBV) infection is a global public health problem, and the number of hepatitis B surface antigen (HBsAg) carriers is ∼250 million (1). Chronic hepatitis B (CHB) can cause gradual aggravation of liver injury, and without intervention, ∼40% of the patients with the infection develop liver cirrhosis (LC). In addition, ∼30% of the patients with LC develop hepatocellular carcinoma (HCC) after 10 years (2). LC was previously considered irreversible; however, it is now known that the condition can be reversed through oral anti-nucleotide drug therapy (3). Liver cancer is a common malignancy of the organ and the fourth most common cause of cancer-related deaths (4). Some liver cancers can progress insidiously in patients with normal liver function, and early diagnosis may not be possible due to non-specific symptoms (5). Therefore, early and accurate diagnosis of LC and liver cancer is important for the choice of appropriate treatment programs. Liver biopsy is the gold standard technique for the assessment of LC and cancer. However, considering its invasiveness, complexity, and potential risks, liver biopsy cannot be performed routinely in most patients (6). Transient elastography is a superior tool to diagnose liver fibrosis; however, it tends to be affected by several factors such as diet, obesity, ascites, and rib gap width (7). The common clinical indicators, alpha-fetoprotein level and liver function, are not ideal for the early diagnosis and prognostication of the conditions. To date, there are no effective markers to predict the progression of chronic liver disease (8).

Adenosine deaminase (ADA), as a key enzyme in purine nucleoside and DNA metabolism, plays an important role in the maintenance and development of the human immune, nervous, and vascular systems (9). Studies have reported higher serum ADA levels in patients with esophageal, gastric, breast, and ovarian cancers than in the healthy population (10). Lactic acid (LAC) is a metabolite of glycolysis produced in the bones, muscles, brain, and red blood cells. The liver is responsible for the clearance of 70% of LAC in humans, and liver damage can cause mitochondrial oxidation, leading to increased LAC levels (11). The lysosomal enzyme, α-L-fucosidase (AFU), is widely present in tissues and body fluids. A study reported a significant increase in AFU levels in patients with liver cancer compared to those with benign liver disease (12). In this study, we aimed to evaluate the applicability of ADA, AFU, LAC, and their combined detection in the early diagnosis of hepatitis B-associated LC and HCC. In addition, we aimed to determine the best cutoff value of the aforementioned markers for LC and early HCC and to provide a reference for the delay in the occurrence as well as for the early diagnosis and timely treatment of the two conditions to improve the quality of life and prolong the survival of patients.

Abbreviations: ADA, adenosine deaminase; AFU, α-L-fucosidase; LAC, lactic acid; CHB, chronic hepatitis B; LC, liver cirrhosis; HCC, hepatocellular carcinoma; NPV, negative predictive value; PPV, positive predictive value; AUC, area under the ROC curve.

MATERIALS AND METHODS

Patients

We conducted a retrospective analysis of hepatitis B-positive liver disease patients admitted to Jiaozuo Fifth People's Hospital, Luoyang Traditional Chinese Medicine Hospital, Dongtai Hospital, affiliated to Nantong University, Yancheng Jianhu People's Hospital, Huzhou Central Hospital, and Shanghai Tongji Hospital from 2015 to 2020. We included 240 patients diagnosed with CHB (CHB group), 281 patients with LC (LC group), and 280 patients with early HCC (HCC group). The diagnoses of CHB and LC were based on the CHB prevention and treatment guidelines (13), and that of HCC was based on the liver cancer diagnosis guidelines (14). The inclusion criteria were as follows: HBsAg positive for more than 6 months, chronic HBV infection confirmed by histopathology, and early HCC (size of the lesion of 3 cm or less than three lesions). The exclusion criteria were patients with other types of liver disease, those who had received drugs that could cause liver damage within 6 months before admission, those with tumors in other parts of the body and/or hematological disease, those who had undergone organ transplantation, and patients with immune deficiencies.

Clinical Information and Laboratory Examination

The following clinical and laboratory data of the included patients were recorded: age, sex, alpha-fetoprotein (AFP), carcinoembryonic antigen (CEA), aspartate aminotransferase (AST), alanine aminotransferase (ALT), total bilirubin (TBIL), direct bilirubin (DBIL), total protein (TP), albumin (ALB), alkaline phosphatase (ALP), gamma-glutamyltransferase (GGT), ADA, AFU, and LAC levels, among other markers.

Statistical Analysis

SPSS 25.0 was used to perform the statistical analyses on the data that met the requirements, and normal test analyses were performed on the measurement data using the Kolmogorov–Smirnov test. Normally distributed data are presented as x ± standard deviation (SD). The measurement data of normal distribution between groups were compared using analysis of variance, and the count data were evaluated by the χ^2-test. The measurement data of skewed distribution are presented as the

TABLE 1 | Comparison of basic clinical data of the three groups of patients.

Project		CHB group ($n = 240$)	LC group ($n = 281$)	HCC group ($n = 280$)	p-value	1VS2	1VS3	2VS3
Gender	Female	133	175	163	0.276	–	–	–
	Male	107	106	117				
Age		37 (24, 55)	35 (23, 61)	34 (22, 57)	0.982	–	–	–
AFP		2.99 (1.89, 4.56)	3.17 (2.06, 4.81)	3.11 (2.23, 4.53)	0.183	–	–	–
CEA		2.40 (1.44, 4.04)	2.69 (1.54, 3.87)	3.17 (1.78, 7.32)	<0.001	0.567	<0.001	<0.001
AST		31 (20, 57)	32 (23, 52)	33 (23, 49)	0.637	–	–	–
ALT		25 (15, 48)	30 (18, 54)	35 (20, 60)	<0.001	0.064	<0.001	0.113
TBIL		18.1 (11.5, 33.1)	19.0 (12.7, 28.9)	17.8 (13.1, 27.5)	0.752	–	–	–
DBIL		7.0 (4.0, 14.7)	6.3 (3.8, 10.9)	5.6 (4.0, 9.4)	0.008	0.181	0.006	0.668
TP		65.0 (57.0, 73.5)	63.8 (57.2, 71.15)	59.5 (54.8, 68.5)	<0.001	0.675	<0.001	<0.001
ALB		35.6 (30.0, 41.1)	32.8 (26.6, 37.8)	30.4 (25.6, 35.7)	<0.001	<0.001	<0.001	0.002
ALP		80 (59, 125)	83 (64, 114)	86 (66,116)	0.301	–	–	–
GGT		36 (19, 112)	42 (21, 91)	42 (21,91)	0.516	–	–	–

median (M) and percentile (P25, P75). The measurement data of skewed distribution were compared by independent sampling using the Kruskal–Wallis H test. Pairwise comparisons were performed using the Bonferroni correction method for groups with differences in the overall test. GraphPad Prism software was used to construct the receiver operating characteristic (ROC) curve of each index and combined test to determine the sensitivity, specificity, optimal cutoff value, Youden index, negative predictive value (NPV), and positive predictive value (PPV) of each index in patients with LC and HCC. The area under the curve (AUC) was used to assess the accuracy of the tests. We performed binary logistic regression analysis to calculate the joint predictors of ADA, AFU, and LAC and the Z-test to compare the area under the ROC curve of each marker. The median (P50) and quartiles (P25, P50, and P75) were considered the cutoff points, and binary logistic regression analysis was performed to evaluate the risk of ADA, AFU, and LAC levels in LC and HCC. Factors with statistical significance in the univariate analysis ($p < 0.10$) were included in the multivariate logistic regression analysis, and binary logistic regression analysis was performed to calculate the single factor, multivariate-adjusted odds ratio, and 95% confidence interval (CI) values based on maximum likelihood estimation. The difference was considered statistically significant when p-value was <0.05.

RESULTS

Characteristics of the Enrolled Patients

The sex, age, AFP, AST, TBIL, ALP, and GGT levels of patients in the three groups were not statistically significant ($p > 0.05$). The ALB level of patients in the LC group was higher than that of patients in the CHB group, and the difference was statistically significant ($p < 0.05$). The CEA level was higher and the TP and ALB levels were lower among patients in the HCC group compared to those in the LC group, and the differences were statistically significant ($p < 0.05$). The TP, ALB, and DBIL levels were lower and the CEA and ALT levels were higher among patients in the HCC group compared to those in the CHB group, and the differences were statistically significant ($p < 0.05$; **Table 1**).

The Expression Levels of ADA, AFU, and LAC Among Patients in the Three Groups

The ADA levels of patients in the CHB, LC, and HCC groups were 14 U/L (11, 22), 20 U/L (17, 27), and 22 U/L (18, 29), respectively. The AFU levels of patients in the CHB, LC, and HCC groups were 24 U/L (19, 32), 31 U/L (26, 37), and 37 U/L (32, 43), respectively, while those of LAC in the three groups were 1.96 mmol/L (1.56, 2.42), 2.87 mmol/L (2.48, 3.33), and 4.34 mmol/L (3.84, 4.78), respectively. The levels of the three markers showed an increasing trend across groups, and the differences were statistically significant (all $p < 0.05$; **Figures 1A–C**).

Diagnostic Performance of the Laboratory-Related Markers Among Patients in the CHB and LC Groups

The hepatitis group logit(P) (LC group = 1, HCC group = 0) was considered the dependent variable, and ADA (X1), AFU (X2), and LAC (X3) were considered the independent variables. Binary logistic regression analysis was performed to calculate the joint predictors of ADA, AFU, and LAC. The regression equation was $\text{logit(P)} = -7.632 + 0.095X1 + 0.037X2 + 1.958X3$, and the joint predictors were considered the three joint test indicators to analyze the results.

GraphPad Prism software was used to construct the ROC curve of each index and combined test, as shown in **Figures 2A–I**. The AUC of CEA was 0.514 when the cutoff was 2.45 mg/L, and the sensitivity, specificity, NPV, and PPV were 54.45, 52.08, 49.4, and 57.1%, respectively. The AUC of ALT was 0.549 when the cutoff was 27 U/L, and the sensitivity, specificity, NPV, and PPV were 55.52, 55.83, 51.7, and 59.5%, respectively. Similarly, the AUC of DBIL was 0.547 when the cutoff was 12 μmol/L, and the sensitivity and specificity were 80.43 and 34.17%, respectively. The NPV and PPV of DBIL were 59.9 and 58.9%, respectively.

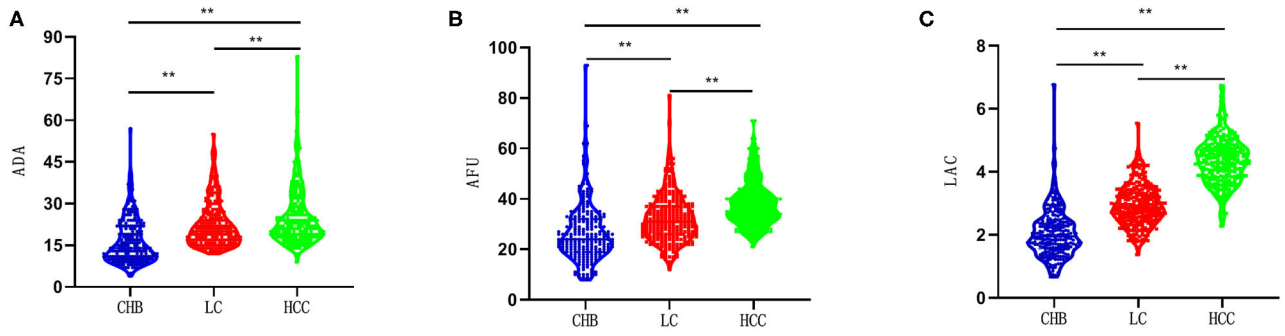

FIGURE 1 | Comparison of the ADA, AFU, and LAC levels between patients in three groups. $^{**}p < 0.01$. CHB, chronic hepatitis B; LC, related liver cirrhosis; HCC, hepatocellular carcinoma; ADA, adenosine deaminase; AFU, α-L-fucosidase; LAC, lactic acid. **(A)** the ADA levels in three groups; **(B)** the AFU levels in three groups; **(C)** the LAC levels in three groups.

The AUC of TP was 0.526 when the cutoff was 73.3 g/L, and the sensitivity, specificity, NPV, and PPV were 82.92, 26.67, 57.1, and 57.0%, respectively. The AUC of ALB was 0.599 when the cutoff was 33.2 g/L, and the sensitivity, specificity, NPV, and PPV were 53.74, 65.00, 54.5, and 64.3%, respectively. The AUC values of ADA, AFU, and LAC were 0.736, 0.694, and 0.834, respectively, when the cutoff values were 13 U/L, 24 U/L, and 2.42 mmol/L, respectively. The sensitivity, specificity, NPV, and PPV of ADA were 96.80, 46.67, 92.6, and 68.0%, respectively, whereas those of AFU were 81.14, 51.67, 70.1, and 66.3%, respectively. The sensitivity, specificity, NPV, and PPV of LAC were 79.00, 75.42, 75.4, and 79.0%, respectively. The AUC of the combined detection of ADA+AFU+LAC was 0.868 when the cutoff was 0.41. The sensitivity, specificity, NPV, and PPV of the combined detection were 91.81, 68.75, 87.8, and 77.5%, respectively. See **Table 2**.

From the data in **Table 2**, it could be concluded that the AUC values of ADA, AFU, LAC, and ADA+AFU+LAC were higher and that the diagnostic performance was superior. MedCalc software was used to compare the AUC values of ADA, AFU, LAC, and ADA+AFU+LAC. There was no statistically significant difference between the AUC values of ADA and AFU ($p > 0.05$). The AUC of LAC was greater than those of ADA and AFU, whereas the value of the combined detection was greater than those of ADA, AFU, and LAC alone, and the difference was statistically significant ($p < 0.05$; **Table 3**).

Diagnostic Performance of the Laboratory-Related Markers Among Patients in the LC and HCC Groups

The hepatitis group logit(P) (HCC group = 1, LC group = 0) was considered the dependent variable, and ADA (X1), AFU (X2), and LAC (X3) were considered the independent variables. Binary logistic regression analysis was performed to calculate the joint predictors of ADA, AFU, and LAC. The regression equation was $\text{logit(P)} = -7.632 + 0.095\text{X1} + 0.037\text{X2} + 1.958\text{X3}$, and the joint predictors were used as the three joint test indicators to analyze the results.

GraphPad Prism software was used to construct the ROC curve of each index and combined test, as shown in **Figures 3A–I**.

The AUC of CEA was 0.605 when the cutoff was 8.32 mg/L, and the sensitivity, specificity, NPV, and PPV were 23.57, 96.80, 56.0, and 88.0%, respectively. The AUC of ALT was 0.532 when the cutoff was 20 U/L, and the sensitivity, specificity, NPV, and PPV were 73.21, 33.81, 55.9, and 52.4%, respectively. The AUC of DBIL was 0.519 when the cutoff was 5.6 μmol/L, and the sensitivity and specificity were 50.71 and 59.07%, respectively. The NPV and PPV of DBIL were 54.6 and 55.3%, respectively. The AUC of TP was 0.578 when the cutoff was 59.5 g/L, and the sensitivity, specificity, NPV, and PPV were 52.14, 66.19, 58.1, and 60.6%, respectively. The AUC of ALB was 0.569 when the cutoff was 29.2 g/L, and the sensitivity, specificity, NPV, and PPV were 48.57, 71.17, 58.1, and 62.7%, respectively. The AUC values of ADA, AFU, and LAC were 0.577, 0.697, and 0.929, respectively, when the cutoff values were 17 U/L, 31 U/L, and 3.54 mmol/L, respectively. The sensitivity, specificity, NPV, and PPV of ADA were 81.19, 31.67, 63.6, and 54.4%, respectively, whereas those of AFU were 78.57, 51.60, 70.7, and 61.8%, respectively. The sensitivity, specificity, NPV, and PPV of LAC were 87.50, 85.05, 87.2, and 85.4%, respectively. The AUC of the combined detection of ADA+AFU+LAC was 0.939 when the cutoff was 0.299. The sensitivity, specificity, NPV, and PPV of the combined detection were 94.29, 79.72, 93.3, and 82.2%, respectively. See **Supplementary Table 1**.

From the data in **Table 2**, it could be concluded that the AUC values of ADA, AFU, LAC, and ADA+AFU+LAC were higher and that the diagnostic performance was superior. MedCalc software was used to compare the AUC values of ADA, AFU, LAC, and ADA+AFU+LAC. The AUC of LAC was greater than that of AFU, whereas the value of AFU was greater than that of ADA. Furthermore, the AUC of the combined detection was greater than that of ADA, AFU, and LAC alone, and the difference was statistically significant ($p < 0.05$; **Supplementary Table 2**).

Risk Assessment of ADA, AFU, and LAC in Predicting LC

Binary logistic regression analysis was performed to evaluate the risk of ADA, AFU, and LAC levels in patients with LC, with the median and quartiles as the cutoff points (two-group and four-group classifications, respectively). First, based on the

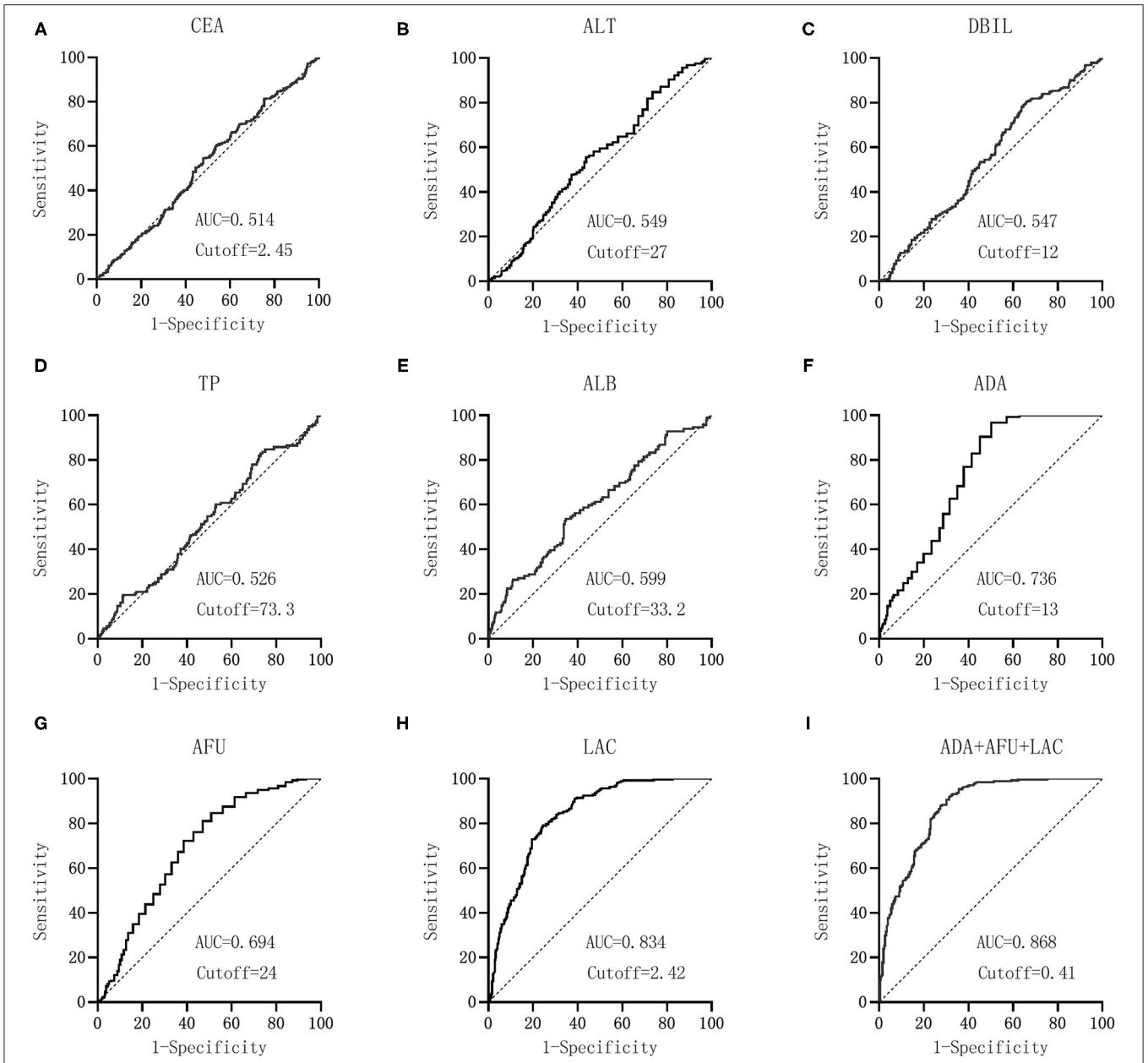

FIGURE 2 | Diagnostic performance (ROC) of laboratory-related indicators in patients between CHB and LC groups. CEA, carcinoembryonic antigen; ALT, alanine aminotransferase; DBIL, direct bilirubin; TP, total protein; ALB, albumin; ADA, adenosine deaminase; AFU, α-L-fucosidase; LAC, lactic acid. GraphPad Prism software was used to the AUC of CEA **(A)**, ALT **(B)**, DBIL **(C)**, TP **(D)**, ALB **(E)**, ADA **(F)**, AFU **(G)**, LAC **(H)**, and ADA+AFU+LAC **(I)**.

median value of ADA (18 U/L), AFU (28 U/L), and LAC (2.49 mmol/L), we divided patients into low-level and high-level groups. Regarding the risk of developing LC, compared to the patients in the low-level group, patients with high ADA levels had an odds ratio (OR) value of 3.290 (95% CI, 2.294–4.719; $p < 0.05$), and the adjusted OR was 3.218 (95% CI, 2.025–5.114; $p < 0.05$). Similarly, compared to the patients in the low-level group, patients with high AFU levels had an OR value of 3.113 (95% CI, 2.174–4.457; $p < 0.05$), and the adjusted OR was 1.859 (95% CI, 1.165–2.965; $p < 0.05$). The OR for the risk of developing LC in patients with high LAC levels was 10.301 (6.859–15.471; $p < 0.05$) compared to those with low levels, and the adjusted OR was 11.474 (95% CI, 7.268–18.114; $p < 0.05$; **Figures 4**, **5**).

Second, based on the quartile values of ADA (Q1 $\leq$ 14; 14 $<$ Q2 $\leq$ 18; 18 $<$ Q3 $\leq$ 24; and 24 $<$ Q4), AFU (Q1 $\leq$ 22; 22 $<$ Q2 $\leq$ 28; 28 $<$ Q3 $\leq$ 35; and 35 $<$ Q4), and LAC (Q1 $\leq$ 1.91; 1.91 $<$ Q2 $\leq$ 2.49; 2.49 $<$ Q3 $\leq$ 3.055; and 3.055 $<$ Q4), the patients were divided into Q1 (lowest), Q2, Q3, and Q4 groups from low to high levels. Compared to that of the group with the lowest ADA level (Q1), the OR values for the risk of developing LC in the Q2, Q3, and Q4 groups were 11.465 (6.390–20.573), 9.616 (5.563–16.624), and 10.975

TABLE 2 | Comparative analysis of the results of laboratory-related indicators in CHB and LC groups.

Indicators	Youden index	Cutoff value	AUC	Sensitivity	Specificity	AUC 95% CI	PPV (%)	NPV (%)
CEA	0.065	2.45	0.514	54.45	52.08	0.470–0.558	57.1	49.4
ALT	0.114	27	0.549	55.52	55.83	0.505–0.592	59.5	51.7
DBIL	0.146	12	0.547	80.43	34.17	0.504–0.591	58.9	59.9
TP	0.096	73.3	0.526	82.92	26.67	0.482–0.569	57.0	57.1
ALB	0.187	33.2	0.599	53.74	65.00	0.556–0.641	64.3	54.5
ADA	0.435	13	0.736	96.80	46.67	0.696–0.773	68.0	92.6
AFU	0.328	24	0.694	81.14	51.67	0.653–0.733	66.3	70.1
LAC	0.544	2.42	0.834	79.00	75.42	0.799–0.865	79.0	75.4
ADA+AFU+LAC	0.606	0.41	0.868	91.81	68.75	0.836–0.896	77.5	87.8

TABLE 3 | Comparison of the AUC of ADA, AFU, LAC, and ADA+AFU+LAC in CHB and LC groups.

Detection indicators	*Z*-value	*p*-value
Combined test and ADA	5.963	<0.001
Combined test and AFU	7.180	<0.001
Combined test and LAC	2.814	0.005
ADA and AFU	1.497	0.135
ADA and LAC	3.279	0.001
AFU and LAC	4.725	<0.001

(6.206–19.408), respectively, and the adjusted OR values were 12.991 (6.261–26.957), 11.456 (5.723–22.933), and 11.350 (5.443–23.667), respectively. Similarly, based on AFU levels, compared to that of the Q1 group, the OR values for the risk of developing LC in the Q2, Q3, and Q4 groups were 3.474 (2.064–5.845), 5.035 (2.999–8.454), and 7.238 (4.164–12.581), respectively, and the adjusted OR values were 3.935 (1.999–7.746), 3.710 (1.925–7.153), and 3.900 (1.919–7.928), respectively. The OR values for the risk of developing LC based on LAC levels in the Q2, Q3, and Q4 groups were 8.569 (4.315–17.015), 30.115 (14.725–61.590), and 54.083 (25.259–115.803), respectively, and the adjusted OR values were 8.209 (3.787–17.792), 31.887 (14.314–71.032), and 64.835 (27.654–152.006), respectively, compared to that in the Q1 group. See **Figures 6**, **7**.

Risk Assessment of ADA, AFU, and LAC in Predicting HCC

We performed binary logistic regression analysis to evaluate the risk of ADA, AFU, and LAC levels in patients with HCC, considering the median and quartiles as the cutoff points (two-group and four-group classifications, respectively). First, based on the median value of ADA (21 U/L), AFU (34 U/L), and LAC (3.58 mmol/L), patients were divided into low-level and high-level groups. Regarding the risk of developing HCC, compared to the patients in the low-level group, patients with high ADA levels had an OR value of 1.440 (95% CI, 1.033–2.009; $p < 0.05$), and the adjusted OR was 0.967 (95% CI, 0.551–1.697; $p > 0.05$). Similarly, compared to the patients in the low-level group, patients with high AFU levels had an OR value of 2.886 (95% CI, 2.048–4.067; $p < 0.05$), and the adjusted OR was 2.365 (95% CI, 1.362–4.105; $p < 0.05$). The OR for the risk of developing HCC in patients with high LAC levels was 38.368 (23.778–61.912; $p < 0.05$) compared to those with low levels, and the adjusted OR was 39.821 (95% CI, 23.729–66.825; $p < 0.05$; **Figures 8**, **9**).

Second, based on the quartile values of ADA (Q1 $\leq$ 17.5; 17.5 $<$ Q2 $\leq$ 21; 21 $<$ Q3 $\leq$ 27; and 27 $<$ Q4), AFU (Q1 $\leq$ 29; 29 $<$ Q2 $\leq$ 34; 34 $<$ Q3 $\leq$ 40.5; and 40.5 $<$ Q4), and LAC (Q1 $\leq$ 2.83; 2.83 $<$ Q2 $\leq$ 3.58; 3.58 $<$ Q3 $\leq$ 4.38; and 4.38 $<$ Q4), we divided the patients into Q1 (lowest), Q2, Q3, and Q4 groups from low to high levels. Compared to that of the lowest ADA level group (Q1), the OR values for the risk of developing HCC in the Q2, Q3, and Q4 groups were 2.049 (1.279–3.280), 1.998 (1.231–3.244), and 2.203 (1.362–3.563), respectively, and the adjusted OR values were 1.974 (0.902–4.322), 1.272 (0.566–2.86), and 1.635 (0.705–3.789), respectively. Similarly, based on AFU levels, compared to that in the Q1 group, the OR values for the risk of developing HCC in the Q2, Q3, and Q4 groups were 3.333 (2.034–5.462), 4.529 (2.738–7.492), and 5.936 (3.597–9.798), respectively, and the adjusted OR values were 2.665 (1.220–5.819), 3.224 (1.516–6.857), and 4.531 (2.028–10.125), respectively. The OR values for the risk of developing HCC based on LAC levels in the Q2, Q3, and Q4 groups were 7.202 (2.916–17.788), 70.000 (28.333–172.943), and 765.000 (211.138–2771.769), respectively, and the adjusted OR values were 10.029 (3.733–26.947), 91.469 (33.631–248.778), and 1068.638 (271.709–4202.974) compared to the Q1 group. See **Figures 10**, **11**.

DISCUSSION

HBV infection poses a major health threat to humans. Infected patients can develop CHB and gradually LC or HCC (15, 16). LC is also a major risk factor for HCC. Therefore, screening and early diagnosis of LC and HCC are significant for patients with CHB and subsequent cirrhosis.

This study mainly discussed the diagnostic value of ADA, AFU, and LAC levels in patients with early-stage CHB, LC, and HCC. The results revealed that ADA levels in patients with CHB, LC, and HCC were 14 U/L (11, 22), 20 U/L (17, 27), and 22 U/L (18, 29), respectively, which showed an increasing trend, and the difference was statistically significant. Similarly, the levels of AFU in patients with CHB, LC, and HCC were 24 U/L (19, 32), 31

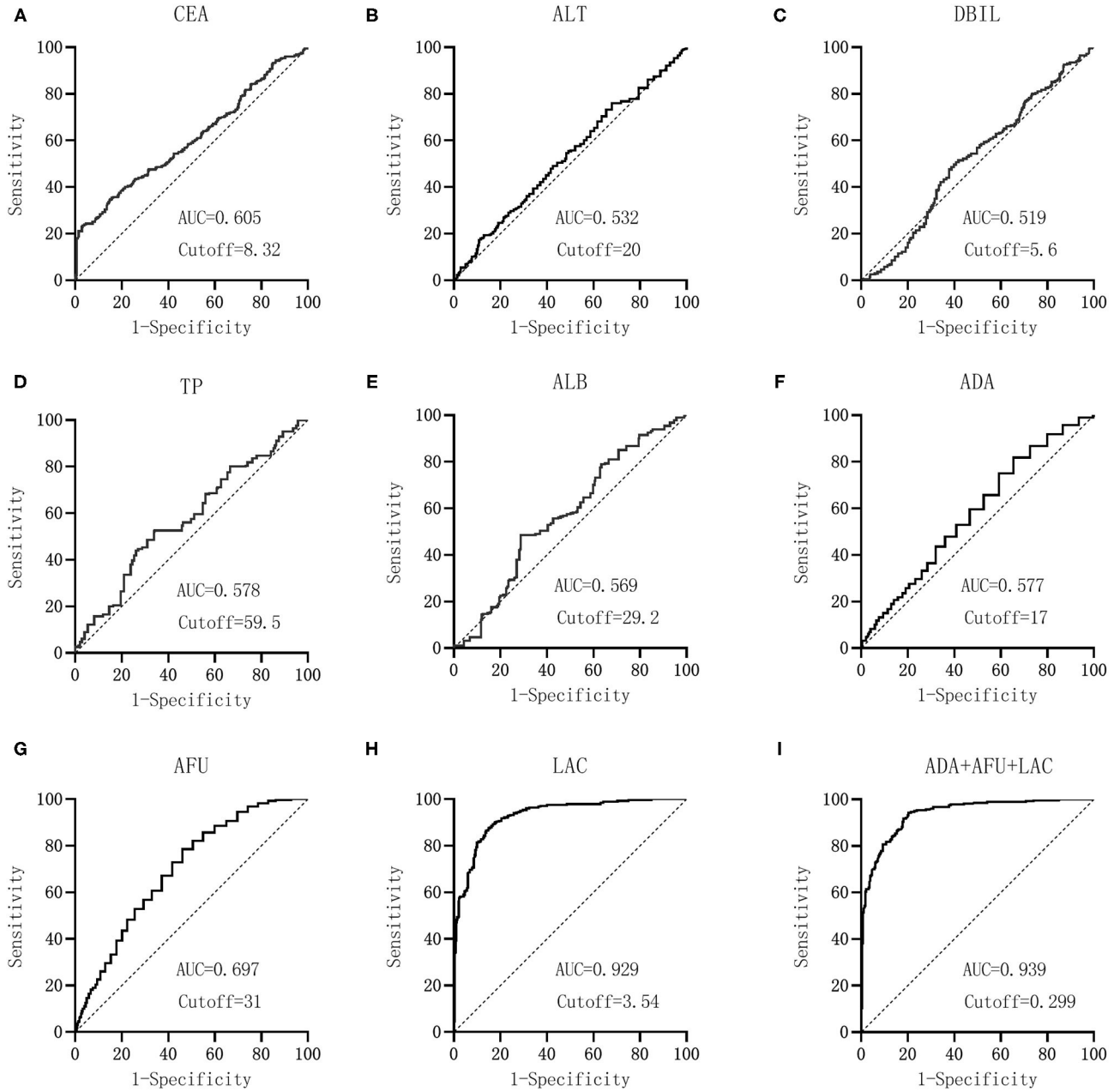

FIGURE 3 | Diagnostic performance (ROC) of laboratory-relevant indicators in patients between CHB and LC groups. CEA, carcinoembryonic antigen; ALT, alanine aminotransferase; DBIL, direct bilirubin; TP, total protein; ALB, albumin; ADA, adenosine deaminase; AFU, α-L-fucosidase; LAC, lactic acid. GraphPad Prism software was used to the AUC of CEA **(A)**, ALT **(B)**, DBIL **(C)**, TP **(D)**, ALB **(E)**, ADA **(F)**, AFU **(G)**, LAC **(H)**, and ADA+AFU+LAC **(I)**. Diagnostic performance (ROC) of laboratory relevant indicators in patients between HCC and LC groups.

U/L (26, 37), and 37 U/L (32, 43), respectively, while those of LAC were 1.96 mmol/L (1.56, 2.42), 2.87 mmol/L (2.48, 3.33), and 4.34 mmol/L (3.84, 4.78), respectively. The values showed an increasing trend, and the differences were statistically significant. The results of our study are in agreement with those reported by Yu et al., who observed a higher expression of ADA in patients with HCC (17). In addition, the present study demonstrated that the level of ADA was higher in patients with LC. The LAC level in the blood can be considered to measure the oxygen metabolism and status of tissue perfusion in the human body. Liver failure caused by liver function metabolism has been shown to increase the level of LAC (18). This observation is consistent with the results of the present study confirming the increase in LAC levels with the progression of liver disease.

The ROC curve of each index and combined test produced by the GraphPad Prism software revealed that the AUC values of ADA, AFU, LAC, and ADA+AFU+LAC in the CHB and LC groups were 0.736, 0.694, 0.834, and 0.868, respectively, and

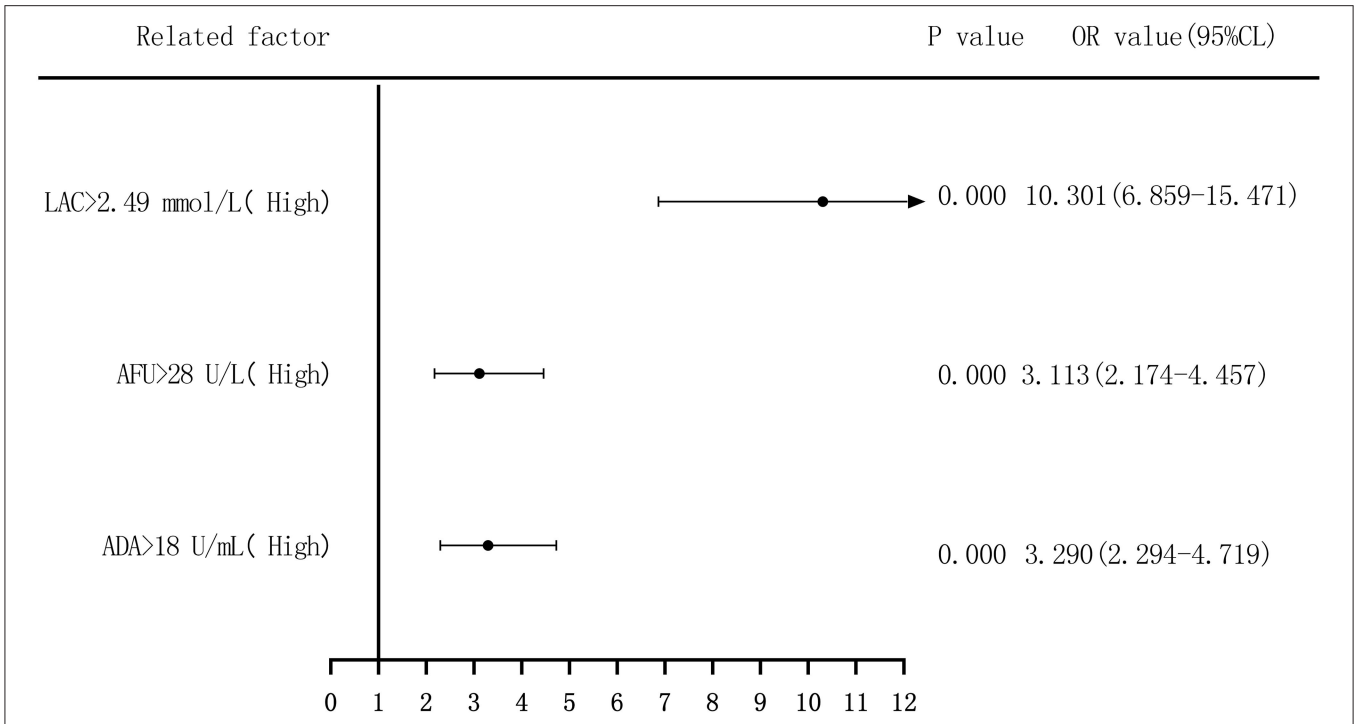

FIGURE 4 | Forest plots of univariate logistic regression analysis of ADA, AFU, LAC and patients (two-group classification) with liver cirrhosis.

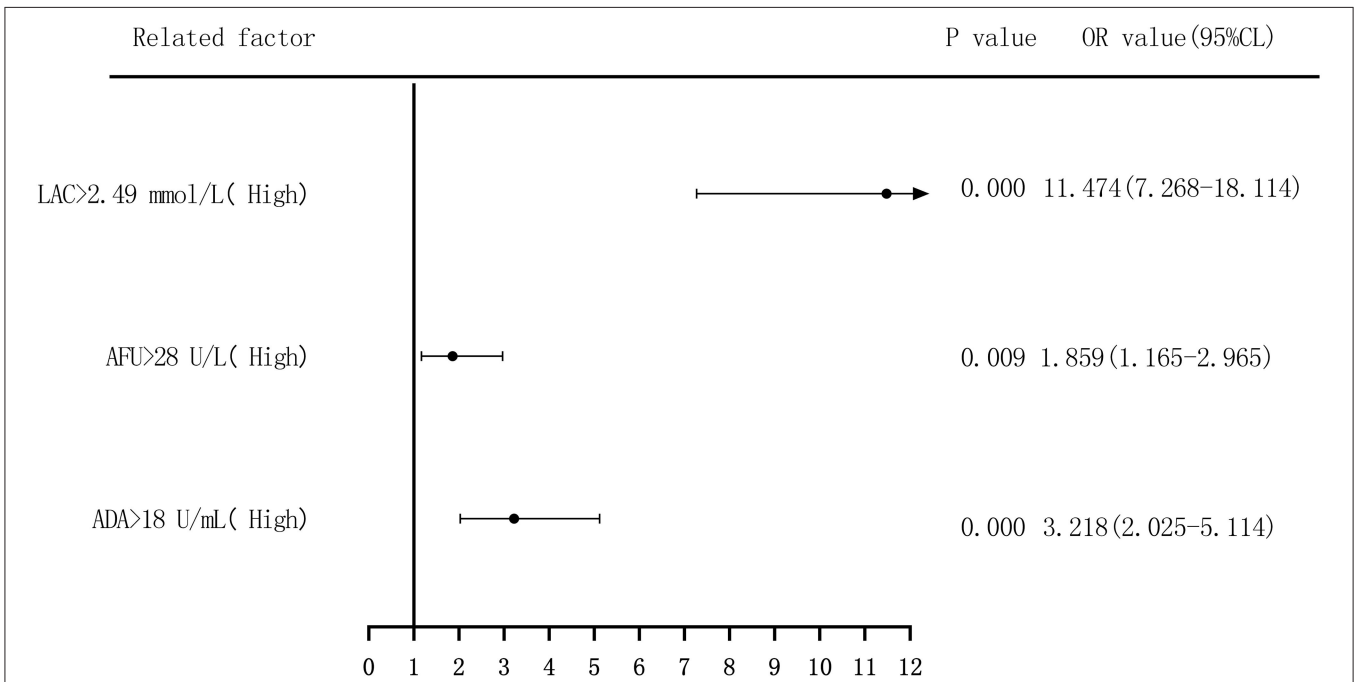

FIGURE 5 | Forest plots of multivariate logistic regression analysis of ADA, AFU, LAC, and patients (two-group classification) with liver cirrhosis (Multi-factor adjustment includes variables ALB, ALT, ADA, AFU, LAC).

that the diagnostic performance was superior. Comparison of the AUC values of ADA, AFU, LAC, and ADA+AFU+LAC revealed that the difference between ADA and AFU was not statistically significant. The AUC of LAC was greater than that of ADA and AFU. The AUC of the combined detection was greater than that of ADA, AFU, and LAC alone, and the difference was statistically

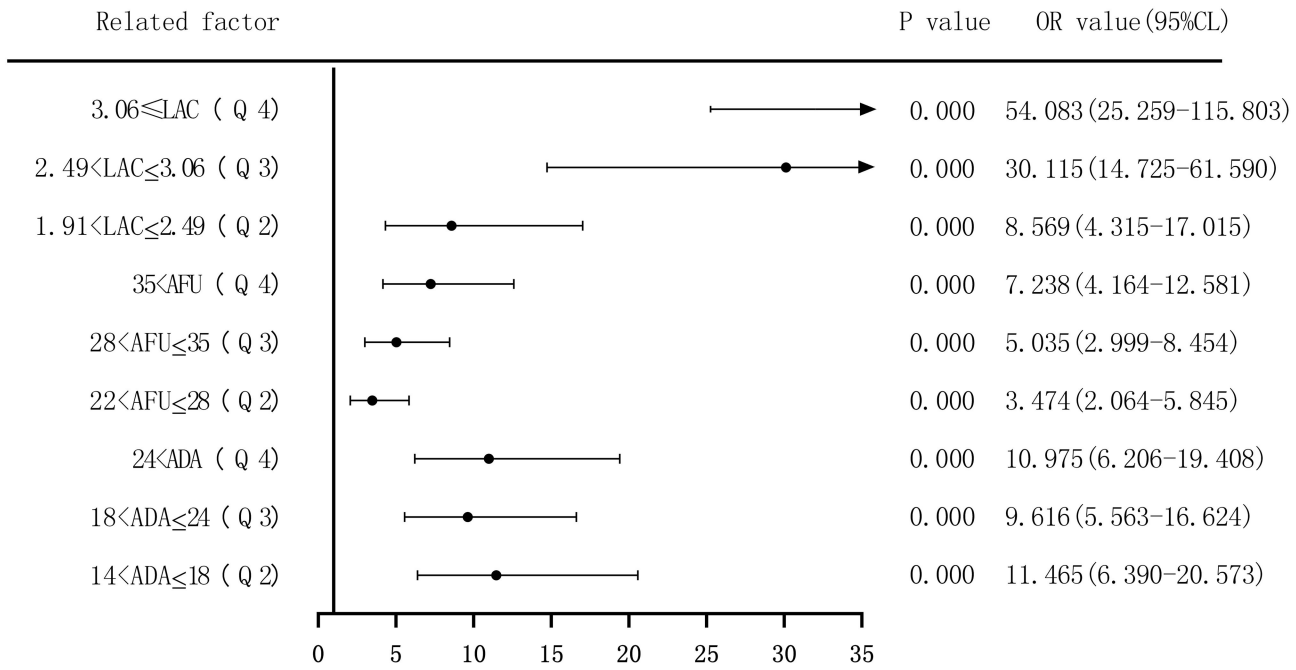

FIGURE 6 | Forest plots of univariate logistic regression analysis of ADA, AFU, LAC, and liver cirrhosis patients (four-group classification).

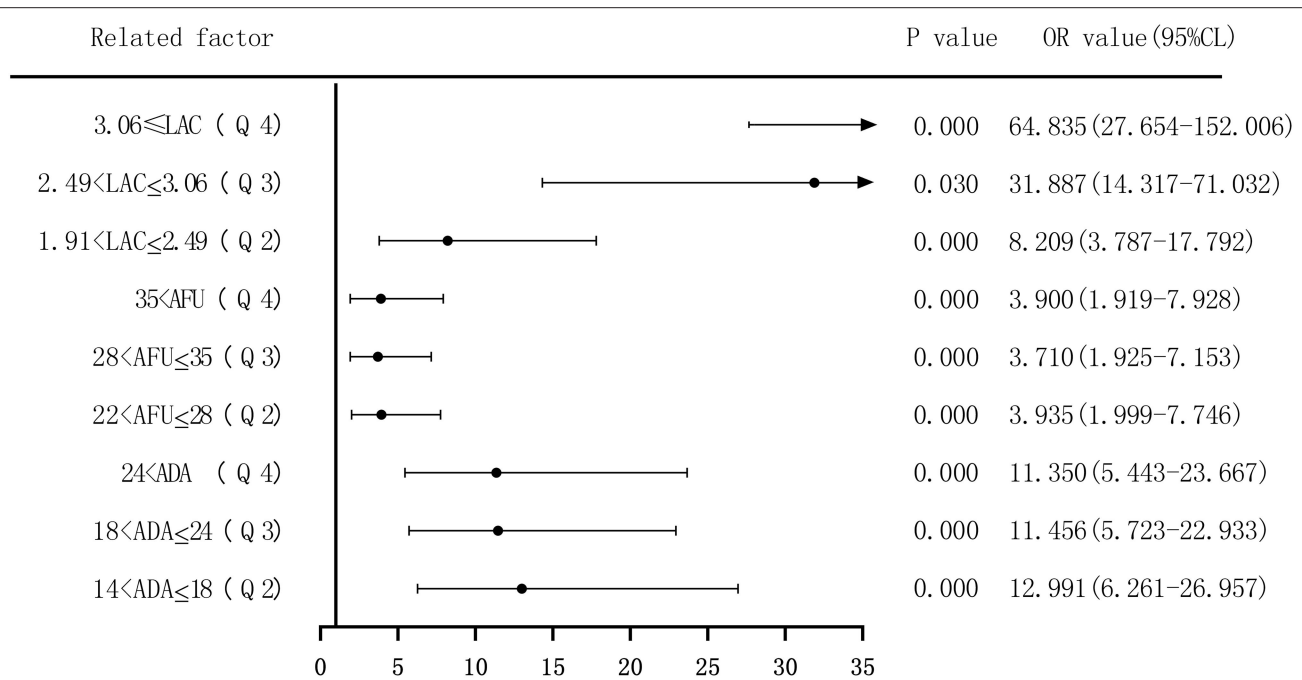

FIGURE 7 | Forest plots of multivariate logistic regression analysis of ADA, AFU, LAC, and liver cirrhosis patients (four-group classification) (Multi-factor adjustment includes variables ALB, ALT, ADA, AFU, LAC).

significant. The results indicated that the diagnostic performance of LAC was superior to that of ADA and AFU; however, there was no advantage between ADA and AFU. Furthermore, the combined detection of ADA+AFU+LAC was superior to single detection for the diagnosis of LC. The AUC values of ADA, AFU, LAC, and ADA+AFU+LAC in the LC and HCC groups were 0.577, 0.697, 0.929, and 0.939, respectively, and the diagnostic performance was superior. The results are consistent with the

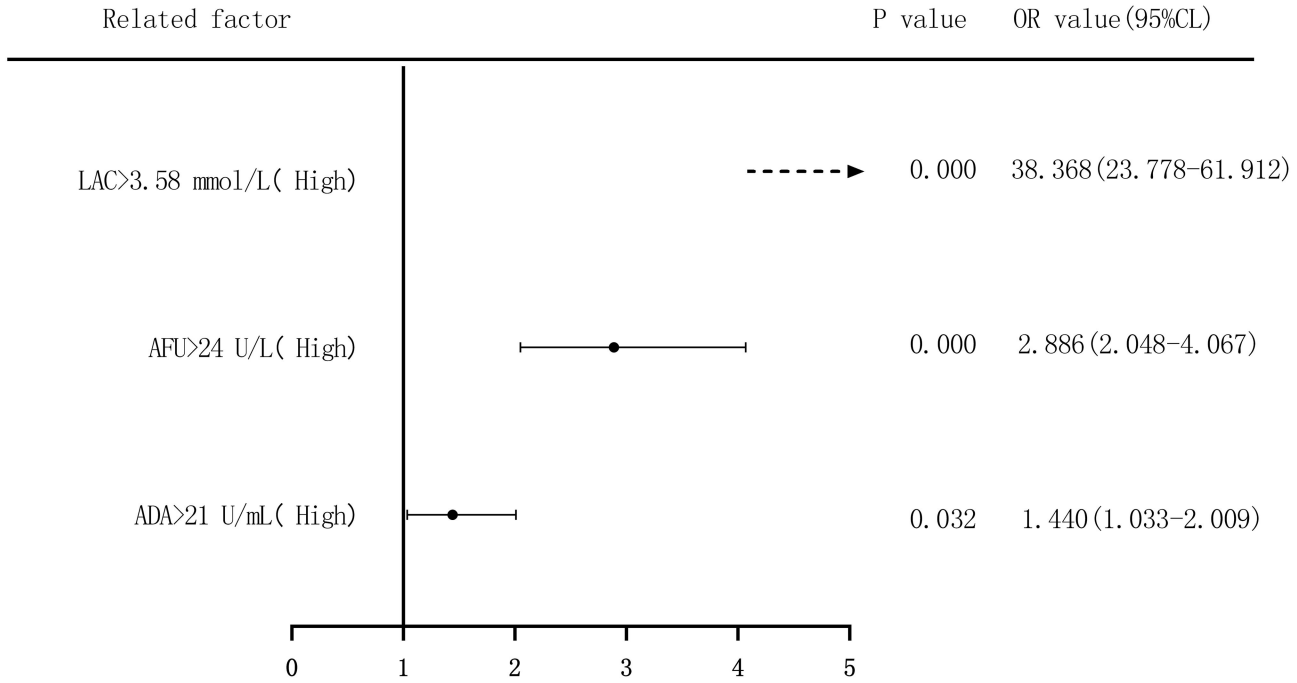

FIGURE 8 | Forest plots of univariate logistic regression analysis of ADA, AFU, LAC, and HCC patients (Note: Dotted line = OR value exceeds the range shown in the figure).

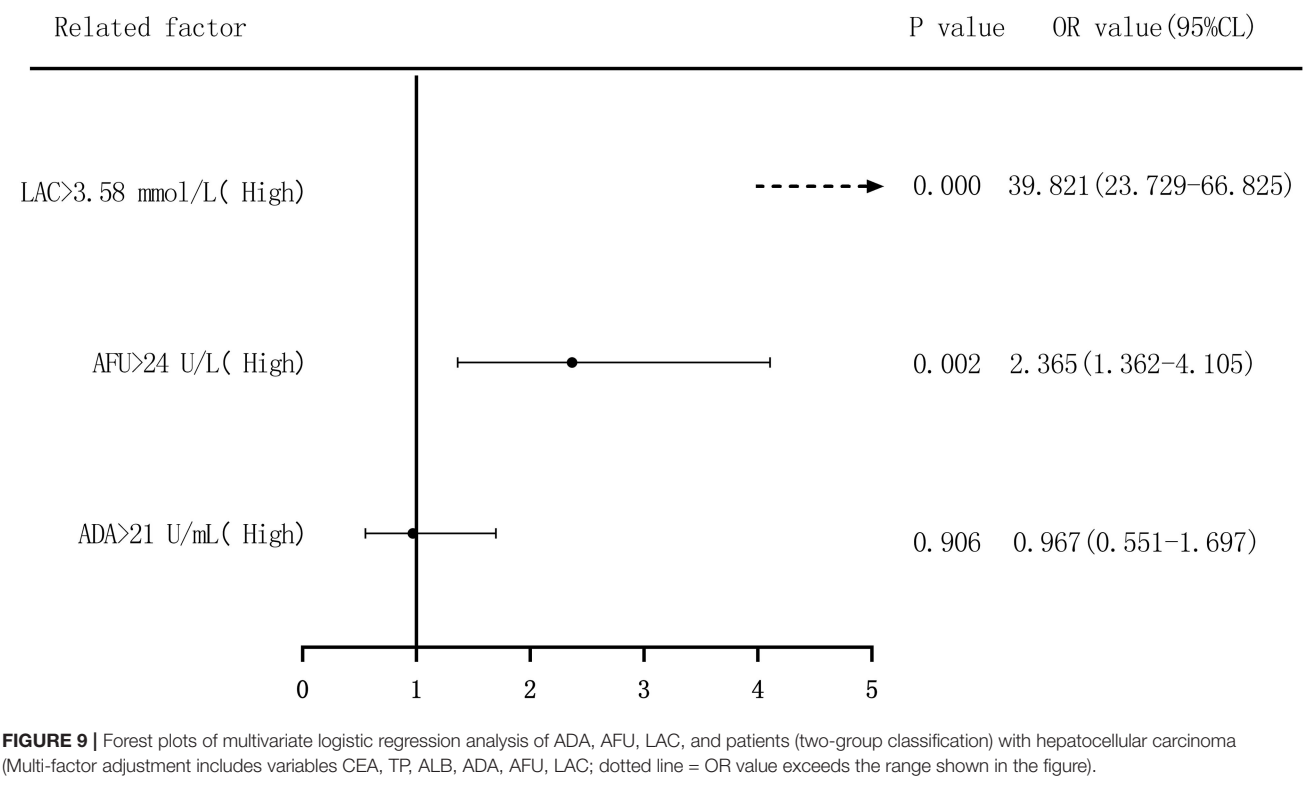

FIGURE 9 | Forest plots of multivariate logistic regression analysis of ADA, AFU, LAC, and patients (two-group classification) with hepatocellular carcinoma (Multi-factor adjustment includes variables CEA, TP, ALB, ADA, AFU, LAC; dotted line = OR value exceeds the range shown in the figure).

value of AFU reported in the study by Xing et al. for the diagnosis of early HCC (19). Analysis of the AUC values of ADA, AFU, LAC, and ADA+AFU+LAC revealed that the AUC of LAC was greater than that of AFU. Moreover, the value of AFU was greater than that of ADA. The AUC of the combined detection was greater than that of ADA, AFU, and LAC alone, and there

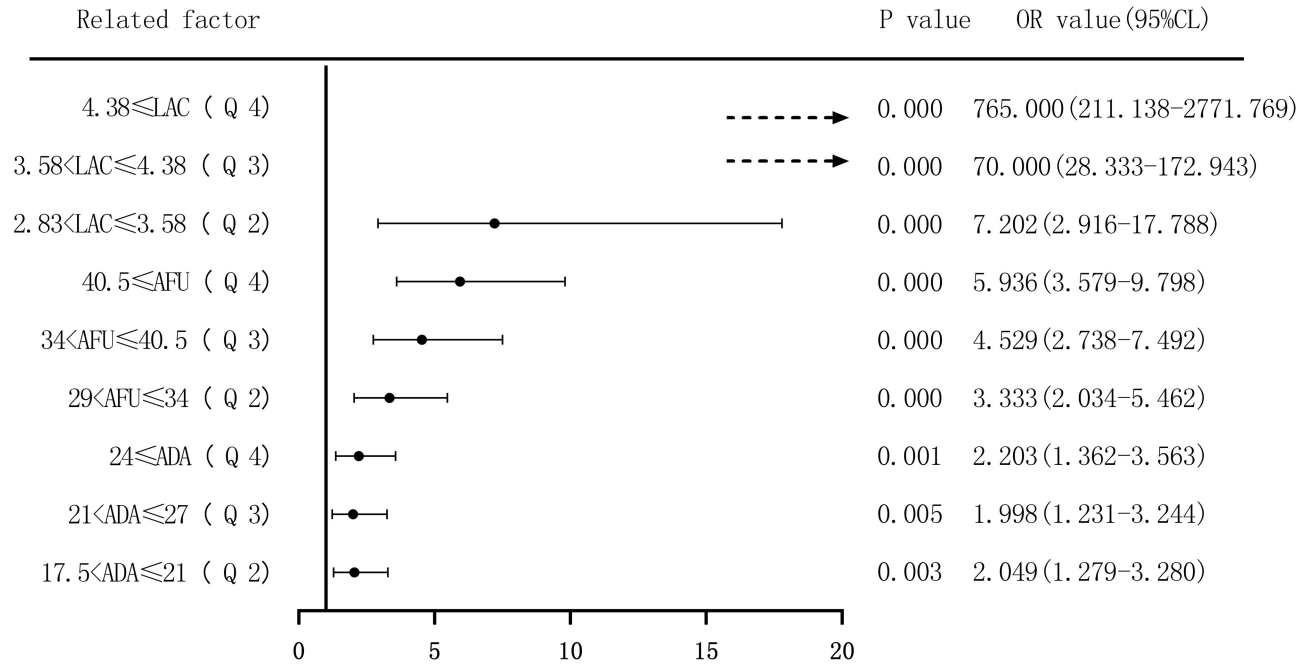

FIGURE 10 | Forest plots of univariate logistic regression analysis of ADA, AFU, LAC, and hepatocellular carcinoma patients (four-group classification) (Dotted line = OR value exceeds the range shown in the figure).

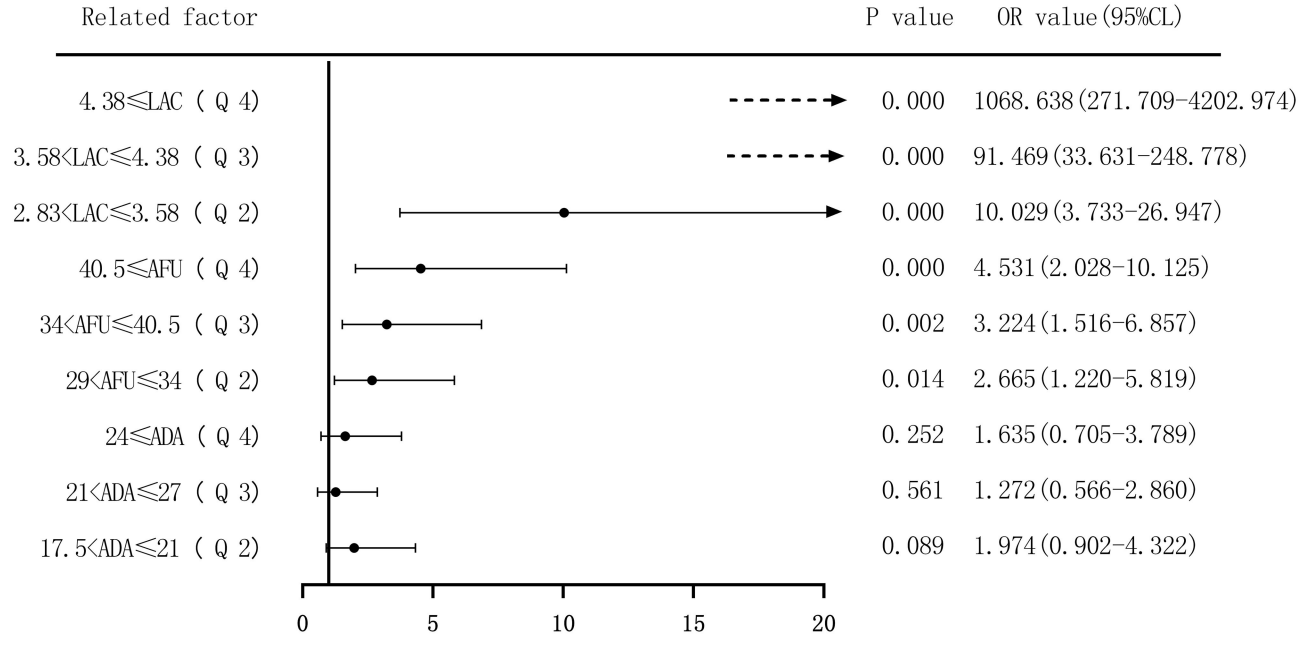

FIGURE 11 | Forest plots of multivariate logistic regression analysis of ADA, AFU, LAC, and hepatocellular carcinoma patients (four-group classification) (Multi-factor adjustment includes variables CEA, TP, ALB, ADA, AFU, LAC; dotted line = OR value exceeds the range shown in the figure).

was a statistically significant difference, indicating that the three markers showed an upward trend in the early diagnosis of HCC. In addition, the combined detection of ADA+AFU+LAC was superior to single detection for the early diagnosis of HCC.

The risk of ADA, AFU, and LAC levels in patients with early LC and HCC was assessed by binary logistic regression analysis, and the median and quartiles were considered the cutoff points (two-group and four-group classifications, respectively).

The risk assessment in patients with LC demonstrated that when the median was considered the cutoff point in the two-group classification, the adjusted OR values of ADA, AFU, and LAC for the risk of developing LC among patients in the high-level group compared to those in the low-level group were 3.218, 1.859, and 11.474, respectively. When quartiles were considered the cutoff point in the four-group classification, the adjusted OR values of ADA, AFU, and LAC were higher than those in the lowest-level group (Q1), and the difference was statistically significant. The results show that ADA, AFU, and LAC can be considered risk predictors of LC. The risk assessment in patients with HCC also showed that when the median was considered the cutoff point in the two-group classification, the adjusted OR values of ADA, AFU, and LAC for the risk of developing LC among patients in the high-level group compared to those in the low-level group were 0.967, 2.365, and 38.368, respectively. When quartiles were considered the cutoff point for the four-group classification, the adjusted OR values of AFU and LAC compared to the lowest-level group (Q1) were higher than that of the Q1 group, and the difference was statistically significant. There was no statistically significant difference between the adjusted ADA level and the lowest-level group (Q1). The results reveal that AFU and LAC, but not ADA, can be considered early risk predictors of HCC.

In summary, detection of ADA, AFU, and LAC has good value in the early diagnosis of LC and HCC. The combined detection of ADA+AFU+LAC is superior to single detection for the early diagnosis of LC and HCC. ADA, AFU, and LAC can be considered risk predictors of LC. Furthermore, AFU and LAC can be considered early risk predictors of HCC; however, the predictive ability of ADA is insufficient. It is worth noting that due to the small sample size and the failure to consider factors such as the use of drugs during the treatment of patients, further research is required with a larger sample size and a prospective study design to investigate the value of various markers in the early diagnosis of LC and HCC.

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 studies involving human participants were reviewed and approved by the Ethics Committees of Jiaozuo Fifth People's Hospital (Approval No. 20150762). Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

WZ, ZC, and CX contributed to study concept and design, acquisition of the data, analysis and interpretation of the data, and drafting of the manuscript. YZ and LW contributed to statistical analysis. JZ contributed to sample collections. SX, JT, and ZP contributed to study concept and design, study supervision, and critical revision of the manuscript. All authors have read and approved the manuscript.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2021.740029/full#supplementary-material

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ORIGINAL RESEARCH
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A Novel Non-invasive Model Based on GPR for the Prediction of Liver Fibrosis in Patients With Chronic Hepatitis B

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Background: Some controversy remains regarding conventional serum indices for the evaluation of liver fibrosis. Therefore, we aimed to combine the existing index with other serum parameters to discriminate liver fibrosis stages in patients with chronic hepatitis B (CHB).

Methods: A total of 1,622 treatment-naïve CHB patients were divided into training (n = 1,211) and validation (n = 451) cohorts. Liver histology was assessed according to the Scheuer scoring scheme. All common demographic and clinical parameters were analyzed.

Results: By utilizing the results of the logistic regression analysis, we developed a novel index, the product of GPR, international normalized ratio (INR), and type IV collagen (GIVPR), to discriminate liver fibrosis. In the training group, the areas under the ROCs (AUROCs) of GIVPR, APRI, FIB-4, and GPR for significant fibrosis were 0.81, 0.75, 0.72, and 0.77, respectively; the AUROCs of GIVPR, APRI, FIB-4, and GPR for advanced fibrosis were 0.82, 0.74, 0.74, and 0.78, respectively; and the AUROCs of GIVPR, APRI, FIB-4, and GPR for cirrhosis were 0.87, 0.78, 0.78, and 0.83, respectively. Similar results were also obtained in the validation group. Furthermore, the decision curve analysis suggested that GIVPR represented superior clinical benefits in both independent cohorts.

Conclusion: The GIVPR constructed on GPR represents a superior predictive model for discriminating liver fibrosis in CHB patients.

Keywords: CHB, liver fibrosis, type IV collagen, INR, GPR

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BACKGROUND

Hepatitis B virus (HBV) infection is a serious public health problem. It is estimated that more than 350 million people are chronically infected worldwide (1). From 1990 to 2013, the mortality rate of liver cirrhosis and hepatocellular carcinoma caused by HBV infection increased by 33% worldwide (2). Based on the outcomes of patients who receive early diagnosis and effective antiviral therapy, the prognosis of CHB can be significantly improved even if the case is histologically advanced fibrosis or cirrhosis (3). Therefore, it is of great importance to assess the risk of early liver fibrosis in CHB patients.

Currently, the gold standard for the assessment of liver fibrosis is still liver biopsy. However, its limitations, such as its invasiveness, sampling errors, cost, intra- and inter-observer discrepancies, and the risk of potentially life-threatening complications, restrict its clinical application (4). Clinical practice requires simple operations or non-invasive and easy methods to diagnose liver inflammation, injury or fibrosis (5). The World Health Organization (WHO) guidelines recommend serologic biomarkers and FibroScan as useful non-invasive methods for evaluating CHB patients (6). However, several factors, including necroinflammatory activity, ascites, cost, and lack of skilled operators, may diminish the clinical use of FibroScan (6, 7). Serum biomarkers are particularly important in these methods because they do not require qualified staff and expensive equipment for evaluation (8). The WHO has recommended the aspartate aminotransferase (AST)-platelet ratio index (APRI) and fibrosis-4 (FIB-4) as non-invasive indices for CHB patients (6). The diagnostic value of these two indices in liver fibrosis has been widely studied, but their sensitivity and specificity are still controversial (9). Recently, a study by Lemonie et al. (10) suggested that the γ-glutamyl transpeptidase to platelet ratio (GPR) was more accurate than APRI or FIB-4, and this study was supported by several studies on Chinese subjects (11, 12). However, there were still a few inconsistent conclusions (13). Therefore, novel non-invasive serum calculations are still needed because the current biochemical markers do not have enough diagnostic accuracy to replace liver biopsy.

Serum collagen, especially type IV collagen, has been confirmed to be a useful, non-invasive marker for measuring the activity of this pathway at a single time point and has been shown to reflect prognosis and responses to a variety of chronic liver diseases (14). INR is a routine serological marker associated with liver function and essentially reflects the progression of liver diseases. Wu et al. reported that the INR was an independent factor for the prediction of significant fibrosis in patients with CHB (6, 15).

More efforts should be dedicated to pursuing simple, safe and reliable non-invasive diagnostic measures to stage liver fibrosis. In this study, we aimed to construct and validate a predictive index consisting of GPR, INR, and type IV collagen to reflect liver fibrosis simply and effectively in CHB patients.

METHODS

Patients

Overall, between January 2014 and January 2021, we retrospectively screened 2,193 consecutive Chinese individuals with chronic hepatitis B who underwent liver biopsy and clinical examination at Shanghai Public Health Clinical Center, Fudan University. CHB was diagnosed when serum hepatitis B surface antigen (HBsAg) was persistently positive for more than 6 months (16). All the patients were >18 years old. Non-alcoholic fatty liver disease (NAFLD) was diagnosed as at least 5% biopsy-proven hepatic steatosis without significant alcohol consumption (17). The exclusion criteria were as follows: antiviral treatment history, coinfection with hepatitis C virus (HCV), hepatitis D virus (HDV), hepatitis E virus (HEV), or human immunodeficiency virus (HIV), significant alcohol consumption (>20 g/d), autoimmune hepatitis, hepatocellular carcinoma, decompensated cirrhosis, inadequate liver biopsy samples (<1.5 cm), and the use of warfarin.

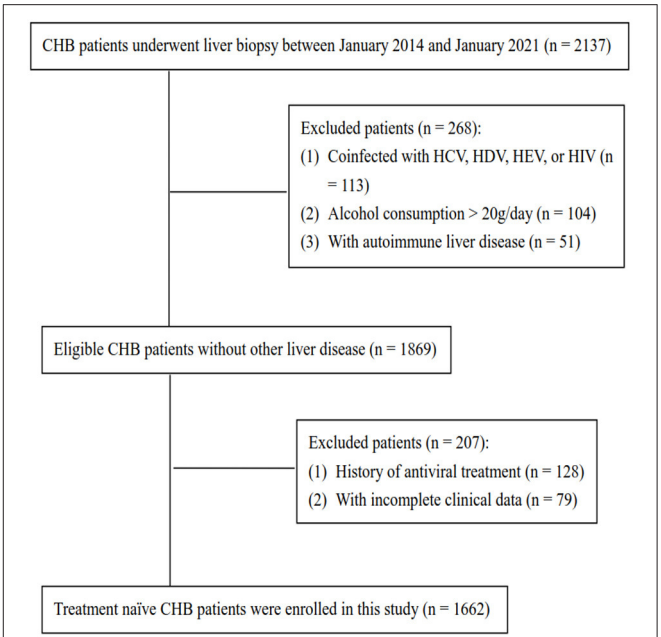

FIGURE 1 | Flow diagram of this study population. CHB, chronic hepatitis B; HCV, hepatitis C virus; HDV, hepatitis D virus; HEV, hepatitis E virus; HIV, human immunodeficiency virus.

We summarized the flow diagram of the study population in **Figure 1**. After excluding patients with coinfection with HCV, HDV, HEV, or HIV ($n = 113$), alcohol consumption (>20 g/d) ($n = 104$), autoimmune hepatitis ($n = 51$), history of antiviral treatment ($n = 128$), and incomplete clinical data ($n = 79$), 1,662 treatment-naïve patients with CHB were included. The population was randomly divided into a training set ($n = 1,211$) and a validation set ($n = 451$) for model development and validation using SPSS software.

Liver Biopsy

Percutaneous liver biopsy was performed using a 16 G needle under ultrasound guidance. Liver samples with a minimum length of 1.5 cm and at least 7 complete portal tracts were fixed in 10% formalin, embedded in paraffin, and stained with HE Masson's trichrome and reticulin for histological analysis. Liver histology was analyzed by two experienced pathologists who were blinded to other clinical and laboratory data and classified according to the Scheuer scoring system (18) as follows: S0 (no fibrosis), S1 (mild fibrosis without septa), S2 (moderate fibrosis with few septa), S3 (severe fibrosis with numerous septa without cirrhosis), and S4 (cirrhosis). In this study, liver fibrosis stage ≥S2 was defined as significant fibrosis, ≥S3 was defined as advanced fibrosis, and S4 was defined as cirrhosis. These definitions represent at minimum significant fibrosis

and affect the management of patients in terms of treatment indications (16, 19).

Laboratory Data

Fasting blood samples were obtained within a week of liver biopsy. Platelets and other blood cells were counted using a Sysmex-XT 4000i automated hematology analyzer. The international normalized ratio (INR) and other coagulation indices were measured using a STAR Max automatic coagulation analyzer. Alanine transaminase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), γ-glutamyl transferase (GGT), hyaluronic acid, laminin, N-terminal propeptide of type III procollagen (PIIINP), type IV collagen, and other serum biochemical parameters were measured using an Architect C16000 automatic biochemical analysis system.

Formulas

The formulas for APRI, FIB-4, and GPR are as follows: APRI = (AST (U/L)/ULN of AST)/platelet count (10^9/L) × 100 (20); FIB-4 = (age (years) × AST (U/L))/(platelet count (10^9/L) × (ALT

TABLE 1 | Clinical characteristics of studied patients with CHB.

Variables	Training set (n = 1,211)	Validation set (n = 451)	P-value
Age, years	37 (31-45)	37 (31-45)	0.419
Male, *n* (%)	779 (64.3)	296 (65.6)	0.649
NAFLD, *n* (%)	120 (9.9)	57 (12.6)	0.109
Serum parameters			
logHBVDNA, IU/ml	5.15 (3.06-7.11)	5.28 (3.16-7.08)	0.584
ALT, U/L	48.00 (26.00-119.00)	54.00 (30.00-134.00)	0.041
AST, U/L	35.00 (23.00-71.00)	38.00 (24.00-79.00)	0.117
ALP, U/L	75.00 (62.00-93.00)	77.00 (63.00-97.00)	0.222
GGT, U/L	32.00 (18.00-67.00)	35.00 (19.00-73.00)	0.078
TBil, μmol/L	14.30 (10.20-19.90)	15.00 (10.50-21.00)	0.054
DBil μmol/L	5.40 (3.90-7.59)	5.60 (4.10-8.00)	0.100
Albumin, g/L	42.40 (39.62-45.09)	42.10 (39.30-45.00)	0.476
FBG, mmol/L,	4.90 (4.55-5.32)	4.95 (4.58-5.40)	0.305
TC, mmol/L	4.19 (3.68-4.85)	4.26 (3.67-4.90)	0.325
TG, mmol/L	0.96 (0.72-1.30)	0.99 (0.74-1.34)	0.184
HDL, mmol/L	1.34 (1.07-1.58)	1.27 (1.01-1.56)	0.059
LDL mmol/L	2.63 (2.14-3.17)	2.73 (2.17-3.18)	0.161
Urea, mmol/L	307.02 (253.48 0-362.49)	300.55 (251.90-366.70)	0.519
Creatinine,μmol/L	65.50 (53.99-74.81)	64.40 (54.30-74.78)	0.725
INR	1.05 (0.99-1.11)	1.04 (1.00-1.11)	0.958
APTT, s	38.40 (35.80-41.20)	38.50 (36.10-40.90)	0.695
Fibrinogen, g/L	2.45 (2.14-2.78)	2.45 (2.15-2.78)	0.963
WBC count, $\times10^9$/L	5.27 (4.36-6.23)	5.21 (4.33-6.16)	0.528
Platelet count, $\times10^9$/L	165.00 (131.00-203.00)	168.00 (132.00-201.00)	0.574
Neutrophils count, $\times10^9$/L	2.87 (2.26-3.63)	2.82 (2.19-3.52)	0.263
Lymphocyte count, $\times10^9$/L	1.75 (1.41-2.16)	1.80 (1.44-2.21)	0.146
Hyaluronic, ng/ml	60.20 (41.00-98.06)	59.82 (42.63-98.49)	0.543
Laminin, ng/ml	25.49 (18.53-38.98)	26.01 (17.79-39.78)	0.667
PIIINP, ng/ml	25.74 (17.95-38.07)	25.69 (18.15-39.27)	0.680
Type IV collagen, ng/ml	26.01 (20.11-36.51)	26.53 (19.47-38.69)	0.267
Non-invasive indexes			
APRI	0.59 (0.33-1.38)	0.64 (0.35-1.29)	0.058
FIB-4	1.28 (0.83-2.07)	1.28 (0.86-1.99)	0.187
GPR	0.40 (0.21-0.95)	0.44 (0.23-1.09)	0.115
Liver pathology			
Scheuer fibrosis stage (S0-1/S2/S3/S4)	525(43.4%)/293(24.2%)/ 138 (11.4%)/255(21.1%)	218 (48.3%)/113(25.1%)/43 (9.5%)/77(17.1%)	0.134
Scheuer activity grade (G0-1/G2/G3/G4)	667 (55.1%)/335(27.7%)/209(17.3%)/0	251(55.7%)/115(25.5%)/85(18.8%)/0	0.590

NAFLD, non-alcoholic fatty liver disease; ALT, alanine transaminase; AST, aspartate aminotransferase; ALP, alkaline phosphatase; GGT, γ-glutamyl transpeptadase; TBil, total bilirubin; DBil, direct bilirubin; FBG, fasting blood glucose; TC, total cholesterol; TG, triglyceride; LDC, low-density lipoprotein; HDL, high-density lipoprotein; INR, international normalized ratio; APTT, activated partial thromboplastin time; PIIIP, N-Terminal procollagen III propeptide; APRI, AST to platelet ratio index; FIB-4, fibrosis-4; GPR, GGT to platelet ratio.

$(U/L))^{1/2}$) (21); GPR = (GGT (U/L)/ULN of GGT)/platelet count (10^9/L) × 100 (10).

Statistical Analysis

Statistical analysis was performed using IBM SPSS Statistics version 26.0 (SPSS Inc., Chicago, USA) and R 4.0.3 (http://www.R-project.org). Continuous variables are expressed as the mean ± standard deviation or median (interquartile range, IQR) and were compared using Student's *t*-test (for normally distributed continuous variables) or the independent Mann–Whitney *U*-test (for non-normally distributed continuous variables). Categorical variables are expressed as proportions and were compared by the chi-square test. Logistic regression models were used to assess the correlations between variables and liver fibrosis. The performances of the non-invasive markers for predicting liver fibrosis were assessed by receiver operating characteristic (ROC) curve analyses. The Delong *Z*-test was used to compare the AUROCs of the serum models. Decision curve analysis (DCA) was used to further evaluate the predictive performances. A two-sided $P < 0.05$ was considered statistically significant.

RESULTS

Clinical Characteristics of the Study Population

A total of 1,662 treatment-naïve CHB patients who had undergone a liver biopsy were enrolled in the study, with median ages of 37 (31–45) and 37 (31–45) years in the training and validation sets, respectively. The clinical data of the studied groups are summarized in **Table 1**. Except for ALT, there were no

TABLE 2 | Variables associated with significant fibrosis and cirrhosis by logistic analysis in training cohort.

Variables	Significant fibrosis (S2-4)				Cirrhosis (S4)			
	Univariate		Multivariate		Univariate		Multivariate	
	OR (95% CI)	*P*-value	OR (95% CI)	*P*-value	OR (95%CI)	*P*-value	OR (95% CI)	*P*-value
Gender, male	0.71 (0.65-1.04)	0.251			0.57 (0.42-0.77)	<0.001		
Age	1.01 (1.00-1.02)	0.105			1.02 (1.01-1.03)	0.001		
NAFLD, yes vs. no	0.53 (0.36-0.78)	0.001			0.51 (0.27-0.89)	0.017		
LogHBVDNA, IU/ml	1.07 (1.02-1.13)	0.013			1.04 (0.97-1.11)	0.285		
ALT, U/L	1.00 (1.00-1.01)	<0.001			1.00 (1.00-1.00)	<0.001		
AST, U/L	1.01 (1.00-1.01)	<0.001			1.00 (1.00-1.00)	<0.001		
ALP, U/L	1.02(1.02-1.03)	<0.001			1.02 (1.01-1.02)	<0.001	1.01 (1.00-1.01)	0.007
GGT, U/L	1.02(1.01-1.02)	<0.001			1.01 (1.01-1.01)	<0.001		
TBil,μmol/L	1.02 (1.01-1.03)	<0.001	0.98 (0.97-0.99)	0.025	1.03 (1.02-1.04)	<0.001		
DBil, μmol/L	1.04 (1.03-1.06)	<0.001			1.03 (1.02-1.05)	<0.001		
Albumin, g/L	0.87 (0.85-0.90)	<0.001			0.81 (0.78-0.84)	<0.001		
FBS	0.96 (0.84-1.08)	0.483			1.04 (0.90-1.19)	0.620		
TC, mmol/L	0.78 (0.69-0.88)	<0.001			0.62 (0.53-0.74)	<0.001		
TG, mmol/L	0.76 (0.63-0.91)	0.004			0.96 (0.77-1.19)	0.682		
LDL, mmol/L	1.00 (0.98-1.03)	0.899			0.62 (0.51-0.74)	<0.001		
HDL, mmol/L	0.92 (0.71-1.21)	0.563			0.54 (0.38-0.76)	<0.001		
Creatinine, μmol/L	1.00 (0.99-1.01)	0.778			1.00 (0.99-1.01)	0.900		
Urea, mmol/L	1.00 (0.99-1.00)	0.216			1.00 (0.99-1.00)	0.978		
INR	2.22 (1.92-2.57)	<0.001	**1.69 (1.39-2.06)**	**<0.001**	2.60 (2.22–3.05)	<0.001	**1.70 (1.40-2.06)**	**< 0.001**
APTT,	1.09 (1.06-1.13)	<0.001			1.04 (1.01-1.08)	0.009		
Fibrinogen, g/L	0.93 (0.82-1.06)	0.271			0.94 (0.77-1.13)	0.488		
WBC count, $\times 10^9$/L	0.81 (0.75-0.88)	<0.001			0.72 (0.65-0.80)	<0.001		
Neutrophils count, $\times 10^9$/L	0.79 (0.71-0.87)	<0.001			0.61 (0.52-0.70)	<0.001		
Lymphocyte count, $\times 10^9$/L	0.91 (0.75-1.10)	0.315			0.79 (0.62-1.00)	0.049	1.42 (1.03-1.97)	0.035
Platelet count, $\times 10^9$/L	0.99 (0.98-0.99)	<0.001	0.99 (0.99-1.00)	<0.001	0.98 (0.98-0.98)	<0.001	0.99 (0.98-0.99)	< 0.001
Hyaluronic, ng/ml	1.00 (1.00-1.01)	<0.001			1.01 (1.00-1.01)	<0.001		
Laminin, ng/ml	1.01 (1.01-1.02)	<0.001			1.01 (1.01-1.02)	<0.001		
PIIINP, ng/ml	1.06 (1.05-1.07)	<0.001			1.04 (1.03-1.04)	<0.001		
Type IV collagen, ng/ml	1.12 (1.10-1.13)	<0.001	**1.10 (1.08-1.12)**	**<0.001**	1.04 (1.04-1.05)	<0.001	**1.03 (1.02-1.04)**	**< 0.001**

NAFLD, non-alcoholic fatty liver disease; ALT, alanine transaminase; AST, aspartate aminotransferase; ALP, alkaline phosphatase; GGT, γ-glutamyl transpeptadase; TBil, total bilirubin; DBil, direct bilirubin; FBG, fasting blood glucose; TC, total cholesterol; TG, triglyceride; LDC, low-density lipoprotein; HDL, high-density lipoprotein; INR, international normalized ratio; APTT, activated partial thromboplastin time; PIIIP, N-Terminal procollagen III propeptide; APRI, AST to platelet ratio index; FIB-4, fibrosis-4; GPR, GGT to platelet ratio. The bold values are independent risk factors in the significant liver fibrosis group and liver cirrhosis.

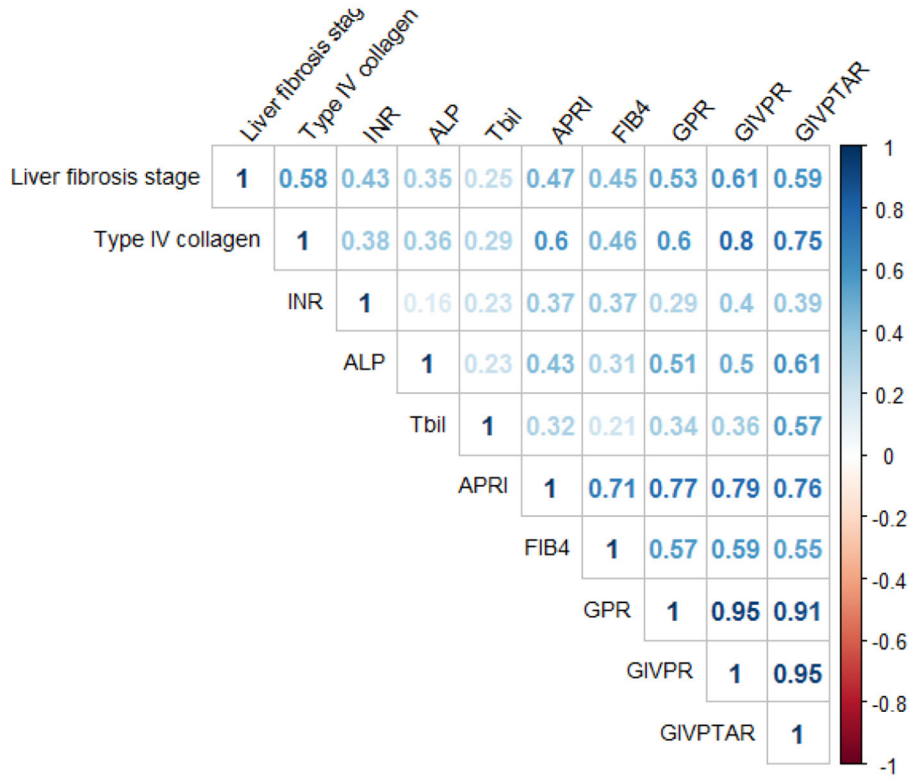

FIGURE 2 | Correlation between the serum indexes and liver fibrosis score.

statistically significant differences in other parameters between the training and validation sets. Additionally, 293 (24.2%) patients were in fibrosis stage S2, 138 (11.4%) were in S3, and 255 (21.1%) were in S4 in the training set, while 113 (25.1%) patients were in S2, 43 (9.5%) were in S3, and 77 (17.1%) were in S4 in the validation set.

Development of the GIVPR Index in the Training Cohort

In the training cohort, a significantly increased odds ratio of stage S2–4 was associated with age, NAFLD, HBV DNA, ALT, AST, ALP, GGT, total bilirubin (TBil), direct bilirubin (DBil), albumin, total cholesterol (TC), triglyceride (TG), INR, activated partial thromboplastin time (APTT), white blood cells (WBC), neutrophils, platelets, hyaluronic, laminin, PIIINP, and type IV collagen. Multivariable analysis identified TBil, INR, platelets, and type IV collagen as independent predictors of significant liver fibrosis. Similarly, a significantly increased odds ratio of stage S4 was associated with sex, age, NAFLD, ALT, AST, ALP, GGT, TBil, DBil, albumin, TC, high-density lipoprotein (HDL), low-density lipoprotein (LDL), INR, APTT, WBC, neutrophils, lymphocytes, platelets, hyaluronic, laminin, PIIINP, and type IV collagen. Multivariable analysis identified ALP, INR, platelets, and type IV collagen as independent predictors of cirrhosis (**Table 2**). Thus, in addition to platelets, both INR and type IV collagen were independent predictors of significant fibrosis and cirrhosis (all $P < 0.01$).

Spearman's correlation analysis showed that type IV collagen ($r = 0.58$), INR ($r = 0.43$), ALP ($r = 0.35$), and TBil ($r = 0.25$) were significantly correlated with liver fibrosis scores (**Figure 2**). Based on these independent predictors, we devised two simple models to amplify the predictive performances of the established non-invasive indices and serum parameters for the progression of liver fibrosis. The models are as follows: GIVPR = GPR × INR × type IV collagen; GIVPTAR = GPR × INR × type IV collagen × TBil × ALP. GIVPR ($r = 0.61$) was significantly positively correlated with the Scheure fibrosis score with a higher correlation coefficient than APRI, FIB-4, GPR, and GIVPTAR ($r = 0.47$, 0.45, 0.53, and 0.59, respectively) (**Figure 2**).

Comparison of GIVPR With Other Non-invasive Indices for Predicting Liver Fibrosis in the Training and Validation Cohorts

Using ROC curve analysis, GIVPR was compared to GIVPTAR, APRI, FIB-4, and GPR for staging liver fibrosis. GIVPR displayed better accuracy in predicting significant fibrosis, advanced fibrosis, and cirrhosis. The ROC curves for the fourth non-invasive serum marker are shown in the training set (**Figure 3**) and the validation set (**Figure 4**). In the training set, for the discrimination of significant fibrosis,

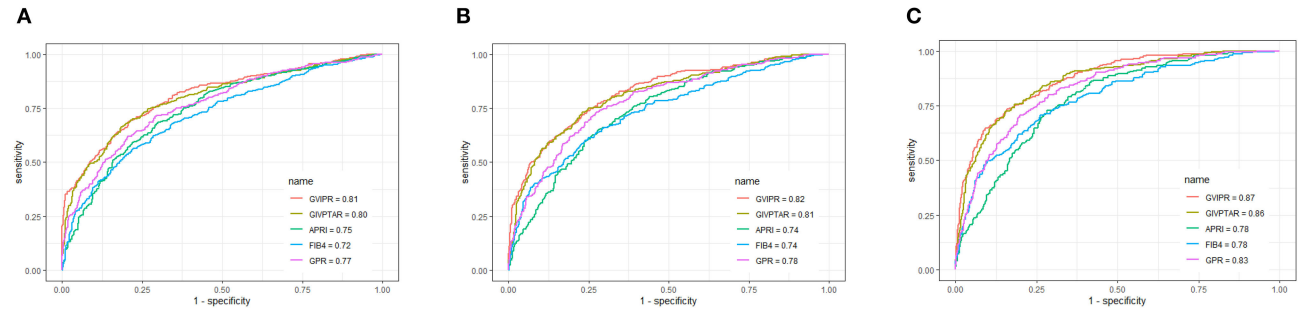

FIGURE 3 | Area under receiver operating characteristic (ROC) comparison of GIVPR, GIVPTAR, APRI, FIB-4, and GPR in training set. **(A)** ROC comparison for predicting significant fibrosis; **(B)** ROC comparison for predicting advanced fibrosis; **(C)** ROC comparison for predicting cirrhosis.

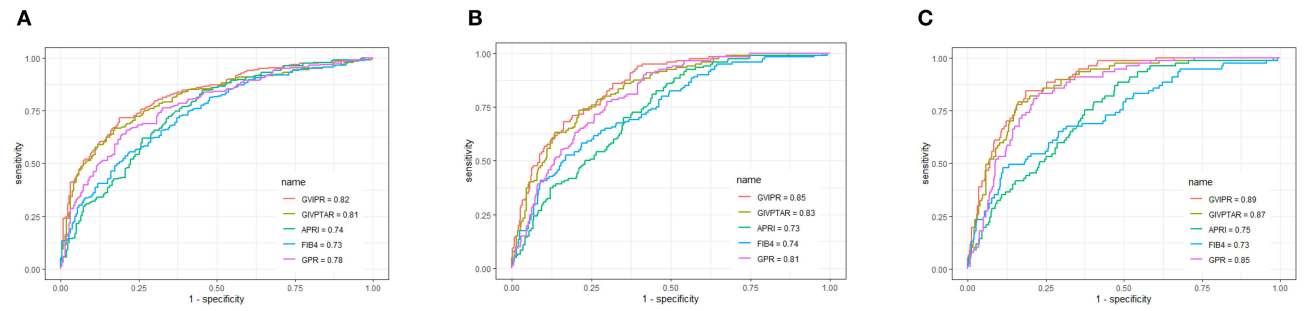

FIGURE 4 | Area under receiver operating characteristic (ROC) comparison of GIVPR, GIVPTAR, APRI, FIB-4, and GPR in validation set. **(A)** ROC comparison for predicting significant fibrosis; **(B)** ROC comparison for predicting advanced fibrosis; **(C)** ROC comparison for predicting cirrhosis.

GIVPR had the highest AUC (0.81, sensitivity 68.95% and specificity 79.23%) compared with GIVPTAR (0.80, sensitivity 69.53% and specificity 78.67%), APRI (0.75, sensitivity 68.37% and specificity 70.10%), FIB-4 (0.72, sensitivity 56.20% and specificity 77.86%), and GPR (0.77, sensitivity 71.37% and specificity 70.86%). When discriminating advanced fibrosis, GIVPR had the highest AUC (0.82, sensitivity 74.81% and specificity 74.57%) compared with GIVPTAR (0.81, sensitivity 75.06% and specificity 75.06%), APRI (0.74, sensitivity 65.14% and specificity 72.00%), FIB-4 (0.74, sensitivity 66.07% and specificity 70.38%), and GPR (0.78, sensitivity 73.03% and specificity 72.62%). For predicting cirrhosis, GIVPR also had the best AUC (0.87, sensitivity 73.33% and specificity 84.21%) compared with GIVPTAR (0.86, sensitivity 75.69% and specificity 81.80%), APRI (0.78 sensitivity 72.94% and specificity 71.86%), FIB-4 (0.78, sensitivity 70.59% and specificity 73.90%), and GPR (0.78, sensitivity 80.00% and specificity 71.44%). The cutoffs of GIVPR for the assessment of significant fibrosis, advanced fibrosis, and cirrhosis were 11.57, 15.45, and 29.07, respectively (**Table 3**).

Similarly, in the validation set, compared to the other four serum indices, GIVPR had the highest AUCs of 0.82 (sensitivity 73.82% and specificity 75.23%) for predicting significant fibrosis, 0.85 (sensitivity 81.67% and specificity 70.09%) for predicting advanced fibrosis, and 0.80 (sensitivity 84.42% and specificity 78.88%) for predicting cirrhosis (**Table 4**). These results suggest that GIVPR is an excellent predictor of liver fibrosis in CHB patients.

DCA for the Clinical Utility of GIVPR

Moreover, we conducted DCA to further investigate the clinical application value of GIVPR, GIVPTAR, APRI, FIB-4, and GPR for predicting liver fibrosis. In the training group, DCAs revealed that from a threshold probability of 20–80%, the application of GIVPR to predict liver fibrosis risk increased the benefit considerably more than the other four scores (**Figure 5**). Regarding the validation group, the DCAs of GIVPR also showed a better net benefit with a wide range of threshold probabilities and better performances for predicting liver fibrosis than GIVPTAR, APRI, FIB-4, and GPR (**Figure 6**).

DISCUSSION

Early diagnosis and accuracy in evaluating liver fibrosis or cirrhosis may play important roles not only in controlling disease progression but also in the treatment of chronic HBV infection (22). Liver biopsy is the gold standard for evaluating liver fibrosis in chronic liver disease. However, although liver biopsy is usually a safe procedure, it has some technical limitations and risks (23). Thus, there is an increasing need for simple and reliable non-invasive predictors for liver fibrosis, some

TABLE 3 | Predictive performances of GIVPR, GIVPTAR, APRI, FIB-4, and GPR for liver fibrosis in CHB patients (Training cohort).

Indexes	AUROC (95%CI)	Cutoff	Se (%)	Sp (%)	PPV (%)	NPV (%)	Accuracy (%)	*P-value
S2-4								
GIVPR	0.81 (0.78-0.83)	11.57	68.95	79.23	81.3	66.1	73.41	–
GIVPTAR	0.80 (0.77-0.82)	11130.67	69.53	78.67	81.0	66.4	73.41	0.028
APRI	0.75 (0.72-0.77)	0.55	68.37	70.10	74.9	62.9	68.79	<0.0001
FIB-4	0.72 (0.69-0.75)	1.49	56.20	77.86	76.8	57.6	65.51	<0.0001
GPR	0.77 (0.74-0.79)	0.37	71.43	70.86	76.2	65.5	71.10	<0.0001
S3-4								
GIVPR	0.82 (0.80-0.85)	15.45	74.81	74.57	58.6	86.0	74.48	–
GIVPTAR	0.81 (0.79-0.83)	16667.88	75.06	75.06	59.1	86.2	75.06	0.008
APRI	0.74 (0.71-0.76)	0.78	65.14	72.00	52.8	81.1	69.61	<0.0001
FIB-4	0.74 (0.71-0.76)	1.49	66.07	70.38	51.7	81.2	68.73	<0.0001
GPR	0.78 (0.76-0.81)	0.52	73.03	72.62	56.2	84.9	72.75	<0.0001
S4								
GIVPR	0.87 (0.85-0.89)	29.07	73.33	84.21	55.3	92.2	81.92	–
GIVPTAR	0.86 (0.84-0.88)	32331.38	75.69	81.80	52.6	92.7	80.51	0.047
APRI	0.78 (0.75-0.80)	0.85	72.94	71.86	40.9	90.9	71.92	<0.0001
FIB-4	0.78 (0.76-0.81)	1.65	70.59	73.90	42.0	90.4	73.11	<0.0001
GPR	0.83 (0.81-0.85)	0.56	80.00	71.44	42.8	93.1	73.08	<0.0001

*AUROC, area under ROC; Se, sensitivity; Sp, specificity. *Compared with GIVPR.*

TABLE 4 | Predictive performances of GIVPR, GIVPTAR, APRI, FIB-4, and GPR for liver fibrosis in CHB patients (Validation cohort).

Indexes	AUROC(95%CI)	Cutoff	Se (%)	Sp (%)	PPV (%)	NPV (%)	Accuracy (%)	*P-value
S2-4								
GIVPR	0.82 (0.78-0.86)	11.57	73.82	75.23	76.0	72.6	74.28	–
GIVPTAR	0.81 (0.77-0.84)	11130.67	74.25	73.39	74.9	72.7	74.06	0.021
APRI	0.74 (0.70-0.78)	0.55	74.68	62.84	68.2	69.9	68.74	<0.0001
FIB-4	0.73 (0.69-0.77)	1.49	54.94	78.44	73.1	62.0	66.08	<0.0001
GPR	0.78 (0.73-0.81)	0.37	76.39	67.43	71.5	72.8	71.84	<0.0001
S3-4								
GIVPR	0.85 (0.81-0.88)	15.45	81.67	70.09	49.7	91.3	73.17	–
GIVPTAR	0.83 (0.79-0.86)	16667.88	80.00	69.18	48.5	90.5	71.84	0.006
APRI	0.73 (0.69-0.77)	0.78	59.23	74.31	71.1	63.0	65.41	<0.0001
FIB-4	0.74 (0.70-0.78)	1.49	65.00	70.69	44.6	84.8	68.96	<0.0001
GPR	0.81 (0.77-0.84)	0.52	77.50	69.79	48.2	89.5	71.62	<0.0001
S4								
GIVPR	0.89 (0.86-0.92)	29.07	84.42	78.88	45.1	96.1	80.27	–
GIVPTAR	0.87 (0.84-0.90)	32331.38	81.82	77.27	42.6	95.4	78.27	0.033
APRI	0.75 (0.70-0.79)	0.85	66.23	66.04	28.7	90.5	65.85	<0.0001
FIB-4	0.73 (0.69-0.77)	1.65	61.04	72.73	31.5	90.1	70.51	<0.0001
GPR	0.85 (0.81-0.88)	0.56	85.71	70.05	37.1	96.0	72.51	<0.0001

*AUROC, area under ROC; Se, sensitivity; Sp, specificity. *Compared with GIVPR.*

of which have been evaluated in multiple studies. However, how their sensitivity and accuracy are affected by various factors is still a matter of debate (24). By combining non-invasive indicators, the overall diagnostic coincidence rate can be improved.

In the present study, we assessed the relationships between serum parameters and non-invasive indices and liver fibrosis in CHB patients. GIVPR and GIVPTAR based on GPR all exhibited excellent capacities to predict the progression of liver fibrosis. However, GIVPTAR, which required more variables, did not obtain higher AUCs than GIVPR and did not improve the predictive performance for liver fibrosis. We also compared the predictive accuracy of GIVPR with APRI, FIB-4, and GPR. Our results showed that in both the training and validation cohorts,

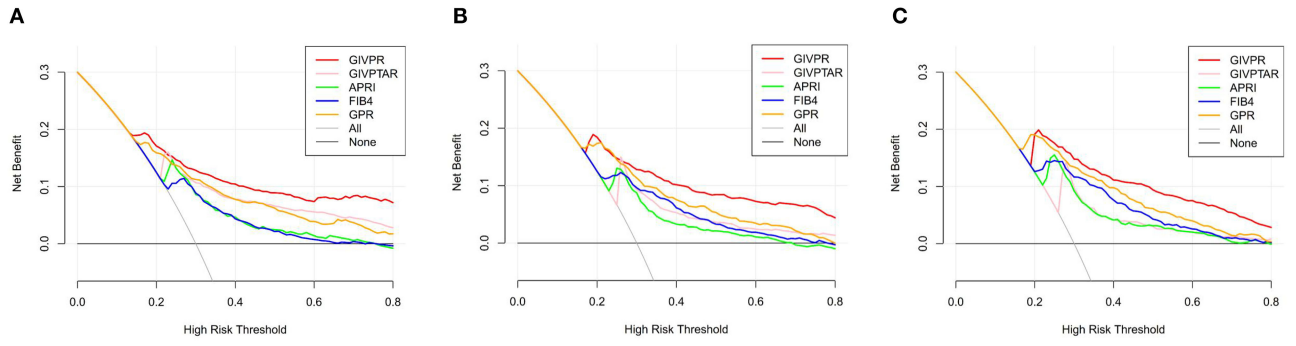

FIGURE 5 | Liver fibrosis decision curve analysis in training set. Decision curve analysis depict the clinical net benefit. GIVPR is compared with GIVPTAR, APRI, FIB-4, and GPR for predicting significant fibrosis **(A)**; GIVPR is compared with GIVPTAR, APRI, FIB-4, and GPR for predicting advanced fibrosis **(B)**; GIVPR is compared with GIVPTAR, APRI, FIB-4, and GPR for predicting cirrhosis **(C)**. Black line, net benefit when no patient will experience the event; gray line, net benefit when all patients will experience the event. The preferred markers is the marker with the highest net benefit at any given threshold.

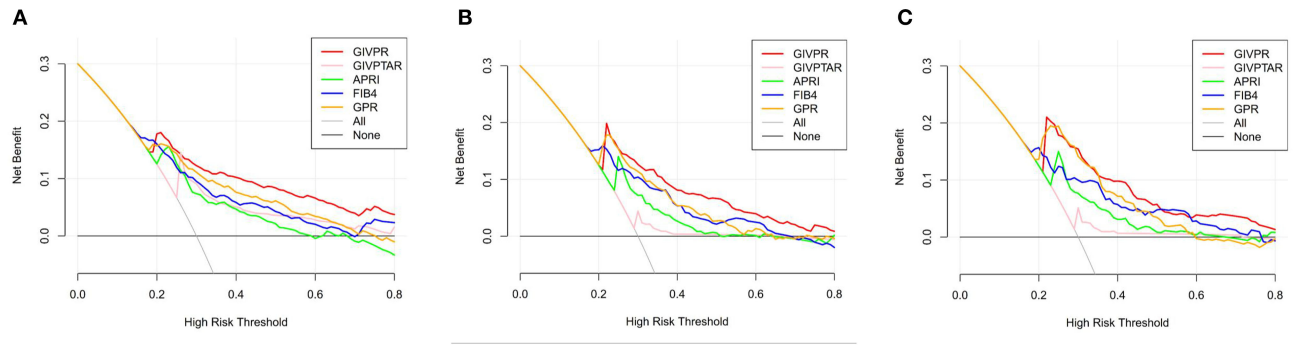

FIGURE 6 | Liver fibrosis decision curve analysis in validation set. Decision curve analysis depict the clinical net benefit. GIVPR is compared with GIVPTAR, APRI, FIB-4, and GPR for predicting significant fibrosis **(A)**; GIVPR is compared with GIVPTAR, APRI, FIB-4, and GPR for predicting advanced fibrosis **(B)**; GIVPR is compared with GIVPTAR, APRI, FIB-4, and GPR for predicting cirrhosis **(C)**. Black line, net benefit when no patient will experience the event; gray line, net benefit when all patients will experience the event. The preferred markers is the marker with the highest net benefit at any given threshold.

GIVPR had the best AUC value for staging significant fibrosis, advanced fibrosis, and cirrhosis. Thus, GIVPR, which requires only GPR, INR, and type IV collagen and is simple to calculate, has a more powerful predictive performance for liver fibrosis in CHB patients.

There were two kinds of serum biomarkers for liver fibrosis progression, indirect serum markers and direct serum markers (25). Indirect serum markers had no direct correlation with liver fibrosis but reflected liver dysfunction or other fibrosis-related symptoms. They are often calculated into mathematical formulas or may be used individually (26). APRI and FIB-4 are the two non-invasive procedures for evaluating liver fibrosis that receive the most attention. They were reported to have a high AUROC to detect significant fibrosis and cirrhosis in CHB patients in East Africa and Asia (27, 28). The WHO CHB guidelines also recommend APRI and FIB-4 for application in resource-limited health care regions (29). However, a meta-analysis suggested that their diagnostic performance was not good enough to discriminate liver fibrosis in CHB patients and could not be used as an ideal replacement for liver biopsy (30). GPR is a novel index to assess liver fibrosis in patients with CHB in West African cohorts. It was shown to be better than the classical models APRI and FIB-4 (10). Additionally, GPR was reported to diagnose significant liver fibrosis and cirrhosis well in a large cohort of HBV monoinfected Gambian patients using FibroScan measures as a reference (31). However, GPR showed a less clear advantage in a Brazilian cohort and other Chinese cohorts (13, 32). In this study, our GIVPR model showed acceptable distinguishing power for the prediction of significant live fibrosis, advanced liver fibrosis, and cirrhosis in the training set, with AUCs of 0.797, 0.815, and 0.844, respectively; similar results were obtained in the validation set. Furthermore, we confirmed significantly better performance for the assessment of liver histological scores compared to the biochemical marker panels APRI, FIB-4, and GPR. Due to the different inflammatory and clinical conditions of patients with chronic hepatitis B and chronic hepatitis C, the effect of etiology on fibrosis progression and clinical biomarkers can explain this result (33, 34).

Moreover, the indirect serum markers evaluated in this study included the measurement of coagulation parameters,

which were found to increase with the progression of liver fibrosis. Among these routine markers, INR was identified as an independent factor for the prediction of significant fibrosis and cirrhosis in CHB patients. Sterling et al. (21) reported that the INR was an independent predictor of liver fibrosis, and its concentration was directly related to liver function. Another study demonstrated that the INR level was associated with liver fibrosis and used INR as a parameter in their King's score, which was closely related to the progression of liver fibrosis (35, 36).

Direct biomarkers of liver fibrosis are fragments of liver matrix components produced in the process of fibrosis. These markers represent the intensity of fibrogenesis or fibrinolysis, such as type IV collagen, laminin, hyaluronic acid and metalloproteinases (37). Serum collagen levels, especially type IV, have been shown to be a useful, non-invasive measure of the activity of this pathway at a single time point and have been shown to reflect prognosis and responses to a variety of chronic liver diseases (14). Type IV collagen is an important component of the normal extracellular matrix. Compared with type I and type III collagen, which are partially hydrolyzed, type IV collagen remains intact in the matrix; therefore, the serum composition of type IV collagen is considered to mainly reflect the degradation of the matrix (38). Serum type IV collagen has been confirmed to be associated with both the progression of liver inflammation and fibrosis, which is in line with our data (26, 39).

This study has several limitations worth considering. First, this was a retrospective study in a single center and should be further confirmed in more patients from other centers. Second, GIVPR was not dynamically observed. We recommend further investigation into the efficacy of GIVPR compared to other non-invasive indices in evaluating fibrosis progression and in predicting liver-related end-stage disease after long-term antiviral inhibition of HBV.

CONCLUSION

In summary, a novel non-invasive calculation, GIVPR, was established from GPR, INR, and type IV collagen. GIVPR demonstrates superior diagnostic accuracy and clinical usefulness compared to conventional serum indices. Although the clinical usefulness of GIVPR warrants future investigation, our findings showing that GIVPR is non-invasive and easily administered indicate that it could be a promising tool for the discrimination of liver fibrosis, especially in resource-limited regions.

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 study protocol and informed consent documents were reviewed and approved by the Ethics Committee of Shanghai Public Health Clinical Center, Fudan University. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

RD designed the study and wrote the manuscript. WL, XZ, and DH collected and analyzed data. YW and LY reviewed the statistical data. XL and WL were involved in critical revision of the paper. SS, ZZ, and LC approved the final manuscript. All authors have read and approved the manuscript.

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Hepatitis E in 24 Chinese Cities, 2008–2018: A New Analysis Method for the Disease's Occupational Characteristics

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Background: The disease burden of hepatitis E remains high. We used a new method (richness, diversity, evenness, and similarity analyses) to classify cities according to the occupational classification of hepatitis E patients across regions in China and compared the results of cluster analysis.

Methods: Data on reported hepatitis E cases from 2008 to 2018 were collected from 24 cities (9 in Jilin Province, 13 in Jiangsu Province, Xiamen City, and Chuxiong Yi Autonomous Prefecture). Traditional statistical methods were used to describe the epidemiological characteristics of hepatitis E patients, while the new method and cluster analysis were used to classify the cities by analyzing the occupational composition across regions.

Results: The prevalence of hepatitis E in eastern China (Jiangsu Province) was similar to that in the south (Xiamen City) and southwest of China (Chuxiong Yi Autonomous Prefecture), but higher than that in the north (Jilin Province). The age of hepatitis E patients was concentrated between 41 and 60 years, and the sex ratio ranged from 1:1.6 to 1:3.4. Farming was the most highly prevalent occupation; other sub-prevalent occupations included retirement, housework and unemployment. The incidence of occupations among migrant workers, medical staff, teachers, and students was moderate. There were several occupational types with few or no records, such as catering industry, caregivers and babysitters, diaspora children, childcare, herders, and fishing (boat) people. The occupational similarity of hepatitis E was high among economically developed cities, such as Nanjing, Wuxi, Baicheng, and Xiamen, while the similarity was small among cities with large economic disparities, such as Nanjing and Chuxiong Yi Autonomous Prefecture. A comparison of the classification results revealed more similarities and some differences when using these two methods.

Conclusion: In China, the factors with the greatest influence on the prevalence of hepatitis E are living in the south, farming as an occupation, being middle-aged or

elderly, and being male. The 24 cities we studied were highly diverse and moderately similar in terms of the occupational distribution of patients with hepatitis E. We confirmed the validity of the new method on in classifying cities according to their occupational composition by comparing it with the clustering method.

Keywords: HEV, occupational classification, new method, cluster analysis, diversity, similarity

INTRODUCTION

Hepatitis E virus (HEV) infection is a great economic burden on Chinese people. Data released by the Chinese Centre For Disease Control and Prevention show that from 2010 to 2020, the proportion of HEV cases among all infectious diseases was one in one thousand (1), especially in rural areas (2). The results of a health economics study in Jiangsu Province showed that the total economic burden of HEV cases accounted for 60.77% of the per capita disposable income (3). Genotypes 1 and 2 are responsible for the majority of acute viral hepatitis infections in endemic areas in South Asia (4), which are limited to humans and non-human primates and have been found in areas with frequent water contamination via fecal-oral transmission, mostly in developing countries with limited access to sanitation. Genotypes 3 and 4 are related to zoonotic diseases, being low-endemic in developed countries, and transmitted by eating infected animal meat or having close contact with animals (5). The World Health Organization (WHO) has set the global target to reduce new viral hepatitis infections by 90% and reduce deaths due to viral hepatitis by 65% by 2030 (6). Therefore, research on HEV has public health significance.

The relationship between occupation and HEV susceptibility remains unclear. Risky occupational populations are present. Studies in Moldova (7) and Cuba (8) and Accra, Ghana (9, 10) have shown that the detection rate of HEV antibodies in people with occupational exposures, such as pig workers, is higher than that in the general non-occupational population. In China, one study found certain occupations to be more at risk for being frequently exposed to pathogens, such as people working in the catering industry, livestock breeders, soldiers, field workers, college students, and migrant workers or business travellers in epidemic areas (11). Another epidemiological survey showed that additional occupational populations at risk are farmers, retirees, domestic workers, and unemployed people (12). Several studies on pig slaughtering and sales workers in Zhejiang Province and Shanghai Municipality of China have confirmed this (13, 14). However, another study in Zhejiang Province showed that the infection rate of HEV among pig slaughterers and pet breeders is not different from that of the general population (15). Existing surveys have the limitations of small sample sizes and restricted survey areas. Therefore, our study aimed to investigate the occupational characteristics of HEV cases in 24 cities across 18 occupations based on the Infectious Disease Report Card of the People's Republic of China. Our objective was to better understand the differences in the occupational classification of HEV cases in China.

Cluster analysis has been proven to be an effective classification method when conducting age stratification of patients with diabetes (16), studying differences in the degree of environmental pollution (17), and recognizing temperature zones in China (18).

The new method (19) was also used for the classification. Previously, we used this new method to analyse the species composition and similarity of malaria vectors (20), and found that the sub-regions of Changsha City shared moderate diversity and high similarity for occupational distribution of hand, foot, and mouth disease (21). The new method supplements the traditional descriptive analysis method and contains six indicators, including richness, diversity, evenness, and similarity analyses. Although the feasibility of this new method has been verified in the above studies, the effectiveness of this classification method has not been verified. In this paper, we compare the classification results of the new method with those of the clustering analysis by analyzing the HEV occupational incidence.

In summary, we aimed to investigate the characteristics of occupational distributions in interregional HEV patients. In addition, we hope to confirm the validity of the new method in classification analysis by comparing it with the clustering analysis.

METHODS

Study Design

This study is divided into four sections. The first section provides a brief overview of HEV epidemiological characteristics in terms of temporal, regional, age, gender, and occupational distributions of reported HEV cases. In the second section, the new method is used for richness, diversity, evenness, and similarity analyses. The results of the cluster analysis are presented in the third section. The final section analyzes the results of classifying cities using the new method and cluster analysis. A research flow diagram is shown in **Figure 1**.

Study Areas

We selected 24 cities in northern, eastern, southern, and southwestern China as the study area (**Figure 2**). To compare the difference among occupations of patients with HEV between the north and the south, we used data from Jilin Province in the north of mainland and Jiangsu Province in the south of mainland. Furthermore, we compared the differences within provinces; therefore, we separately compared and analyzed the occupational incidence of HEV infection in nine cities in Jilin Province and 13 cities in Jiangsu Province. In addition, to compare the differences among different cities, we added the data from Xiamen City of

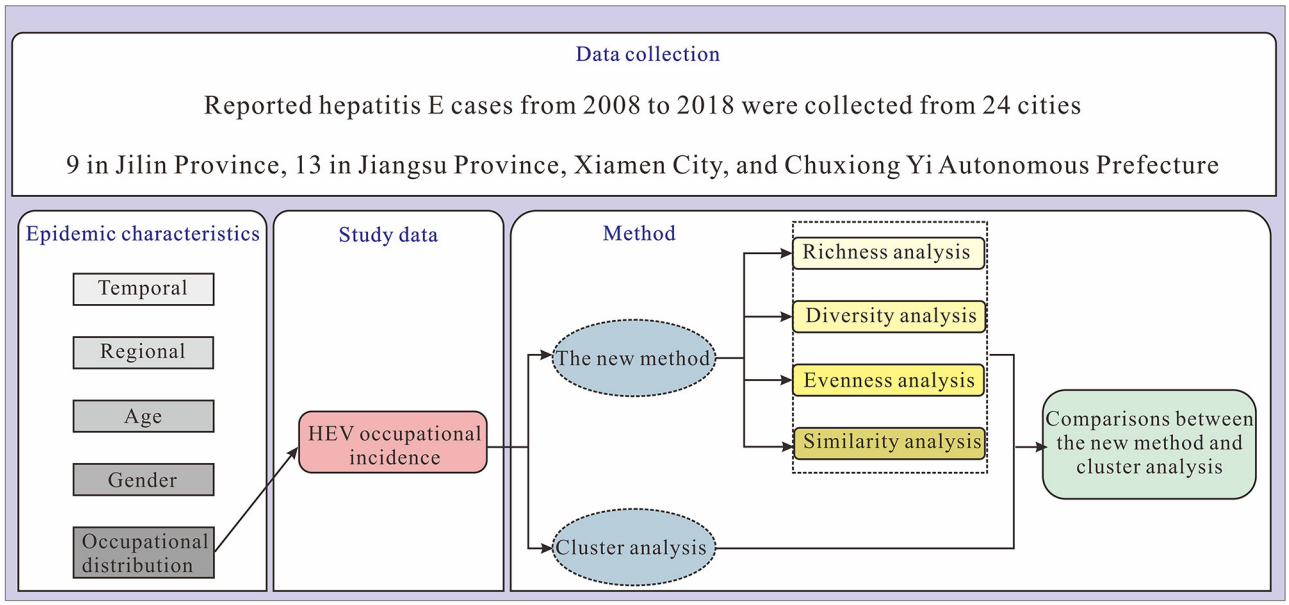

FIGURE 1 | Research design flow diagram.

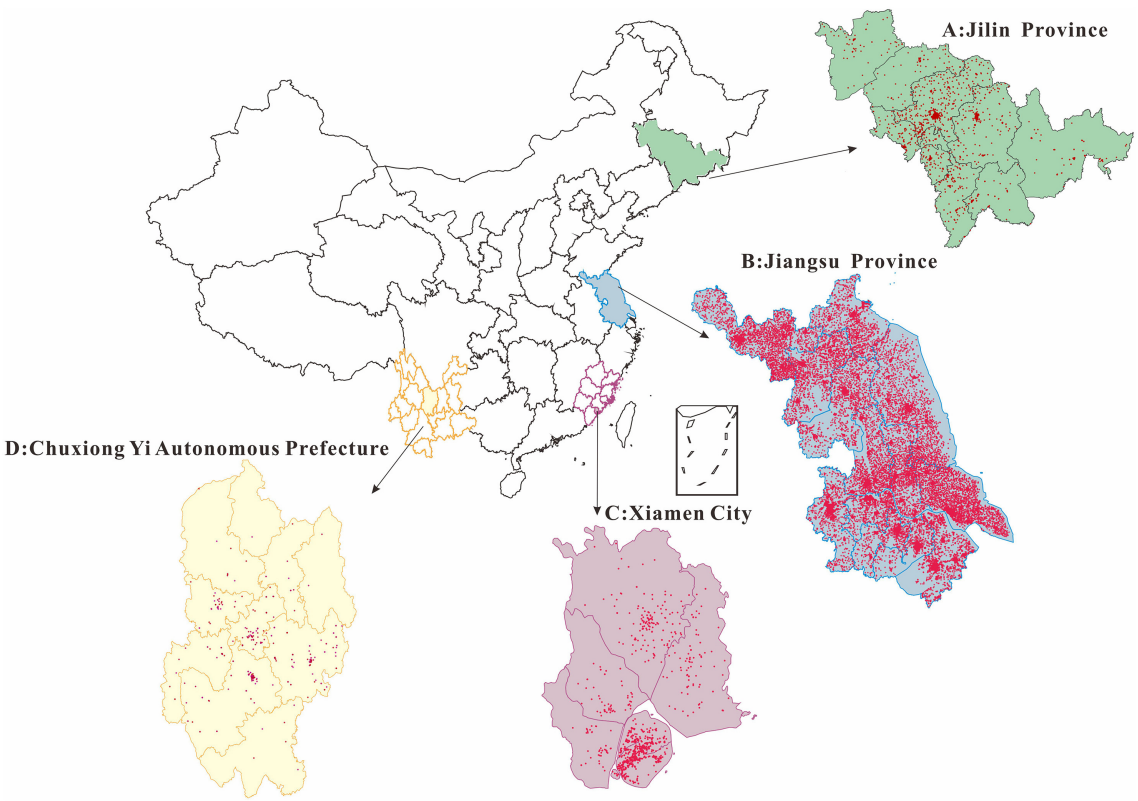

FIGURE 2 | Punctuation map of incidence in our study areas (2008–2018). **(A)** Jilin Province (in the north of China). **(B)** Jiangsu Province (in the east of China). **(C)** Xiamen City (in the south of China). **(D)** Chuxiong Yi Autonomous Prefecture (in the southwest of China). The map depicted in this figure was taken from Wikimedia Commons (http://commons.wikimedia.org/wiki/Main_Page).

Fujian Province and Chuxiong Yi Autonomous Prefecture of Yunnan Province to those of the 22 cities under Jilin and Jiangsu Provinces for comparative analysis.

Jilin Province (40°52–46°18′*N*, 121°38–131°19′*E*, northern) has jurisdiction over nine prefecture-level administrative regions, including eight prefecture-level cities and one autonomous prefecture (Changchun City, Jilin City, Siping City, Liaoyuan City, Tonghua City, Baishan City, Songyuan City, Baicheng City, and Yanbian Korean Autonomous Prefecture).

Jiangsu Province (30°45–35°20′*N*, 116°18–121°57′*E*, eastern) governs 13 prefecture-level administrative regions (The southern part: Nanjing City, Suzhou City, Wuxi City, Changzhou City, Zhenjiang City; The central part: Yangzhou City, Taizhou City, Nantong City; The northern part: Xuzhou City, Huaian City, Suqian City, Lianyungang City, Yancheng City).

Xiamen City (24°26′*N*, 118°04′*E*, southern), with a total area of 170,061 square kilometers, is located at the southeast end of Fujian Province.

Chuxiong Yi Autonomous Prefecture (24°13–26°30′*N*, 100°43–102°30′*E*, southwestern) is located in the middle of Yunnan Province, with a total area of 29,000 square kilometers.

Data Collection

A dataset of symptomatic cases of HEV reported in 24 cities from 2008 to 2018 was created, including date of onset and type of occupation, age, sex, current address, disease classification, and excluding disease severity. In China, hepatitis B and C cases are classified as acute or chronic. However, hepatitis E patients were all acute, and the disease classification column of our dataset was unclassified, which actually refers to acute hepatitis E. Disease data were obtained from the Centre for Disease Control and Prevention of Jilin Province, Jiangsu Province, Xiamen City, and Chuxiong Yi Autonomous Prefecture separately. Demographic data for the 24 cities were obtained from the National Statistics Bureau.

Occupational classification was based on the Infectious Disease Report Card of the People's Republic of China stipulated in the Law of the People's Republic of China on prevention and control of infectious diseases (22), which came into force on December 1, 2004. The 18 occupations were classified as childcare (for kindergarten children), diaspora children (for children raised at home, who have not yet been to school), students (including students in primary, secondary, and high school or in college), teachers, caregivers and babysitters, catering industry, business services, medical staff, workers, migrant workers (farmers working outside of their town of origin), farmers, herders, fishing (boat) people, cadre staff, retiree, housework and unemployment, others, and unknown.

Diagnostic Criteria

According to the "Code of Practice for the Treatment of Viral Hepatitis E" formulated by the Infectious Disease Physicians Branch of the Chinese Medical Association in July 2009 (23), a comprehensive diagnosis was made based on the epidemiological history, symptoms, signs, and laboratory examination results. Diagnostic criteria for acute HEV infection include positive anti-HEV IgM, 4-fold or greater increase in anti-HEV IgG positive conversion or content, and positive serum and/or fecal HEV RNA. In general, the positive results of any of these three indicators can be used as the basis for the clinical diagnosis of acute HEV infection, and the diagnosis can be confirmed if multiple indicators have positive results. Anti-HEV IgM detection reagents developed at home and abroad relying on conformational antigens have been developed (24), and anti-HEV IgM has become the most important diagnostic indicator of acute HEV infection in clinical practice; IgM antibody positivity and IgG antibody positivity can generally be diagnosed.

Statistical Methods

First, the traditional descriptive epidemiological method was adopted to analyze the temporal and regional, age and gender, and occupational distributions of the reported cases. Second, the new method (19) was used to describe the similarity and diversity of HEV, including six indices: richness index (*N*), Simpson diversity index (*D*), Shannon diversity index (*H*), Berger–Parker dominance index (*d*), Shannon evenness index (*E*), and Morisita–Horn similarity index (*C*). *N* represents the number of occupational classifications. The p_i refers to the proportion of the *ith* classification, and the maximum of p_i is the index *d*, which measures occupational dominance. Occupational diversity and evenness were evaluated using three indices: *D*, *H*, and *E*. If *D* is closer to 1 or *H* is larger, the diversity will be greater. The closer *E* is to 0.5, the better the equitability. The larger *d* is, the stronger the dominant occupation. Similarities among the different study areas was measured using index *C*. The closer *C* is to 1, the greater the similarity. The indices *D* and *H* were calculated from the proportion of each occupation; *E* was calculated by dividing *H* by the richness index, and *C* was calculated by the number of individuals in each occupation and the total number of populations by region. These indices are represented by the following equations:

$$D = 1 - \sum_{n=1}^{N} p_i^2$$

$$H = 1 \sum_{n=1}^{N} p_i \ln p_i$$

$$E = \frac{H}{\ln N}$$

$$C = \frac{2 \sum n_{1i} n_{2i}}{(\lambda_1 + \lambda_2) M_1 M_2}, \lambda_i = \frac{\sum n_{ji}^2}{M_j^2}$$

Third, we used the cluster analysis method (16), which is a multivariate statistical analysis method. The between-group linkage method was used to calculate the distance between classes. By comparing the properties of various samples, those with similar properties are classified into one category, and those with different properties are divided into different categories (25). The clustering method regards *N* samples as *N* classes at the beginning, and then merges them step by step until *N* samples are merged into one class. In this study, each of the 24 study cities was regarded as a sample, and clustering was carried out according to the incidence of 18 occupational reports (for example, farmers, students, herders) of patients with HEV from 2008 to 2018.

Microsoft Excel 2019 software (Microsoft Corp, USA) was used for data entry, sorting, drawing, and calculation of the six indices. The data were analyzed using IBM SPSS Statistics for Windows, version 26.0 (IBM Corp., Armonk, N.Y., USA) for *Q*-type clustering analysis. The statistically significant level was set at $P < 0.05$. DataMap 6.2 software (Microsoft Corp, USA), was used to create punctuation maps.

RESULTS

Distributions of Traditional Descriptive Epidemiological Method

Temporal and Regional Distributions of Reported HEV Cases

Figure 3 depicts the incidence of HEV infection in 24 cities from 2008 to 2018. The incidence of HEV infection in northern China (Jilin Province) was similar to that in western China (Chuxiong Yi Autonomous Prefecture) and southern China (Xiamen City) but was lower than that in southern China (Jiangsu Province).

We found that the dynamics of prevalence varied within the provinces. In Jilin Province, the incidence of HEV in the four cities increased, while that in the other five cities showed an annual decreasing trend. In Jiangsu Province, with the exception of the incidence of HEV in the two cities that were on the rise, the other 11 cities showed a downward trend. The incidence in Xiamen City, a coastal city, has been increasing annually, while the incidence in Chuxiong Yi Autonomous Prefecture, an inland city, is decreasing.

Age and Gender Distributions of Reported Incidence of Patients With HEV

According to the radar map of age distribution in the 24 cities (**Figure 4**), the majority of patients with HEV were in the 41–50 and 51–60 age ranges. The proportions for the 60–70 years and the 31–40 years age groups were medium. The proportion of those over 70 years of age and under the age of 20 years was lower.

In terms of sex, there were significantly more males than females (**Figure 4**), and the sex ratio ranged from 1.6 to 3.4, with the highest sex ratio of 3.4, in Suqian City, Jiangsu Province, and the lowest sex ratio of 1.6, in Suzhou City, Jiangsu Province.

Occupational Distribution of Reported HEV Cases

Table 1 shows the percentage of cumulative HEV cases in 18 occupations. In summary, farmers accounted for the largest proportion of occupation types among all cities, followed by housework and unemployment, retirees, and workers. The top two occupational types among HEV cases in Jilin Province were farmers and retirees. Among the 13 cities in Jiangsu Province, farmers were the highest occupational type, followed by houseworkers and the unemployed, retirees and workers. We found that the main occupation types in Xiamen City were the other types (not the above-mentioned 17 types) and farmers. Farmers and retirees accounted for the highest proportions in the Chuxiong Yi Autonomous Prefecture of Yunnan Province. For 24 cities, we observed few or no records of caregivers and babysitters among the patients, and few cases were seen among diaspora children, childcares, herders, and fishing (boat) groups.

The Results of the New Method on Occupational Types in the 24 Cities

The Richness Analysis by the New Method Using Index N

The *N*-value was highest in Xuzhou City ($N = 17$) and Lianyungang City ($N = 17$) of Jiangsu Province and was the lowest in Suzhou City ($N = 2$) and Nantong City ($N = 2$) of Jiangsu Province. There were more than 10 occupational types among patients with HEV in other cities, such as Jilin City ($N = 16$) in Jilin Province, Xiamen City ($N = 14$), and Chuxiong Yi Autonomous Prefecture ($N = 14$; **Table 2**).

The Diversity Analysis by the New Method Using Index D and H

In Jilin Province, the Simpson diversity index ($D = 0.805$) and Shannon diversity index ($H = 1.887$) of Liaoyuan City were the highest. In Jiangsu Province, the two indices were the highest in Nanjing City ($D = 0.921$, $H = 1.726$) and the lowest in Nantong City ($D = 0.001$, $H = 0.002$). In Xiamen City, Fujian Province, the Simpson diversity index ($D = 0.991$) was close to 1, while the Shannon diversity index ($H = 0.929$) was high. The Simpson diversity index ($D = 0.993$) of Chuxiong Yi Autonomous Prefecture in Yunnan Province was close to 1, while the Shannon diversity index ($H = 0.379$) was low (**Table 2**).

The Evenness Analysis by the New Method Using Index E and d

The evenness index of most cities in Jiangsu Province was close to 0.5, for example, Lianyungang City ($E = 0.497$), Zhenjiang City ($E = 0.508$), and Taizhou city ($E = 0.484$). However, the *E*-values of Nantong City ($E = 0.004$) and Suzhou City ($E = 0.179$) were the opposite. All nine cities in Jilin Province ($E = 0.088$–0.697) were away from 0.5. Similarly, the same was true for Xiamen City ($E = 0.352$) and Chuxiong Yi Autonomous Prefecture ($E = 0.144$; **Table 2**).

The farmer group was the occupational type with the largest Berger-Parker dominance index *d* in most cities, such as Siping City, Suzhou City, and Nantong City ($d = 0.999$–1.000). However, the *d*-values of Baicheng City ($d = 0.255$) and Nanjing City ($d = 0.255$) were much lower.

The similarity analysis by the new method using index *C* **Table 3** shows the similarity coefficient matrix of HEV infection among the 24 cities from 2008 to 2018. In Jilin Province, Baicheng City and Changchun City have the highest similarity ($C = 0.990$), while Baishan City and Songyuan City have the lowest *C*-values ($C = 0.511$). Except for the above cases, the similarity coefficients among the cities in Jilin Province were higher than 0.8. The similarity coefficient between cities in Jilin Province and Chuxiong Yi Autonomous Prefecture was moderate ($C = 0.506$–0.901), and the similarity with Xiamen City was a little higher ($C = 0.800$–0.905). The similarity of occupational composition of HEV cases between the remaining 11 cities in Jiangsu Province was largely above 0.9, except for Nantong City and Suzhou City. Suzhou and Nantong Cities had the lowest similarity with most cities in Jiangsu Province ($C < 0.4$). The cities of Nanjing City and Wuxi City in Jiangsu Province had higher similarity coefficients with cities of Jilin Province ($C = 0.8497$–0.960), and Xiamen

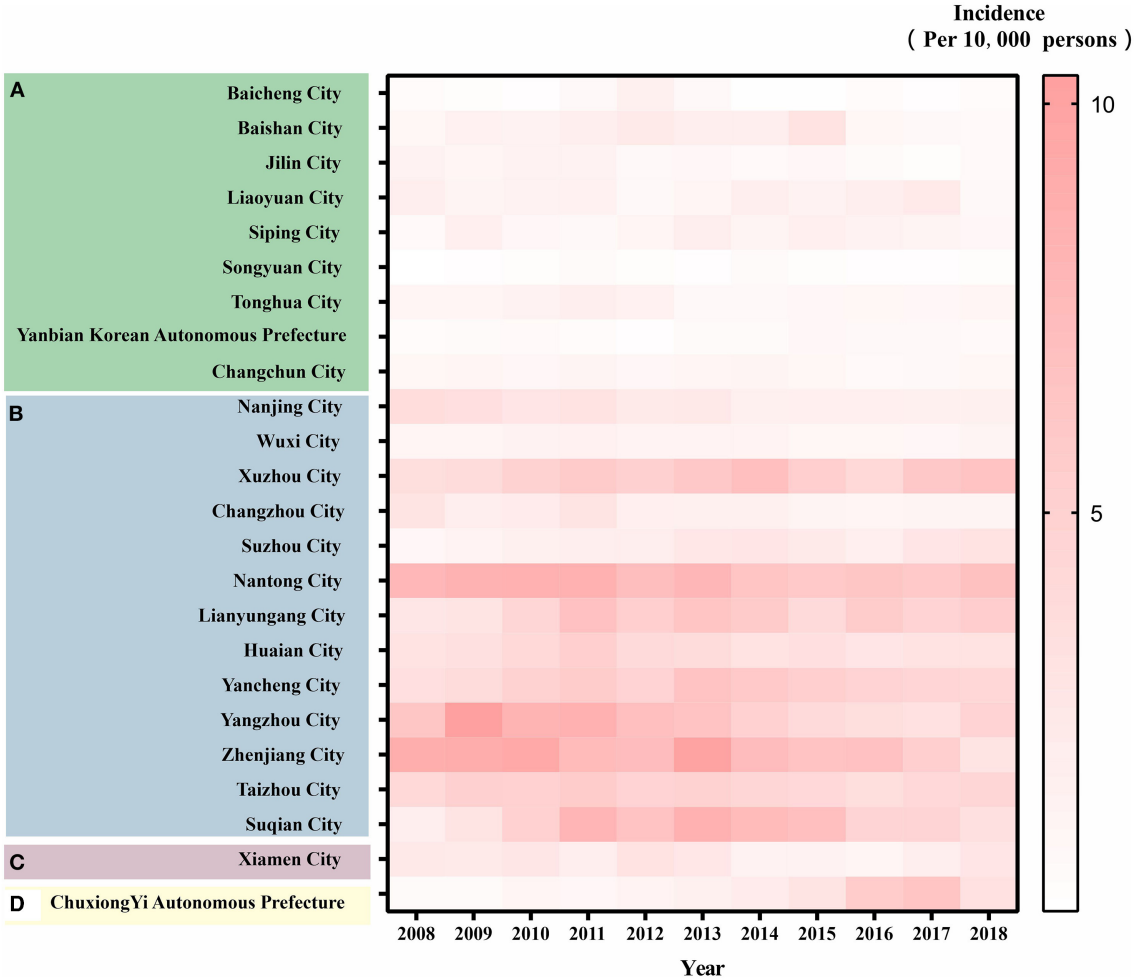

FIGURE 3 | Heat map of reported incidence of HEV cases in 24 cities (2008-2018). **(A)** Jilin Province (in the north of China). **(B)** Jiangsu Province (in the east of China). **(C)** Xiamen City (in the south of China). **(D)** Chuxiong Yi Autonomous Prefecture (in the southwest of China). The following is the same.

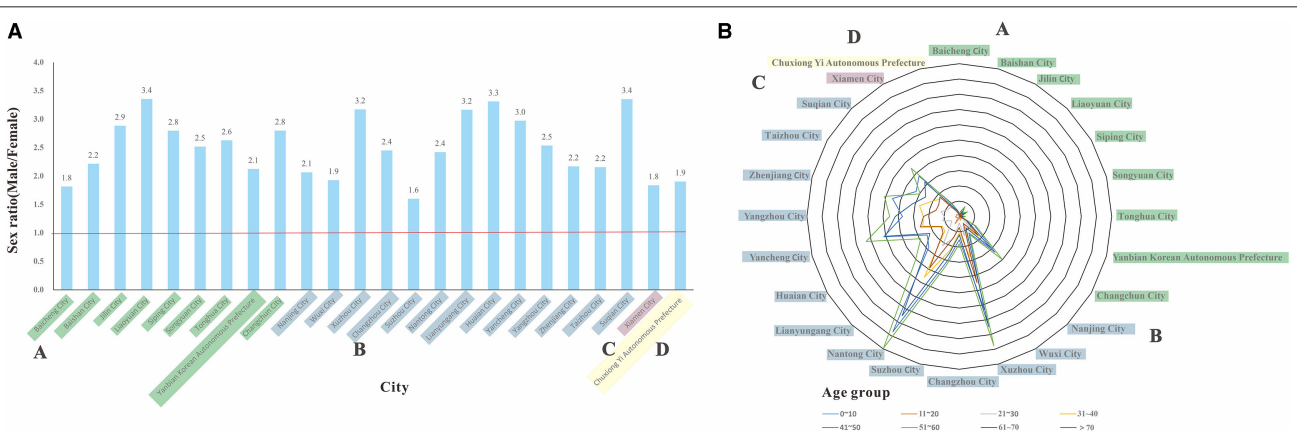

FIGURE 4 | Sex and age distribution of reported HEV cases in 24 cities (2008-2018). **(A)** sex ratio distribution of the study regions. **(B)** distribution of HEV cases across the study regions by age groups.

TABLE 1 | Occupational distribution of reported HEV cases in 24 cities (2008–2018).

City	Baicheng City	Baishan City	Jilin City	Liaoyuan City	Siping City	Songyuan City	Tonghua City	Yanbian Korean Autonomous Prefecture
Occupation								
Business services	4	3	12	1	3	2	7	3
Cadre staff	7	13	27	14	11	5	13	14
Caregiver and babysitter	0	0	0	0	0	1	1	0
Catering industry	0	1	2	3	1	1	1	0
Childcare	0	0	0	0	0	0	0	0
Diaspora children	0	0	1	1	0	0	0	0
Farmer	39	28	158	48	233	60	115	23
Fishing (boat) people	0	0	0	0	0	0	0	0
Herder	0	0	1	1	1	0	0	0
Housework and unemployment	26	61	76	66	90	10	53	53
Medical staffs	1	2	2	2	3	0	0	1
Migrant workers	0	1	5	2	2	1	2	1
Others	8	17	3	11	10	11	18	22
Retired	21	39	82	26	33	6	21	16
Student	1	1	4	1	1	1	4	2
Teacher	0	6	6	2	0	0	3	2
Unknown	5	28	5	2	60	9	23	3
Worker	9	26	52	29	15	2	29	13

City of Fujian Province (C = 0.892–0.924). The occupational distribution among the 11 cities in Jiangsu Province was similar to that of Chuxiong Yi Autonomous Prefecture ($C > 0.9$). The similarity index values between Xiamen City and Chuxiong Yi Autonomous Prefecture differed significantly ($C = 0.455$).

The Results of Cluster Analysis on Occupational Types in the 24 Cities

From the clustering result chart of **Figure 5**, when the cities were divided into two categories, Nantong City was in its own group, while the other 23 cities were in another group.

When the cities were divided into three categories, Nantong City was still divided into a separate group, nine cities in Jilin Province, Wuxi City, Nanjing City, Changzhou City, Xiamen City, and Chuxiong Yi Autonomous Prefecture were grouped together, and the remaining nine cities, including Yancheng City and Xuzhou City in Jiangsu Province, were grouped together.

When the cities were divided into four categories, the results were consistent with those of when they were divided into three categories; the only difference was that Baicheng City was classified as a separate group.

The Comparisons of Results Between New Method and Cluster Analysis

Most of the cities in Jilin province were close in similarity and diversity and were classified into the same group. The similarity coefficients between Nanjing and Baishan, Jilin, Liaoyuan, and Changchun ($C > 0.8$), and Nanjing and Xiamen ($C = 0.924$) are similar, which is consistent with the results of the cluster analysis.

In the cluster analysis, Baishan City and Songyuan City, which do not have high diversity and similarity, were nevertheless placed in the same category, and it was the same for Xiamen City and Chuxiong Yi Autonomous Prefecture. The similarity index between Xiamen, a coastal city, and Chuxiong Yi Autonomous Prefecture, an inland region, was not high ($C = 0.455$), yet they were grouped together in the clustering. Nantong city and Suzhou city had the highest similarity ($C = 1.000$); however, the cluster analysis did not provide enough information about why these two cities were not grouped into the same category. Similarly, 11 cities in Jiangsu Province have similar occupational distribution ($C > 0.9$) to Chuxiong Yi Autonomous Prefecture, yet Chuxiong Yi Autonomous Prefecture is grouped with three other cities in Jiangsu Province, namely Nanjing, Wuxi, and Changzhou, by cluster analysis.

DISCUSSION

Epidemiological Characteristics Analysis

The incidence of HEV infection varied among the 24 cities in the four regions. One study (12) also confirmed that the incidence of HEV infection was lower in the central (Jilin Province) and western (Chuxiong Yi Autonomous Prefecture) regions than in the eastern region (Jiangsu Province, Xiamen City) in China from 2004 to 2017. There are more river systems and frequent floods in the southern region, which may contribute to transmission via dirty water. In addition, it may also be related to the improvement of surveillance levels, the popularization of diagnostic reagents (26) and HEV mutation (27) in southern China, where economic and demographic structures are more

TABLE 2 | Analysis of richness, diversity and uniformity of 24 cities (2008–2018).

Year	N	Diversity index		d	E
		D	H		
Baicheng City	10	1.000	0.203	0.255	0.088
Baishan City	12	0.993	0.750	0.304	0.302
Jilin City	16	0.999	0.312	0.665	0.113
Liaoyuan City	15	0.805	1.887	0.462	0.697
Siping City	13	0.992	0.522	0.999	0.203
Songyuan City	12	1.000	0.085	1.000	0.034
Tonghua City	13	0.998	0.432	0.588	0.169
Yanbian Korean Autonomous Prefecture	12	0.999	0.321	0.767	0.129
Changchun City	12	0.993	0.679	0.734	0.273
Nanjing City	15	0.921	1.726	0.255	0.637
Wuxi City	14	0.887	1.758	0.304	0.666
Xuzhou City	17	0.653	1.063	0.665	0.375
Changzhou City	16	0.732	1.728	0.462	0.623
Suzhou City	2	0.235	0.124	0.999	0.179
Nantong City	2	0.001	0.002	1.000	0.004
Lianyungang City	17	0.702	1.408	0.588	0.497
Huaian City	16	0.541	1.061	0.767	0.383
Yancheng City	16	0.595	1.104	0.734	0.398
Yangzhou City	16	0.695	1.274	0.646	0.459
Zhenjiang City	16	0.866	1.408	0.448	0.508
Taizhou City	16	0.669	1.343	0.633	0.484
Suqian City	14	0.472	0.974	0.780	0.369
Xiamen City	14	0.991	0.929	0.780	0.352
Chuxiong Yi Autonomous Prefecture	14	0.993	0.379	0.780	0.144

complex. The high incidence in middle-aged and elderly people may be associated with the natural history of the disease. This is consistent with the finding that population antibody levels increased with age (28, 29). According to the WHO report, the infection rate in children is low, and the affected population is mainly adults (30). The majority of HEV patients were male, as expected, since males had fewer chores than women; thus, they had less exposure to dirty water and animals.

We found that farmers accounted for a large proportion of patients. This is consistent with the results of several epidemiological studies (12, 31). Farmers are easily exposed to contaminated water and are in close contact with animals for living in rural areas. HEV contamination of pig manure and water sources can be accompanied by potential transmission of contaminated agricultural or seafood to humans through the food chain. Recently, a systematic review identified that living in rural areas is a risk factors for anti-HEV IgG positivity (32). Housework and unemployed people are also exposed to animal viscera, and sewage during cooking, so a significant number of groups could be infected. Retirees and elderly individuals are easily infected because of their poor status and immunity (33).

Several occupational incidences are at an intermediate level for balancing occupational exposure and hygiene prevention Compared with farmers, migrant workers live in cities and have less direct contact with animals. Medical staff, teachers, and students have a relatively small incidence of infections due to the implementation of disinfection measures at hospitals and schools. The routes of environment-to-human and animal-to-human transmissions are difficult to achieve.

We found that there were few or no records of several occupational types. For people in the catering industry, caregivers, and babysitters, they undergo health examinations by the local Centre for Disease Control and Prevention before entering their work, which prevents the spread of HEV to some extent. We did not observe any diaspora children or childcare group since they are not as susceptible as adults and have access to the meticulous care; herder is rare occupation, and there may exist many unreported cases. The fishing (boat) group is not susceptible to HEV, indicating that being exposed to seafood is not as contagious as other animals.

Analysis of HEV Occupational Incidence in the 24 Cities by the New Method

The occupational distribution of HEV cases in Jiangsu Province was more balanced than in the other three regions. Unlike most cities, Baicheng and Nanjing cities have developed economies

TABLE 3 | Matrix of occupational similarity in the number of cases of hepatitis E in 24 cities (2008–2018).

Year	Baicheng	Baishan	Jilin	Liaoyuan	Siping	Songyuan	Tonghua	Yanbian	Changchun	Nanjing	Wuxi	Xuzhou	Changzhou	Suzhou	Nantong	Lianyungang	Huaian	Yancheng	Yangzhou	Zhenjiang	Taizhou	Suqian	Xiamen	Chuxiong
Baicheng City	1.000																							
Baishan City	0.852	1.000																						
Jilin City	0.975	0.775	1.000																					
Liaoyuan City	0.929	0.914	0.891	1.000																				
Siping City	0.888	0.636	0.891	0.762	1.000																			
Songyuan City	0.834	0.511	0.846	0.658	0.658	1.000																		
Tonghua City	0.953	0.747	0.947	0.865	0.865	0.926	1.000																	
Yanbian Korean Autonomous Prefecture	0.846	0.916	0.748	0.948	0.948	0.543	0.543	1.000																
Changchun City	0.990	0.834	0.969	0.910	0.910	0.825	0.825	0.834	1.000															
Nanjing City	0.903	0.886	0.877	0.827	0.675	0.627	0.781	0.768	0.921	1.000														
Wuxi City	0.940	0.810	0.961	0.901	0.821	0.781	0.929	0.768	0.947	0.885	1.000													
Xuzhou City	0.731	0.377	0.793	0.562	0.900	0.942	0.823	0.401	0.720	0.529	0.707	1.000												
Changzhou City	0.925	0.647	0.957	0.820	0.951	0.939	0.970	0.670	0.915	0.744	0.918	0.896	1.000											
Suzhou City	0.539	0.214	0.596	0.385	0.766	0.825	0.651	0.253	0.525	0.336	0.510	0.938	0.729	1.000										
Nantong City	0.539	0.214	0.596	0.384	0.765	0.825	0.651	0.252	0.525	0.335	0.509	0.937	0.729	1.000	1.000									
Lianyungang City	0.827	0.484	0.875	0.665	0.949	0.974	0.905	0.508	0.815	0.625	0.799	0.982	0.957	0.868	0.868	1.000								
Huaian City	0.692	0.338	0.749	0.523	0.886	0.935	0.798	0.374	0.680	0.476	0.662	0.996	0.868	0.959	0.959	0.968	1.000							
Yancheng City	0.708	0.359	0.763	0.538	0.903	0.947	0.818	0.385	0.693	0.493	0.682	0.996	0.881	0.949	0.949	0.975	0.998	1.000						
Yangzhou City	0.765	0.408	0.823	0.593	0.918	0.964	0.860	0.434	0.758	0.564	0.750	0.995	0.924	0.908	0.907	0.993	0.987	0.991	1.000					
Zhenjiang City	0.883	0.601	0.931	0.727	0.944	0.949	0.946	0.556	0.878	0.747	0.897	0.915	0.970	0.753	0.753	0.963	0.886	0.903	0.943	1.000				
Taizhou City	0.772	0.423	0.830	0.606	0.925	0.966	0.874	0.443	0.763	0.570	0.767	0.990	0.932	0.897	0.897	0.993	0.981	0.988	0.998	0.952	1.000			
Suqian City	0.667	0.319	0.721	0.498	0.877	0.928	0.781	0.353	0.653	0.449	0.637	0.989	0.848	0.969	0.969	0.956	0.998	0.997	0.979	0.870	0.973	1.000		
Xiamen City	0.809	0.838	0.701	0.764	0.633	0.620	0.757	0.822	0.822	0.829	0.763	0.432	0.651	0.288	0.288	0.533	0.409	0.428	0.480	0.622	0.493	0.396	1.000	
Chuxiong Yi Autonomous Prefecture	0.730	0.370	0.794	0.548	0.893	0.939	0.814	0.386	0.722	0.539	0.699	0.998	0.889	0.931	0.931	0.980	0.992	0.991	0.992	0.914	0.985	0.984	0.427	1.000

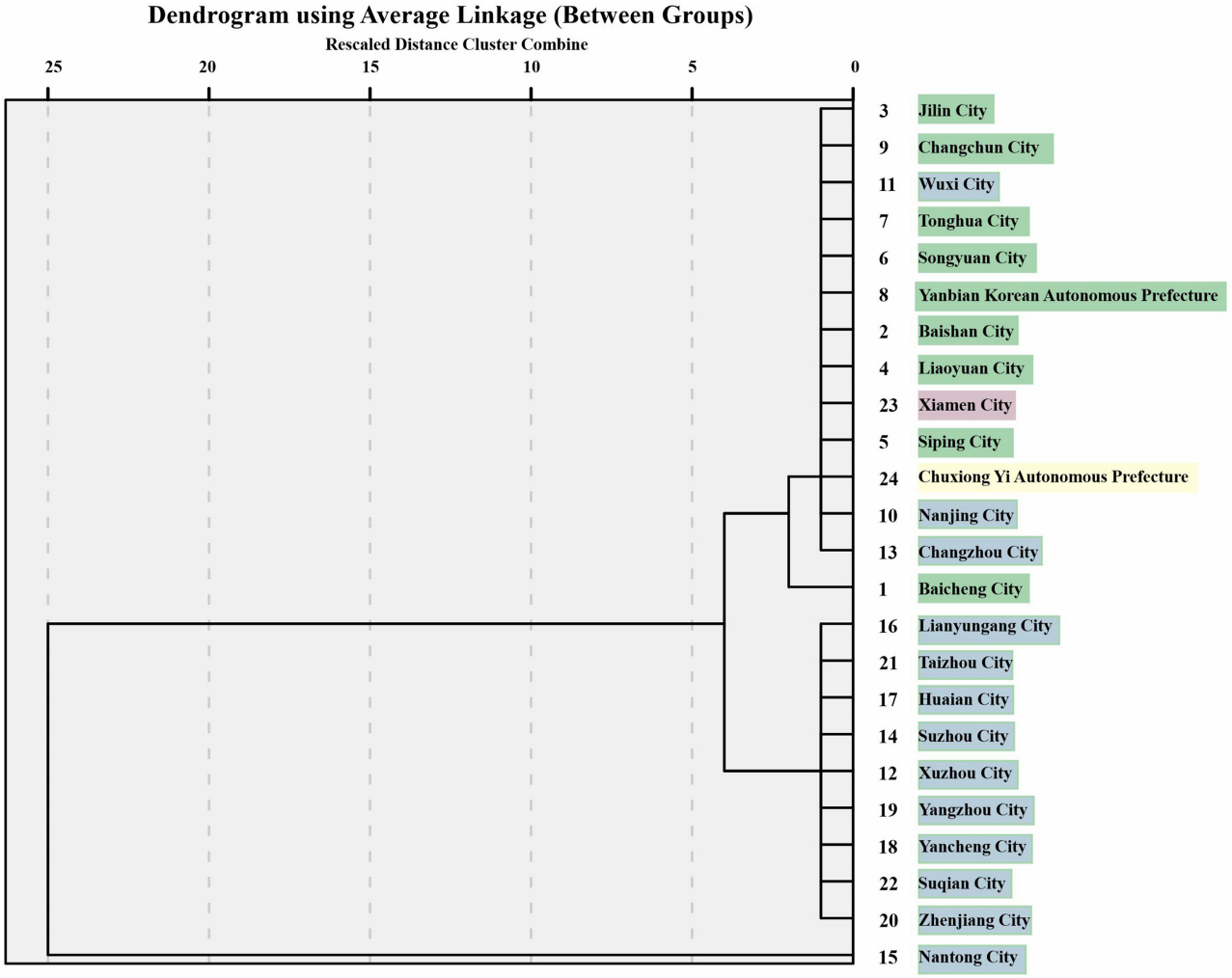

FIGURE 5 | Diagram of cluster analysis results of occupational incidences in 24 cities (2008-2018).

and large populations, so the dominant occupational type is not farmers. The relatively even distribution of HEV-affected occupations in Liaoyuan, Nanjing, and Xiamen cities may be due to demographic reasons, and the cities are richer in occupational types.

We believe that the economic environment is a key factor in determining occupational similarity. Nanjing and Wuxi, two cities located in the southern part of Jiangsu province, have a developed economy and fewer people are engaged in HEV-related high-risk occupations, such as agriculture, compared to other cities. Similarly, Xiamen has a well-developed economy and a high degree of similarity with the Nanjing and Wuxi cities. The less economically developed Chuxiong Yi Autonomous Prefecture had lower occupational similarity with Nanjing, Wuxi, and Xiamen cities. The high incidence in the 24 cities was concentrated in the central urban areas, where there are more employment opportunities, more frequent human contact, and therefore a greater potential for transmission.

As far as the differences in *N*-values within Jiangsu province, such as with only two occupations in the HEV-affected population in Suzhou and Nantong, we speculate that this may be related to inaccurate and under-reported disease reporting, as well as the uneven degree of development within the same province. The results showed that the occupational distribution among HEV cases was more diverse in Liaoyuan, Nanjing, and Xiamen cities. In contrast, the opposite was true for the Nantong and Chuxiong Yi autonomous prefectures.

The Cluster Analysis of HEV Occupational Incidence in the 24 Cities

When each city is divided into two categories, it shows the difference in the distribution of occupational morbidity. Nantong City is classified as a separate category, because the incidence

of hepatitis E in Nantong is entirely contributed by the peasant population. When divided into three categories, we can see the differences between cities within the same province. For example, three cities in Jiangsu Province were classified in one category with all cities in Jilin province, Xiamen city, and Chuxiong Yi Autonomous Prefecture, while the remaining 10 cities in Jiangsu Province were in another category. These cities all had similar levels of hepatitis E prevalence and similar occupational compositions of high and low prevalence.

When the cities were divided into four categories, we can also find small differences in cities within the same province. For example, the city of Baicheng in Jilin Province is separated from the other eight cities in the same province, probably because Baicheng has only 10 occupations for the HEV-affected population, while all other cities have 12 or more types.

From the results of the cluster analysis, we were able to classify the cities according to the incidence of the type of occupation, as well as with increasing grouping, we were able to find differences between cities within the same province.

Comparisons Between the New Method and Clustering Analysis

When analyzing the occupational composition of hepatitis E across regions and thus classifying cities, both methods based on the cluster analysis method and the new method yielded similar results in most cases and a few opposite results. We believe there may be several reasons for this. First, the principles of the two methods are different. The clustering method we used was analyzed in the form of defining the distance between classes, and the results obtained may be concise. The new method is a comprehensive analysis of diversity, balance, similarity, and other levels with the help of six major indicators to obtain rich results. Second, the new method does not establish a good connection between the values of each indicator and the specific criteria. For example, for the similarity coefficient *C*-value, we think that when the *C*-value is higher than 0.8, then the two cities are more similar; if the *C*-value is lower than 0.5, then the similarity is lower. Further research could focus on the criteria system to improve classification accuracy.

We confirmed the validity in cross-regional disease occupational composition analysis, which is an extension of the method from microbial level classification to population level. The method has good feasibility and applicability, and more detailed outcome indicators can be obtained.

Suggestions for Prevention and Control Measures

First, we need to strengthen health education on hepatitis E prevention and control for various occupational risk groups. Awareness of hepatitis E is significantly lower than that of hepatitis B and C, especially for key occupational groups, such as farmers engaged in livestock (pig) and poultry-related farming or slaughtering, as well as retirees, housework and unemployment groups.

Second, it is necessary to control the transmission of viral hepatitis by frequently testing HEV in workers of related occupations. For example, the rate of positive IgM antibodies to HEV can be used as a signal indicator.

Third, the main strategies to deal with HEV in China at this stage are the development of HEV vaccines and the improvement of laboratory diagnosis rates. One study considered the strategy of HEV vaccination in women of childbearing age (34), and public health professionals recommended promoting HEV vaccine in Shanghai (35). We believe there is a need to consider the strategy of HEV vaccination in high-risk occupational groups, such as farmers, to effectively reduce the disease burden.

LIMITATIONS

First, the regions we chose were not random, which may lead to deviations. This was a preliminary study. In the future, if possible, we will use the disease data from more regions for in-depth studies.

Second, because not all cases were genotyped in the laboratory, we could not include genotypes for a detailed study due to the availability of data.

Third, we could not analyze the disease severity of hepatitis E. Cases of hepatitis E are largely common and mild, with few critical illnesses and deaths. The latter tends to be common in pregnant women and in the older age group. If pregnant women are infected, serious consequences are associated not only with high mortality in the late fetal period, but also with the occurrence of preterm birth and a high probability of vertical transmission to offspring (36, 37). However, the incidence in these populations is low.

CONCLUSIONS

In China, the factors influencing the prevalence of hepatitis E are living in the south, working as farmers, being middle-aged or elderly, and being male. The 24 cities we studied were highly diverse and moderately similar in terms of the occupational distribution of patients with hepatitis E. We confirmed the validity of the new method in classifying cities according to their occupational composition by comparing it to the clustering analysis.

DATA AVAILABILITY STATEMENT

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

AUTHOR CONTRIBUTIONS

TC, XZ, and QZ designed the study. SY, JR, XC, ZZ, CL, SL, YZ, YW, JX, MY, and XL collected the data. SY, JR, XC, ZZ, MW, and ZL analyzed the data. SY and JR wrote the manuscript. All authors have read and approved the final manuscript.

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REVIEW
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Advances in Multi-Omics Applications in HBV-Associated Hepatocellular Carcinoma

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Hepatitis B virus (HBV) specifically infects liver cells, leading to progressive liver cirrhosis and significantly increasing the risk of hepatocellular carcinoma (HCC). The maturity of sequencing technology, improvement in bioinformatics data analysis and progress of omics technologies had improved research efficiency. The occurrence and progression of HCC are affected by multisystem and multilevel pathological changes. With the application of single-omics technologies, including genomics, transcriptomics, metabolomics and proteomics in tissue and body fluid samples, and even the novel development of multi-omics analysis on a single-cell platform, HBV-associated HCC changes can be better analyzed. The review summarizes the application of single omics and combined analysis of multi-omics data in HBV-associated HCC and proposes the importance of multi-omics analysis in the type of HCC, which provide the possibility for the precise diagnosis and therapy of HBV-associated HCC.

Keywords: hepatitis B virus, hepatocellular carcinoma, metabolomics, proteomics, genomics, transcriptome, non-coding RNA

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INTRODUCTION

Globally, 830,180 people died of cancers in 2020, and liver cancer became the third causative factor of cancer-associated death (8.3% of 9.9 million deaths) (1). Hepatocellular carcinoma (HCC) is the most dominant primary liver cancer, which can be caused by hepatitis B virus (HBV), hepatitis C virus (HCV), alcohol abuse, and so on (2, 3). Although acquired HBV infection has been well-controlled by vaccines, HBV remains the main cause of HCC due to nearly 300 million individuals with chronic HBV (CHB) infection worldwide (4). It is estimated that 8–20% of untreated patients with CHB infection will progress to liver cirrhosis within 5 years (5), and ∼2–8% of the patients with liver cirrhosis can be transformed into HCC (6). Persistent HBV infection or active HBV replication results in liver injury, fibrosis, cirrhosis, and liver cancer, leading to most of the end-stage liver diseases (7, 8). Up to one-third of patients with HBV-associated HCC will develop cirrhotic tumors (2). Additionally, inactive HBV carriers with serum alanine aminotransferase (ALT) levels in the normal range have substantial risk of HCC compared to those without HBV infection (9).

Nucleos(t)ide analogs (NAs) and PEG-interferon are recommended antiviral treatments in routine medicine (such as lamivudine, adefovir, dipivoxil, entecavir, and tenofovir) that can prevent

viral replication and CHB progression. However, these drugs don't affect the HBV genome in the host liver cells, which has always been in the form of covalently closed circular DNA (cccDNA) (10). Additionally, HBV-associated HCC presents more chemoresistance than non-HBV tumors. Thus, current treatment regimens are not curative, and the primary objective of therapy for CHB infection is to permanently inhibit HBV replication, followed by lifelong therapy (11). Given the above limits in treatment and huge scale of HBV infection worldwide, new therapeutic strategies are necessary.

CHB infection is dynamic interactions among the hepatocytes, virus and immune system of the host. In recent years, significant progress has been made in genome and proteomic analysis, clinical data management, next generation sequencing data mining, machine learning and deep learning algorithms. "Omics" technologies are used to mainly detect all protein, transcripts, and metabolites for mining for available data in the biological sample. These high-throughput technologies play critical roles in describing gene and/or protein expression profiles, and their effects on HBV-associated HCC (12–14). Although many biomarkers for diagnosis and prognosis have been identified through omics analysis of HBV infection, the previous studies focus on a single aspect of the natural history of CHB. However, most studies on systematic omics are based on genomics, transcriptomics and proteomics. The integration of multi-omics data analysis is critical for providing novel insights into the transitions and molecular mechanisms in related diseases (15, 16).

With the technological advances of platforms, multi-omics analysis will be more crucial for molecular therapies and precision medicine. Integrative analysis of multi-omics platforms mainly relies on innovative technology platforms including genomics, metabolomics, and proteomics. Multi-omics studies have been successfully exploited to elucidate the pathogenic mechanism of infectious diseases, such as helicobacter pylori (HP)-associated gastric carcinoma (17), COVID-19 (18), and herpes simplex virus-1 (HSV-1) infection (19). The promise of the multi-omics approach has been well-described in more complex diseases, and several studies have proposed potential biomarkers for HCC using omics resources. Although "omics-level" studies have been very useful in understanding the mechanism of HCC manifestation, few are available to integrate different omics data.

Herein, we review recent advances in multi-omics applications, including genomics, epigenetics, transcriptomics, proteomics, and metabolomics. Overall, this review will highlight the omics advances in HBV-associated HCC to provide novel insights into immunotherapies based on specific biomarkers in the future (**Figure 1**).

EPIDEMIOLOGY

Hepatitis B virus is a DNA virus with a partial double-stranded relaxed circular DNA genome, containing four open reading frames (ORFs) with *P, pre-S/S, pre-C/C,* and *X* genes, and *Pre-S/S* comprises the *pre-S1, pre-S2*, and *S* genes. The *P* region encodes DNA polymerase and RNase H, which is associated with virus replication. *Pre-C/C* encodes hepatitis B core antigen (HBcAg) and hepatitis B e antigen (HBeAg), and *X* region encodes a hepatitis B x (HBx) non-structural protein, which is involved in viral replication and oncogenic activity. A recent report showed that the HBx protein could promote the degradation of the structural maintenance of chromosome (SMC) 5/6 protein complexes to increase HBV replication and indirectly proves the association with the occurrence of HCC again (20).

Mutations of the viral genome causing biological behavior changes may have crucial effects on HBV pathogenicity and are closely associated with the malignant transition of liver cancer (21). The occurrence of HBV-associated HCC is a complex process. HBV infection can promote HCC through direct or indirect mechanisms, including HBV gene integration, genomic instability, and activation of cancer-associated signaling pathways. Additionally, new insights into the mechanism of HCC-related pathway activation, including epigenetics, autophagy, exosomes, metabolism, and immune responses (22–25), are being continuously focused on. Previous studies also showed that HBV-associated HCC individuals displayed distinctive profiles including chromosomal alterations and β-catenin mutations (26). Furthermore, genetic alterations in subgroups of HCC cases were remarkably associated with HBV-DNA levels (27). Additionally, novel biomarkers, such as DNA mutations, DNA or RNA methylation, long non-coding RNAs (lncRNAs), microRNAs (miRNAs), and circular RNAs (circRNAs), are under investigation and can be considered for future clinical practice of HCC.

Thus, integrated multi-omics analysis must be performed to obtain a better understanding of the pathogenesis of HBV-associated HCC. The combined application of genomics, epigenomics, and transcriptomics to illustrate the mechanism of virus-associated carcinogenesis is required.

GENOMICS CHARACTERISTICS

In contrast to HCV, HBV can integrate the viral genome into the host hepatocyte genome. A thorough understanding of the pathogenesis of virus-associated carcinogenesis is critical for early diagnosis, treatment and prevention of HCC. Recent advances in deep sequencing technologies including next-generation sequencing, nanopore sequencing, and single-cell sequencing, contribute to revealing the landscape of genetic and epigenetic changes in tumor tissues and chronic liver damage caused by hepatitis virus infection, particularly HBV (28). Persistent suppression and/or eradication of HBV/HCV can contribute to reducing the incidence of HCC, but multi-centric tumors in patients often arise after viral clearance (29–31). Additionally, the accumulation of viral genetic alterations negatively affects the epigenetic transformation of normal cells into cancer cells (32). NGS technologies, including whole-exome sequencing (WXS), RNA sequencing (RNA-seq), and whole-genome sequencing (WGS), form the basis of current genomics research. Therefore, vast amounts of sequencing data have been

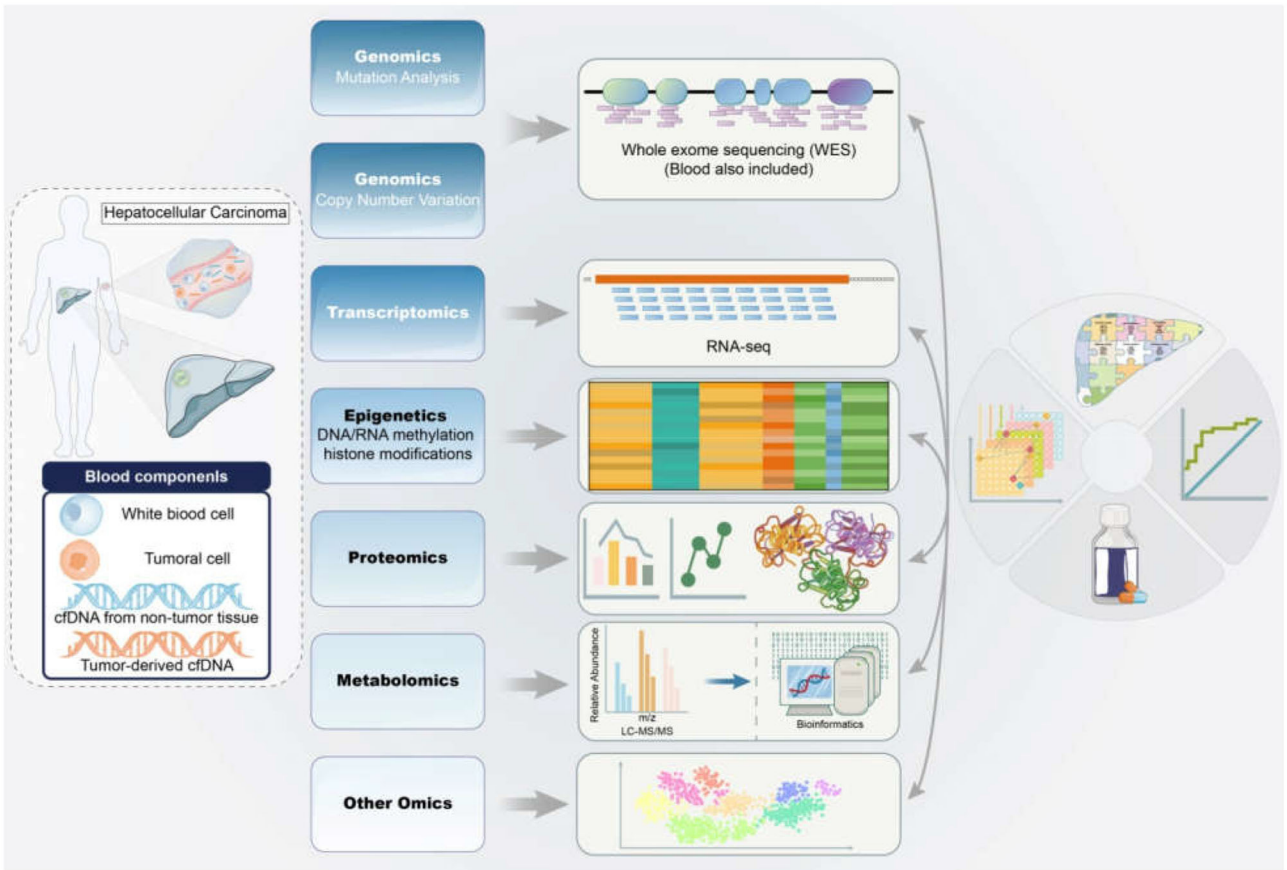

FIGURE 1 | Application of multi-omics in the diagnosis and therapy of HBV-associated HCC. The samples from HBV-associated HCC subjects were detected and analyzed by mitiomics technology including genomics, transcriptomics, epigenetics, proteomics, metabolomics and other omics to accurately promote biomarker-driven treatments and immunotherapies for HCC patients. HCC, hepatocellular carcinoma; HBV, hepatitis B virus; RNA-seq, RNA sequencing; LC-MS/MS, liquid chromatograph-mass spectrometer/mass spectrometer.

shared in global databases, allowing researchers to synthesize analyses that lead to new findings.

Host Profile

Genetic aberrations comprise nucleotide changes and structural variations (STVs) (33–35). The accumulation of somatic genomic alterations in primary tissues is the major cause of HCC. An average of 40–60 somatic alterations is detected in the protein coding regions of the genomes from HCC patients (36) (**Table 1**). Based on WES and single-nucleotide polymorphism (SNP) array analysis, how these mutated genes and the copy number of their alterations are involved in regulating these pivotal pathways, including cell cycle control, telomere maintenance, chromatin modification, and receptor tyrosine kinase, which have been reported (51). Among these mutations, a few genomic alterations are considered to be directly involved in the activation of the important signaling pathways for hepatocarcinogenesis.

The integration of viral genome is a unique molecular characteristic of HCC. Notably, the integration of HBV genome affects gene expression near integration sites. Multiple recurrent genetic aberrations and the disruption of the host genome due to HBV-DNA integration are important for the hallmarks of HBV-associated HCC. By large-scale genome sequencing analysis of HCC, the core drivers (*TERT*, *TP53*, and *CTNNB1/AXIN1*) have been identified as initial molecular events and other low-frequency drivers including therapeutically targetable drivers. These genes regulate some pathways, including cell cycle (*p53*, *p16*), apoptosis (*bcl2*), cell proliferation and differentiation (*b-catenin*, *c-myc*, *APC*, *E-cadherin*), metastasis (*MMP4*, *MMP9*, *Topoisomerase*, *Rb*, *Cyclin D1*, *Osteopontin*), angiogenesis (*VEGFR-2*, *Angiopoietin-2*), and other growth factor signaling components (*IGF-II*, *TGF*, *EGFR*, *HGF/c-MET*, *PTEN*, *K-RAS)* (37, 42). These findings indicate that HCC is not caused by a specific driver mutation but involves in the multiple carcinogenic pathways that enhances extremely heterogeneous of HCC.

By next-generation sequencing, somatic mutations in *TP53*, *TERT* promoter, and *CTNNB1* have often been reported in HCC patients (38). Somatic mutations are abundant in *TERT* gene promoters and occur in more than 50% of the patients with HCC, while the protein alterations caused by gene mutations are often observed in *CTNNB1* genes and *TP53*. Additionally,

TABLE 1 | The common somatic genomic alterations and HBV integration events in HCC.

Genomic aberration	Aberration frequency (% of patients)	HBV integration events	Pathway	Biological roles	References
TP53	3–40% (mutations); 2–15% (loss)	No	P53 pathway	Tumor suppressor	(37, 38)
CTNNB1	11–41%	No	Wnt pathway	Regulation in cell adhesion, growth, and differentiation	(38, 39)
ARID1A	5–15%	No	Chromatin remoedling	Transcriptional activation of selective genes and inhibition of chromatin remodeling	(39)
ARID2	3–15%	No	Chromatin remoedling	Tumor suppressor gene in the transcriptional activation and inhibition of specific genes	(40, 41)
JAK1	7.70%	No	JAK1/STAT3 pathway	Good prognostic marker for survival of HCC patients	(25)
AXIN1	5–19%	No	Wnt pathway	As signal transducer to regulate cell adhesion, growth, and differentiation.	(37, 42)
CDKN2A	7–8%	No	cell cycle	Tumor suppressor genes that promote cell cycle arrest in G1 and G2.	(39)
KEAP1	2–8%	No	Oxidative stress pathway	Proteinase adaptor	(14)
ARID2	3–15%	No	Chromatin remodeling	Growth hormone receptor	(40, 41)
FGF family members (FGF3, FGF4, FGF19)	4–5.6%	No	FGF pathway	Mitogenic and cell survival activities	(36)
TERT	∼60%	Polymerase, X protein, Precore/core protein	Telomere maintenance	Telomere repeat (TTAGGG) was added to the end of chromosome;the erosion of telomere protective end was compensated.	(39, 40, 43, 44)
MLL4	3%	Polymerase, X protein	Chromatin regulators	Epigenetic modification	(45, 46)
CCNE1	10%	X protein, Precore/core protein, S	TP53 /cell-cycle pathway	Strong CcnE1 overexpression is correlating with poor prognosis of HCC patients.	(47, 48)
FN1	No	Precore/core protein, X protein, polymerase	No	FN1 promotes the migratory and invasive of hepatoma cells.	(40, 45, 47, 49)
CDK15	No	S, Polymerase, X protein, Precore/core protein	No	Protein Coding gene	(41)
ROCK1	No	X protein, S	No	Stable overexpression of ROCK2 remarkably promoted cell motility and invasiveness in HCC cells.	(50)
ApoA2	No	Polymerase, X protein, S	No	Apolipoprotein family	(50)

somatic structure variants (SVs) affect gene expression in cancers. The Pan-Cancer Analysis of Whole Genomes (PCAWG) Consortium revealed that 100-kb SV breakpoints for hundreds of genes were associated with their altered expressions by aggregating whole-genome sequencing data from a cohort of 1,220 cancer individuals. For most of these genes, SVs result in increased expression rather than decreased expression, and the up-regulated cancer-related genes included *TERT*, *CDK4*, *MDM2*, *ERBB2*, *PDCD1LG2*, and *IGF2* (39). Simultaneously, WGS analysis demonstrated several important types of SVs in the genome of liver cancer, including *TERT*, *APC*, *CDKN2A*, *ARID1A*, and new genes such as *TTC28*, *LRP1B*, and *MACROD2*, and these SVs affected their expressions (39).

The increased copy number of HBV-DNA at HBV breakpoint locations indicates that chromosomal instability is associated with HBV genome integration (52). Hama et al. identified the structural rearrangement that integrated the viral genome by WGS analysis in HBV-associated HCC (50). Therefore, the structural instability of the integrative viral genome is periodic and may be related to the chromosomal instability of the host hepatocyte genome.

HBV Profile

Approximate 350–400 million people worldwide are infected by HBV, and persistent HBV infection leads to more than 50% of HCC patients. HBV plays an important role in the development of HCC by integrating the HBV genome into the host genome. Several high-throughput sequencing studies have reported that HBV genome integration occurs in a high rate of HBV-associated HCC patients (**Table 1**) (52). Although HBV can randomly and repeatedly integrate into the host genome including *TERT and MLL4,* suggesting functional consequences for the host by HBV integration events (45). Some studies have also identified that the region between 1,600 and 1,900 nucleotides within

the viral genome, corresponding to the 3′-end of HBx gene and 5′-end of precore gene, is not only preferentially involved in structural alterations within the viral genome, particularly deletion and inversion events, but also significantly related with the insertion into the host genome (43, 53). In a previous study, HBV genome integration was significantly enriched on the q-arm of chromosome-10 in a cohort of 48 HCC cases, and the event was related with poorly differentiated tumors (43). In several studies, HBV has been reported to integrate into the *CCNE1* and *TERT* genes. However, *CDK15, ROCK1, FN1, ApoA2*, and *MLL4* have rarely been reported as HBV integration sites (40, 47, 49). In 76 samples of HBV-associated HCC cases, 4 cases of HBV integration within *CCNE1* were reported, resulting in the high expression of *CCNE1* (47). Multiple high-throughput genomic studies have found that repeated integration sites on *TERT* promoters are the most frequent integration sites (40). Disruption of the *telomerase reverse transcriptase (TERT)* promoter may result in the dysregulation of *TERT* expression (44). The mRNA expression of *TERT* is increased when HBV binds to the *TERT* transcription start site, which implies HBV sequences as enhancers for *TERT* mRNA expression (43). In a Chinese cohort of forty-four HBV-associated HCC tissues, 8 fusion transcripts of *HBx/MLL* were found, leading to high expression of the *MLL4* gene (46). Furthermore, multiple transcripts with HBV-CDK15 fusion were observed in an HCC case, including one in-frame fusion, which induced CDK15 overexpression (41). Hence, the genes of *CCNE1, ANGPT1,* and *TERT* are not only mutated in somatic cells, but also integrated in viruses (48).

EPIGENETICS CHARACTERISTICS

Viruses can alter the chromatin structure by redirecting the modifications of chromatin and consequently affecting host cell transcription, which may contribute to oncogenesis (54, 55). Epigenetics refers to changes of gene expression without altering the underlying DNA sequence, and comprises three major components: histone modifications, DNA methylation, and non-coding RNA mechanisms (56). Hepatocellular carcinoma is caused by the somatic mutations leading to the abnormalities of chromatin regulations and epigenetic characteristics (57). In the cases with HBV infection, the disorders of DNA/RNA methylation and histone modifications have been reported, but which have focused on specific genes or pathways, and genome-wide mapping of the epigenetic alterations is rare.

DNA Methylation

DNA methylation in many tumor suppressor genes is related with carcinogenesis. Through an array-based platform, the genomic DNA methylation pattern of nearly 200 patients with HCC further revealed the different cancer-specific DNA hypermethylation clusters (58). DNA methylation is significantly different among HCV-related HCC, HBV-associated HCC and normal tissue (**Table 2**) (66). However, some evidences support more prominent DNA methylation alterations in HCV-associated HCC than in HBV-associated HCC (67). Similar to other cancers, HCC is characterized by the global DNA hypomethylation and promoter hypermethylation, which are related with the up-regulated tumor-promoting genes (68, 69). High frequencies of aberrant DNA hypermethylation of specific genes (*GSTP1*, *RASSF1A*, *DOK1*, and *CHRNA3*) in HCC were reported, and these genes was suggested as a prognostic marker of HCC combined with clinicopathological data (59, 60). Furthermore, a recurrent hypomethylated enhancer of CCAAT/enhancer-binding protein-beta (C/EBP-β) promoted HCC tumorigenicity through global transcriptional reprogramming (70). Methylation of the *APC, RASSF1A*, and *GSTP-1* genes is associated with HCC (61–64). Apart from methylation at gene promoters and CpG islands, epigenetic regulation and genome-wide enhancer hypomethylation patterns in primary human HCCs must be elucidated by whole-genome sequencing. In HCC patients, the latest three reports identified 6 CpG sites in white blood cell (WBC) DNA and showed that DNA methylation at those sites could distinguish HCC from healthy blood in prospective samples taken before diagnosis (71–73). Additionally, compared with hepatitis and cirrhosis liver tissues, increased DNA methylation of CpG island 3 in the HBV genome indicated HBV methylation in HBV-associated HCC pathogenesis (74). Furthermore, HBV infection induces gene methylation in HCC (75). HBV infection promotes the activity of DNA methyltransferase, which causes the simultaneous methylation of host CpG islands and HBV-DNA in cell experiments (76).

However, the potential for these markers to be used for clinical application is low because biopsies are unsuitable for early diagnosis. Because blood contains circulating tumor DNA, blood may be a promising material for carrying the same DNA methylation signals of markers as tumor tissue (71, 77). With the important role of "liquid biopsy" in identifying specific molecular signals in nucleic acids released by cancer cells, some studies have found that by detecting the methylation level of specific sites of circulating tumor DNA(ctDNA) in a small amount (4–5 ml) of peripheral blood, it can be used to accurately diagnose HCC early and to predict the curative effect and prognosis (78).

RNA Methylation

N6-methyladenosine (M6A) is present in most eukaryotic messenger RNAs (mRNAs) and is the most commonly modified form of mammalian RNA. Recently, some studies have reported that hepatocarcinogenesis is closely related with abnormal m6A modifications (79, 80). They found that high expression of the m6A methylase METTL3 in HCC patients leads to high levels of m6A in SOCS2 mRNA, resulting in the rapid degradation of SOCS2 and HCC occurrence (79), while METTL14 had no significant effect on HCC, and down-regulation of METTL14 expression was related with a poor prognosis in HCC patients without recurrence (80). Although the relationship between DNA and RNA m6A remains unclear, at least one independent way can verify the m6A modification sites predicted by big data (81). By transcriptome sequencing, some genes related with m6A in HCC, particularly METTL3 and YTHDF2, had been confirmed to be a risk signature (79). The processing of miR-126 maturation is mediated by the methylation transferase METTL14 in HCC, and the reduced expression of miR-126

TABLE 2 | DNA methylation in HBV-associated HCC.

DNA methylation	Methylation status	Biological functions in HCC	References
RASSF1A	Hypermethylation	As a diagnostic and prognostic non-invasive biomarker for HCC.	(59, 60)
GSTP1	Hypermethylation	As a diagnostic marker, GSTP1 methylation can obviously enhance the risk of HBV-associated HCC patients with cirrhosis.	(59–61)
DOK1	Hypermethylation	A tumor suppressor gene, and methylation level of *DOK1* is inversely related with gene expression.	(59, 60)
APC	Hypermethylation	Methylation of *APC* could involve in early stages of HBV-related HCC, coupled with RASSF1A.	(61–65)
p16	Hypermethylation	As a diagnostic marker, P16 methylation in promoter region could obviously increase the risk of HBV-associated HCC in patients with cirrhosis.	(37)
MGMT	Hypomethylation	Loss of methyl-cytosine at the MGMT gene promoter may be considered as an early and transient biomarker of hepatocarcinogenesis.	(60)

maturation will cause HCC metastasis (80). Thus, regulators of m6A modification can become potential biomarkers for prognosis in HBV-associated HCC.

Histone Modifications

In the hepatocyte nucleus, HBV-cccDNA assembles with the histone proteins of host cells to form minichromatin, which is dynamically regulated through histone post-translational modifications (PTMs) to promote the expression of viral genes. Previous reports have revealed a series of histone modifications on HBV cccDNA, such as H3K4me2, H3K4me3, H3K9ac, H3K27ac, H3K36me3, and H3K9me3. H3K4me2, H3K4me3, H3K9ac, H3K27ac, and K3K36me3 are associated with activating gene expression, while H3K9me3 is related to gene silencing (82). A highly sensitive technique, 3C-high-throughput genome-wide translocation sequencing (3C-HTGTS), was used to identify the interactions of HBV-DNA and host DNA in which H3K4me1 histone modification is enriched by kmt2c/d, while H3K4me1 histone modification contributes to activate the transcriptional activity of HBV. They found that histone modifications not only strongly affected HBV transcription on minichromosomes of HBV cccDNA but also affected host gene expression (83). Recently, Alvarez-Astudillo et al. found that the histone variant H3.3 was assembled from the histone chaperone HIRA to the HBV-cccDNA, and this assembly was correlated with increased levels of the active H3K4me and activation of HBV transcription (84). The first genome-wide maps of PTMs obtained by chromatin immunoprecipitation sequencing (ChIP-Seq) have revealed that high levels of PTMs associated with transcription activation are enriched at specific sites in the HBV genome, whereas very low levels of PTMs are related with transcriptional inhibition, even at silent HBV promoters (82). Herein, the effect of transcriptional and active PTMs may open the possibility of chromatin regulating HBV-cccDNA transcription, providing a new way to treat chronic hepatitis B virus infection.

TRANSCRIPTOMICS CHARACTERISTICS

Host-HBV Transcription

Hepatitis B virus plays a crucial role in HCC progression by integrating the viral genome into the host genome, and genome integration events are observed in HBV-associated HCC patients using high-throughput sequencing (43, 47, 52, 85). Pregenomic RNA (pgRNA) of 3.5 kb, an RNA intermediate, is critical for HBV replication. Thus, the level of HBV replication in tumors or adjacent non-tumors is assessed by the presence of intact pgRNAs in liver tissue, but few intact pgRNAs are observed, particularly in the tumor tissues of HCC patients.

Most somatic mutations in HCC are in the coding regions with potential functional effects. Five thousand four hundred and eleven tumor-specific mutations were identified with an average of 230 somatic mutations in each HCC patient, and these somatic mutations were significantly different in the distribution of the different genomic regions and their predicted functions. Moreover, deep transcriptome sequencing of HCC patients provides information on RNA expression, transcriptional mutations and characteristics of HBV-human chimeric transcripts (45). The integration characteristics of HBV were identified by RNA sequencing, and the preferred integration sites near the telomeres were reported (52). The integration sites in the structural changes within HBV and host genome have been well-described at the genomic level, but the status of HBV transcripts in HBV-associated HCC has not been comprehensively analyzed (86). To Target different HBV transcripts in depth, Stadelmayer et al. have developed an HBV full-length 5′RACE (rapid amplification of cDNA ends) method, which significantly contributes to the understanding of HBV transcription and may guide the development of new therapies targeting HBV-cccDNA (87).

HBV fusion sequences are significantly enriched on chromosome 10 (43). More HBV-human fusions (161 fusions) in non-tumorous tissues were observed in the HCC transcriptome of 22 HCC patients than in matched HCC tissues (33 fusions)

(41). Notably, the data obtained through transcriptome analysis showed that most chimeric transcripts in tumors fuse with gene sequences more than at the genomic level, HBV was fused with the repetitive sequences, particularly the LINE and SINE families of the repetitive sequences in 40% of the chimeric transcripts (43).

Although transcriptome sequencing can provide valuable insights into the characteristics of HBV-associated HCC patients, most studies have focused on host transcripts rather than viral transcripts (88–90). More than 90% of HBV-associated HCC contains transient HBV-DNA integration, which does not produce all the HBV antigens, and these transient HBV-DNA fragments encode epitopes that can be recognized by activate T cells (47, 91–93). The HBV transcriptomes of the HCC cells can be used for individualized immunotherapy with engineered T cells and as a treatment measure for a wider range of HBV-associated HCC patients (93). The transcriptome similarities and differences in $CD8^+$ T cell dysfunction were explored in both chronic HBV infection and HCC patients through high-throughput RNA-seq, and the results demonstrated that $CD8^+$ T cell dysfunction in the two groups shared high similar characteristics, but each had its own characteristics in specific genes and signal pathways (94).

Non-coding RNA

Non-coding RNAs (ncRNAs) are functional RNAs that cannot encode proteins. ncRNAs make up a significant proportion of cellular RNAs, accounting for more than 90% of human RNAs. Recent reports have shown that ncRNAs play an important role in multiple cellular processes including cell proliferation, apoptosis, migration, and angiogenesis. Many tumor cells including liver cancer cells, also release specific circRNAs, microRNAs (miRNAs), long non-coding RNAs (lncRNAs), and extracellular vesicles containing proteins, lipids, RNAs and miRNAs in peripheral blood (95–98). Non-coding variants are closely linked to human cancers and are even involved in drug resistance in HCC. Herein, we focused on miRNAs, lncRNAs and circRNAs implicated in the pathogenesis of HCC (**Table 3**).

miRNAs

miRNAs are one kind of important non-coding RNAs (ncRNAs), ∼22 nucleotides in length, and are highly expressed in many types of tumors associated with HCC progression or suppression (101, 102). miRNAs may act as tumor suppressor genes or oncogenes by silencing and targeting mRNAs involved in carcinogenesis. Recent studies showed that miRNA expression is more valuable than mRNA-based profiling to identify tissue types of tumor origin, and cancer treatments targeting miRNAs are currently in clinical trials as early detection markers of HCC (100, 121). In the past 2 years, many reports showed that miRNAs were closely related with hepatocarcinogenesis (116). A recent study showed that miR-154 as a tumor inhibitor could suppress cell proliferation and metastasis, and the miR-154 expression was downregulated in HCC (99). Feng et al. found that lncRNA PCNAP1 promoted HBV replication by regulating miR-154/PCNA/HBV-cccDNA signal and PCNAP1/PCNA signal, which drived the growth of both HBV-associated HCC and HBV-free HCC (98). Some studies have considered that miRNA expressions play an important in the pathogenesis of HCC by the downregulation of miRNAs that upregulate oncogenes or the upregulated miRNAs that target tumor suppressor genes (122). In the "Expert consensus on early screening strategy for liver cancer in China," serum miRNAs as a potential diagnostic marker have made some progress. In a study of 934 subjects, including groups of healthy people, patients with CHB, liver cirrhosis and HBV-associated liver cancer, 723 miRNAs were screened on a large scale in plasma samples. The results showed that seven specific miRNAs were selected to construct the diagnosis model of HCC and can distinguish liver cancer from healthy people (sensitivity for 83.3%, specificity for 93.9%), liver cancer from hepatitis (sensitivity for 79.1%, specificity for 76.4%), and liver cancer from liver cirrhosis (sensitivity for 75.0%, specificity for 91.1%) (123). At the same time, other studies have also proven that miRNAs have important value in the diagnosis of HCC, but the sensitivity and specificity of this technology must be further improved. Furthermore, the application values of miRNAs require large-scale samples and multicenter clinical verification, which can be used as a supplement for individualized diagnosis.

LncRNAs

Benefiting from advances in the transcriptome sequencing, lncRNAs, transcripts more than 200 bp in length without encoding proteins, play roles in different physiological and pathological processes and affect cellular functions (106, 124). To date, most of lncRNAs play important roles in regulating specific cellular processes, particularly in the expressions of protein-coding genes at the epigenetic, transcriptional and post-transcriptional levels in cancer including HCC (125). Previous studies have shown that the lncRNAs MALAT1, H19, HOTAIR (HOX transcript antisense intergene RNA), HULC, and PRNCR1 are abnormally expressed in various human cancers, particularly HCC (104). LncRNAMALAT1 induces murine HCC experimentally, H19 expression is upregulated in HBV-associated HCC, HOTAIR is overexpressed in tumor tissues from HCC patients and in liver cancer cell lines, and is related with poor prognosis of HCC, HBx upregulates lncRNAHULC by inhibiting P18 and promoting the occurrence of HCC (103, 105, 107, 108). Additionally, the up-regulated expressions of lncRNA-HEIH and HOTTIP promoted tumor progression and significantly associated with tumor progression and disease outcome in HCC patients (109, 110). Low expression of lncRNA-MEG3 was observed in HCC tissues and cells, and overexpression of lncRNA-MEG3 could inhibit the proliferation, migration and invasion of HCC cells (111). LncRNA Low Expression in Tumor (lncRNA-LET) and lncRNA-SRHC were generally downregulated in HCC, which was associated with hepatic invasion and abdominal metastases (112, 113). Exosomal miR-21 can inhibit the expression of the lncRNA-PTENp1 to promote HCC growth, miR-21 inhibitors or lncRNA-PTENp1 overexpression can weaken the role of exosomal miR-21, which indicates that PTENp1 can repress the tumorigenic properties of HCC cells (114). These findings indicate that lncRNAs play critical regulatory roles in the proliferation, migration and invasion of HCC cells.

TABLE 3 | Non-coding RNAs in HCC.

	Non-coding RNAs	Expression change	Biological function	References
microRNAs	miRNA Panel (miR-122, miR-192, miR-21, miR-223, miR-26a, miR-27a and miR-801)	Up-regulation	The miRNA panel can differentiate HBV-associated HCC from healthy subjects, CHB and cirrhosis, respectively.	(99)
	miR-154	Down-regulation	As a tumor suppressor, the miR-154 expression is down-regulated in HCC.	(98, 100)
	miR-519d, miR-595, miR-939, miR-494 and miR-21	Up-regulation	miR-939, miR-595 and miR-519d could differentiate cirrhotic patients with and without HCC. Moreover, miR-519d, miR-494 and miR-21 were related with the progression of HCC.	(101)
	miR-21 and miR-10b	Up-regulation	The exosomal miR-21 and miR-10b promote cancer cell proliferation and metastasis in HCC, and may serve as prognostic markers and therapeutic targets for HCC.	(102)
	miR-15a/miR-16-1	Down-regulation	HBx transcript directly drives the down-regulation of miR-15a/miR-16-1 by the miRNA targeting sequences in the viral RNA.	(103)
	miR-204, miR-1236	Down-regulation	miR-204 and miR-1236 can inhibit HBV replication involved in two different mechanisms.	(100)
LncRNAs	H19	Up-regulation down-regulation	Promote HCC growth, inhibit migration and invasion of HCC cells.	(104, 105)
	HULC	Up-regulation	Triggering autophagy *via* stabilizing Sirt1; Promoting HCC growth	(104, 106, 107)
	HOTAIR	Up-regulation	Activating STAT3/ABCB1 pathway and promoting HCC growth	(104, 108)
	MALAT1	Up-regulation	Associated with tumor metastasis, recurrence	(103, 104)
	HEIH	Up-regulation	Associated with HBV-HCC and prognosis	(109)
	HOTTIP	Up-regulation	Associated with tumor progression and disease outcome	(110)
	MEG3	Down-regulation	Associated with methylation and Inhibit cell growth	(111)
	LncRNA-LET	Down-regulation	Reduces hepatic invasion and abdominal metastases	(112)
	SRHC	Down-regulation	Inhibit cancer proliferation	(113)
	PTENP1	Down-regulation	Suppress the tumorigenic properties of HCC cells	(114)
circRNAs	circ_104075	Up-regulation	Circ_104075 as a ceRNA can upregulate YAP expression by absorbing miR-582-3p, and may provide new insights in HCC diagnosis and therapy.	(115)
	cSMARCA5	Down-regulation	It has diagnostic value for patients with alpha-fetoprotein <200 ng/mL.	(97)
	circ_0009582, circ_0037120, circ_0140117	Up-regulation	The high sensitivity and specificity of the combination of three circRNAs and AFP could be used to distinguish HBV-infected patients with and without cancer.	(116)
	circ_KIAA1429	Up-regulation	Post-transcriptional modification of circ_KIAA1429 may also play a role in influencing translation.	(117)
	circMET	Up-regulation	Inducing development and immune tolerance of HCC by the Snail/DPP4/CXCL10 axis.	(118)
	circPTGR1	Up-regulation	Associated with the clinical stage and prognosis.	(119)
	circ_0051443	Down-regulation	Potential therapeutic target for HCC	(120)

CircRNAs

CircRNAs (circular RNAs), is a new type of non-coding RNA with a closed circular structure without a 5′-end cap and a 3′-end poly A tail. Most of circRNAs are formed by exon loops encoding polypeptides, but some are lariat structures formed by intron loops without encoding ability. Currently, the biological functions of circRNAs are recognized as miRNA sponges, regulatory protein binding, regulation of gene transcription, and coding functions (126). Currently, the circRNAs in human body fluid have been identified in human disease including cancers, autoimmune diseases and infectious diseases. For example, circ-KIAA1244 serves as a novel circulating biomarker to detect gastric cancer (115). CircRNA_0001178 and circRNA_0000826 are considered potential diagnostic biomarkers for liver metastases from colorectal cancer (117). Differential expression of circRNAs and lncRNAs is found in recurrent COVID-19 patients (118). These reports indicated that circRNAs could serve as biomarkers for the diagnosis and therapeutic intervention of human diseases.

To date, the most common mechanism by which circRNAs act as miRNA sponges and interact with certain mRNAs and miRNAs is *via* competing endogenous RNAs (ceRNAs) (127,

128). RNA sequencing revealed that circRNA cSMARCA5 is downregulated in HCC, inhibiting the growth and migration of hepatocellular carcinoma cells, and is associated with a poor prognosis (97). Furthermore, Wu et al. reported that the combination of circ_0009582, circ_0037120, circ_0140117, and AFP has a high sensitivity and specificity to predict HCC (116). Zhang et al. reported that hsa_circ_0001445 levels in plasma are significantly downregulated, which had high specificity (94.2%) and sensitivity (71.2%) in HCC patients, and the efficient combination of plasma hsa_circ_0001445 and AFP levels can be used for HCC diagnosis rather than each parameter alone (129). Furthermore, another circRNA, hsa_circ_104075, was significantly increased in serum from HCC patients, and the AUC value of hsa_circ_104075 (0.973) suggested high sensitivity of 96.0% and specificity of 98.3% (119). Plasma three circRNAs (circ_0009582, circ_0037120, and circ_0140117) were overexpressed in HCC patient, and the combination of the three circRNAs and AFP acquired both valuable positive predictive value (PPV) and negative predictive value (NPV) of 95%, suggesting that these three circRNAs can predict HBV-associated HCC patients or healthy individuals (116).

In addition to the typical miRNA sponging mechanism, post-transcriptional modification of circRNAs may influence translation. The phenomenon was confirmed by overexpressed circ_KIAA1429 in HCC (120). Some circRNAs are associated with drug resistance in HCC treatment. For PD-1 antibody-mediated immunotherapy, circMET makes HCC cells resistant to PD-1 by enhancing the therapeutic microenvironment of immunosuppressive tumors (130). The mechanism revealed that circMET as a sponge of miR-30-5p could promote SNAIL-mediated dipeptidyl 4 (DPP4) expression, leading to CXCL10 degradation, and CXCL10 is a key chemokine in driving intratumor infiltration of effector T cells and may cause subsequent resistance to anti-PD-1 therapy.

Currently, exosomal circRNAs play significant roles in HCC progression, and several exosomal circRNAs function as either diagnostic/prognostic biomarkers or oncogenic/tumor-suppressive factors in HCC (65, 131). The first study in 2015 demonstrated the presence of abundant circRNA inexosomes (132). Previous studies have shown that three exosomes secreted by HCC cells with high metastatic potential, the circPTGR1 subtype, can enhance HCC metastasis with low metastatic potential *via* the miR449a-Met signaling pathway (133). The exosomal circ-0051443 is produced from normal cells and transferred into HCC cells to inhibit the progression of HCC through competitive combination with miR-331-3p and aggravating apoptosis and cell cycle arrest of HCC cells (134). Therefore, recently described properties of circRNAs can not only help us improve understanding but also contribute to the clinical diagnosis and treatment of HCC.

PROTEOMICS CHARACTERISTICS

Proteomics are a large-scale study to unveil the profile of proteins expressed under certain biological conditions (135). Recently, proteomics has been used to analyze the overall level of proteins to investigate the pathogenesis, cellular patterns, and functional practices of HBV-associated HCC. With alterations in protein expression in the progression of HCC, some proteins can be considered as potential biomarkers for diagnosis and therapy (136). In a validation study, 28 proteins could separate acute-on-chronic liver failure (ACLF) from CHB patients, the proteomic features developed in this study reflected deficiencies of important hematologic functions in patients with HBV-ACLF, and demonstrated the potential for diagnosis and risk prediction of HBV-ACLF, complementing current clinical-based parameters (137). Compared with serum sample, urine sample is non-invasive and easy to collect, making it more suitable for HCC surveillance in high-risk patients who require frequent examination. Seven protein features were selected in a previous study; among them, HPX, APOH, APCS and PLG were upregulated in HCC urine samples, and GOT1, GLRX, and NCR3LG1 were downregulated (138).

Presently, mass spectrometry is considered a means of protein identification. Pollination mass spectrometry has developed rapidly with the emergence of electrospray ionization mass spectrometry (ESI) and matrix-assisted laser desorption and ionization time of flight mass spectrometry (MALDI-TOF-MS), which provide technical support for proteomics research. Because of its high throughput and sensitivity, MALDI-TOF-MS has provided an optimal response surface for proteomics research as an advanced technique in recent years. The application of MALDI-TOF-MS by the translocation of boron effectively detects the differential serum proteins of HBV-associated HCC, providing important support for diagnosis and treatment of HBV-associated HCC. Tandem mass tag (TMT), isobaric tags for relative and absolute quantification (iTRAQ), stable isotope labeling by amino acids in cell culture (SILAC), and liquid mass spectrometry are used to identify differential proteins. Among them, iTRAQ is considered as one of the most robust quantitative proteomics techniques (139). Compared with 2D gel electrophoresis, iTRAQ technology has many advantages including recognition of low-abundance proteins and high-throughput capabilities. Based on iTRAQ quantitative comparative proteomics, researchers have utilized liquid chromatograph-mass spectrometer/mass spectrometer (LC-MS/MS) to recognize and quantitate differential proteins in HepG2 cell lines stably containing different functional domains of HBx, and p90 ribosomal S6 kinase 2 (RSK2) has been identified as a new host protein that plays a key role in HBx enhancing HBV replication (140). Plasma fibronectin was demonstrated to be related with serum clearance of HBsAg and may be a potential predictor of "functional cure" of CHB by iTRAQ-based quantitative proteomics (141), meanwhile the TMT isobaric labeling-based technology was used to quantitatively characterize the renal proteome of HBV transgenic mice, and to elucidate the pathogenesis of HBV-associated glomerulonephritis (HBV-GN) (142). Additionally, proteomic analyses of formalin-fixed paraffin-embedded (FFPE) HCC graft samples, conducted using a label-free proteome mass spectrometry workflow, were used to characterize the global quantitative analysis of protein expression profiles after gene therapy and to identify differentially expressed proteins (143). Thus, a proteomic strategy to identify HCC

candidate biomarkers requires more integrated analysis, and no single methodology can perform this function.

At the same time, the application of proteomics also plays a role revealing the mechanism of the regulation of HBV viral protein in HCC progression, such as HBx, HBs, and HBc. High expressions of GNA13 and GNAi3, belong to the members of the guanine nucleotide-binding protein subunit α (GNA) protein family, are involved in the development of liver cancer through positive and negative regulatory mechanisms, respectively (144, 145). Additionally, recent studies found that the HBx protein promotes the expression of the DNA methylation enzymes DNMT1 and DNMT3A, thereby increasing the methylation level of CpG islands in the promoter region of GNA14 and inhibiting GNA14 expression (146). Endoplasmic reticulum (ER) dysfunction is closely associated with malignant transformation, particularly liver transformation (147). Reticulon (RTN), which is located in the ER, is important for ER maintenance (148). Relevant research results show that HBsAg promotes HCC development by inducing non-mutagenic inactivation of the p53 signaling pathway through the interacting protein RTN3, and proteomic analysis of HBV core protein (HBc) interactions in the nucleus of HepaRG cells revealed that the interaction of HBc with multiple RNA-binding proteins (RBPs) that regulate viral mRNA metabolism provides a new perspective to develop novel host-targeted antiviral strategies (149). Ribosome profiling (RiboSeq) is a novel technology, which could accurately locate the position of ribosomes on mRNA. By combining with RNA-ribosome profiling and proteomics, novel post-translational events hereby detected were then characterize. One study integrated multi-omics analysis, such as RNA-seq, Ribosome profiling and quantitative mass spectrometry, uncovered that an RNA element derived from HBV enhancer I forms a stem-loop which suppresses HBV translation (150). Furthermore, 11 RBPs (RAN, BRIX1, SMG5, DYNC1H1, PRKDC, GTPBP4, and so on) are associated with the overall survival of HCC patients by integrating RNA sequencing and proteomic data (13). These RBPs bind to various RNAs, such as mRNAs, rRNAs, ncRNAs, play a critical role in post-transcriptional gene regulation (PTGR) and are associated with RNA splicing, transport, maturation, degradation, stability, and translation (151). Therefore, they may be drug targets that will help optimize future clinical therapies.

The role of post-translational modifications (PTMs) includes modification events of biochemical functional groups, such as phosphorylation, glycosylation, ubiquitination and so on, which also play an key role on regulating development of HCC. Studies have proved that PTMs are very rich, and the same protein may be modified at multiple sites, which contributes to the diversity of protein structure and function (152). Recent studies have shown that HBV proteins can be modified by different types of PTMs, which affect their protein-protein interaction, subcellular localization and function (153). Recently, multi-omics platforms have performed to systematically interrogate HBV-host interactions. At the transcriptome, proteome and phosphoproteome levels of liver cancer tissues, it was observed that the key enzymes of glycolysis pathway (HK2, ALDOA, PKM2) were significantly up-regulated, indicating that liver cancer has an increased demand for glucose metabolism, and phosphorylation of glycolytic enzymes including ALDOA, may drive metabolic reprogramming and proliferation in liver cancer with CTNNB1 mutation (14). A proteomic analysis had identified that SRSF10as a RNA-binding proteins (RBPs) could be able to alter its phosphorylation and then to regulate HBV RNA metabolism (154). Using high-resolution mass spectrometry, 22,539 phosphorylation sites on 5431 proteins had identified in an HBx-transgenic mouse model of HCC, and these phosphoproteome data highlight potential mechanisms of kinase regulation, especially kinase activities of Src family kinases (SFKs), PKCs, MAPKs, and ROCK2 in HCC (155). Hu et al. revealed the relationship between metabolic reprogramming and antiviral innate immunity against HBV infection usingLC-MS/MS. And O-linked-N-acetylglucosaminylation (O-GlcNAcylation) was proved to regulate host antiviral response against HBV, and O-GlcNAcylation of SAMHD1, as an effector of innate immunity, could stabilize samhd1 structure and enhance host antiviral activity (156). Protein glycosylation is a well-known post-translational modifications and analysis of which based on MS technology commonly. It was reported that the change of glycan heterogeneity in HCC promotes the occurrence, progression and metastasis of tumor, and N-glycosylation is related to the development and progression of HBV-related HCC (157, 158). They showed that altered N-glycopeptide may be part of the unique glycan characteristics, indicating the IgA mediated mechanism and providing potential diagnostic clues for HBV-related HCC. Interferon-α (IFN-α) signaling is crucial for antiviral response. Through high-throughput RNAi screening, Chen et al. identified that the methylation of STAT1 catalyzed by methyltransferase SETD2 was determined to be IFNα-dependent antiviral immunity and showed the potential of SETD2 in controlling HBV infection (159). In the process of viral infection, ubiquitin system is an important part of cellular defense mechanism. Recent studies have shown that ubiquitination may be involved in the degradation of host protein after HBV integration, and there is a negative correlation between the whole proteome and ubiquitin group by performing an Ubiscan quantifcation analysis based on stable isotope labeling of amino acids in cell culture (SILAC) of HepG2.2.15 and HepG2 cell lines (145). Overall, HBV infection mediated changes in post-translational modifications will provide valuable data for further study of the pathogenesis of HBV-related HCC.

METABOLOMICS CHARACTERISTICS

The liver is an internal organ in the human body and is responsible for substrate metabolic and detoxification activities. As a hepatotropic virus, the infectious status of HBV affects liver metabolic function. Understanding how HBV infection relates to hepatic metabolism may provide new insights into the pathogenesis of HBV infection.

Metabolomics is the study of the profile of metabolites (e.g., amino acids, lipids, sugars, and hormones) that are detectable under certain conditions. Tumors from HCC patients may alter metabolic pathways, and the resulting changes in

nutritional supply are essential to overcome nutritional hunger and changes in environmental conditions (160). Compared with other "omics," metabolomics not only provides the most direct snapshot of the actual functional and physiological state of biological networks but also establishes a key technique to investigate metabolic alterations in carcinogenesis (161, 162). Currently, no standard or routine screening test exists for liver cancer. X-ray computed tomography scan, ultrasound and α-fetoprotein (AFP) are the typical tests used to screen for liver cancer, while liver biopsy is used as the gold standard (163). Metabolomics studies uncover new insights into the biological understanding of HCC and reveal particular implications related to clinical and therapeutic plans. The main techniques applied to metabolomics are nuclear magnetic resonance spectroscopy (NMR), gas chromatography-mass spectrometry (GC-MS) and LC-MS (164). Recent studies based on mass spectrometry and next-generation sequencing unveiled the active status of signaling pathways and reprogramming of hepatic metabolism in HBV-associated HCC at the genomic and proteomic levels (14, 38). MS-based technologies can provide measures of the global changes in protein abundance related to the deregulation of signaling and metabolic pathways in HCC. NMR spectroscopy-based metabolomics provide a non-targeted, quantitative snapshot of global metabolite abundance to provide additional biological insights that cannot be deciphered by proteomics alone (165). Additionally, the combination of GC-MS- and NMR-based metabolomic platforms is promising because the application of multi-metabolomics platforms yields a superior biomarker panel to diagnose bipolar disorder (166). Previously, in the field of metabonomics, substantial efforts have been made to search for biomarkers of HCC, some of which are candidate biomarkers (167). However, how the metabolic phenotype is driven remains unclear in HBV-associated HCC.

The metabolomics profile identified in HCC offers unprecedented opportunities to screen candidate metabolites for early diagnosis and treatment. From the perspective of metabolomics, lipid, energy and amino acid metabolism may be affected in the progress of HCC (168). Glycolysis-related metabolites, TCA cycles and pyrimidine synthesis change in tumor tissues at different stages. Carbohydrates that are energy sources of hepatocytes and carbohydrates, such as mannose, galactose, and arabinose, are significantly reduced in the serum of HCC patients and other liver diseases (169). The reductional feature of carbohydrates in HCC is also consistent with most cancer cells, in which they can produce energy by undergoing high speed glycolysis followed by lactic acid fermentation in the cytoplasm instead of using oxidative phosphorylation in mitochondria (170). Dysregulation of amino acid metabolism is associated with liver disease and HCC development (171). Because of increased tumor protein synthesis and energy demand of amino acids in malignant tumor cells (172), multiple amino acids, such as proline, lysine, ornithine, phenylalanine serine, and tyrosine, are upregulated significantly in HCC and HBV-cirrhosis patients (165). Additionally, because of aggressive cell proliferation in HCC, the energy supply and cell membrane synthesis must increase fatty acids including arachidonic acid, which is at a higher level in HBV-cirrhosis and HCC patients (173, 174). Thus, the fatty acids may be involved in the pathogenesis of HCC.

During the viral life cycle, HBV is associated with hepatic metabolism. This evidence of the involvement of cell metabolism in HBV-associated cancer prognosis raises interest in metabolic enzymes-targeted cancer therapy (175). In the study of the metabolic pathway, omics evidence also shows that immune patterns between HBV and the host are closely associated with the disease progression of patients infected with viruses, and metabolic alterations can be regulated by HBV protein in HCC cells. Through multiomic analysis, Xie et al. demonstrated that HBV core protein (HBc) increased the secretion of metabolites and expression of metabolic enzymes in HCC cells, and activated the amino acid and glycolysis metabolism pathways (176). Similar to previous studies, HBc can bind to human gene promoters to mediate primary metabolic processes (177). The metabolic components of liver microenvironment are actively involved in the occurrence and development of HBV infection, and hot-spot mutations in HBc, including L60V, I97L, and S87G, affect viral replication, persistence and immune pathogenesis in CHB infection (178, 179). Additionally, HBs, HBx, and HBc integrate into human genes to affect patient survival (47). Cellular retinoid X receptor alpha (RXRα), a key transcription factor for monitoring hepatic lipid metabolism, regulates HBV infection, and the arachidonic acid (AA)/eicosanoid biosynthesis pathway may be involved in the regulation of HBV infection (180). Therefore, hepatic lipid homeostasis is critical to modulate viral infection.

MICROBIOME CHARACTERISTICS

Microbiome is becoming a potentially key regulator of cancer development, especially in gut and liver microbiomes. Because the microbial group is mainly located in the intestines, the gut microbiome is the most studied and associated with a variety of human diseases, including Alzheimer's disease, cardiovascular disease, diabetes, arthritis and cancer, which is not surprising (181). Actually, gut bacteria play a key role in maintaining gut-liver axis health, and intestinal flora disorders occurred in 20–75% of patients with chronic liver disease (182). One study carried out 16S rRNA analyses in 35 individuals with HBV related HCC (B-HCC). Compared with 22 individuals with non-HBV/non-HCV (NBNC) related HCC (NBNC-HCC), the species richness of fecal microbiota of B-HCC patients was much higher. The results showed that there are differences in the number of bacteria involved in different functions or biological pathways (183). Zheng et al. also showed that gut microbiota disorder was more common in patients with liver cirrhosis-induced HCC, however, hepatitis virus infection was not associated with intestinal microbial imbalance. The data indicated that butyrate-producing genera was decreased and genera producing-lipopolysaccharide (LPS) was increased in liver cirrhosis-induced HCC (184). In HBV induced tumors, this tumor inhibitory effect is inferred based on the down-regulation of microorganisms that induce cancer and stem cell pathway. Using next-generation RNA-sequencing against HBV-related HCC patients and adjacent

normal liver tissues, the results of this study suggest that both heavy drinking and HBV infection may use the tumor microbiome to promote the development of cancer, however, only HBV infection could downregulate microorganisms that may promote stem cell function (185). They suggested strains of *Escherichia coli* were to be potentially important to HCC progression. The change of liver microenvironment in HCC patients may lead to the change of bacterial level in gut. Overall, gut-liver-axis could be used to monitor and prevent the progress of liver disease and liver cancer.

CONCLUSIONS

The initiation and progression of liver cancer are involved in multisystem and multilevel pathological changes. Single-omics analysis plays a key role in the diagnosis and therapy of diseases in modern society. However, with the development of research technologies and needs, single-omics is not sufficiently comprehensive; one type of omics change can't represent the overall status of the disease, only 10–20% of transcriptome changes are associated with proteomic data (186). Abnormal gene expression is also a risk factor leading to tumor cell formation. Changes in DNA nucleotide sequences and epigenetic mechanisms may result in aberrant gene expression profiles. The entire regulatory network may be clearly illustrated by more advanced and sensitive high-throughput omics technologies. Single-omics research is crucial, and this method often has some limitations. By contrast, the integrated analysis of multi-omics data can better describe the overall changes in liver cancer, thus achieving more valuable data in the diagnosis and development of therapeutic targets in human diseases. The latest advances in analytical technology, including ultra deep sequencing, have made multi-omicsmulti-omics analysis faster, more accurate and simpler. Many omics technologies are widely used in cancer research. The introduction of omics technology to analyze the pathogenesis or treatment of HBV-associated HCC may help not only to identify biomarkers for clinical use but also to explore the experimental research background of the pathogenesis of various diseases. Several studies have detected exome sequencing or whole genome sequencing and have used genome sequencing to identify driver gene mutations in liver cancer. The application of currently rapidly developing omics technology will promote the development of knowledge-based diagnosis and treatment strategies. In the future, more cohort studies will explore the prognostic factors of HCC patients, and candidate genes related to prognosis or recurrence of HCC will be identified using omics technology.

AUTHOR CONTRIBUTIONS

YW, DC, and JX conceived the topic. YW and DC conducted literature review, drafted the manuscript, designed the figures, and tables. JX, WL, JW, and DJ polished the manuscript. All authors approved the submitted version.

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Genetic Mutations in *TNFSF11* Were Associated With the Chronicity of Hepatitis C Among Chinese Han Population

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Background: Recently, several studies have reported that the host immune response can be related to the RANKL/RANK/OPG signaling pathway. However, the associations of *TNFSF11*, *TNFRSF11A*, and *TNFRSF11B* gene polymorphisms in the RANKL/RANK/OPG pathway with hepatitis C virus (HCV) infection outcomes remain unclear.

Methods: In this case-control study, 768 persistent HCV infection and 503 spontaneous HCV clearance cases, and 1,259 control subjects were included. The Taman-MGB probe method was utilized to detect *TNFSF11* rs9525641, *TNFRSF11A* rs8686340, and *TNFRSF11B* rs2073618 genotypes. The distribution of three single nucleotide polymorphisms (SNPs) genotypes was analyzed using stata14.0.

Results: SNPs rs9525641, rs8086340, and rs2073618 genotype frequencies followed the Hardy-Weinberg natural population equilibrium ($p = 0.637$, 0.250, and 0.113, respectively). Also, rs9525641 was significantly associated with HCV chronicity risk in recessive (OR = 1.203, 95% CI: 1.018–1.420, $p = 0.030$) and additive models (OR = 1.545, 95% CI: 1.150–2.075, $p = 0.004$). The stratified analysis showed that rs9525641 variant genotypes were associated with HCV chronicity among people older than 50 years (OR =1.562, 95% CI: 1.079–2.262, $p = 0.018$), females (OR = 1.667, 95% CI: 1.145–2.429, $p = 0.008$), ALT < 40 U/L (OR = 1.532, 95% CI: 1.074–2.286, $p = 0.018$), and AST < 40 U/L (OR = 1.552, 95% CI: 1.095–2.201, $p = 0.014$).

Conclusion: *TNFRSF11* rs9525641 was significantly associated with HCV chronicity in the Chinese population.

Keywords: gene polymorphism, hepatitis C virus, TNFSF11, chronicity, bioinformatics

INTRODUCTION

With the introduction of all-oral direct-actingantiviral therapy, a substantial breakthrough has been made in chronic HCV infection treatment during the past decades (1). However, due to HCV's significant heterogeneity and high variability, virus reinfection following successful treatment remains an important public health problem (2). The HCV pathogenesis and progression are complex and interact with its biological characteristics, environmental behavior factors, host immunity, and genetic background.

The activation of NF-κB and NF-κB-dependent inflammatory pathways are important to chronic HCV infection and its related cirrhosis and HCC. The NF-κB ligand (RANKL) receptor activator, a 316-amino acid transmembrane protein, is highly expressed in different immune cells including T or dendritic cells. RANKL can be induced by inflammatory factors such as interleukin 1, tumor necrosis factor α, and transforming growth factor β (3). Besides, RANK and osteoprotegerin (OPG) are RANKL receptors, and the RANKL/RANK/OPG pathway is important for cellular immune responses such as cell death and proliferation, inflammation, and immunity (4). The communication pathways mediated by TNFSF/TNFRSF are essential for numerous developmental, homeostatic, and stimulus-responsive processes. Both innate and adaptive immune cells are controlled by TNFSF/TNFRSF members in a manner that is crucial for the coordination of various mechanisms driving either co-stimulation or co-inhibition of the immune response (5). Different cellular immune responses can be triggered by genetic differences between different individuals. During the past decades, many studies have identified that immune cytokines SNPs were significantly associated with HCV spontaneous clearance and virological response, except for viral and environmental factors (6–9). Studies have shown that multiple TNFSF and TNFRSF gene SNPs are related to autoimmune diseases, suggesting that these SNPs play an important role in immunity. rs8086340 and rs2073618 are closely related to the occurrence of rheumatoid arthritis (10), and rs9525641 may affect the susceptibility and severity of AS disease (11). However, no research has addressed *TNFSF* and *TNFRSF* genetic polymorphisms' impacts on HCV-related chronic liver diseases. Considering that China has the largest number of HCV infections (about 10 million patients), we examined the relationships between OPG-RANKL-RANL pathway genes SNPs rs9525641, rs2073618, and rs8686340, and HCV infection outcomes in a high-risk Chinese population.

METHODS

Study Participants

This study included three HCV infection high-risk groups. In the present study, 2,800 subjects were recruited from 2008 to 2016, including 722 hemodialysis patients from 9 hemodialysis centers in southern China, 459 drug users from a Nanjing compulsory detoxification center, and 1,619 paid blood donors from 6 Zhenjiang villages. All research objects voluntarily signed the informed consent. The exclusion criteria were: (1) patients under 18 years and over 80 years; (2) patients with interferon treatment history; (3) patients co-infected with HBV and HIV; (4) patients who suffer from autoimmune diseases or malignant tumors; (5) patients with other liver diseases. All patients were diagnosed with patient's clinical symptoms and biochemical examination indicators by experienced doctors, strictly following international standards. Patients were grouped according to their HCV antibodies and viral load. The participants were categorized into three groups: (1) A, uninfected control (anti-HCV and HCV RNA negative); (2) B, spontaneous HCV clearance (anti-HCV positive and HCV RNA negative); (3) C, persistent HCV infection cases (anti-HCV and HCV RNA positive).

This study was conducted strictly under the "Declaration of Helsinki" and was approved by the Ethics Committee of Nanjing Medical University (2017445).

We interviewed each participant with trained personnel and collected demographic data and environmental exposure history information through a structured survey. All participants were informed and agreed to participate in this study before recruitment.

Data and Blood Sample Collection

After the interview, we collected venous blood samples (∼10 mL) from each participant, separated the plasma and white blood cells, and stored them at −80°C until assays. The detection of subjects' anti-HCV antibodies, HCV RNA, and HCV genotypes was performed with Jurong City People's Hospital and Yixing City People's Hospital, using third-generation enzyme-linked immunosorbent assay (ELISA) (Architect Anti-HCV assay, Abbott Laboratories, Abbott Park, IL, USA), Trizol LS reagent (Takara Biotech, Tokyo, Japan) and the murex HCV serotype ELISA kit (Abbott, Wiesbaden, Germany), respectively. The HCV RNA load detection limit was 1×10^3 IU/ml and all serological tests were performed with the same analytical systems.

SNP Selection and Genotyping

Candidate *TNFSF11*/*TNFRSF11A*/*TNFRSF11B* gene Tag SNPs were selected by searching the 1,000 Genomes Project (http://www.1000genomes.org/) or the HapMap (http://www.hapmap.org/) databases. The selected SNPs were filtered with the following criteria: (1) minor allele frequency >5% in Han Chinese population, acquired from the Haploview software (version 4.2; Broad Institute, Cambridge, MA, USA); (2) Hardy-Weinberg equilibrium test $p \geq 0.05$; (3) reported SNPs from previous studies associated with immune-related disorders; (4) combined bioinformatics data from Regulome DB (http://regulome.stanford.edu/). Finally, three SNP candidates (rs9525641, rs8086340, and rs2073618) were chosen for genotyping. Primers and probes are presented in **Supplementary Table 1**.

Genomic DNA was isolated from subjects' peripheral blood leukocytes using protease K digestion, phenol-chloroform extraction, and ethanol precipitation. SNPs genotyping was performed with a Taman allelic discrimination assay on the LightCycler® 480 IIReal-Time PCR System (Roche, Switzerland). All genotyping was performed without knowing the subjects' case or control status. Each SNP accordance

rate was 100% for the repeated experiments of 10% random samples. Additionally, the genotyping success rates for these polymorphisms were above 95%. All tests were carried out following the manufacturer's instructions and were performed with the same analytical systems.

In silico Analysis

The function of SNPs was predicted using the Regulome DB online database, HaploReg database (https://pubs.broadinstitute.org/mammals/haploreg/haploreg.php) and Vienna RNA Web Servers (http://rna.tbi.univie.ac.at/cgi-bin/RNAWeb/Suite/RNAfold.cgi). The Regulome DB online database annotated SNPs with known and predicted regulatory elements in *Homo sapiens* genome intergenic regions was used to obtain SNPs' Regulome DB scores. Different Regulome DB score represents different combinations of the above and detailed information on all Regulome DB scores (**Supplementary Table 2**). The HaploReg database can be used for exploring chromatin states, conservation, and regulatory motif alterations within a set of genetically linked variants. Moreover, RNA secondary structures were predicted using the Vienna RNA Web Servers based on its latest package (Version 2.3.1).

Statistic Analysis

The demographic and clinical data distribution among the three groups was compared using the χ^2 test. HWE was assessed among control subjects by the goodness-of-fit χ^2 test. Logistic regression, with age, gender, and high-risk population adjustments, was used to analyze the relationship between the three SNPs and HCV infection outcome according to four genetic models, showing odds ratio and 95% confidence intervals. Hierarchical analysis was used to control confounding factors' effects on the results and Q tests were used to determine heterogeneity between subgroups. Statistical analyzes were performed using stata14.0, and a two-sided $p < 0.05$ was considered statistically significant.

RESULTS

Demographic and Clinical Characteristics of Participants

According to the HCV antibody and RNA, subjects were divided into three groups. The demographic and clinical characteristics distribution among the HCV-uninfected control, the spontaneous HCV clearance, and the persistent infection groups are presented in **Table 1**. No significant differences in age or gender distribution among groups were detected ($p = 0.185$ and 0.095, respectively). On the other hand, alanine aminotransferase (ALT), aspartate aminotransferase (AST), infection routes, and HCV genotype differed ($p < 0.001$).

SNPs rs9525641, rs8086340, and rs2073618 genotype frequencies in the HCV-uninfected control followed the Hardy-Weinberg equilibrium ($p = 0.637$, 0.250, and 0.113, respectively). This indicated that the control was a representative group.

Associations Between SNP Candidates and HCV Infection Outcomes

The genotype distribution rs9525641, rs8086340, and rs2073618 among groups are shown in **Table 2**. To analyze the association between these SNPs and HCV infection susceptibility, we first combined patients from groups B and C in an HCV-infected group and compared it with the control (A). However, no significant association was observed in the logistic regression analyses between these three SNPs and HCV infection susceptibility ($p > 0.05$). To determine the association between these SNPs and HCV chronicity, the B group was selected as a control and compared to C. The regression analysis of a co-dominant model—corrected for age, gender, and high-risk population—showed that patients carrying the rs9525641-C gene were significantly associated with HCV chronic diseases (adjusted OR = 1.518, 95% CI: 1.079–2.136, $p = 0.017$), as for a recessive (adjusted OR = 1.203, 95% CI: 1.018–1.420, $p = 0.030$), and additive models (adjusted OR = 1.545, 95% CI: 1.150–2.075, $p = 0.004$). However, no correlation was observed between the two other genotypes and HCV infection chronicity ($p > 0.05$).

Stratified Analysis

To control age, gender, high-risk population, and HCV genotypes bias in each population, we performed a stratified analysis to explore the association between the rs9525641 genotype and HCV chronicity using a recessive model (**Table 3**). Results showed that the rs9525641 variant genotypes were significantly associated with an increased chronic HCV infection risk among people ≥50 years (adjusted OR = 1.562, 95% CI: 1.079–2.262, $p = 0.018$), females (adjusted OR = 1.667, 95% CI: 1.145–2.429, $p = 0.008$), ALT < 40 U/L (adjusted OR = 1.532, 95% CI: 1.074–2.286, $p = 0.018$), and AST < 40 U/L (adjusted OR = 1.552, 95% CI: 1.095–2.201, $p = 0.014$). Additionally, considering data heterogeneity in different subgroups, we performed a heterogeneity test. Results showed no significant heterogeneity between groups ($p > 0.05$).

Bioinformatics Analysis

The rs9525641 genotype had a Regulome DB score of 5, suggesting its potential functions such as transcription factor binding or DNase peak. Based on the HaploReg database, rs9525641 overlaps promoter histone marks, enhancer histone marks, and DNase and FXR motifs. Furthermore, 22 SNPs were in linkage disequilibrium with rs9525641 in the Asian population ($r^2 > 0.8$) (available at HaploReg database). Results of the 22 SNPs are presented in **Supplementary Table 3**. The in?uence of those SNPs on the RANKL mRNA secondary structure was predicted using the RNAfold Web Server. Six SNPs presented local structure changes (rs17458177, rs1325799, rs17536328, rs7984870, rs9533155, and rs3742257) (**Supplementary Figures 1–6**). Moreover, rs17458177-C and -T alleles showed a difference in the lowest free energy (−18.40 vs. −18.90 kcal/mol), suggesting that mutations might affect RANKL transcription. Specific information for the other SNPs can be found in the **Supplementary Figures 1–6**.

TABLE 1 | Demographic and clinical characteristics among HCV control, spontaneous clearance, and persistent infection groups.

Variables	Group A (%) $n = 1,529$	Group B (%) $n = 503$	Group C (%) $n = 768$	*P*
Age (years)				0.185
<50	561 (36.69)	193 (38.37)	312 (40.63)	
≥50	968 (63.31)	310 (61.63)	456 (59.38)	
Gender				0.095
Male	615 (40.22)	194 (38.57)	273 (35.55)	
Female	914 (59.78)	309 (61.43)	495 (64.45)	
ALT (U/L)				<0.001
<40	1,437 (94.91)	393 (78.29)	443 (57.83)	
≥40	77 (5.09)	109 (21.71)	323 (42.17)	
AST (U/L)				
<40	1,438 (95.11)	401 (81.17)	454 (59.97)	<0.001
≥40	74 (4.89)	93 (18.83)	303 (40.03)	
High-risk population				<0.001
HD	555 (36.30)	91 (18.09)	76 (9.90)	
IVDU	181 (11.84)	138 (27.44)	140 (18.23)	
PBD	793 (51.86)	274 (54.47)	552 (71.88)	
HCV genotype				<0.001
1b	–	42 (26.25)	223 (46.07)	
Non-1b	–	118 (73.75)	261 (53.93)	

Group A: controls; Group B: spontaneous clearance subjects; Group C: persistent infection patients.
Non-1b means viral strains other than 1b, including genotype 1a, 2, and 3 (either solely or mixed infection).
HCV, hepatitis C virus; SD, standard deviation; ALT, alanine transaminase; AST, aspartate transaminase; HD, hemodialysis patients; IVDU, Intravenous drug user; PBD, paid blood donors.

DISCUSSION

The RANKL message is detected in the peripheral lymph nodes and bone marrow, thymus, spleen, Peyer's patches, brain, heart, skin, skeletal muscle, kidney, liver, lung, and mammary tissues (12). The RANKL/RANK system has been shown to play a critical role in the immune system, including lymph-node development, lymphocyte differentiation, dendritic cell survival, and T-cell activation and tolerance induction (13). RANKL can regulate lymph-node organogenesis, T- and B-lymphocyte development, and osteoclast differentiation. Some studies also indicated that RANKL regulates the thymus microenvironment by autoimmune regulators expression activation (14). Additionally, at the molecular level, RANK interacts with RANKL to activate the transcription factor NF-κB along with TNF receptor-related factor family signaling molecules (15). Considering that the NF-κB function is related to RANKL/RANK, and NF-κB has been linked to chronic hepatitis C (16–18), we hyphothesized that the RANKL/RANK pathway polymorphisms would affect the HCV infection outcome.

RANKL, RANK, and OPG are encoded by *TNFSF11* (gene map locus 13q14), *TNFRSF11A* (gene map locus 18q22.1), and *TNFRSF11B* (gene map locus 8q24), respectively (19). The *TNFSF11* gene structure is highly conserved among mammals, consisting of five exons that span 33.9 kb in humans (10). SNPs located near *TNFSF11*, *TNFRSF11A*, and *TNFRSF11B* have been reported to be closely associated with Paget's disease (20), osteoporotic fractures (21), cardiovascular diseases (22), ankylosing spondylitis (11), and breast (23), and esophageal cancers (24). In this study, we showed that subjects who carried the rs9525641-C allele were more likely to develop HCV chronicity than those with the rs9525641-T allele. Furthermore, in the stratification analyses based on age, gender, and high-risk population, we found that the rs9525641-C allele was associated with HCV chronicity among elders, females, and persons with ALT and AST < 40 U/L. Interestingly, RANKL can cause various degenerative bone diseases, such as rheumatoid arthritis and osteoporosis. These diseases are mostly female. Our research shows that RANKL is related to the chronicity of HCV in women. This suggests to some extent the combined effect of gender factors and the RANKL system in disease progression, which is worthy of further discussion. However, the heterogeneity test showed no significant heterogeneity in any pair-wise comparison ($p > 0.05$), indicating that these variables did not materially affect the results.

Although rs9525641 is located in the *TNFSF11* intronic region, this variant might play an important role in gene transcription regulation or might be in linkage disequilibrium with other functional SNPs, such as rs17458177, rs1325799, rs17536328, rs7984870, rs9533155, and rs3742257. We also calculated the degree of linkage between the candidate SNPs, $r^2 < 0.1$ indicates that there is no linkage disequilibrium among these three SNPs. The bioinformatics analysis indicated that these variants could regulate gene transcription, mRNA

TABLE 2 | Genotypes distributions of three SNPs among persistent infection, spontaneous clearance and control group.

SNPs (genotype)	Group A *n* (%) *n* = 1,529	Group B *n* (%) *n* = 503	Group C *n* (%) *n* = 768	OR(95%CI)[a]	*P*[a]	OR (95%CI)[b]	*P*[b]
rs9525641				0.037			
TT	406 (26.71)	144 (29.63)	209 (27.68)	1.00	–	1.00	–
TC	768 (50.53)	260 (53.50)	365 (48.34)	0.946 (0.788–1.137)	0.556	0.973 (0.743–1.274)	0.842
CC	346 (22.76)	82 (16.87)	181 (23.97)	0.892 (0.715–1.114)	0.313	**1.518 (1.079–2.136)**	**0.017**
Dominant model				0.930 (0.782–1.105)	0.409	1.104 (0.855–1.425)	0.448
Recessive model				0.925 (0.767–1.115)	0.412	**1.545 (1.150–2.075)**	**0.004**
Additive model				0.945 (0.846–1.055)	0.313	**1.203 (1.018–1.420)**	**0.030**
rs8086340				0.267			
GG	621 (41.40)	212 (43.71)	332 (43.92)	1.00	–	1.00	–
GC	704(46.93)	209 (43.09)	322 (42.59)	0.878 (0.743–1.036)	0.124	0.987 (0.769–1.265)	0.916
CC	175 (11.67)	64 (13.20)	102 (13.20)	1.140 (0.887–1.465)	0.306	1.024 (0.713–1.472)	0.896
Dominant model				0.929 (0.794–1.086)	0.355	0.995 (0.788–1.257)	0.970
Recessive model				1.219 (0.963–1.543)	0.100	1.031 (0.733–1.450)	0.860
Additive model				1.007 (0.898–1.130)	0.902	1.005 (0.850–1.188)	0.952
rs2073618				0.149			
GG	854 (56.33)	266 (53.41)	409 (53.46)	1.00	–	1.00	–
GC	552 (36.41)	207 (41.57)	305 (39.87)	1.131 (0.962–1.330)	0.137	0.933 (0.735–1.185)	0.572
CC	110 (110)	25 (5.02)	51 (6.67)	0.841 (0.613–1.155)	0.286	1.318 (0.791–2.197)	0.289
Dominant model				1.082 (0.927–1.264)	0.316	0.975 (0.774–1.227)	0.827
Recessive model				0.799 (0.586–1.090)	0.157	1.358 (0.823–2.239)	0.230
Additive model				1.015 (0.896–1.149)	0.817	1.029 (0.851–1.243)	0.770

CI, confidence interval; HCV, hepatitis C virus; OR, odds ratio; SNP, single nucleotide polymorphism.

Group A: controls; Group B: spontaneous clearance subjects; Group C: persistent infection patients. Group (B+C): Infected individuals.

[a] *The P-value, OR and 95% CIs of Group (B + C) vs. Group A were calculated on the basis of the logistic regression model, adjusted by gender, age, and high-risk population.*

[b] *The P-value, OR and 95% CIs of Group C vs. Group B were calculated on the basis of the logistic regression model, adjusted by gender, age, high-risk population.*

Bold type indicates statistically significant results.

TABLE 3 | Stratified analysis the association of rs9525641 with HCV chronicity.

Subgroups	Group A *n* (CC/CA/AA)	Group B *n* (CC/CA/AA)	Group C *n* (CC/CA/AA)	OR (95%CI)[a]	P^a	P^b
Age						
<50	145/284/126	51/100/28	80/155/65	1.482(0.900–2.442)	0.122	0.872
≥50	261/484/220	93/160/54	129/210/116	**1.562 (1.079–2.262)**	**0.018**	
Gender						
Male	179/300/133	58/92/33	81/126/64	1.330 (0.823–2.150)	0.245	0.474
Female	277/468/213	86/168/49	128/239/117	**1.667 (1.145–2.429)**	**0.008**	
ALT (U/L)						
<40	383/722/326	115/203/63	124/211/100	**1.532 (1.074–2.186)**	**0.018**	0.884
≥40	21/37/16	29/56/19	85/152/81	1.455 (0.826–2.562)	0.176	
AST (U/L)						
<40	380/729/321	112/213/65	130/210/106	**1.552 (1.095–2.201)**	**0.014**	0.736
≥40	22/30/21	30/43/17	79/147/73	1.373 (0.751–2.510)	0.303	
High-risk population						
HD	135/286/131	26/44/17	21/37/17	1.337 (0.610–2.928)	0.468	0.614
IVDU	46/93/38	37/74/15	41/67/32	2.263 (1.152–4.445)	0.018	
PBD	225/389/177	81/142/50	147/261/132	1.419 (0.983–2.047)	0.062	
HCV genotypes						0.708
1b	—	7/24/9	72/99/51	0.996 (0.347–2.858)	0.994	
Non-1b	—	38/54/19	57/136/58	1.302 (0.643–2.637)	0.464	

CI, confidence interval; HCV, hepatitis C virus; OR, odds ratio; HD, hemodialysis patients; IVDU, Intravenous drug user; PBD, paid blood donors; Non-1b, viral strains other than 1b, including genotype 1a, 2, and 3 (either solely or mixed infection).

Group A: controls; Group B: spontaneous clearance subjects; Group C: persistent infection patients. Group (B+C): Infected individuals.

[a] *The P-value, OR and 95% CIs of Group C vs. Group B were calculated on the basis of the logistic regression model, adjusted by gender, age, high-risk population.*

[b] *P-value for the heterogeneity test.*

Bold type indicates statistically significant results.

export, and protein translational efficiency. In the present study, we found two possible *TNFSF11* biological processes using the STRING database website prediction (https://string-db.org/cgi/network.pl?taskId=kGEafB5GcPa7:) extracellular signal-regulated kinase 1 (ERK1) and ERK2 cascade positive regulation via *TNFSF11*-mediated signaling; and the tumor necrosis factor-mediated signaling pathway. It has been reported that the HCV non-enveloped particles' intriguing cellular internalization properties can activate the ERK1/2 pathway that could be important in the HCV life cycle and infection pathogenesis (25). Additionally, Fletcher et al. reported that several TNF superfamily members – including TNF-α, TNF-β, TWEAK, and LIGHT – can promote HCV entry via NF-κB-mediated activation of myosin light chain kinase and tight junctions disruption (26). Therefore, *TNFSF11* may be involved in these pathways and contribute to the hepatitis C chronic process. However, these hypotheses are based on computer simulations and functional evaluations using biological assays. Thus, it should be warranted in future studies.

RANK is an intrinsic hematopoietic cell surface receptor that stimulates NF-κB receptor activation, plays a central role in T and dendritic cells, and promotes lymph node development (27). Previously, several studies have found that the TNFRSF11 rs8680340 was closely related to the anti-citrullinated peptide antibody and age at natural menopause (28). Additionally, the *TNFRSF11B* rs2073618 was significantly associated with Type 2 Diabetes (29), rheumatoid arthritis (30), and volumetric bone mineral density (31). However, in this study, no significant association was observed between *TNFRSF11A* rs8086340, *TNFRSF11B* rs2073618, and HCV infection outcome.

Our study also has some limitations. First, this study was performed with the Chinese Han population, requiring further reproduction in different ethnic populations. Second, we did not collect immune markers data in the RANKL/RANK pathway and could not check the association between immune markers data and the target SNPs. Third, we did not collect complete virus subtypes and viral load data and could not check the association between virus data and the target SNPs. Hence, we should improve the collection of this part of the data in the later stagethe and possible immune mechanism requires to be further studied and verified. Furthermore, we did not explore how the functional mechanisms of these SNPs affect HCV chronicity using molecular biology approaches. Then, functional studies are required in the future to explore how these polymorphisms impact chronic HCV infection.

Overall, our findings suggested that the rs9525641 *TNFSF11* polymorphism might affect HCV chronicity in a high-risk Chinese population. Larger well-designed epidemiological studies with ethnically diverse populations and functional

evaluations are warranted to confirm these findings before the effect of these variants can be fully and accurately evaluated.

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 studies involving human participants were reviewed and approved by the Ethics Committee of Nanjing Medical University. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

SY and R-BY: conceptualization. Y-QH: formal analysis, investigation, and writing–original draft. PH: funding acquisition and methodology. Y-DW and J-JW: resources. J-JW and FZ: software. SY: supervision. Y-QH and X-YY: writing–review and editing. 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/fmed.2021.743406/full#supplementary-material

Supplementary Figure 1 | The influence of rs17458177 on the RANKL mRNA secondary structures. The arrow indicates the position of the mutation (50 bases upstream and 50 bases downstream of the mutation). The minimum free energies for the C and T allele of rs17458177 were estimated at −18.40 and −18.90 kcal/mol, respectively, by RNAfold Wed Server.

Supplementary Figure 2 | The influence of rs1325799 on the RANKL mRNA secondary structures. The arrow indicates the position of the mutation (50 bases upstream and 50 basesdownstream of the mutation). The minimum free energies for the G and A allele of rs1325799 were estimated at −11.80 and −14.00 kcal/mol, respectively, by RNAfold Wed Server.

Supplementary Figure 3 | The influence of rs17536328 on the RANKL mRNA secondary structures. The arrow indicates the position of the mutation (50 bases upstream and 50 basesdownstream of the mutation). The minimum free energies for the C and T allele of rs17536328 were estimated at −17.4 and −16.60 kcal/mol, respectively, by RNAfold Wed Server.

Supplementary Figure 4 | The influence of rs7984870 on the RANKL mRNA secondary structures. The arrow indicates the position of the mutation (50 bases upstream and 50 basesdownstream of the mutation). The minimum free energies for the G and C allele of rs7984870 were estimated at −14.40 and −15.50 kcal/mol, respectively, by RNAfold Wed Server.

Supplementary Figure 5 | The influence of rs9533155 on the RANKL mRNA secondary structures. The arrow indicates the position of the mutation (50 bases upstream and 50 basesdownstream of the mutation). The minimum free energies for the C and G allele of rs9533155 were estimated at −14.80 and −15.50 kcal/mol, respectively, by RNAfold Wed Server.

Supplementary Figure 6 | The influence ofrs3742257 on the RANKL mRNA secondary structures. The arrow indicates the position of the mutation (50 bases upstream and 50 basesdownstream of the mutation). The minimum free energies for the T and C allele of rs3742257 were estimated at −31.70 and −27.80 kcal/mol, respectively, by RNAfold Wed Server.

Supplementary Table 1 | Probes and primers of investigated *TNFSF* and *TNFRSF* SNPs for Taman assay.

Supplementary Table 2 | The detailed information on different RegulomeDB scores.

Supplementary Table 3 | Annotation of variannts with stong linkage disequilibrim with SNP rs 9525641 in HaploRegV4.1.

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Distinct Bile Acid Profiles in Patients With Chronic Hepatitis B Virus Infection Reveal Metabolic Interplay Between Host, Virus and Gut Microbiome

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Hepatitis B virus (HBV) can hijack the host bile acids (BAs) metabolic pathway during infection in cell and animal models. Additionally, microbiome was known to play critical role in the enterohepatic cycle of BAs. However, the impact of HBV infection and associated gut microbiota on the BA metabolism in chronic hepatitis B (CHB) patients is unknown. This study aimed to unveil the distinct BA profiles in chronic HBV infection (CHB) patients with no or mild hepatic injury, and to explore the relationship between HBV, microbiome and BA metabolism with clinical implications.

Methods: Serum BA profiles were compared between CHB patients with normal ALT (CHB-NALT, $n = 92$), with abnormal ALT (CHB-AALT, $n = 34$) and healthy controls (HCs, $n = 28$) using UPLC-MS measurement. Hepatic gene expression in CHB patients were explored using previously published transcriptomic data. Fecal microbiome was compared between 30 CHB-NALT and 30 HCs using 16S rRNA sequencing, and key microbial function was predicted by PICRUSt analysis.

Results: Significant higher percentage of conjugated BAs and primary BAs was found in CHB patients even without apparent liver injury. Combinatory BA features can discriminate CHB patients and HCs with high accuracy (AUC = 0.838). Up-regulation of BA importer Na+ taurocholate co-transporting peptide (NTCP) and down-regulation of bile salt export pump (BSEP) was found in CHB-NALT patients. The microbial diversity and abundance of *Lactobacillus*, *Clostridium*, *Bifidobacterium* were lower in CHB-NALT patients compared to healthy controls. Suppressed microbial bile salt hydrolases (BSH), 7-alpha-hydroxysteroid dehydrogenase (hdhA) and 3-dehydro-bile acid Delta 4, 6-reductase (BaiN) activity were found in CHB-NALT patients.

Conclusion: This study provides new insight into the BA metabolism influenced both by HBV infection and associated gut microbiome modulations, and may lead to novel strategy for clinical management for chronic HBV infection.

Keywords: chronic hepatitis B, metabolomics, bile acid metabolism, gut microbiome, NTCP

INTRODUCTION

Human hepatitis B virus (HBV) infection remains a major public health problem, affecting over 250 million population globally (1). Unfortunately, there was no curable option for HBV infection. Series of recent discoveries have revealed complex interplay between HBV infection and host bile acids (BA) metabolism which may lead to future antiviral therapies.

The Na+ taurocholate co-transporting polypeptide (NTCP, encoded by SLC10A1), originally known as a major hepatic transporter for BAs (2–4), was recently discovered as the receptor permitting the hepatotropic entry of HBV (5–7). This interesting dual role of NTCP played in both HBV entry and BA transportation was further confirmed by a report showing inhibition of taurocholate uptake by HBsAg pre-S1 polypeptide in NTCP-expressing HepG2 cells (8). Furthermore, HBV was found able to induce the key rate-limiting enzyme for BA synthesis, cholesterol 7α-hydroxylase (CYP7A1), possibly via a FXRα-dependent fashion (9–11). Normally, the hepatic BA metabolism is tightly regulated by a group of factors orchestrated by FXRa which was kept activated by BA binding. The active FXRa is required for the expression of conjugation enzymes UGT2B4 and SULT2A1 (12, 13), detoxification enzymes ADH1A/B (14) and CYP3A4 (15), efflux pumps BSEP and ABCB4 (16), while conversely, suppresses CYP7A1, NTCP and FXRa itself via a SHP-mediated negative feedback loop (17, 18). In HBV infection cell models, inadequate hepatocellular BA level due to NTCP obstruction by HBV binding can lead to inactivation of FXRa which in turn up-regulates CYP7A1, NTCP and FXRa (11). Moreover, the inactive FXRa appeared to be a proviral factor that can transcriptionally enhance the HBV activity (10, 11). Therefore, the competitions between HBV and BAs for host factors occur both in viral entry and post-entry stages, and HBV is capable of hijacking BA regulatory network for its own benefit. Additionally, the host inflammatory response to the HBV activity also modulates the BA metabolism, as excessive cytokine activities was found to suppress NTCP and leads to cholestasis and jaundice in CHB patients (19–21). Therefore, caution should be taken to investigate the interaction of BA metabolism and HBV in patients with exacerbated hepatic inflammation.

Other than the interplay between HBV infection and BA metabolism in the liver, one cannot ignore the increasingly recognized role of gut microbiota (22). The enterohepatic circulation of BAs within the gut-liver axis requires microbial biotransformation before BA being reabsorbed into the portal vein (22–24). This process depends on intestinal bacteria containing bile salt hydrolases (BSHs) which transformed conjugated BA (CBAs) to unconjugated forms (UCBAs), as well as dehydrogenase and oxidoreductases which convert primary BAs into their secondary counterparts (23). Importantly, the links between liver diseases and gut microbiota have been widely documented (25–27). Particularly, fecal samples from CHB patients were found to have a marked decrease in bifidobacteria and lactic acid bacteria (25). However, little was known whether such modulation of gut microbiota could influence the BA metabolism in CHB patients.

The potential clinical implications of BA modulation in HBV infected patients are multifold. Bile acids play essential roles in glucose and cholesterol homeostasis, intestinal absorption of nutrients (28). In addition, BAs activate a variety of nuclear receptors and signaling pathways (29). Abnormal accumulation of BAs could cause hepatic and biliary injury and inflammation, which are associated with fibrosis and cirrhosis eventually leading to liver failure or liver cancer (30–34). Therefore, BA metabolism has been advocated as a novel therapeutic target for liver diseases treatment (35–37). However, comprehensive profiles of BAs in liver disease at very early or mild stages of CHB are still lacking.

This study aimed to investigate the association between HBV infection, microbiome modulation and BA metabolism in CHB patients with mild hepatic inflammation. We first compared the fasting serum profiles of BA in healthy controls and CHB patients with or without hepatic inflammation. For the first time, we show distinct pattern of BAs can be found in CHB patients even with normal ALT level. On top of this, the roles of hepatic genes and gut bacteria related to BA metabolism were investigated in CHB patients.

Abbreviations: (NTCP), Na+ taurocholate co-transporting peptide; (HBV), hepatitis B virus; (BA), bile acid; (CHB), chronic hepatitis B; (ALT), alanine aminotransferase; (AST), aspartate aminotransferase; (CHB-NALT), chronic hepatitis B with normal alanine aminotransferase; (CHB-AALT), chronic hepatitis B with abnormal alanine aminotransferase; (UPLC), ultra-performance liquid chromatography; (MS), mass spectrometry; (PICRUSt), phylogenetic investigation of communities by reconstruction of unobserved states; (BSH), bile salt hydrolases; (BSEP), bile salt export pump; (HBsAg), hepatitis B surface antigen; (HBeAg), hepatitis B e antigen; (GGT), gamma-glutamyl transpeptidase; (ALB), albumin; (UCBA), unconjugated bile acids; (CBA), conjugated bile acids; glyco-conjugated (GCBA), (TCBA), bile acids; tauro-conjugated bile acids; (PBA), primary bile acids; (SBA), secondary bile acids.

MATERIALS AND METHODS

Patients Enrollment

A total of 126 HBV-infected patients and 28 healthy subjects were recruited from the First Affiliated Hospital, School of Medicine, Zhejiang University (Hangzhou, China). Routine biochemical parameters including aspartate transaminase (AST), alanine transaminase (ALT), alkaline phosphatase (ALP), γ-glutamyltransferase (GGT), creatinine (Cr), albumin

(ALB), γ-globulins (GLB), total bilirubin (T-Bil), direct bilirubin (D-Bil), prothrombin time (PT), blood urea nitrogen (BUN), uric acid (UA), triglyceride (TG), total cholesterol (TC), low-density lipoprotein (LDL), glucose (GLU), serum HBV-DNA and hepatitis viral antigens (HBsAg, HBeAg) were measured in the clinical laboratory of the First Affiliated Hospital, School of Medicine, Zhejiang University. Chronic HBV infection is defined as HBsAg sero-positive status for at least 6 months according to 2015 APASL guidelines (38). The upper limit of normal laboratory reference (ULN) of ALT was 40 IU/mL (38). Exclusion criteria including: (1) cases complicated with other infectious diseases or liver diseases such as alcoholic hepatitis, non-alcoholic fatty liver disease, biliary diseases, liver cancer, liver cirrhosis, liver failure; (2) patients with history of using UDCA supplements, or drugs lead to cholestasis such as ademetionine and silymarin, antibiotics or probiotics or herbal medicine within 24 weeks; (3) cases with gastrointestinal tract abnormalities; (4) pregnant or lactating women. Detailed definitions for clinical criteria can be found in **Supplementary Table 1**. This study was carried out with the approval of the Ethics Committee of the First Affiliated Hospital, School of Medicine, Zhejiang University, and all subjects have signed informed consent. Since liver injury due to inflammation was known to alter BA profiles, we therefore divide CHB patients into two subgroups, CHB-NALT with normal ALT level (<40 IU/mL), and CHB-AALT with abnormal ALT level (≥40 IU/mL) indicating hepatic injury.

Serum Bile Acids Measurement

Serum samples were obtained using their fasting blood in the early morning. To extract circulatory BAs, 50 μL of serum samples were allowed to thaw on ice and were subsequently spiked with 150 μL cold acetonitrile. Samples were vortexed 3 × 10 s and maintained at −20°C for 20 min to precipitate proteins. After centrifugation at 10,000 g for 10 min at 4°C, 100 μL supernatants were transferred to clean tubes and dried in a Speed Vac concentrator (Labconco). The residue was reconstituted in 50 μL of 50% methanol: water and was centrifuged at 10,000 g for 10 min at 4°C.

The following 15 most common human BA species were determined by a LC-MS/MS method: cholic acid (CA), chenodeoxycholic acid (CDCA), deoxycholic acid (DCA), lithocholic acid (LCA), ursodeoxycholic acid (UDCA), glycocholic acid (GCA), glycochenodeoxycholic acid (GCDCA), glycodeoxycholic acid (GDCA), glycolithocholic acid (GLCA), glycoursodeoxycholic acid (GUDCA), taurocholicacid (TCA), taurochenodeoxycholic acid (TCDCA), taurodeoxycholic acid (TDCA), taurolithocholic acids (TLCA), and tauroursodeoxycholic acid (TUDCA). BAs were separated by Waters ACQUITY UPLC BEH C18 column (2.1 mm × 10 cm, 1.7 μm, 130 Å) installed in a 1,290 UHPLC system (Agilent) at a flow rate of 0.5 mL/min. The BA profile was analyzed by an Xevo TQ-S mass spectrometer (Waters) operated under the multiple reaction monitoring (MRM) mode as detailed in the **Supplementary Materials**. Data acquisition parameters for each BA species in MRM experiment can be found in **Supplementary Table 2**. The typical chromatogram can be found in (**Supplementary Figure 1**).

BA Related Gene Expression Analyses

Expression level of 31 hepatic genes related to BA metabolism (**Supplementary Table 3**) from CHB patients and healthy controls (dataset GSE83148) (39) were downloaded from NCBI GEO database. The affymetrix chip-based expression signals were log10 transformed prior to statistical analysis.

Fecal Microbiome Profiling

Fecal samples of 30 CHB-NALT patients and 30 healthy volunteers (**Supplementary Table 4**) were used for 16S rDNA profiling. Detail about sample preparation, 16S rDNA library building and sequencing, OTUs assembly, and microbial community diversity analyses can be found in **Supplementary Materials**. To predict bile salt hydrolase gene content in the samples, sequencing data were mapped to OTUs defined in the 13_5 release of the Greengenes database. Relative abundances of those OTUs were used to predict the abundances of genes corresponding to KEGG orthology K01442 (cholylglycine hydrolase), K00076 (7-alpha-hydroxysteroid dehydrogenase, hdhA), K07007 (3-dehydro-bile acid Delta4, 6-reductase, baiN) with PICRUSt algorithm (40).

Statistical Analyses

Variables following a normal distribution were presented as mean ± SEM and were compared by parametric *t*-test. BA profiles and ratios did not fit normal distribution according to the Shapiro-Wilk test (all $p < 0.05$), therefore were compared by nonparametric Mann–Whitney U test. Logistic regression models were built by stepwise method, using $P < 0.05$ for entering the model and $P > 0.10$ for removing from the model. Area under the curve (AUC) in the receiver operating characteristic (ROC) analysis was used to estimate the predictive power of indicators. All statistical tests were two-sided, and $P < 0.05$ were considered as statistically significant. The statistical analyses were performed by using R (v3.2.0).

RESULTS

Clinical and Laboratory Characteristics of the Patients and HCs

Characteristics of the study subjects are summarized in **Table 1**. Compared to healthy controls, CHB-NALT patients had significantly higher levels of serum GLB, ALT, AST, and TBIL levels (all $p < 0.05$). The other biochemical parameters of CHB-NALT patients and healthy controls were within the reference ranges. In comparison, CHB-AALT patients had significantly higher level of ALT, AST, GGT, and TBIL (all $p < 0.05$) than CHB-NALT patients as expected. There was no difference of TP, ALB, ALP, Cr, BUN, UA, TG, TC, LDL, and GLU levels among all groups.

TABLE 1 | Baseline characteristics of study subjects for serum examination.

	HC ($n = 28$)	CHB-NALT ($n = 92$)	CHB-AALT ($n = 34$)
Gender (male/female)	14/14	52/40	24/10
Age (years)	46.00 ± 10.73	39.78 ± 10.56*	40.35 ± 11.61
HBV-DNA (copies/mL)	/	3.96 ± 10.99 E+07	1.70 ± 4.96 E + 07
HBsAg (IU/mL)	/	15408.15 ± 26749.88	5711.73 ± 13339.52
HBeAg (PEIU/mL)	/	89.38 ± 140.82	62.74 ± 119.83
TP (g/L)	72.62 ± 3.19	73.96 ± 4.69	72.74 ± 6.14
ALB (g/L)	48.13 ± 2.72	47.33 ± 2.99	46.00 ± 5.58
GLB (g/L)	24.51 ± 2.23	26.64 ± 3.72*	26.74 ± 3.84#
ALT (U/L)	15.46 ± 7.99	22.33 ± 8.35*	123.18 ± 143.59#†
AST (U/L)	18.36 ± 4.09	21.76 ± 6.38*	66.12 ± 54.04#†
ALP (IU/L)	69.54 ± 44.98	67.43 ± 18.63	75.94 ± 25.98#
TBIL (μmol/L)	11.11 ± 4.88	14.27 ± 7.95	23.85 ± 40.56#
GGT (U/L)	27.68 ± 23.12	19.08 ± 11.37	57.00 ± 37.82#†
Cr (μmol/L)	71.93 ± 13.04	72.30 ± 16.70	72.74 ± 14.57
BUN (mmol/L)	5.26 ± 1.35	4.87 ± 1.04	5.00 ± 0.98
UA (μmol/L)	294.54 ± 71.79	308.95 ± 80.74	311.44 ± 90.15
TG (mmol/L)	1.41 ± 1.35	1.18 ± 0.67	1.13 ± 0.53
TC (mg/dL)	4.71 ± 0.77	4.34 ± 0.93*	4.11 ± 1.07#
LDL (mg/dL)	2.58 ± 0.72	2.49 ± 0.68	2.27 ± 0.80
GLU (mmol/L)	5.05 ± 1.21	5.04 ± 0.70	5.33 ± 1.67

All data were presented by mean ± SEM. HC, healthy control; CHB-NALT, ALT normal patient; CHB-AALT, ALT abnormal patient; The p values of categorical and quantitative variables were determined by χ^2 and Mann–Whitney U test. Significant difference ($P < 0.05$) between HC and CHB-NALT, HC and CHB-AALT# or between CHB-NALT and CHB-AALT† were indicated.*

The Distinct Bile Acid Profiles in Chronic Hepatitis B Patients

The overall BA profiles across all subjects are visualized in **Figure 1** and summarized in **Table 2**. In brief, BA profiles were highly heterogeneous in CHB patients (**Figure 1A**). Although there was no significant difference in total BAs levels (**Figure 1B**, all $p > 0.05$), alteration of BA composition can be found among all three groups. Significantly, the percentage of total DCA, adding both unconjugated and conjugated forms, was lower in both CHB groups (**Figure 1C**). Lowest median level of all UCBAs was found in CHB-NALT patients, while highest median level of all UCBAs was found in healthy controls (**Figure 1D**). Gradual increases of conjugated forms of primary BAs, i.e. GCA, TCA, GCDCD, TCDCA were observed in CHB-NALT and CHB-AALT patients (**Figures 1E,F**). In addition, higher GLCA, TDCA, TLCA and TUCDA levels were found in CHB-AALT patients than CHB-NALT (**Figures 1E,F**).

Despite the heterogeneity of individual BA profiles across different groups, the relatively concerted change of unconjugated and conjugated BA groups prompted us to investigate the relevance of UCBA/CBA ratios with CHB progression. We found the total UCBA/CBA ratios were significantly lower in CHB-NALT and CHB-AALT patients than in HCs (both $P < 0.001$, **Figure 2A**). Moreover, the ratio of glycine/taurine-conjugated BAs gradually decreased from NCs to CHB-NALT and CHB-AALT patients. Similar trends can be found in UCBA/CBA ratio for each individual BA species (**Figure 2B**). For CHB-NALT patients, decreasing UCBA/CBA ratio compared to HCs can be found in CA, CDCA (both $P < 0.001$), DCA and UDCA (both $P < 0.0001$). For CHB-AALT patients compared to NCs, decreasing UCBA/CBA ratio can be found in CA ($P < 0.0001$), CDCA, DCA (both $P < 0.01$), LCA ($P < 0.05$) and UDCA ($P < 0.001$).

In addition to changes in UCBA/CBA ratios, we also identified modulation of primary/secondary ratios of BAs particularly in CHB-NALT patients. Although no significant changes of total primary BAs were found among three groups, total secondary BAs were significantly lower in CHB-NALT patients as compared to HCs ($P < 0.01$, **Figure 2C**), which correspondingly led to significantly higher total primary/secondary BA ratios in CHB-NALT patients ($P < 0.05$, **Figure 2D**). Moreover, compared to individual BAs that generally showed limited value to indicate CHB (**Supplementary Table 5**), we found total unconjugated BA (UCBAs) level, and its ratio to the conjugated BAs (UCBAs/CBAs), as well as total secondary BAs (SBAs) level have high AUC value (all >0.7) to distinguish CHB from healthy controls (**Supplementary Figure 2A**). Moreover, we found the ratio of unconjugated/conjugated CA, CDCA, DCA and UDCA also have higher AUC value (all >0.7) than individual BA species (**Supplementary Figure 2B**). These results then encouraged us to further build a combinatory model including total UCBAs, UCBAs/CBAs ratio and U/C ratio of CDCA by logistic regression (**Supplementary Table 5**) with higher diagnostic ability (AUC = 0.838, **Supplementary Figure 2C**) than conventional markers such as ALT, AST, ALP, GGT and TBIL (**Supplementary Table 5**). These results indicated BA based signatures can be used in junction with current biochemical markers to monitor CHB progress in very early stages.

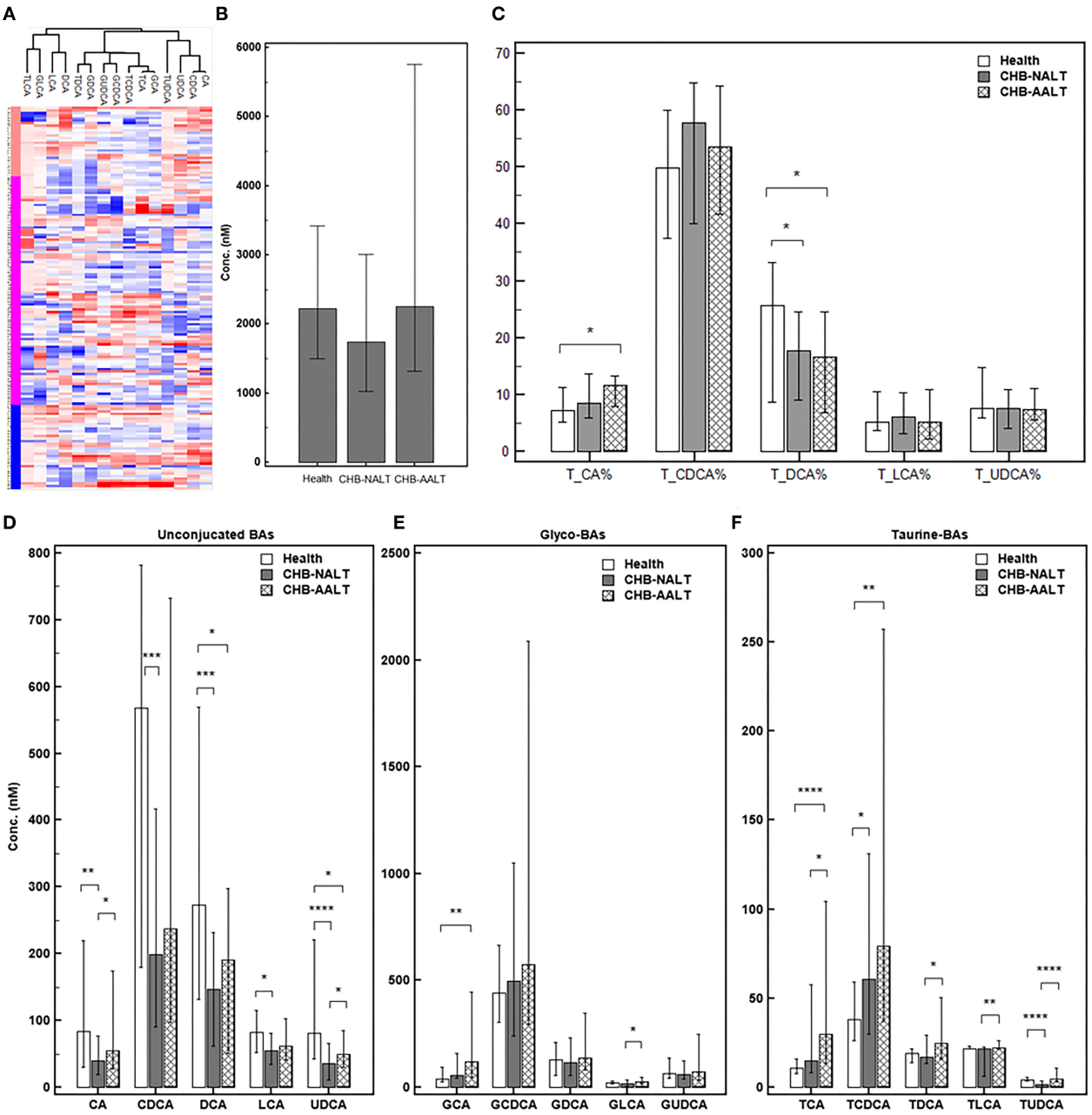

FIGURE 1 | Summary of BA profiles. **(A)** Bile acid profiles across all samples were visualized by heatmap. Concentration of each BA in Log2 scale were transformed into Z-score by columns. The columns were clustered using the K-mean algorithm. The levels of **(B)** total BA, **(C)** percentage of total CA, CDCA, DCA, LCA and UDCA, and the levels of **(D)** each unconjugated, **(E)** glyco-conjugated and **(F)** taurine-conjugated BAs measured in different groups were summarized. Error bars represented 25–75% quartile. *P* values were determined by the Mann–Whitney *U* test, significant differences were noted by $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$. CHB-NALT: ALT normal patient group, CHB-AALT: ALT abnormal patient group.

Modulated Expression of Hepatic Genes Related to BA Transport and Synthesis in CHB Patients

Previously documented liver transcriptomic data comparing NALT-CHB and AALT-CHB patients with healthy controls (GEO dataset GSE83148) (39) were reanalyzed for genes related to BA transport and hepatic *de novo* synthesis (**Figure 3**). As expected, BA importer NTCP but not OATP was shown upregulated in CHB-NALT patients, but both were suppressed in AALT-CHB patients. Surprisingly, the key rate-limiting

TABLE 2 | The BAs concentrations in serum measured in different groups.

BA (nM)	HC	CHB-NALT	CHB-AALT
CA	153.68 ± 159.64	78.06 ± 99.87*	197.71 ± 402.73†
CDCA	654.78 ± 660.43	329.35 ± 391.39*	518.80 ± 675.81
DCA	402.76 ± 350.11	163.01 ± 116.83*	219.69 ± 176.24#
LCA	94.95 ± 57.21	82.65 ± 107.05*	85.16 ± 70.34
UDCA	130.83 ± 114.96	67.53 ± 182.53*	76.23 ± 72.59#†
GCA	72.86 ± 93.69	145.75 ± 205.31	611.88 ± 1957.62#
GCDCA	649.19 ± 648.55	897.40 ± 1087.79	2405.02 ± 5935.38
GDCA	156.39 ± 149.88	163.27 ± 154.70	249.83 ± 298.31
GLCA	25.26 ± 15.82	32.06 ± 51.98	34.18 ± 23.41†
GUDCA	128.04 ± 178.12	148.76 ± 433.65	1379.11 ± 5822.72
TCA	18.56 ± 29.40	505.00 ± 2451.09	280.77 ± 1083.32#†
TCDCA	54.28 ± 69.18	226.64 ± 749.23*	704.94 ± 2707.97#
TDCA	24.62 ± 22.68	27.34 ± 31.35	42.62 ± 51.00†
TLCA	22.82 ± 19.29	24.06 ± 28.99	26.81 ± 13.82†
TUDCA	6.24 ± 5.63	14.62 ± 62.54*	268.95 ± 1427.16†
Total CBA	1140.60 ± 1057.12	2164.78 ± 3176.61	5967.35 ± 18853.30
Total UCBA	1436.99 ± 930.21	720.59 ± 635.21*	1097.60 ± 1073.55#
Total primary BA	1603.35 ± 1155.52	2182.19 ± 3068.23	4719.12 ± 11560.92
Total secondary BA	974.24 ± 503.56	703.18 ± 713.98*	2345.83 ± 7268.26
Total BA	2577.59 ± 1460.36	2885.37 ± 3312.46	7064.95 ± 18767.43

Table represent the mean±SEM. HC, healthy control group; CHB-NALT, ALT normal patient group; CHB-AALT, ALT abnormal patient group. P values were determined by Mann–Whitney U test, significant difference ($P < 0.05$) between HC and CHB-NALT, HC and CHB-AALT# or between CHB-NALT and CHB-AALT† were indicated, respectively.*

enzyme CYP7A1, only shown slight upregulation in CHB-NALT patients without statistical significance, but was found significantly inhibited in AALT-CHB patients (**Figure 3A**). On the contrary, its counterpart in the alternative pathways, CYP7B1, was found overexpressed in CHB patients (**Figure 3B**). Significant upregulation of none-rate-limiting genes, ACOT8, ACOX2, HSD3B7, SLC27A5, CH25H, CYP27A1 and CYP8B1 in the neutral pathway of BA synthesis were also recorded in CHB-NALT patients. Almost all synthesis related genes, except CH25H, were down-regulated in CHB-AALT patients. Enzymes related to BA conjugation, bile acid-CoA synthase (BACS) or bile acid-amino acid transferase (BAAT) did not show significant changes in CHB patients. Downregulation of BA exporter BESP was found in both NALT and AALT patients (**Figure 3C**).

Distinct Gut Microbiome Profiles in Patients With CHB-NALT

To explore possible roles of microbiome related to the changes of BA composition in the early stage CHB, fecal microbiota profiles from CHB-NALT patients and healthy controls were analyzed by 16S rRNA sequencing. Overall, the analyses revealed significant decreased ecological diversity, as measured by Shannon index, in CHB-NALT (**Figure 4A**, $P < 0.01$). Analysis of bacterial population at the phylum level revealed that *Proteobacteria* and *Bacteroides* increased, while *Firmicutes* decreased in CHB-NALT patients compared to HCs (**Figure 4A**). Using LDA score > 4, the LEfSe analysis revealed that CHB patients had more *Bacteroidales* which belongs to *Bacteroidia*, *Selenomonadales* which belongs to *Negativicutes*, and *Gammaproteobacteria* which belongs to *Proteobacteria*, but had less *Lachnospiraceae* and *Ruminococcaceae* which both belong to class *Clostridia* and phylum *Firmicutes* (**Figure 4B**). Using LDA score > 2, the LEfSe analysis further provided more detailed list of 192 OTUs altered in CHB-NALT with their evolutionary tree summarized by cladogram (**Figure 4C**).

To reveal possible links to the distinct BA profiles in CHB-NALT patients, we first focused on BSH-harboring bacteria genera documented by previous studies, such as *Lactobacillus*, *Bifidobacterium*, *Enterococcus*, *Clostridium*, and *Bacteroides* spp (23, 41–44). Compared to HCs, the relative abundances of *Lactobacillus,* particularly the *Lactobacillus salivarius* was significantly lower in CHB-NALT (**Figure 4E**). Bifidobacterium and Clostridium were also shown decreasing level in CHB-NALT patients albeit with less statistical significance ($p < 0.1$). Despite that *Bacteroidia* was more abundant, one key BSH containing member *Bacteroides fragilis* (43) was found significantly suppressed in CHB-NALT patients (**Figure 4E**). In addition, the only member of archaeon harboring BSH activity, *Methanobrevibacter smithii* (44), was also found significantly lower in CHB-NALT patients. Predicted by PICRUSt analysis, BSH gene abundance in the CHB-NALT group was significantly lower than that of the HCs (**Figure 4D**, $P = 0.006$). These results indicated that the decreased microbial BA deconjugation activity might have contributed to the relatively higher percentage of UCBA in CHB-NALT patients. Analogously using PICRUSt estimation, we showed two critical secondary BA conversion enzyme groups: hdhA and baiN gene abundance was significantly

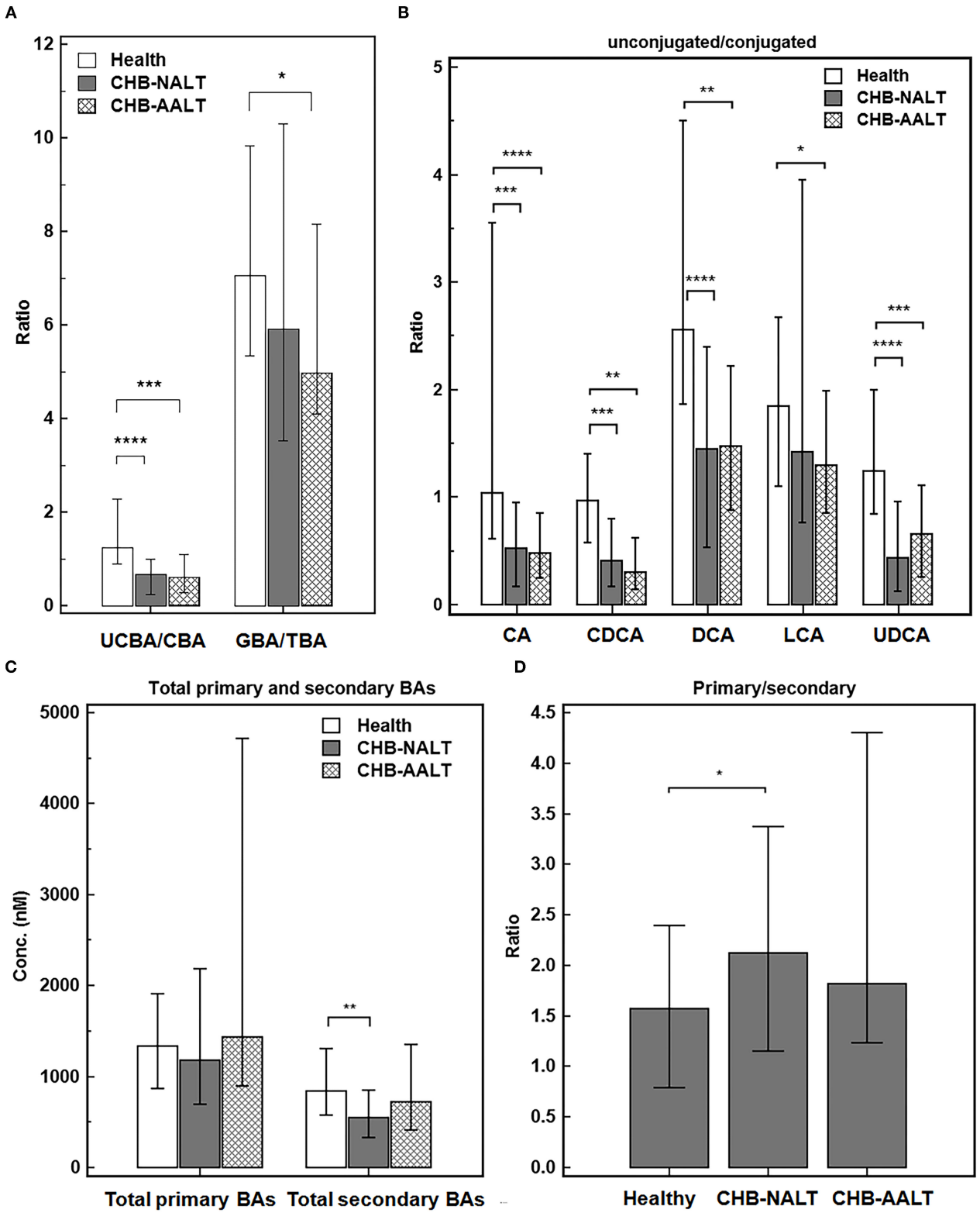

FIGURE 2 | Alteration of BA ratios in CHB patients. The ratios of **(A)** total unconjugated BAs vs. total conjugated BAs, and total glyco-conjugated vs. total taurine-conjugated BAs, **(B)** ratios of unconjugated vs. conjugated individual BAs, **(C)** total primary BAs vs. secondary BAs levels and **(D)** ratios of total primary BAs vs. secondary BAs across all three groups were summarized. Error bars represented 25–75% quartile. *P* values were determined by the Mann–Whitney *U* test, significant differences were noted by $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$. CHB-NALT: ALT normal patient group, CHB-AALT: ALT abnormal patient group.

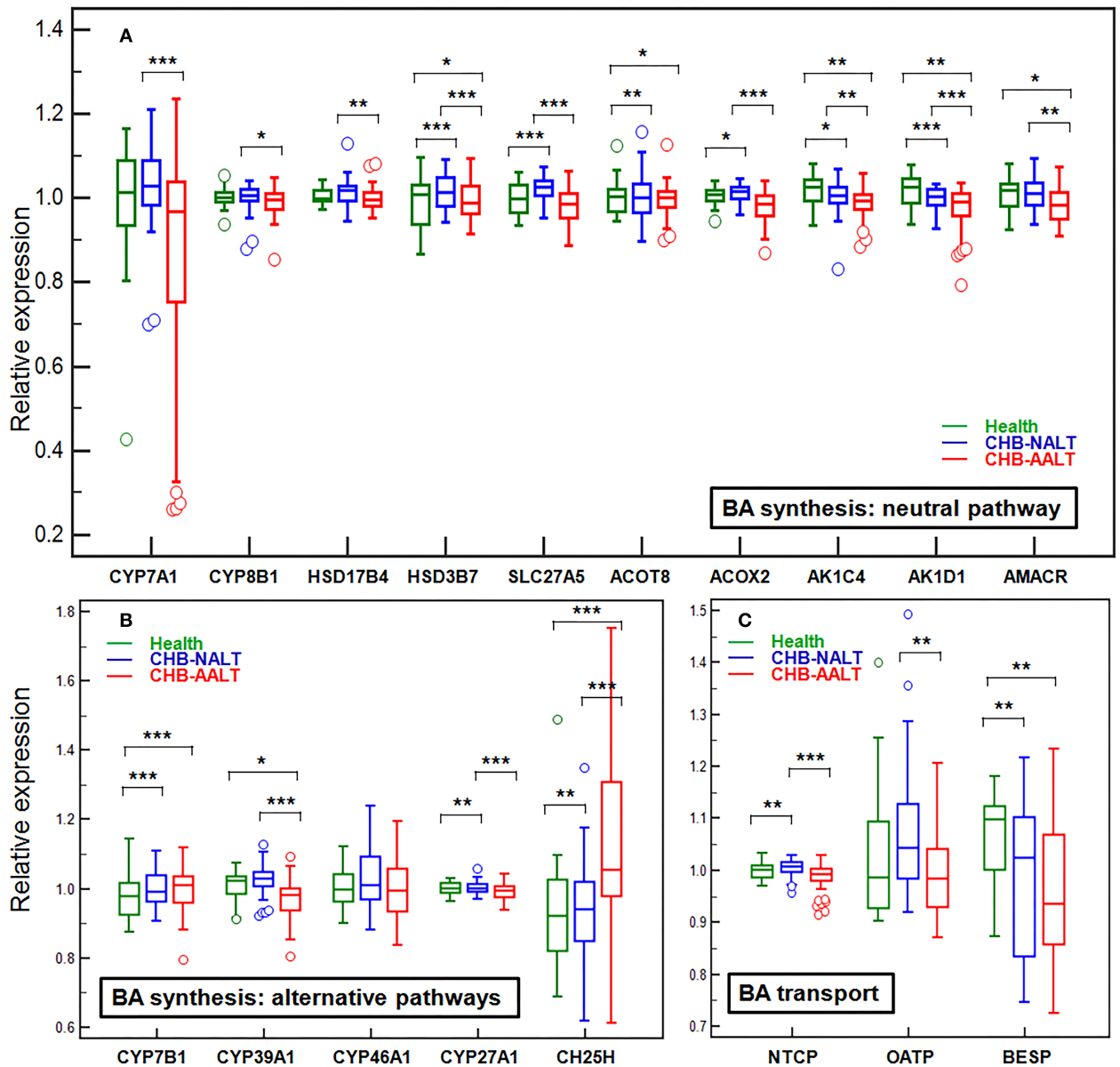

FIGURE 3 | Alteration of hepatic genes related to BA transport and synthesis in CHB patients. The expression levels across all three groups were compared for genes related to *de novo* synthesis by **(A)** main neutral pathway, **(B)** alternative acidic or 24/25-hydrolyase pathways and **(C)** BA transport. All expression levels were normalized against the medium level. Error bars represented 25–75% quartile. *P* values were determined by the Mann–Whitney *U* test, significant differences were noted by $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. CHB-NALT: ALT normal patient group, CHB-AALT: ALT abnormal patient group.

lower in the CHB-NALT groups (**Figure 4D**, $P = 0.006$, $P = 0.0006$, respectively), which can be partly attributed to the decreasing level of class *Clostridia*.

DISCUSSION

Bile acids (BAs) play an important role in a wealth of physiological and pathological processes. Most prominently, bile acids are also key regulators of energy expenditure, glucose and lipid metabolism, thyroid hormone signaling, and cellular immunity (23, 45). The enterohepatic BA cycle is vital to the intestinal absorption of lipids and fat-soluble vitamins, and the elimination of cholesterol. Bactericidal BAs also play role to maintain healthy gut microbiome against bacterial overgrowth. Previous studies have revealed that HBV pre-S1 domain competing with NTCP blocks the uptake of the conjugated BAs (8) in HepG2-hNTCP cells and remarkably

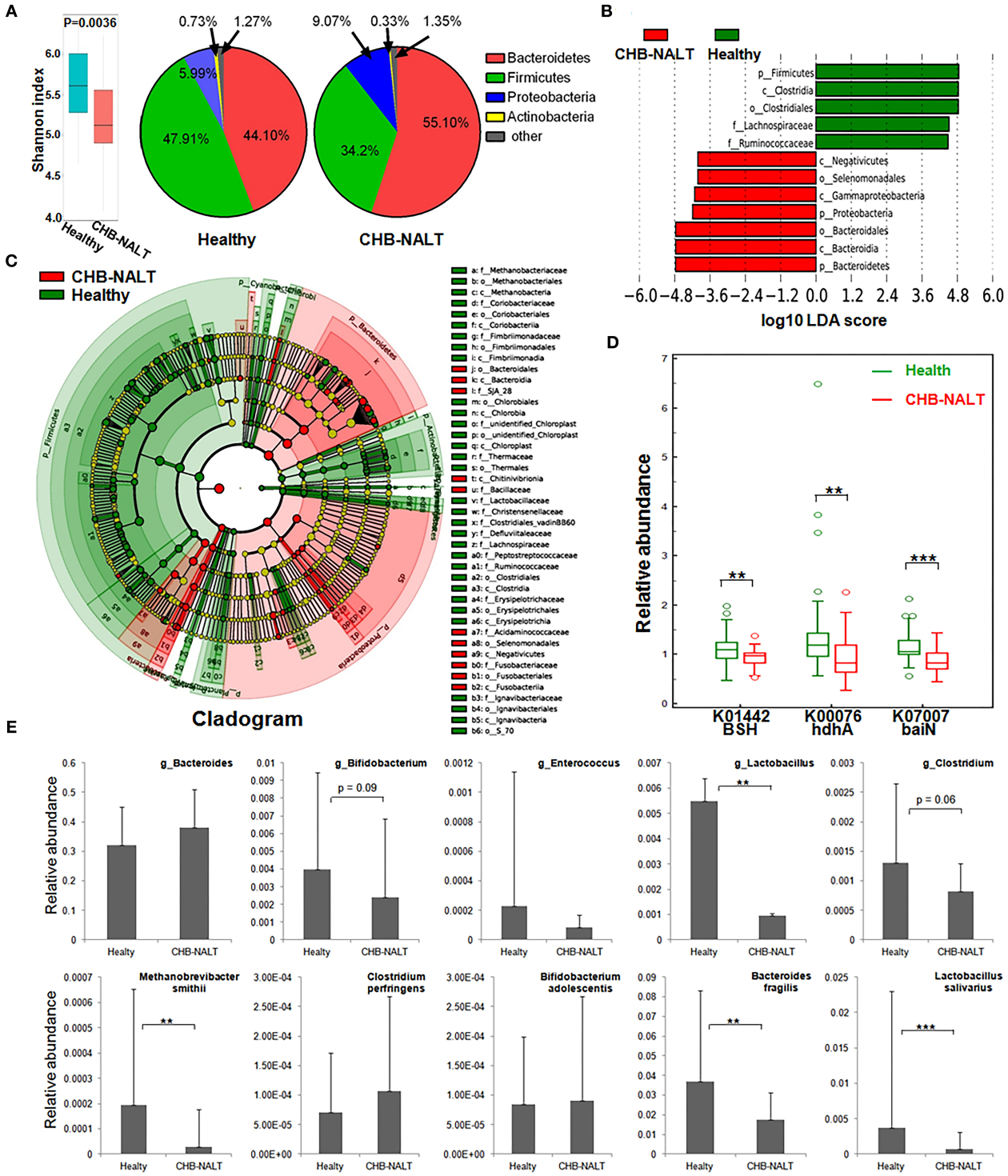

FIGURE 4 | Modulation of fecal microbial profiles and functionalities related to CHB-NALT. **(A)** Shannon index of microbial ecological diversity and microbiota constituents at phylum level shown difference between groups. Linear discriminant analysis (LDA) coupled with effect size measurements identifies the taxons most differentially abundant between the groups. **(B)** Taxa with LDA significant cutoff >4 were shown. **(C)** Taxa with LDA > 2 were summarized by cladogram. **(D)** Taxa overrepresented in CHB-NALT (red) or healthy controls (green) were highlighted according to LEfSe analysis. The predicted gene abundance of BSH (KEGG orthology K01442), hdhA (K00076) and baiN (K07007) between the groups. **(E)** The relative abundances of BSH containing genus (top panel) and bacteria species (bottom panel) between the groups. *P* values were determined by the Mann–Whitney *U* test, significant differences were noted by $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. Data were presented by median ± quantiles.

enhanced the expression of BA synthesis genes in HBV-infected human chimeric mice liver (9), suggesting that HBV binding to NTCP may increase the serum BA concentration. In contrary to this assumption however, we found that overall BA level did not change in NALT-CHB patients. In fact, transcriptomic data also did not support significant enhancement of hepatic CYP7A1 activity in CHB patients. Therefore, it is doubtful if the *de novo* synthesis of BAs is substantially enhanced in CHB patients. However, since HBV binding is known to deactivate FXRa (11), which suppress the expression of NTCP while maintains that of the BA exporter BESP (10, 11), therefore might explain the upregulation of NTCP and downregulation of BESP in CHB patients. This reciprocal expression change of BA importer and exporter suggested a compensatory accumulation of intrahepatic BAs in response to HBV infection.

Several studies have indicated that the composition of BAs is more relevant to progression of liver disease compared with the absolute level of total or individual BAs (33, 46–48). When looking at the detailed profile of BAs, we found that both CHB subgroups shared a decreasing ratio of unconjugated/conjugated BAs. As previous study have suggested, such change might be linked to the competitive binding of HBV to BA transporter NTCP (8, 9), which were mainly responsible for hepatic uptake of conjugated BAs, while OATP showed preference for unconjugated BAs (2, 49–51). Therefore, higher proportion of conjugated BAs was observed both in mouse with defective NTCP and human treated with NTCP inhibitor myrcludex B (2, 52). Our data, in agreement with those reports, suggested blocking of NTCP by HBV also caused relatively higher proportion of circulating conjugated BAs in CHB patients. To rule out the possibility of enhanced liver BA conjugation activity, we checked previous transcriptomic data (39) which revealed no significant changes of hepatic bile acid-CoA synthase (BACS) or bile acid-amino acid transferase (BAAT) in CHB patients (data not shown).

Other than hepatic factors, it is generally accepted that unconjugated BA levels can also be influenced by gut microbiome which harbors BSH enzymes for BAs deconjugation (23). Disrupted microbiome, for instance by means of antibiotic-treatments, resulted in host BAs profile dominated by conjugated species (53). Human microbiome studies also confirmed the impact of gut flora on BA compositions in various liver diseases, including liver cirrhosis, alcoholic liver disease, nonalcoholic fatty liver disease (NAFLD) (48, 54). Regarding this, we also found the gut microbiota diversity were lower and BSH expressing *Lactobacillus*, *Clostridium*, *Bifidobacterium*, *Enterobacteriaceae* were suppressed in CHB patients (**Figure 4**). This results is partially in agreement with previous microbiome study on CHB patients, which found lower abundance of *Bifidobacterium* and *Lactobacillus* (25). Therefore, caution should be taken to extrapolate findings by cellular or animal model in the scenario of HBV infection in human, in which both HBV-NTCP interaction and gut microbiome modulation were likely to influence BA compositions.

Gut microbiome modulation might also lead to the surged ratio of primary to secondary (p/s) BA ratio in the CHB-NALT patients. Previous microbiome profiling studies have found suppressed *Bifidobacterium*, *Clostridium*, and *Bacteroides* species in CHB patients (25, 55, 56). Similar observation was documented previously in cirrhotic patients which were also characterized by the deficiency of key gut microbiome taxa (48). Obviously, insufficient deconjugation activity will lead to lower level of substrates for secondary BA conversion, but we also found enzymes specifically responsible for secondary BA generation were impaired in CHB patients. These included 7-alpha-hydroxysteroid dehydrogenase (hdhA), which oxidizes the 7-alpha-hydroxy group of primary BA and is majorly found in *Bacteroides*, *Clostridia* and *Ruminococcus* spp (23, 57–60). One important member of hdhA producing bacteria, *B. fragilis* (58), was found significantly lower in CHB patients. The BA intermediates generated by HSDs are further processed by a group of bacterial enzymes encoded by the bile acid inducible (*bai*) operon, which catalyze only unconjugated bile acids and are highly conserved in both *Cl. scindens* and *Cl. hylemonae* strains (23, 61). In our analysis, we found 3-dehydro-bile acid delta-4,6-reductase (baiN) in the CHB patients was significantly lower than healthy controls. Taken together, the decreasing UCBA/CBA ratio and increasing p/s BA ratio indicate a deficient role of microbiome in the enterohepatic cycle of BAs in CHB patients, which is in line with previous data showing strikingly lower level of total fecal BAs in CHB patients even without cirrhosis (25). More importantly, such microbiota-mediated BA profile changes in NALT-CHB patients could be the prelude to more drastic changes in patients at advanced stages of CHB. During the completion of this study, another two studies of CHB patients with liver damages (ALT $\geq$ 40 IU/mL) and fibrosis have found interesting association of serum BA parameters with advanced fibrosis stages (62). Their data also confirmed the higher proportion of conjugated and primary BAs were in CHB patients and possible association with microbiome modulation (46, 62), which is in line with our founding. Moreover, previous study has observed that lower level of total, secondary, secondary/primary BA ratios, and higher primary BAs levels in feces correlated with the stage of liver cirrhosis, while serum primary BAs were also higher in patients with advanced cirrhosis (48). Major findings in our data and possible mechanism underlying the distinct profiles of CHB patients were summarized in **Figure 5**.

It is worthy to argue that there are likely a three-way mutual relationship among HBV, host BA metabolism and microbiota, as both BA and HBV also exerted their effects to remodel gut microbiota. As a group of amphipathic and bactericidal components, the BAs were well-documented as key regulator of gut microbial composition (23). Importantly, it is till recently the differential impact of different BA components on gut microbial species start to draw attention (63). Therefore, the distinct BA profiles could in turn contribute to the distinct microbial profiles in the CHB patients. For instance, the lower level of DCA in CHB patients might explain the higher percentage of *Bacteroides* which were shown to be inhibited by DCA produced majorly by *Firmicutes* (24). Previous studies have also reported gut flora alterations related to immune environment modulations during HBV infection. Mucosal immunity associated inflammatory cytokines (25, 64) such as the nuclear factor kappa B (NF-kB), tumor necrosis factor-alpha (TNF-a), and the secretory IgA

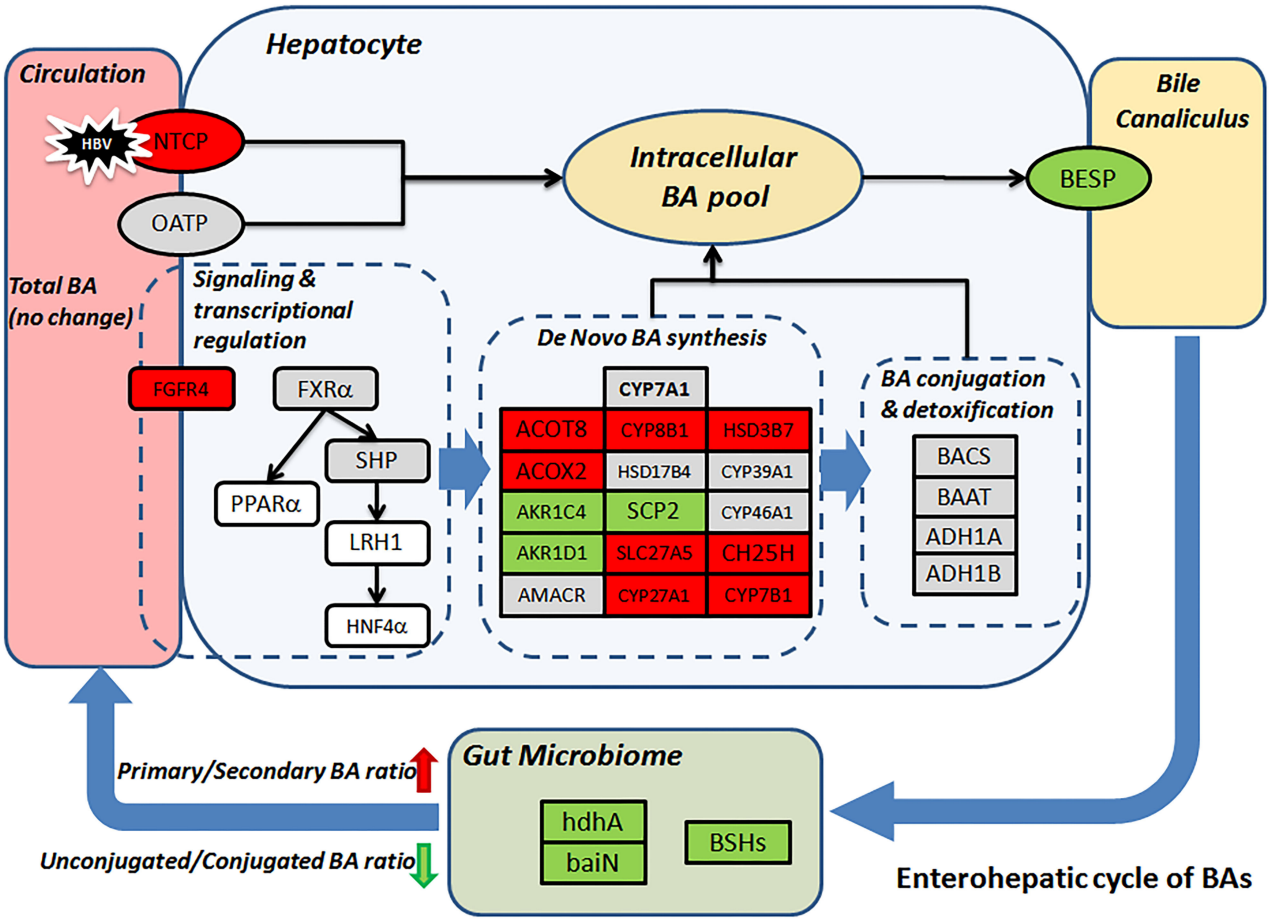

FIGURE 5 | Proposed mechanism leads to the distinct BA profile in CHB patients. Change level of BA related genes (rectangular) according to transcriptomic data was visualized by color. Red/green represent up-/down-regulation, gray, and white indicate no-changes and missing data, respectively. BSHs, hdhA and baiN levels were predicted based on microbiome 16S sequencing data.

(sIgA) were found significantly increased in CHB patients (65). Such alterations in host mucosal environment and immunity eventually affect intestinal microbial homeostasis. In short, our understanding of the complex relationship between HBV, host BA metabolism and microbiota is still incomplete thus warrant further investigation.

Currently, ALT and AST are two commonly used biomarkers to assess liver injury and to identify CHB patients for antiviral therapy. However, both markers lack organ specificity, and numerous studies have suggested that approximately 50–90% CHB patients with apparently normal ALT levels still have chronic inflammation by liver biopsy (66). Aminotransferases should therefore be argued as imperfect surrogate marker for active liver disease. Considering their higher liver specificity, BAs were proposed to predict liver necroinflammation (67). While BA signatures have been proposed as alternative biomarkers to gauge liver malfunctions to monitor end-stage liver diseases (30–33), we found the modulation of BA composition occurred at very early or mild stages of chronic HBV infection. Despite the highly heterogeneous profile of individual BA species found in all groups, our data showed that combinatory BA features such as total UCBAs, UCBAs/CBAs ratio and U/C ratio of CDCA could facilitate the diagnosis of chronic HBV infection with high accuracy. In parallel to our discovery, a recent study concluded that though limited changes in total serum BAs were found between NAFLD vs. non-NAFLD, or between nonalcoholic fatty liver vs. nonalcoholic steatohepatitis, the increased ratios of conjugated/unconjugated and primary/secondary BAs were associated significantly with liver fibrosis progression (47). Also, increased ratios of serum conjugated/unconjugated BAs were found in hepatocellular carcinoma patients (68). Therefore, we believe our preliminary data was encouraging, thus future longitudinal studies should be conducted in larger CHB cohorts with biopsy records to further determine whether BA signatures reflects fibrotic or necrotic development, or whether they prelude different clinical outcomes of CHB, including cirrhosis and hepatocellular carcinoma.

In summary, we found distinct patterns of serum BAs majorly featured by significant higher ratio of conjugated and primary BA species in CHB patients at early stages. These changes were likely to be the results of interaction of HBV with NTCP plus distinct gut microbiome alteration during HBV infection. Our findings provided a new insight into the complex relationship among HBV, BA and the gut microbiota in human, and suggested potential benefits of BA pathway or microbiota targeted interventions in the CHB patients.

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 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

ZS, CH, MZ, and ZY: study concept and design. CH, YS, RW, JF, and YY: samples and clinical data collection. ZZ, KZ, and ZS: LC-MSMS experiments. ML and QN: microbiome profiling. ZS, CH, and ZY: analysis and interpretation of data. ZS, CH, and ZY: statistical analysis. ZC, MZ, and ZY: critical revision of the manuscript. ZS, CH, and ZY: drafting of the manuscript. 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/fmed.2021.708495/full#supplementary-material

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REVIEW
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HBV/HIV Coinfection: Impact on the Development and Clinical Treatment of Liver Diseases

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Hepatitis B virus (HBV) infection is a common contributor to chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma. Approximately 10% of people with human immunodeficiency virus (HIV) also have chronic HBV co-infection, owing to shared transmission routes. HIV/HBV coinfection accelerates the progression of chronic HBV to cirrhosis, end-stage liver disease, or hepatocellular carcinoma compared to chronic HBV mono-infection. HBV/HIV coinfection alters the natural history of hepatitis B and renders the antiviral treatment more complex. In this report, we conducted a critical review on the epidemiology, natural history, and pathogenesis of liver diseases related to HBV/HIV coinfection. We summarized the novel therapeutic options for these coinfected patients.

Keywords: hepatitis B virus, human immunodeficiency virus, coinfection, liver disease, clinical treatment

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INTRODUCTION

Chronic hepatitis B virus (HBV) infection affects ∼250 million people worldwide and can cause progressive liver fibrosis and hepatocellular carcinoma (HCC) (1, 2). Over 50% of HCC cases globally have been attributed to HBV infection (3, 4). Owing to their similar transmission patterns, HBV/HIV coinfection is relatively common in endemic areas (5). About 10% of HIV-infected individuals have been found to be chronically infected with HBV (6, 7). Compared with HBV mono-infection, HBV/HIV coinfection complicates the natural course and increases the risk of deterioration of diseases (8). Although some current therapeutic strategies are considered effective options in treating single virus infections, HBV/HIV coinfection has altered the natural history of the virus, requiring novel individualized therapeutic forms. This paper reviews the epidemiology, natural history, and pathogenesis of liver diseases in HBV/HIV coinfection. We also highlighted the individualized therapeutic options in these patients.

METHODOLOGY

We performed a comprehensive literatures search in Pubmed, Embase, and Web of Science with the key words of "hepatitis B virus," "HBV," "human immunodeficiency virus," "HIV," "coinfection," "liver disease," "epidemiology," "pathogenesis," "treatment," and "clinical treatment." Clinical trials (http://clinicaltrials.gov/) was also searched for the important clinical trials about the HBV/HIV coinfection therapies. Literatures with no full text, describing only protocol design or preliminary results were excluded.

EPIDEMIOLOGY

HBV, HIV, and HBV/HIV coinfection are caused by several means including unsafe drug injection, inappropriate medical practices, unsafe therapeutic injections, and high-risk unprotected heterosexual and man-man sexual acts. Overlapping transmission routes contribute to the prevalence of HBV, HIV, and HBV/HIV coinfection (9). Nevertheless, the prevalence varies in different geographic regions, ranging from 10 to 28% (10–13). Based on the prevalence of chronic HBV infection, it can classify the endemicity to high, intermediate, and low endemicity areas in geography (14). In the high endemicity area, such as sub-Saharan Africa and east Asia, ∼10% of HIV-infected individuals have been observed to be concurrently infected with HBV (15). In Vietnam, the rate could be as high as 28% in the unsafe drug injection populations (11). In these regions, perinatal transmission, close household contact during childhood, or cultural procedures are the most common transmission routes (10, 16). However, in areas of low endemicity, such as North America, Western Europe, and Australia, HBV/HIV coinfection is usually recorded in adolescents or adults *via* unsafe drug injection or sexual transmission (15) with the estimated infection rate 6–14% (17). In the highest risk group of unsafe male homosexuals, the estimated rate ranges from 9 to 17% (14).

Nevertheless, prevalence of HBV/HIV infection showed a slightly decreased trend based on several studies published recently. An analysis developed by the North American Cohort Collaboration on Research and Design collected information covering 12 clinical sites from 1996 to 2010 and revealed that the prevalence of chronic HBV infection in HIV cohort was only 7% (18). Another research from the US Military HIV Natural History Study found that the overall incidence of chronic HBV infection was 4.3% (19). The most prevalence was appeared in 1995 with an obvious decrease in 2008, according to the evaluation of cross-sectional incidence (19). Even so, the health threat posed by HBV/HIV coinfection still cannot be ignored.

NATURAL HISTORY

Acute HBV infection in adults is difficult to clinically detect. Spontaneously recovery is commonly observed among the most immunocompetent adults whose antibodies against hepatitis B surface antigen (anti-HBs) can be detected (20). Approximately 5–10% of immunocompetent adults will progress to chronic infection (21), 20% of individuals with chronic infection are likely to develop cirrhosis within 1–13 years (21). HCC and decompensated liver diseases occur in 6 and 23% of patients with cirrhosis, respectively (22).

The natural history of HBV factors are associated to the characteristics of virus, host, and environment. HBV/HIV coinfection accelerates the progression of HBV infection by impacting the immune response of the host (23). People infected with HIV have a high risk of contracting chronic HBV infection (24). Lower rates of HBeAg and/or HBcAg clearance and anti-HBe and anti-HBs seroconversion with higher rates of HBV replication were observed in HBV/HIV coinfected persons (25, 26). The acceleration of the process of cirrhosis and HCC is the most serious consequence in the liver-related damages (27). HBV/HIV-coinfected individuals have approximately five- to six-fold higher risk of HCC incidence with the presence of cirrhosis (28–30). Additionally, HIV/HBV-coinfected accelerate the progress to liver cirrhosis (31). Host $CD4^+$ T cells are vital to the recognition of viral antigens presented by Kupffer cells and the regulation of the activities of $CD8^+$ cytotoxicity T cells, antibody producing B cells, and secreting cytokines cells. Host immunosuppression as manifested by the depletion of $CD4^+$ T cells may be the key for HIV to alter the natural course of HBV which associated to an increase in liver-related mortality (5, 32–34).

PATHOGENESIS OF LIVER DISEASES

The mechanism by which HIV facilitates liver-related damage has not been completely delineated. HIV-induced immunodeficiency seems to enhance HBV-related hepatotoxicity, which is mediated by the immune response (15). Depletion of $CD4^+$T cells is an important feature of HIV infection which suppress the antigen presentation of liver resident macrophages (Kupffer cells) and the cytokine secretion of lymphocytes, in resulting the host immunosuppression (32). The inhibition of the host immune response enhances HBV replication substantially to further cause severe liver damage (35, 36). HBV infected hepatocytes is found non-cytopathic, without distinct cellular damage and viral cytopathic effects. However, HBV/HIV coinfection persons shows fibrosing cholestatic hepatitis (37, 38). HIV/HBV coinfection causes changes in the hepatic cytokine environment (15, 39, 40). It has been reported that HIV glycoproteins stimulate the hepatocyte to express the tumor necrosis factor related apoptosis inducing ligand (TRAIL) to induce hepatocyte apoptosis (41, 42). HIV envelope protein activates the caspase-independent apoptosis in Huh7 cells (43). HIV infection induces hepatocyte apoptosis through phagocytosed by macrophages or hepatic stellate cells and contributes to the inflammation and fibrosis of the liver (44). The increase of hepatocyte apoptosis has been observed among HBV/HIV coinfected patients compared with HBV mono-infected patients (45). HIV gp120 has been demonstrated to stimulate the hepatic expression of IL-8 to mediate the hepatic inflammation (46). Elevation of HBV load increases the X protein of HBV (HBx), which can also transactivate the expression of IL-8 *via* NF-κB and C/EBP-like cis-elements (47). As a leukocyte chemotactic molecule, IL-8 plays a crucial part in maintaining the inflammatory environment and HCC development (48). Furthermore, the content of IL-8 is positively correlated with the degree of liver damage (49).

HBx can also stimulate the expression of cyclooxygenase-2 (COX-2), which is overexpressed in liver cirrhosis (50, 51). Moreover, COX-2 expression can be activated by IL-8 through CREB and C/EBP (47). Accumulating evidence shows that HBV proteins activate IL-8 and COX-2 to maintain the inflammatory environment (47). The inflammatory hepatocytes secrete C-X-C motif chemokine 10 (CXCL10) which linked to the severity of liver damage involving viral hepatitis (52, 53). Once

CXCL10 binds to its receptor, chemokine receptor 3 (CXCR3), immunocytes such as natural killer cells, and activated T cells and B cells are attracted to the inflammatory sites (54). Elevated CXCL10 was found in HBV/HIV coinfected patients but not in HBV mono-infected patients. This observation indicates that CXCL10/CXCR3 in the liver contributes to the acceleration of liver diseases (55–57).

In another hypothesis of mechanisms of the pathogenesis of liver diseases in coinfected patients, the depleted $CD4^+$ T cells in the gastrointestinal tract contribute to the increase in microbial translocation and enhance the levels of circulating lipopolysaccharides (LPS) (58). When LPS binds to Toll-like receptor 4 and stimulates the NF-κB pathway or other pathways, it induces the secretion of pro-inflammatory cytokines (56). Although the relationship between microbial translocation and liver cirrhosis is reportedly closed, a similar evidence has never been found in HBV/HIV coinfected patients, including the direct relationship between circulating LPS and liver cirrhosis (56, 59). However, studies on simian immunodeficiency virus-infected rhesus macaques indicated that microbial load is capable of triggering the secretion of chemokines and enhancing the infiltration of $CXCR6^+$-activated NK cells, thereby resulting in liver fibrosis (60). Further research is warranted to confirm this theory.

TREATMENTS AGAINST HBV AND HIV

Mechanism of Current and Experimental Dual Antiviral Therapies

Antiviral therapies should be initiated for HBV/HIV coinfected patients as soon as possible regardless of the clinical stage of the disease and the count of $CD4^+$ cells (61, 62). This recommendation is based on the evidence that the effects of anti-HBV treatment might be reduced following the deterioration of immunodeficiency (63). Moreover, utilization of agents against HBV only can lead to drug resistance to HIV. Therefore, the optimal therapeutic options should include agents possessing dual anti-HBV and anti-HIV activity (64, 65). Current dual antiviral choices can be classified into virus-based agents and host-based agents. The former includes nucleoside/nucleotide reverse transcriptase inhibitors (NRTIs) and cyclophilin inhibitors. The latter consists of immunomodulators and monoclonal antibodies. Here, we provide an overview of the antiviral mechanism of these drugs.

The life cycle of a virus can be a potential target for antiviral agents. HIV is an RNA virus with the ability of reverse transcribing into DNA, which could be integrated into the host genome (66). By contrast, HBV is an enveloped DNA virus (67). Given that they belong to different types, HBV and HIV undergo different life cycles (**Figure 1**). Nevertheless, similarities in their life cycles are important in the development of dual antiviral drugs for HBV and HIV. According to a recent study, the polymerase of HBV and the reverse transcriptase of HIV have similar structures and functions, indicating that agents targeting these proteins have the ability to interrupt the life cycle of both HBV and HIV (68, 69). NRTIs are prodrugs that must be phosphorylated into active forms by cellular kinases (69, 70). Activated NRTIs are capable of disturbing the functions of both HIV reverse transcriptase and HBV polymerase by competing with natural nucleotide substrates for joining into DNA chains (69). In general, owing to the lack of 3′-OH, NRTIs work as chain-terminators, thereby interrupting DNA synthesis (69, 71, 72). Furthermore, protein priming activity, which is absent in HIV reverse transcriptase, is considered as another target for NRTIs (69). Inhibiting protein priming also substantially interferes with HBV replication (69). Cyclophilin A (Cyp A) belongs to the cyclophilin family with a peptidyl–prolyl isomerase activity (73, 74). Regarded as an acceleration factor for protein folding and assembly, Cyp A plays a crucial role in the replication of various viruses, including HBV and HIV. Moreover, it is linked to the pathogenesis of virus infection (75, 76). By interacting with the Gap protein of HIV or the small surface protein of HBV, Cyp A facilitates the replication and infectivity of viruses, suggesting that blocking Cyp A could be a potential anti-HIV and anti-HBV strategy (77, 78).

In response to viral invasion, pattern-recognition receptors, including Toll-like receptors (TLRs), are activated, leading to the production of interferon (IFN) (79). Detection of viral DNA or RNA is crucial in triggering the innate immune response, culminating in the activation of transcription factors and release of antiviral cytokines, such as IFN (80, 81). When secreted, IFN interacts with its cognate receptors (i.e., IFNAR2) and activates receptor-associated kinases (i.e., JAK1 and Tyk 2), thereby contributing to the activation of the STAT family to form a transcription factor complex or a homo-/heterodimer (82–84). The transcription factor complex and the homo-/heterodimer bind to the ISRE and GAS promoter elements, respectively, and encode numerous viral restriction factors with potent inhibition potential on viral replication (**Figure 2**) (84–86). Consequently, immunomodulators (i.e., IFN), which can enhance the host's innate immune response, offer a rational option for treating viral infection, including HBV and HIV. The adaptive immune response is also a promising target for novel therapeutic interventions owing to the key position of T cells in viral infection control (66, 87). Regardless of HBV or HIV infection, virus-specific T cells have been found to have a distinct dysfunction, which is supposed to be linked to the expression disorder of programmed cell death protein (PD-1) and its ligand (PD-L1) (88). PD-1 and PD-L1 have been observed to be upregulated during viral infection, including HIV, HBV, and HCV, confirming that the PD-1/PD-L1 axis plays an important role in the pathogenic process of viruses (89). Considering that high expression levels of PD-1 and PD-L1 are usually related to unsatisfactory immune response, agents based on the PD-1/PD-L1 axis might be able to reverse this immune suppression consequence and exert corresponding antiviral effects, including on HBV and HIV (90, 91). Hence, immune checkpoint inhibitor targeting the PD-1/PD-L1 axis can be another option for treating HBV/HIV coinfection.

Virus-Targeted Therapeutic Options

Specialized treatment and management of coinfected patients demand multidisciplinary cooperation. Although the life

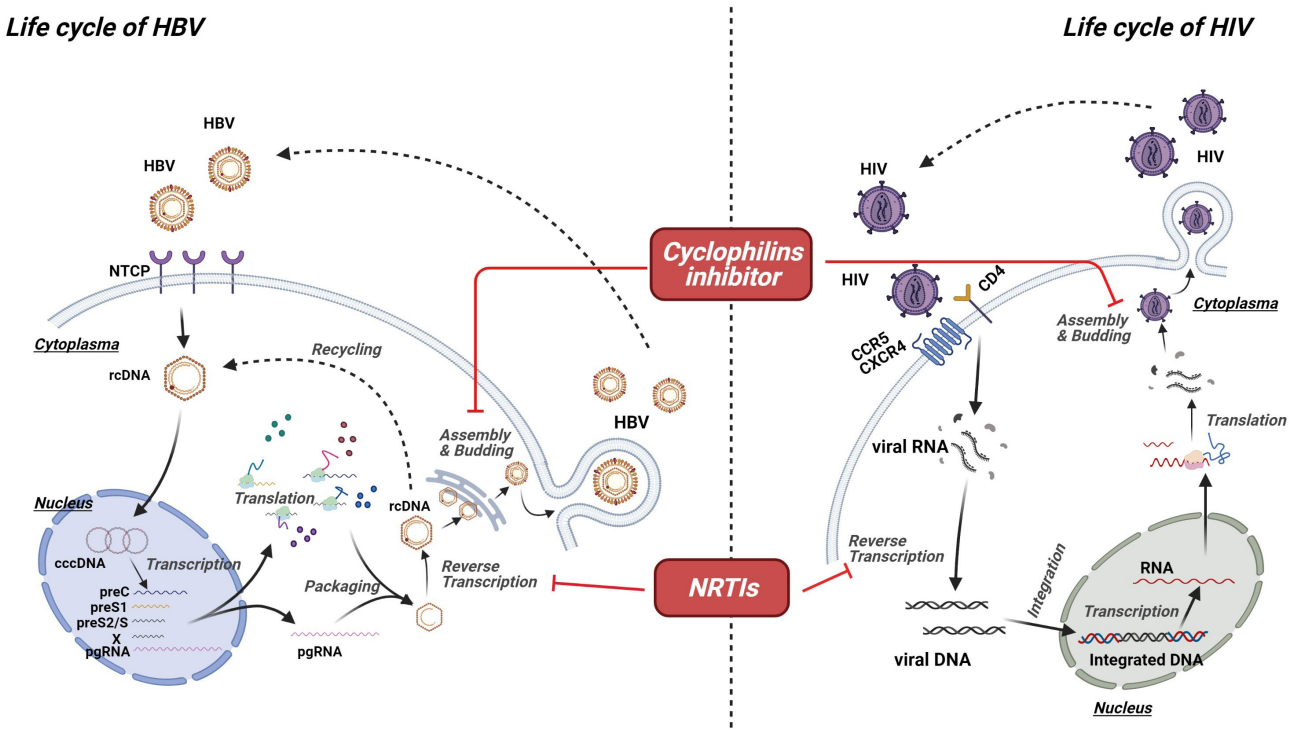

FIGURE 1 | Mechanism of virus-targeted agents. Though undergoing different life cycles, HBV and HIV share some similarities in their life cycles, which are important for the development of dual antiviral drugs for HBV and HIV. Activated nucleoside/nucleotide reverse transcriptase inhibitors (NRTIs) are capable of disturbing the functions of both HIV reverse transcriptase and HBV polymerase by competing with natural nucleotide substrates for joining into DNA chains, resulting in the chain termination and viral replication. Cyclophilin A (Cyp A) plays a crucial role in the replication of various viruses, including HBV and HIV. Thus, cyclophilins inhibitor could be a potential option for anti-HIV and anti-HBV strategy.

expectancy of HIV-infected individuals has been prolonged due to antiviral therapy (ART), liver injury induced by HBV has become the main cause of death in coinfected people. The primary objective of anti-HBV therapy is to suppress the replication of HBV, reduce the activity of inflammation, and halt the progression of liver damage. Several antiviral drugs have been approved for the clinical treatment of HBV, some of which are described as dual antiviral agents against HBV and HIV (**Table 1**). Combination antiviral therapies (cART) are commonly adopted in treating coinfected cases, and clinical trials have been conducted to explore their effectiveness in the treatment of coinfected populations (**Table 2**).

NRTIs

Lamivudine

Lamivudine (3TC) is a dideoxynucleoside cytosine analog with an antiviral effect on both HBV and HIV (92). It exerts its antiviral ability by terminating the chain and suppressing the replication of virus, leading to the reduction in viral load and remission of disease symptoms (92, 93). Amelioration of liver fibrosis and suppression of liver disease progression can be achieved in patients with chronic HBV receiving lamivudine treatment (94–96). Furthermore, a remarkably improved virologic response was detected after 10 years of lamivudine treatment. HBV DNA was undetected among all patients, and 14 and 11% of patients achieved HBsAg seroconversion and loss, whereas 83 and 42% of patients achieved HBeAg seroconversion and loss, respectively (94).

As the first-line NRTI, lamivudine had been approved for HIV treatment in 1995 and had been proposed as part of fix-dose combinations in antiretroviral therapy (97, 98). Preclinical studies have revealed the potent antiviral efficacy of lamivudine; its half inhibitory concentration in infected cell lines of diverse HIV strains ranges from 0.002 to 1.14 μM (99, 100). Previous clinical trials also confirmed that single-tablet regimen (STR) containing lamivudine has persistent viral suppression and favorable safety in patients with HIV (93, 101). Dolutegravir is the most commonly used agent in combination with lamivudine for HIV clinical treatment. With regard to HBV/HIV coinfected individuals, lamivudine-based ART regimens achieved 30–60% HBV DNA suppression after 48 weeks of treatment (102–106).

Although lamivudine can be tolerated well and despite its outstanding antiviral effect, its application was restricted because of high rates of resistance, a character frequently observed in nucleoside analogs (107). Based on the guideline published in 2017 from the clinicalinfo.HIV.gov, lamivudine in combination with other antiviral agents, such as TAF or TDF, could be an alternative option for HIV-infected individuals with confirmed HBV infection (108). Nevertheless, regimens containing 3TC is hardly recommended to the treatment for coinfected patients,

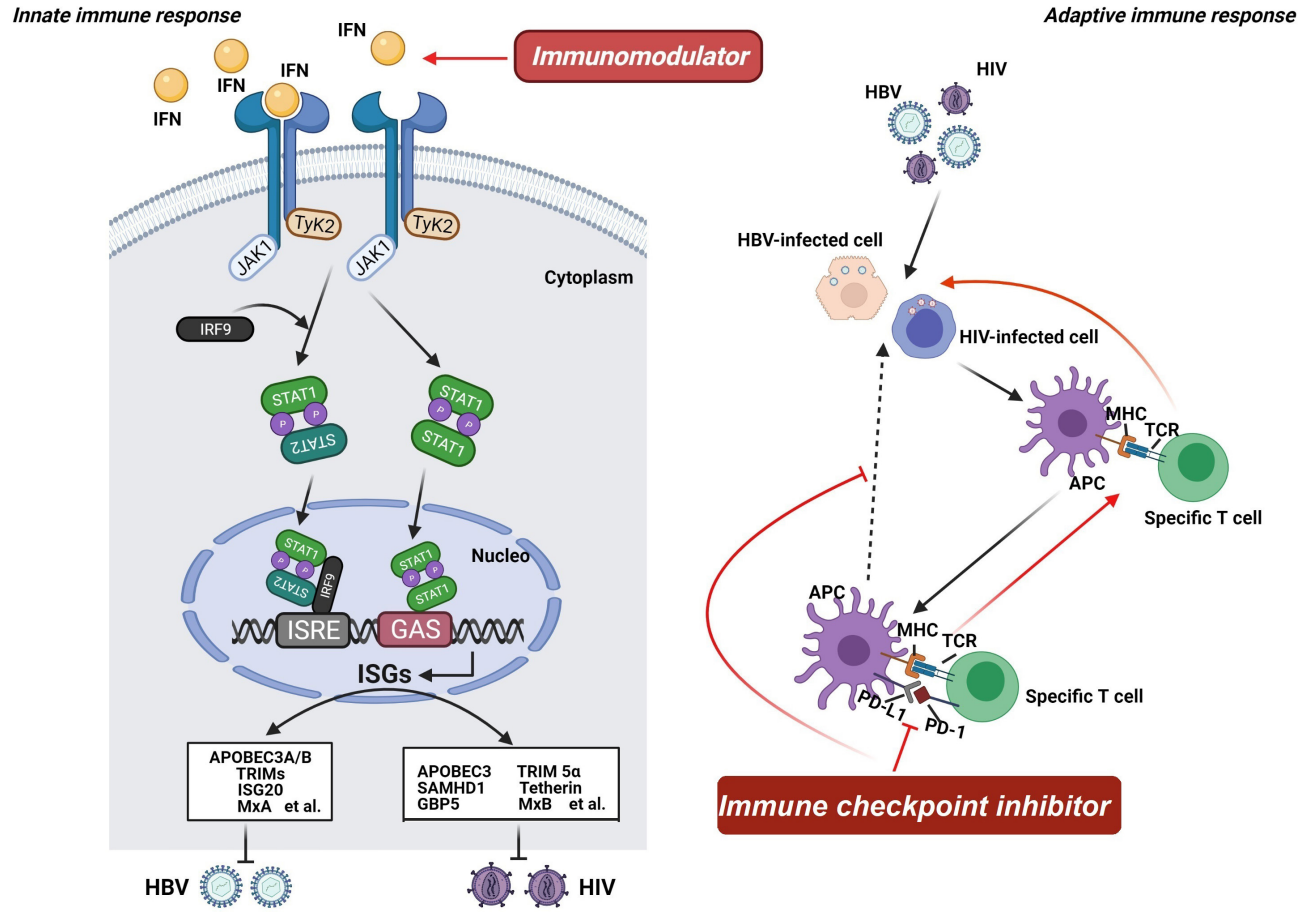

FIGURE 2 | Mechanism of host-targeted agents. Pattern-recognition receptors are activated when the virus invades, leading to the production of interferon (IFN). IFN interacts with its cognate receptors and activates receptor-associated kinases, contributing to the activation of the STAT family to form a transcription factor complex or a homo-/heterodimer. The transcription factor complex and the homo-/heterodimer bind to the ISRE and GAS promoter elements, respectively, and encode numerous viral restriction factors with potent inhibition potential on viral replication. Consequently, immunomodulators offer a rational option for treating HBV and HIV infection. Additionally, the adaptive immune response is also a promising target for novel therapeutic interventions owing to the key position of T cells in viral infection control. Regardless of HBV or HIV infection, virus-specific T cells have been found to have a distinct dysfunction. And the PD-1/PD-L1 axis plays an important role in the pathogenic process of viruses. Considering that high expression levels of PD-1 and PD-L1 are usually related to unsatisfactory immune response, agents based on the PD-1/PD-L1 axis might be able to reverse this immune suppression consequence and exert corresponding antiviral effects. Hence, immune checkpoint inhibitor targeting the PD-1/PD-L1 axis can be another option for treating HBV/HIV coinfection.

according to the latest guideline from British HIV association (BHIVA) and European AIDS Clinical Society (EACS) (109, 110).

Emtricitabine

Similar to lamivudine, emtricitabine (FTC) is a nucleoside with dual HBV/HIV inhibitory effects (111). Apart from HIV treatment, emtricitabine, as combined drug, has been approved by Food and Drug Administration (FDA) for the prevention of HIV infection (111). Although it is not an FDA-approved agent for HBV treatment, emtricitabine has an outstanding antiviral value against HBV; it can notably decrease HBV DNA in serum and achieve normal ALT in patients with HBV at the recommended dose of 200 mg/day (112). According to a preclinical study, emtricitabine is superior to lamivudine in terms of intracellular half-life (113). Nevertheless, both of them are considered clinically equivalent (114). A phase III clinical trial (NCT02607930) has demonstrated that emtricitabine-based STR was non-inferior in HIV virological suppression comparable to that of lamivudine-containing regimens (115). Moreover, this regimen involving emtricitabine, bictegravir, and tenofovir alafenamide affords guideline-recommended therapeutic strategy for HBV/HIV coinfected cohorts (115). Other clinical studies have also reached the same conclusion, confirming the clinical value of emtricitabine (114, 116).

Although limited studies are available to confirm the efficacy of emtricitabine in HIV/HBV coinfection cohorts, its combination with other antiviral agents are superior to emtricitabine monotherapy in reducing HBV DNA (117). A

TABLE 1 | Antiviral agents applicated in HBV/HIV coinfection.

Type	Agent	Mechanism	Antiviral spectrum	Status
Immunoregulator	Interferon	Inhibiting replication of HBV	HBV	Approved
	GS-9620	Antagonizing the TLR-7 and improving the host immune response, leading to the suppression of HBV and clearing of HIV.	HBV, HIV	Clinical trial
Nucleoside/Nucleotide reverse transcriptase inhibitor (NRTI)	Lamivudine	Terminating the chain and suppressing the replication of virus	HBV, HIV	Approved
	Emtricitabine	Terminating the chain and suppressing the replication of virus	HBV, HIV	Approved
	Tenofovir	Facilitating the HBeAg seroconversion of HBeAg and suppressing the replication of HBV	HBV, HIV	Approved
	Adefovir	Competing with deoxyadenosine triphosphate for integration in the synthesizing HBV DNA, resulting in the blockage of the viral DNA polymerase and termination of the chain	HBV	Approved
	Entecavir	Competing with guanosine for integration into the synthesizing HBV DNA, contributing to the blockage of viral DNA polymerase and chain termination	HBV	Approved
Cyclophilins inhibitor	CRV 431	Blocking the interaction of Cyp A with Gap protein of HIV as well as small surface protein of HBV, leading to the inhibition of viral replication.	HBV, HIV	Clinical trial
Immune checkpoint inhibitor	Pembrolizumab	Inhibiting the PD-1/PD-L1 axis and enhancing the immune response against virus infection.	HBV, HIV	Approved

HBV, Hepatitis B virus; HIV, human immunodeficiency virus; TLR-7, Toll-like receptor-7; Cyp A, Cyclophilin A; PD-1, programmed cell death protein; PD-L1, programmed cell death protein ligand.

TABLE 2 | Important clinical trials of HIV/HBV therapies.

Trial number	Phase	Status	Sample size	Design
NCT01924455	IV	Completed	138	Maraviroc/Placebo
NCT00192595	IV	Completed	36	Tenofovir/Zidovudine, lamivudine, efavirenz
NCT01751555	IV	Completed	100	TDF/3TC/EFV
NCT03115736	II	Completed	24	Tenofovir Alafenamide
NCT03547908	III	Recruiting	240	B/FTC/TAF or DTG+FTC/TDF
NCT00476463	II	Completed	24	Emtricitabine
NCT03797014	IV	Recruiting	60	B/FTC/TAF
NCT00127959	IV	Completed	24	Tenofovir/emtricitabine/zidovudine/efavirenz
NCT03425994	–	Active, not recruiting	275	Elvitegravir/Cobicistat/Emtricitabine
NCT00033163	II	Completed	90	Adefovir dipivoxil/Tenofovir disoproxil fumarate
NCT00013702	II	Competed	30	Adefovir
NCT00023153	III	Completed	100	Adefovir dipivoxil
NCT01125696	II	Completed	45	Zidovudine/lamivudine/lopinavir-ritonavir or Tenofovir/lamivudine/lopinavir-ritonavir
NCT00391638	II/III	Completed	56	Peg-interferon Alpha 2a/Tenofovir /Emtricitabine
NCT02071082	III	Completed	79	E/C/FTC/TAF

All the detailed information of clinical trials was registered on the website (ClinicalTrials.gov).
TDF, tenofovir disoproxil fumarate; 3TC, lamivudine; EFV, efavirenz; B, bictegravir; FTC, emtricitabine; TAF, tenofovir alafenamide; DTG, dolutegravir; E, elvitegravir; C, cobicistat.

study also indicated that a combination of emtricitabine and tenofovir disoproxil fumarate (TDF) has excellent outcomes with 14% of patients achieved seroconversion to anti-HBe and 94% of them had undetected HBV DNA in the serum (118). Nevertheless, this study had a small sample size. Thus, multicenter and large-scale trials are needed to confirm the value of emtricitabine in HIV/HBV coinfection treatment. Currently, co-formulated FTC and TDF is recommended as part of a suppression combination regimen applicated in HIV-infected individuals with confirmed or presumed sensitive HBV (108, 109).

Tenofovir

Tenofovir is an adenosine nucleotide analog that has been approved for treatment of HIV infection (119, 120). Owing to its poor bioavailability, it is usually available commercially as TDF

and tenofovir alfenamide (TAF) (119, 121). The former releases tenofovir in the bloodstream, whereas the latter releases tenofovir after entering the cells. Together with emtricitabine, tenofovir is used in HIV treatment and pre-exposure prophylaxis (PrEP) (122, 123). Furthermore, tenofovir-containing PrEP strategies are applicable for HIV-negative nursing mothers. In HBV treatment, tenofovir has been discovered to be capable of overcoming resistance to lamivudine and adefovir dipivoxil in HBV treatment (124, 125). Patients with HBV were found to benefit from tenofovir therapy, including TDF and TAF (126). Results showed that 73 and 75% of the HBeAg-positive individuals who received TDF or TAF achieved HBV DNA levels of <29 IU/mL at 96 weeks, respectively (126). Furthermore, no distinct difference was observed between TAF and TDF regimens in terms of loss rate and seroconversion of HBeAg and HBsAg (126). Two landmark studies also confirmed the therapeutic effects of TDF and TAF on HBeAg-positive or HBeAg-negative cohorts. Moreover, a low dose of TAF (25 mg/day) achieved a response similar to that of TDF (300 mg/day) at 48 weeks (127, 128). Akin to HBV, TAF exhibits potent anti-HIV effects *in vivo* at the low dose of 10 mg, which is 30-fold lower than that of TDF (129, 130).

Owing to their dual antiviral ability, tenofovir-containing regimens are extensively used to treat concurrent HBV and HIV (125, 131, 132). A study that enrolled 110 patients coinfected with HBV and HIV revealed that regimens containing TDF are superior to lamivudine therapy in the seroconversion rate of HBeAg (133). Result of this study showed that the proportion of patients displayed seroconversion was 57% in the group of TDF combined with FTC, 50% in the TDF group and 21% in the lamivudine group, respectively (133). Moreover, suppression of HBV replication was observed in 91% of the individuals during the median observation period. According to a meta-analysis of 23 studies involving a total of 550 patients with concurrent HBV and HIV and receiving TDF treatment noted persistent viral suppression, and the ratio of patients who achieved suppression of viral replication was 57.4, 79, and 85.6% after 1, 2, and 3 years, respectively (134). In addition, virus rebound had been rarely reported in TDF treatment. Therefore, all of the patients with HBV/HIV coinfection should receive tenofovir-based antiretroviral treatment unless history of tenofovir intolerance, according to the guideline of EACS (110).

Although rare, renal impairments, including tubular dysfunction, increase in serum creatinine, and acute renal failure, could be substantially induced by TDF. Hence, renal functions should be regularly monitored during TDF treatment (135).

Other NRTIs Used in HBV/HIV Coinfection Treatment

Not all NRTIs have potent antiviral effects on HBV and HIV. Apart from the agents discussed above, several other NRTIs, including adefovir, entecavir, and telbivudine, are active against HBV but display minimal activity against HIV (136–138). Consequently, their application in HIV treatment is rare. However, considering the potent suppressive effects of adefovir, entecavir, and telbivudine on HBV replication, experts proposed that these NRTIs might be of value in the treatment of HBV/HIV coinfection when combined with other antiviral agents.

As the first nucleotide analog approved for HBV treatment, adefovir strongly inhibits HBV replication with a low incidence of resistance (107). However, the dose of adefovir used in HIV treatment is usually linked to nephrotoxicity (31). According to a pilot study on HBV/HIV coinfection, adefovir can postpone the deterioration of liver diseases, enhance HBeAg seroconversion, and normalize ALT levels by suppressing HBV DNA (139). Several clinical trials have been conducted to estimate the value of adefovir in treating individuals with concurrent HBV and HIV (NCT00033163, NCT00013702, and NCT00023153). A prospective study (ACTG A5127) involving HBV/HIV coinfected patients revealed that either TDF or adefovir treatment results in evident decrease in serum HBV DNA; moreover, results showed that these NRTIs were well-tolerated (140). Benhamou (141) also indicated that treating with emtricitabine-containing regimen plus adefovir for 144 weeks decreased serum HBV DNA levels in 45% of HBV/HIV coinfected subjects, which was lower than that in HBV monoinfection (56%).

Entecavir, a guanosine analog, is superior to emtricitabine and adefovir in suppressing serum HBV DNA (142–144). Moreover, it is effective against not only wild-type HBV but also emtricitabine-resistant and adefovir-resistant HBV (31). Although entecavir was once considered as an inactive agent to HIV, a study uncovered a remarkable phenomenon showing that entecavir could result in evident reduction in serum HIV RNA in three HBV/HIV coinfection patients (145). However, such residual antiviral activity might be able to induce resistant changes in HIV (71). Hence, the FDA warned that entecavir should not be used in the absence of antiretroviral therapy in HBV/HIV coinfected cohorts (137). Numerous clinical trials have been conducted to explore the potential value of entecavir in treating patients coinfected with HBV and HIV (**Table 2**).

Cyclophilin Inhibitors

CRV 431

CRV431, which was previously called CPI-431-32, is a non-immunosuppressive cyclophilin inhibitor–cyclosporin A analog (146). Previous studies have confirmed the efficacy of cyclophilin inhibitors against HIV and HCV (146, 147). HBV DNA, HBsAg, and HBeAg could be effectively reduced by CRV 431 by interrupting the interaction of CypA with HBsAg or HBeAg, as well as by blocking the entry of HBV (148). In addition, an *in vivo* study of transgenic mice reported that CRV 431 can lower serum HBsAg levels and HBV DNA loads in the liver in a dose-dependent manner (149). Moreover, the viral inhibitory effect was enhanced when low CRV 431 dose (10 mg/kg/day) was added 5 mg/kg/day of tenofovir exalidex, a prodrug of tenofovir (149). Furthermore, liver fibrosis and tumor burden in a non-alcoholic steatohepatitis mouse model were ameliorated, highlighting the potential of CRV 431 as a novel therapy for liver disease (150). An ongoing clinical trial is assessing the safety and tolerability of CRV 431 in healthy volunteers (NCT 03596697).

CsA analogs have been confirmed to be effective against HIV by blocking cyclophilins and the HIV capsid to form complexes (151, 152). Hence, CRV 431, which belongs to CsA analogs, could be another promising agent with anti-HIV activity. According

to its metabolization, CRV 431 is speculated to not interact with other NRTIs (153). Therefore, CRV431 might be a potential agent for the treatment of patients suffering from both liver diseases and HIV. Coformulation of CRV 431 and current drugs could achieve favorable outcomes in HBV/HIV coinfected patients.

Host-Targeted Therapeutic Options

Immunoregulator

Interferon-α and PEGylated Interferon-α

Interferons (INFs), a cluster of signaling proteins, are secreted by host cells in response to pathogenic invasion (154, 155). Moreover, they are the first class of agents approved for the treatment of chronic hepatitis B. Interferon-α (INF-α) used to be the standard choice but was eventually replaced by PEGylated interferon-α because the latter has a longer half-life and a stronger potency than the former (31, 156). INF-α and PEGylated INF-α can effectively inhibit HBV replication *in vitro via* stimulated-INF genes and augment host immunity to defend against HBV infection. IFN therapy has shown remarkable efficacy among HBeAg+ HBV infection patients with the characteristic of elevated alanine aminotransferase and low serum HBV DNA (157–159). Nevertheless, limited benefits and increased toxicity were discovered in HIV/HBV coinfected patients probably because of abnormal immunity (25, 160). Hence, such agents might be applied to non-decompensated patients who have a good response to INFs (161). In general, the period of treatment lasts for 12 months. The guideline of BH *via* in 2013 gave the recommendation that PEG-IFN should be used only in HBsAg+ individuals with repeatedly raised ALT and low level of HBV DNA, regardless of the status of HBeAg (162). Nevertheless, the place of PEG-IFN therapies is not mentioned in neither the latest EACS nor BHIVA.

GS-9620

GS-9620, also known as vesatolimod, is an oral small-molecule antagonist of toll-like receptor 7 (TLR-7) with outstanding anti-HBV potency; moreover, it is considered in the clinical treatment of chronic HBV infection (163, 164). Preclinical studies reported sustained reduction of HBV DNA, HBV RNA, and HBsAg levels in HBV-infected cells administered with GS-9620 through an IFN-dependent manner (163, 165, 166). According to a study on a chimpanzee model of chronic HBV, GS-9620 participates in the accumulation of $CD8^+$ T cells and B cells in the portal regions of liver, thereby playing a role in wiping out HBV-infected cells or restricting HBV infection (167). Another study on woodchucks with chronic HBV infection found reduced levels of cccDNA and risk of HCC due to GS-9620 (165). Having achieved favorable HBV suppressive effects in preclinical studies, GS-9620 is undergoing clinical trials to definitively establish its therapeutic efficacy in patients with chronic HBV. Currently, GS-9620 is considered to be safe in and well-tolerated by individuals with chronic HBV (168–170).

Existing HIV therapeutic strategies can achieve HBV suppression to undetectable levels rather than complete removal of viruses, which leads to lifelong treatment (171). To overcome this difficulty, experts have focused on the induction of latent HIV expression and the enhancement of the viral recognition ability of the immune system to eliminate latent HIV. On the basis of the viewpoint that TLR can induce HIV expression from infected cells, scientists have explored the value of GS-9620 in HIV treatment (171–173). Results were consistent with this assumption, confirming that GS-9620 has the ability to activate HIV from the peripheral blood mononuclear cells of HIV-infected patients receiving anti-HIV treatment, thereby improving immune functions and enhancing HIV clearance (171). HIV replication was also observed during GS-9620 treatment (174). A clinical trial (NCT02858401) reported that GS-9620 is safe in and well-tolerated by HIV-infected cohorts (175). Owing to its potential dual antiviral capability, GS-9620 is a promising novel agent that can be applied in the combination therapy of HBV/HIV coinfection.

Immune Checkpoint Inhibitor

Pembrolizumab

Pembrolizumab, a checkpoint inhibitor that targets PD-1, has been approved for treatment of various carcinomas, including lung cancer (176). On account of the critical function of the PD-1/PD-L1 axis in the pathogenesis of chronic diseases, including HBV and HIV, PD-1 inhibitors have been speculated to be effective in disease treatment (90, 91, 177, 178). Several studies have evaluated the safety and feasibility of pembrolizumab in the therapy of patients suffering from various types of carcinoma concurrently with HIV (179–182). Treatment with pembrolizumab has an impact on HIV-specific T cell response and HIV load, showing as a transient increase of $CD8^+$ T cell activation and a transient reduction of HIV DNA (180, 182). No serious adverse effects were observed during the treatment, indicating that pembrolizumab is safe and well-tolerated by the patients (181). Among patients with tumor and HBV infection, pembrolizumab was found to be safe (183–185). Moreover, several studies have been developed to explore the efficacy of pembrolizumab in HBV infection, suggesting that it might enhance the host immune status (182, 184, 185). However, further research is needed to assess the efficacy and safety of pembrolizumab in HBV treatment. On the basis of the application of pembrolizumab in the treatment of patients with cancer and HIV or HBV, experts assumed that it might be effective in HVB/HIV coinfected individuals. However, the evidence remains inadequate because patients with HIV or HBV are usually excluded from research on immune inhibitors because of the immune reconstitution inflammatory syndrome (186). Few studies have indicated that PD-1 inhibitors have proviral effects on HBV infection. However, they are regarded to be able to strengthen the immune function and may be a potential option for HIV treatment (88). Combination of PD-1 inhibitors with other agents might be a reasonable strategy for viral coinfection treatment.

Immune Reconstitution Inflammatory Syndrome

Though current guidelines suggest treatment of HBV/HIV coinfected patients with dual antiviral regimen targeting HBV and HIV, immune reconstruction-related hepatic flare following the ART should be noted (187). IRIS is considered as

a complication induced by the initiation of highly active antiretroviral therapy (ART) in HBV/HIV coinfected patients. It is an inflammatory disorder related to the worsening status of existing infection (188).

As to the coinfected patients receiving ART, elevation of liver enzymes is common, most of which are mild and do not require modification of treatment (189, 190). It is uncommon to develop into severe hepatotoxicity, manifesting as liver enzymes higher than 10 times the upper limit of normal (191). Moreover, the acute liver failure is also rare (192). Unfortunately, high proportion of mortality can be observed among acute liver failure patients, maybe owing to the potential impairment of liver (191, 193). According to a recent research, 20–25% of the coinfected patients might appear HBV flares (HF) after the start of ART (194). Currently, little is known about the impact of IRIS induced HF on the natural history of HBV infection. Patients undergoing HF presented an increase of CD4 T cell counts, a peak level of serum alanine aminotransferase (ALT) and a decrease of HBV DNA (195, 196). A recent study revealed that HBsAg loss was more common in patients developed IRIS induced HF compared with those who did not, suggesting that IRIS induced HF after ART was closely linked with the loss of HBsAg (197). The researchers also raised that younger age and higher HBV DNA titer at baseline were related with the development of IRIS induced HF (197). However, the occurrence mechanism of HF has never been illustrated clearly. It is speculated that the exploration of immune response of IRIS induced HF might be benefit to the treatment of HBV/HIV coinfection. Further studies on such aspect are warranted.

CONCLUSION

Accumulating evidence indicates that the coinfection of HBV and HIV place a heavy burden to the society (148, 198, 199). Coinfection is capable of accelerating the progression of liver diseases (200). Treatment with dual antiviral agents must be initiated as soon as possible (61, 62). However, several factors increase the difficulty of treatment. Agents with a single antiviral effect could induce drug resistance during the duration of therapy. For instance, agents against HBV only could lead to drug resistance to HIV. Hence, combination therapeutic strategies with dual antiviral effects are important (65). Drug-related side effects must also be considered when formulating therapeutic regimens. Renal dysfunction is the most common adverse effect, thus it should be considered before choosing drugs, especially tenofovir, for treatment (135). Damages to important organs might limit the application of existing regimens. Therefore, novel dual antiviral agents with less adverse effects must be developed. In-depth research on disease mechanisms has identified several critical pathogenic mechanisms, providing new approaches for disease treatment. PD-1/PD-L1 participates in the pathogenicity of viruses, including HBV and HIV (88–90). The inhibitors that antagonize the PD-1/PD-L1 axis might be a promising drug for HBV/HIV coinfection treatment. Moreover, immunoregulators with the ability to enhance the innate immune response against HBV and HIV are acceptable. Regardless of the type of agents applied for the treatment of HBV/HIV infection, drug-related adverse effects should be closely monitored.

The efficacy and safety of many strategies for the treatment of HBV/HIV coinfection are being assessed in clinical trials. Several agents remain at the preclinical phase and are not yet available for the clinical treatment of HBV/HIV coinfection. More research and clinical trials are required to definitively establish the value of such agents for the therapy of HBV/HIV coinfection. Finally, novel agents with potent antiviral effects on both HBV and HIV are the ideal approaches.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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MINI REVIEW
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Sirtuins as Potential Therapeutic Targets for Hepatitis B Virus Infection

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Sirtuins (SIRTs) are well-known histone deacetylases that are capable of modulating various cellular processes in numerous diseases, including the infection of hepatitis B virus (HBV), which is one of the primary pathogenic drivers of liver cirrhosis and hepatocellular carcinoma. Mounting evidence reveals that HBV can alter the expression levels of all SIRT proteins. In turn, all SIRTs regulate HBV replication *via* a cascade of molecular mechanisms. Furthermore, several studies suggest that targeting SIRTs using suitable drugs is a potential treatment strategy for HBV infection. Here, we discuss the molecular mechanisms associated with SIRT-mediated upregulation of viral propagation and the recent advances in SIRT-targeted therapy as potential therapeutic modalities against HBV infection.

Keywords: HBV infection, HBx, sirtuins, therapy, molecular mechanisms

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INTRODUCTION

Chronic infection with hepatitis B virus (HBV), which can cause cirrhosis and hepatocellular carcinoma (HCC), remains a serious global public health problem (1, 2). To date, two types of drugs-namely, interferon-α (IFN-α) and nucleos(t)ide analogs-have been approved for treating diseases caused by HBV (1, 3). Although clinical evidence has shown that these drugs have the capability to suppress viral replication and improve liver histology, current standard antiviral treatment strategies rarely cure HBV infection; moreover, drug resistance and disease recurrence after therapy cessation remain prevalent. Given the fact that the interaction between HBV and host cells determines the clinical outcomes of HBV infection, targeting cellular factors that contribute to viral replication is a clinically viable strategy to eliminate the virus. Sirtuins (SIRTs) are nicotinamide adenine dinucleotide $(NAD)^+$-dependent histone deacetylases that regulate various biological processes, including stress responses, apoptosis, and metabolism (4, 5). In particular, emerging evidence shows that SIRTs play a significant role in chronic HBV infection (6–16). Here, we present a review on the role of HBV in the alteration of SIRTs, function of SIRTs in the modulation of viral replication, and therapeutic potential of SIRT-targeting strategies in the suppression of HBV infection.

EFFECT OF HBV ON SIRTS

The SIRT protein was first discovered in *Saccharomyces cerevisiae* as a silent information regulator 2 (SIR2) that contributes to extension of life span under different types of stress (17–19).

To date, a total of seven mammalian SIRT proteins have been identified. All seven SIRTs are present in all living organisms but with diverse intracellular localizations. SIRT1 and SIRT2 are present in the cell nucleus and cytoplasm; SIRT3, SIRT4, and SIRT5 are primarily localized in the mitochondria; and SIRT6 and SIRT7 are mainly present in the nucleus. Additionally, all SIRTs are members of class III histone deacetylases. In addition to their constitutive deacetylase activity, some SIRTs have alternative enzymatic activities, including ADP ribosyltransferase (SIRT4 and SIRT6), demalonylase (SIRT5), desuccinylase (SIRT5 and SIRT7), and demyristoylase (SIRT6) (18, 19). Given their diverse localizations and enzymatic activities, SIRTs are known to play a significant role in a wide range of biological functions. Owing to their ability to target various cellular substrates, SIRTs are crucial for numerous biological processes, including DNA repair, proliferation, mitochondrial energy homeostasis, and metabolism (17, 19). In particular, increasing evidence shows that SIRTs are implicated in the persistence and pathogenesis of viral infections (20) caused by human immunodeficiency virus, influenza A virus, herpes simplex virus 1, and human papillomavirus (18, 19). Considering the potential importance of SIRTs in viral infections, an improved understanding of the interaction between SIRTs and viruses may help researchers to develop alternative novel antiviral therapeutic agents.

To date, the effect of HBV on all proteins in the SIRT family has been extensively investigated by several groups. Notably, current evidence shows that the expression of all SIRT proteins can be modulated by HBV. In particular, expression levels of SIRT1 (6), SIRT2 (7), SIRT5 (8), and SIRT7 were found to be increased in HBV-infected cells (8, 9), whereas those of SIRT3, SIRT4, and SIRT6 were decreased in HBV-infected hepatocytes (10–12, 21). Of note, studies have also demonstrated that changes in the expression level of SIRT1 (14), SIRT2 (7), SIRT3 (11), SIRT4 (10), and SIRT7 are associated with a non-structural viral protein, HBx (9). Specifically, Wang et al. showed that, compared with those in the hepatoma cell line HepG2, the levels of SIRT1 mRNA and its protein were increased in HepG2.2.15 (HepG2 harboring HBV genome). Additionally, the HBV-mediated alteration in SIRT1 expression was further confirmed in HepAD38 cells, in which HBVs were continuously produced in the presence of tetracycline (14). Among the HBV-encoded proteins, only HBx has been found to increase SIRT1 expression at the mRNA and protein levels (14). The expression of HBx could also be upregulated by SIRT1 in the HBV-expressing hepatocytes. However, the cellular factors associated with the upregulation of SIRT1 mediated by viral proteins are still unknown.

Similar to the expression of SIRT1, SIRT2 and SIRT7 are increased in HBV-expressing hepatocytes (7, 9) although the mechanisms underlying the upregulation of SIRT2 and SIRT7 expression by the viral protein HBx is different. HBx-induced overexpression of SIRT2 mRNA and its proteins has been observed. Furthermore, HBx is capable of enhancing SIRT2 transcription by activating its promoters in the HBV-infected hepatoma cells (7). HBx has no effect on the expression of SIRT7 mRNA in hepatoma cells. However, the viral protein can interact and co-localize with SIRT7 (primarily in the nucleus), collectively enhancing SIRT7 stability by restraining its degradation that is regulated *via* the ubiquitin-proteasome pathway (9).

HBx suppresses the expression level of SIRT3 and SIRT4 in hepatoma cells (10, 11). A study showed that SIRT3 inhibition could increase the release of reactive oxygen species (ROS) induced by HBx (11). HBx-mediated SIRT4 suppression is related to an increase in cell cycle progression and the inhibition of apoptosis in hepatoma cells (10). Nevertheless, the detailed mechanisms of HBx-mediated SIRT3 and SIRT4 expression alterations are yet to be fully elucidated. Moreover, although HBV can modulate the expression levels of SIRT5 and SIRT6 (8, 21), the exact virus-encoded proteins that are responsible for the virus-mediated modification of SIRT5 and SIRT6 expression are still unknown.

FUNCTION OF SIRTS IN HBV REPLICATION

The replication cycle of the small enveloped double-stranded circular DNA of HBV has been fully deciphered (**Figure 1**) (3, 22). The covalently closed circular DNA (cccDNA) mini-chromosome of HBV acts as the central replication and transcription template and thus plays a central role in its replication (3). Although all viral transcripts are derived from HBV cccDNA, elements such as viral proteins, transcriptional regulators, and epigenetic modulators are known to regulate the transcription of cccDNA. Specifically, the HBV cccDNA mini-chromosome is stably maintained in host hepatocytes to facilitate the persistence of HBV infection. In particular, current studies have shown that not only can SIRT1 interact with HBx (13) but also can both SIRT1 and HBx be recruited to the viral cccDNA, resulting in a more robust production of cccDNA, pregenomic RNA, as well as surface antigen. To date, various research groups have investigated the effect of SIRT1 on HBV replication. Li et al. showed that SIRT1 facilitates HBV replication by activating all viral promoters located in the HBV cccDNA, including the core, X, preS1, and preS2 promoters (15). Moreover, previous studies have indicated that SIRT1 can sensitize the FXRα, PGC-1α, and c-Jun to enhance viral transcription *via* the activation of HBV core promoter (6, 16). In addition, C/EBPα and PPARα, which contribute to the activation of the core, X, preS1, and preS2 promoters (23), participate in the SIRT1-mediated HBV replication (15, 24) (**Figure 1**).

Autophagy is an important non-selective self-degradation physiological process, by which cell constituents are sequestered in autophagosomes that consecutively fuse with lysosomes to facilitate substrate degradation (25). A growing body of evidence shows that SIRT1 induces autophagy by directly deacetylating autophagy-related molecule markers, including ATG5, ATG7, and ATG8 (26). Of note, studies have demonstrated that autophagic response is crucial for HBV replication (27), and particularly, Yang and Gao reported that SIRT1 facilitates HBV replication through viral-induced autophagy (28, 29). However, miR-141 and miR-224 may play a role of suppressing autophagy-induced HBV replication by targeting SIRT1 (**Figure 1**). Yamai et al. showed that SIRT1 is capable of promoting

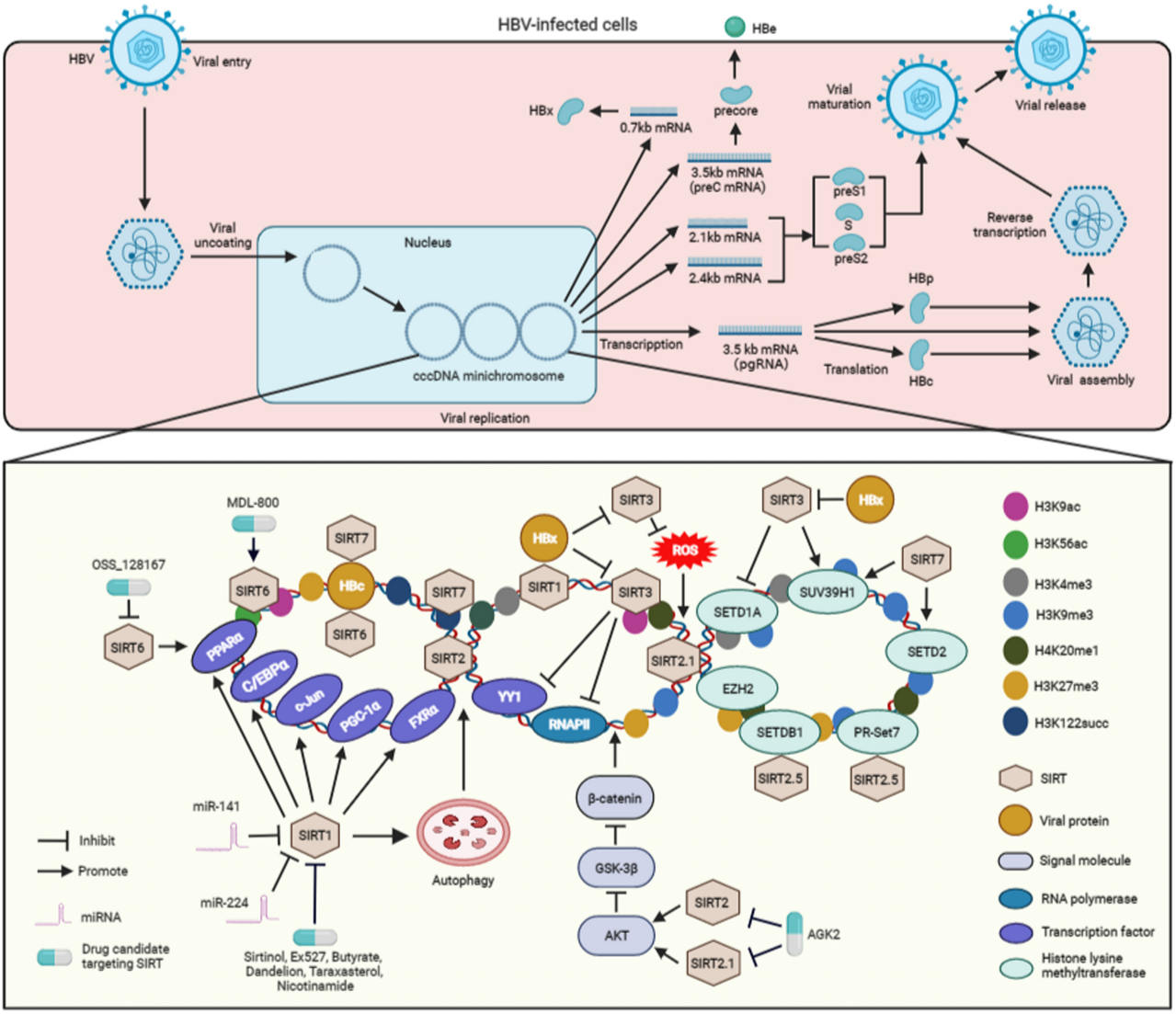

FIGURE 1 | The molecular mechanisms associated with the regulation SIRT-mediated HBV replication and the SIRT-targeting antiviral agents to inhibit viral replication. After entering hepatocytes, HBV is uncoated and transferred into the nucleus and its genome is converted into cccDNA. Subsequently, the cccDNA forms a mini-chromosome and is transcribed into various viral mRNA, including pregenomic RNA (pgRNA) and preC mRNA, two envelope mRNAs, and X mRNA. The pgRNA serves as a translation template for HBc and viral polymerase proteins (HBp). The preC mRNA encodes pre-core protein, which is secreted as HBe antigen. X mRNA is translated to HBx protein. The two envelope mRNAs encode preS1, S, and preS2 domains to construct the various viral surface proteins. The pregenomic RNA is encapsulated into viral particles, and reverse-transcribed into viral DNA. The enveloped HBV particles are secreted from the HBV-expressing hepatocytes. During the cccDNA mini-chromosome-dependent HBV replication, SIRT1 interacts with HBx and then recruited to cccDNA. SIRT1 activates the transcription factors, inculding FXRα, PGC-1α, c-Jun, C/EBPα, and PPARα, which can promote the activation of HBV promoters, to facilitate viral replication. In addition, SIRT1 also enhances HBV replication via autophagy. However, miR-141 and miR-224 inhibit SIRT1 to block autophagy-mediated viral replication. SIRT2 and SIRT2.1 increase the replication of HBV via activating AKT, inhibiting GSK-3β, and then sensitizing the β-catenin signaling. SIRT2.5 recruits histone methyltransferases, including SETDB1, EZH2, SUV39H1, PR-Set7, and H3K27me3, H3K9me3, and H4K20me1 to cccDNA. The interactions of PR-Set7 and SETDB1 with SIRT2.5 are increased upon HBV replication. Upon recruitemnt to HBV mini-chromosome, SIRT3 can lead to an increase in histone lysine methyltransferases SUV39H1 but a decrease in SETD1A on viral cccDNA, which results in increased recruitment of H3K9me3 but decreased binding of H3K4me3 to HBV cccDNA. SIRT3-mediated viral cccDNA transcriptional repression is similar to the decreased recruitment of RNAPII and transcription factor YY1 on the viral cccDNA. HBx enhances cccDNA transcription by suppressing the expression of SIRT3 and inhibiting the recruitment of proteins to cccDNA. In addition, SIRT3 overexpression inhibits viral replication by reducing cellular ROS level. HBx can induce ROS production to promote viral replication, which is associated with the inhibition of SIRT3. SIRT6 activates the HBV promoters via upregulating PPARα expression. However, SIRT6 is able to repress HBV replication via interacting with HBc and downregulating H3K56 acetylation (H3K56ac) and H3K9 acetylation (H3K9ac) on the viral cccDNA mini-chromosome. SIRT7 binds to cccDNA by interacting with HBc and inducing the decrease of H3K122 succinylation (H3K122succ). Specifically, SIRT7 cooperates with SETD2 and SUV39H1 to induce the inhibition of viral transcription. The inhibitors, including sirtinol, Ex527, butyrate, dandelion, taraxasterol, and nicotinamide, can suppress viral replication via SIRT1-targeting. The inhibitor AGK2 suppresses HBV replication by blocking SIRT2 and SIRT2.1. OSS_128167, a SIRT6 inhibitor, can repress HBV replication. Whereas, MDL-800 can suppress viral replication by activating SIRT6. ROS, reactive oxygen species; RNAPII, RNA polymerase II; H3K9me3, trimethyl-H3K9; H4K20me1, monomethyl-H4K20; H3K27me3, trimethyl-H3K27; H3K4me3, trimethyl-H3K4; H3K9ac, H3K9 acetylation; H3K56ac, H3K56 acetylation; H3K122succ, succinylation of histone H3K122; SETDB1, SET domain bifurcated 1; EZH2, enhancer of zeste homolog 2; SETD1A, SET domain containing 1A; SUV39H1, suppressor of variegation 3–9 homolog 1; cccDNA, covalently closed circular DNA.

autophagy-induced HBV replication, the role of SIRT in viral replication is independent of autophagy (30). Therefore, further investigation is required to clarify the exact role of autophagy in SIRT1-dependent HBV replication. In addition to SIRT1, studies have demonstrated that other SIRTs, including SIRT2, SIRT3, and SIRT 6, can also induce autophagy *via* alternative molecular mechanisms (5, 31). However, it remains ambiguous whether these SIRTs can regulate HBV replication *via* autophagy, and therefore, warranting further studies.

A study from Piracha et al. indicates that HBV can increase the expression level of SIRT2 as well as its alternatively spliced transcripts SIRT2.1 and SIRT2.5 (32). It has been demonstrated that the activation of AKT, a core molecule in the PI3-K pathway, is involved in the replication of HBV (33). As a deacetylase, SIRT2 interacts with AKT to enhance its activation *via* deacetylation (34). In particular, Piracha et al. showed that, *via* their interaction with AKT, SIRT2 and SIRT2.1 induce AKT activation to downregulate GSK-3β, which in turn upregulates β-catenin expression to increase the transcriptional activity of HBV (35). Unlike SIRT2.1, SIRT2.5 could only slightly activate the AKT/GSK-3β/β-catenin signaling pathway. However, SIRT2.5 plays an exact opposite role of SIRT2.1, which is to reduce the expression level of HBV cccDNA and mRNAs. Studies have further revealed that, unlike SIRT2.1, SIRT2.5 is primarily recruited to HBV cccDNA (32). The recruitment of histone lysine methyltransferases, such as enhancer of zeste homolog 2 (EZH2), suppressor of variegation 3–9 homolog 1 (SUV39H1), SET domain bifurcated 1 (SETDB1), and PR-Set7, and the methylation of distinct histones, including trimethyl-H3K9 (H3K9me3), monomethyl-H4K20 (H4K20me1), and trimethyl-H3K27 (H3K27me3), are also increased in the cccDNA of SIRT2.5-overexpressing HBV (32). Among these histone lysine methyltransferases, SIRT2.5 could interact with PR-Set7 and SETDB1 on cccDNA, and these interactions are increased upon the replication of HBV (**Figure 1**). As mentioned earlier, Cheng et al. demonstrated that SIRT2 expression could be upregulated by HBx to promote HBV replication (7). However, Piracha et al. showed that SIRT2-mediated upregulation of HBV replication are independent of HBx (32, 35). Taken together, the findings on the mediating effect of SIRT2 on HBV replication obtained by different researcher groups remain inconclusive. Therefore, further investigation is vital to examine the exact role of SIRT2 and its alternatively spliced transcripts in HBx-mediated HBV replication.

Unlike SIRT1 and SIRT2, SIRT3 exerts an inhibitory effect on HBV replication by targeting the cccDNA *via* epigenetic regulation. Specifically, SIRT3 can induce a decrease in H3K9 acetylation (H3K9ac) on viral cccDNA, leading to an increase in histone lysine methyltransferase SUV39H1 and a decline in the SET domain containing 1A (SETD1A) on cccDNA. The recruitment of SIRT3 to viral cccDNA also results in an increase in H3K9me3 and a decrease in trimethyl-H3K4 (H3K4me3) on HBV cccDNA. Furthermore, the transcriptional repression of HBV cccDNA mediated by SIRT3 is also related to a decrease in RNA polymerase II (RNAPII) and the transcription factor YY1 on cccDNA (**Figure 1**). However, HBx can relieve cccDNA transcriptional repression by restricting the expression of SIRT3 and blocking the recruitment of the proteins to cccDNA (12). As a major mitochondrial deacetylase, SIRT3 plays a crucial role in the regulation of ROS by targeting proteins involved in mitochondrial functions and antioxidant defenses (36). Ren et al. reported that SIRT3 can also inhibit HBV replication by decreasing ROS levels in the HBV-expressing hepatocytes. In contrast, HBx can induce ROS to promote viral replication, a process similar to viral protein-mediated SIRT3 inhibition (11).

Findings from two separate studies by Deng et al. and Jiang et al. showed that SIRT6 can promote HBV replication and activate the HBV core promoter *via* upregulating the expression of transcription factor PPARα (13, 37). However, the latest evidence from Yuan et al. indicated that SIRT6 is a restricting factor of HBV. SIRT6 can interact with HBV core protein (HBc) and suppress viral replication through its deacetylase activity by inhibiting H3K56 acetylation (H3K56ac) and H3K9 acetylation (H3K9ac) on the HBV cccDNA mini-chromosome (21) (**Figure 1**). The reasons for the contrasting effect of SIRT6 on HBV replication by different researchers are unclear. Therefore, further investigations are warranted to examine the exact role of SIRT6 in HBV replication.

Although SIRT7 is involved in the modulation of HBV replication, results on the effect of SIRT7 on HBV replication reported by different groups remain inconclusive. Deng et al. showed that SIRT7 contributes to HBV replication (13), but the molecular mechanisms that contribute to the replication of HBV remains ambiguous. Yu et al. reported that SIRT7 can bind to cccDNA by interacting with the HBc protein. Due to its desuccinylase activity, SIRT7 induces a decrease in histone H3K122 succinylation (H3K122succ) on the cccDNA (**Figure 1**). Furthermore, SIRT7 can act cooperatively with histone lysine methyltransferase SUV39H1 and SETD2 to modulate the chromatin structure of cccDNA and facilitate the inhibition of HBV transcription (38). Similar to SIRT6, further studies are vital to investigate the exact role of STIR7 in HBV replication. Although SIRT4 and SIRT5 can enhance HBV replication (13), the exact molecular mechanisms mediated by these two SIRTs are still not fully clarified.

SIRTS AS POTENTIAL THERAPEUTIC TARGETS IN HBV INFECTION

Owing to the growing evidence on the importance of SIRTs in HBV infection, targeting SIRTs using antiviral agents is an appropriate strategy to attenuate the replication of HBV (**Figure 1**). For example, inhibition of SIRT1 with sirtinol and Ex527 has a significant antagonistic effect on the replication of HBV (13). Emerging data indicate that butyrate can inhibit HBV replication by targeting SIRT1 (39). In addition, dandelion and taraxasterol, which have the role of targeting SIRT1, also exert a significant inhibitory effect on HBV replication (40). Additionally, nicotinamide, another SIRT1 inhibitor, could suppress HBV replication *in vitro* and *in vivo* (15).

As mentioned above, SIRT2 and SIRT2.1 have been demonstrated to accelerate viral replication (32, 35), and AGK2 could suppress HBV replication by targeting SIRT2

and SIRT2.1 *in vitro* and *in vivo* (35, 41). Jiang et al. and Yuan et al. reported contradicting effect of SIRT6 on the replication of HBV (21, 37). The inhibitor or activator of SIRT6 exhibited an inhibitory effect on HBV replication in these two studies. Jiang et al. demonstrated that the selective inhibitor OSS_128167 could target SIRT6 to restrict viral replication in both HepG2.2.15 cells and HBV-transgenic mice (37). However, data from Yuan et al. indicated that a specific activator of SIRT6, MDL-800, can suppress the replication of the virus *in vitro* and *in vivo* (21) (**Figure 1**). Therefore, further study is vital to examine the exact effect of SIRT6 inhibitors or activators on HBV replication.

CONCLUSION AND FUTURE PERSPECTIVES

Currently, the standard treatment regimens for HBV are mainly limited to IFN-α and nucleos(t)ide analogs. IFN-α treatment strategy is primarily employed to elicit cytokine-induced antiviral immune response *via* the expression and antiviral activity of IFN-stimulated genes. Nucleos(t)ide analogs inhibit viral replication *via* their accumulation on the viral genome and thereby disrupt the transcription of viral polymerase (1, 42). However, these two drugs cannot cure the HBV-induced diseases and often result in serious side effects, drug resistance, and disease recurrence. Therefore, novel molecular targets for HBV treatment are urgently needed. Based on this review, we infer that HBV can alter the expression of SIRTs and that SIRTs are capable of promoting viral replication *via* multiple pathways. First, SIRTs regulate HBV replication by controlling the activity of viral promoters that rely on various transcription factors, including FXRα, PGC-1α, and c-Jun. Second, SIRTs can modulate many biological processes, including the induction of autophagy, increase in ROS production, and activation of signaling pathways to regulate HBV replication. Third, SIRTs can also modify the chromatin structure of HBV cccDNA *via* epigenetic regulation based on histone methylation, acetylation, and succinylation to modulate viral replication. Besides these, the utilization of different inhibitors or activators associated with SIRTs can effectively inhibit HBV replication *in vitro* and *in vivo*. Therefore, targeting SIRTs, as a means to disrupt the biological processes that benefit viral replication *via* suppressing the activation of HBV promoters and altering the chromatin structure of viral cccDNA, is a promising treatment strategy against HBV infection.

In the search for potential novel SIRTs antagonists, many significant breakthroughs have been attained in recent years (43, 44). Importantly, several preclinical and clinical trials have been conducted to assess the effect of SIRT-targeting drug candidates for the treatment of various diseases, including diabetes, obesity, and cancer (43, 45). Nevertheless, the use of SIRT-targeting drug candidates may cause other side effects attributed by the interacting effect between the drugs on other biological processes in which SIRTs are involved. Although some SIRT-targeting agents, including EX-527 and resveratrol, are reported to be safe and well-tolerated in various clinical trials (45–47), mild adverse effects, including dizziness, headache, and epididymitis, have been frequently reported in some participants administered with resveratrol (45). Therefore, to better assess whether targeting SIRTs is a novel promising treatment strategy against HBV-induced diseases, large scale clinical trials in the near future are vital. In addition, not only the clinical efficacy but also the safety and tolerability of the SIRT-targeting antagonist candidates in patients with HBV infection should be verified.

HBx is a non-structural viral protein that plays a vital role in the replication of HBV (22). Our review provides clear evidence that the HBV-mediated overexpression of SIRTs to regulate viral replication is primarily HBx-dependent. However, the exact molecular mechanisms underlying the HBx-induced alteration of SIRT expression are still not fully elucidated. To better understand the exact effect of SIRTs on HBV replication regulated by the viral proteins, further investigation on the interactions between HBx and SIRTs is needed. In this review, our analysis of current data was primarily focused on the effect of SIRTs on HBV replication; the role of SIRTs in the development of cirrhosis and HBV-induced HCC remains poorly understood. Therefore, it is crucial to further examine the functions and molecular mechanisms of SIRTs in regulating the development of HBV-induced diseases.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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Peripheral Immune Cells Exhaustion and Functional Impairment in Patients With Chronic Hepatitis B

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After infection of hepatitis B virus (HBV), the virus induces a variety of immune disorders in the host, leading to immune escape and, finally, the chronicity of the disease. This study investigated immune cell defects and functional impairment in patients with chronic hepatitis B (CHB). We analyzed the percentage, function, and phenotypes of various immune cell subpopulations in the peripheral blood along with the concentrations of cytokines in the plasma. We compared the results between patients with CHB and healthy individuals. It was found that in patients with CHB, the cell function was impaired and, there was increased expression of inhibitory receptors, such as NKG2A and PD-1 in both NK and T cells. The impairment of function was mainly in cytokine secretion, and the cytotoxicity was not significantly diminished. We also found that the proportion of dendritic cells (DC) decreased and regulatory B cells (Breg) increased in CHB. In addition, the Breg cells were negatively correlated with T cell cytokine and positively correlated with ALT and HBV viral load. Taken together, various disorders and functional impairments were found in the immune cells of peripheral blood in CHB patients, especially NK and T cells. These cells showed exhaustion and the increase of regulatory B cells may be one of the reasons for this phenomenon.

Keywords: chronic hepatitis B, immune cells, exhaustion, cytokines, functional impairment

INTRODUCTION

Despite the availability of highly effective preventive vaccines and oral antiviral drugs, chronic infection with hepatitis B virus (HBV) still affects more than 240 million people worldwide and causes 620,000 deaths annually (1, 2). HBV is a non-cytopathic hepatophilic double-stranded DNA virus (3). Most people develop an acute self-limiting disease with host immunity after infection with the virus. However, the virus can use various strategies to evade host surveillance (4, 5), and those patients who fail to clear the virus to develop chronic infection.

In patients with chronic hepatitis B (CHB), the host immune response is like a double-edged sword. On one hand, it achieves clearance of the hepatitis B virus by destroying infected hepatocytes, and on the other hand, the immune response causes liver inflammation and aggravates liver damage, leading to liver fibrosis and hepatocellular carcinoma (6, 7). In addition, the HBV virus can induce host immune dysfunction, causing immune imbalance and functional defects.

The exhausted immune cells could accelerate infected hepatocytes to achieve immune escape and promote disease development because of their inability to perform immune monitor (8–11).

The immune system is extremely complex, and there is still no clear explanation on the antiviral inflammation of the body after HBV infection and on the immune escape of the virus. This study intends to investigate the possible dysfunction of the immune system in patients with chronic hepatitis B through the analysis of the percentage, phenotype, and function of peripheral immune cells in 48 patients with chronic HBV infection. Our purpose is to provide clinical data for a more in-depth understanding of the mechanisms of immunodeficiency associated with chronic hepatitis B infection and, furthermore, to offer a theoretical basis for immunotherapy in hepatitis B patients.

TABLE 1 | Clinical characteristics of 4 subgroups of patients with CHB.

Characteristics	IT	IC	LR	RA
Number	13	12	13	10
Age (years)	36 (25–68)	35 (17–72)	43 (28–59)	45 (16–72)
Gender (M/F)	7/6	8/4	8/5	7/3
ALT (IU/L)	26.8 (11–39)	174.6 (73–537)	24.8 (10–35)	233.3 (67–575)
HBV DNA log10 IU/mL	7.8 (7.4–8.2)	5.7 (3.8–8.6)	2.5 (2.1–3.3)	4.4 (3.5–7.1)
HBeAg positive/negative	13/0	12/0	0/13	0/10
ALT raised/normal	0/13	12/0	0/13	10/0

IT, immune tolerant; IC, immune clearance; LR, lower replicative; RA, reactivation.

TABLE 2 | Clinical characteristics of CHB patients and HC.

Characteristics	CHB	HC
Number	48	15
Age (years)	36 (16–72)	38 (23–48)
Gender (M/F)	30/18	9/6
ALT (IU/L)	106.2 (10–575)	21.3 (11–35)
HBV DNA log10 IU/mL	5.1 (2.1–8.6)	NA
HBeAg positive/negative	25/23	NA
ALT raised/normal	22/26	0/15

CHB, chronic hepatitis B; HC, healthy control; M/F, male/female; ALT, alanine transaminase; HBeAg, hepatitis B e antigen; HBV, hepatitis B virus; NA, not applicable.

MATERIALS AND METHODS

Patients and Controls

A total of 48 patients, which included 30 male and 18 female patients, with chronic hepatitis B (CHB) were recruited from the Department of Hepatology in Tongde Hospital of Zhejiang Province. The age of patients ranged from 16 to 72 years old with the mean age of 39 ± 14 years. These patients were HBsAg positive for longer than 6 months, volunteered to participate in the study, and had complete case information. None of the CHB patients had a history of liver surgery, coinfection with another viral hepatitis, autoimmune hepatitis, or human immunodeficiency virus (HIV) infection, and there were also no pregnant or lactating women. The patients were divided into immune tolerant (IT) phase ($n = 13$), immune clearance (IC) phase ($n = 12$), lower replicative (LR) phase ($n = 13$), and reactivation (RA) phase ($n = 10$) according to the Asian Pacific Association for the Study of Liver guidelines (12) (**Table 1**). In addition, 15 healthy control specimens from the health checkups, all of whom had excluded HBV infection, HCV infected or HIV infected, and had normal serum alanine aminotransferase (ALT) levels, were included in the study. CHB and healthy control characteristics are detailed in **Table 2**. All specimens were enrolled after obtaining informed consent from the patients or their families. The study was approved by the Ethics Committees of the Tongde Hospital of Zhejiang Province [identification nos. HMU (Ethics) 2017-K044].

Specimen Processing

The 2 ml peripheral blood was collected using the EDTA anticoagulated or heparin anticoagulated (for intracellular cytokine assay) tubes. The peripheral blood mononuclear cells (PBMC) were obtained by density gradient centrifugation by Ficoll separation. In addition, 5 ml of procoagulated blood was centrifuged at 1,000g for 10 min, the plasma was obtained and stored at −80°C for testing. All treatments were completed within 24 h.

Analysis of Cell Surface Molecule Expression by Flow Cytometry

All analysis was performed in four protocols. Antigen-presenting cell protocol, CD16-FITC, CD11c-PE, CD56-PC5, CD123-PC7, CD14-APC, CD3-APC750, HLA-DR-PB, CD45-KO, B cell protocol, CD38-FITC, IGD-PE, CD19-PC5, CD CD27-PC7, CD24-APC, T cell protocol, CD45RA-FITC, CD127-PE, CD25-ECD, PD-1-PC5, CD45-PC7, CD4-APC, CD8-APC700, CD3-APC750, CXCR5-PB. NK cell, CD16-FITC, CD8-PE, CD56-PC5, CD45-PC7, NKG2A-APC, CD3-APC750, and NKp30-PB. All antibodies were added with 5 ul. Then each protocol was added 50 ul PBMC, incubated for 20 min in the dark, added 500 ul hemolysin, hemolyzed for 5 min, centrifuged, and discarded supernatant before resuspended. Flow cytometry assay and set up isotype control were performed. All assays were completed within 1 h after isolation of PBMC.

Test of Granzyme and Perforin

Firstly, the cell surface staining was performed by taking 50 ul of PBMC, adding 5 ul to each of CD56-PC5, CD45-PC7, CD4-APC, CD8-APC-700, and CD3-APC-750 antibodies, respectively, and incubating for 20min in the dark. Then, hemolysin, fixative, and membrane breaker were added in order, with 20 min of

Abbreviations: HBV, hepatitis B virus; CHB, chronic hepatitis B; IT, immune tolerant; IC, immune clearance; LR, lower replicative; RA, reactivation; PBMC, peripheral blood mononuclear cells; APC, antigen-presenting cells; DC, dendritic cells; MFI, mean fluorescence intensity; SD, standard deviation; PDC, plasma dendritic cell; MDC, myeloid dendritic cell; cTfh, circulating T follicular helper cell; Breg, regulatory B cells.

incubation and centrifugation after the addition of each agent. Finally, 5 ul of perforin -FITC and granzyme-PE antibody was added into each mixture, vortexed, and incubated for 20 min in the dark.

Intracellular Cytokine Staining

We analyzed intracellular cytokines in 31 of the 48 CHB patients. Cells were stimulated for 3 hours in disposable dry powder tubes precoated with stimulating agents (PMA, ionomycin, brefeldin) at 37°C in 5% CO_2, then stained with surface markers CD45-PC7, CD3-APC-750, CD4-APC, CD8-APC700, and CD56-PC5 while being protected from light for 15 minutes. These were then fixed and broken to stain intracellular cytokines IFN-γ-FITC, IL-21-PE, and IL-17-PB, before finally being detected by flow cytometry.

Multiplex Cytokine Cytometric Bead Array

Plasma concentration of cytokines was tested in the 31 patients with CHB. Plasma (standard), buffer, mixed capture beads, and detection antibody, each with affinity 25 ul was mixed and incubated for 2 h in the dark. Then, streptavidin with fluorescein was added (SA-PE) and mixed in the dark for half an hour before being centrifuged. The supernatant was then discarded before 100 ul of PBS was added. Finally, the flow cytometer was tested. The results were quantified in the software LEGEND plex V8.0 for analysis.

Instrument and Reagents

Antibodies: CD279 (PD1) (Clone PD1.3 Beckman Coulter), CD45RA (Clone ALB11 Beckman Coulter), CD185 (CXCR5) (Clone J252D4 Biolegend), CD4 (Clone 13B8.2 Beckman Coulter), CD20 (Clone B9E9 Beckman Coulter), CD19 (Clone J4.119 Beckman Coulter), CD38 (Clone HB-7 Biolegend), CD27 (Clone M-T271 Biolegend), IGD (Clone IA6-2 Biolegend), CD11c (Clone BU15 Beckman Coulter), CD14 (Clone RMO52 Beckman Coulter), CD16 (Clone 3G8 Beckman Coulter), CD24 (Clone ALB9 Beckman Coulter), CD45 (Clone J.33 Beckman Coulter), CD3 (Clone UCHT1 Beckman Coulter), CD8 (Clone B9.11 Beckman Coulter), CD56 (Clone N901 Beckman Coulter), CD57 (Clone NC1 Beckman Coulter), CD25 (Clone B1.49.9 Beckman Coulter), CD127 (Clone R34.34 Beckman Coulter), CD159a (NKG2A)(Clone S19004C Biolegend), CD337 (NKp30) (Clone P30-15 Biolegend), IFN-γ (Clone 45.15 Beckman Coulter), IL-21 (Clone 3A3-N2 Biolegend), IL-17 (Clone BL168 Beckman Coulter), granzyme (Clone QA16A02 Biolegend), perforin (Clone B-D48 Biolegend),

Flow Cytometer: Navios, Beckman Coulter

Cytometric Bead Array: RAISECARE; Analysis Software: Kaluza, LEGEND plex.

Laboratory Indices

Alanine aminotransferase activity in serum was measured by an auto biochemical analyzer (AU5800, Beckman Coulter), with the reference range of 9–50 IU/L. The HBV markers, HBsAg and HBeAg, were determined by commercially available enzyme-linked immunosorbent assays (Shanghai KH Biology). Serum HBV DNA was quantitated by real-time quantitative polymerase chain reaction using a commercially available kit (Anadas9850, Amplly), the lower detection limit was 30 IU/ml.

Statistical Analysis

Statistical analysis was performed using SPSS 22.0 Software (IBM, USA). Data were expressed as the mean ± standard deviation and n (%). Student's t-test or One-way ANOVA was conducted to compare two groups and comparisons between three or more means. Correlations between variables were calculated with the Spearman rank correlation test. In our studies, $p < 0.05$ were considered significant.

RESULTS

The Proportion or Function of Antigen-Presenting Cells Decreased in Patients With CHB

To determine whether deficiency of antigen-presenting cells (APC) occurs in patients, we investigated the percentage of dendritic cells (DC) in patients with CHB and compared it with the healthy controls. The identification of DC by flow cytometry was shown in **Figure 1**. Our result indicates that the plasma dendritic cell (PDC) in patients with CHB was lower than those in the healthy group ($p < 0.001$). However, CD16+ and CD16– myeloid dendritic cells (MDC) do not show a significant difference between these two groups. In order to explore whether there are changes between different stages of CHB, we further compared the DC in CHB with different stages, and we found that CD16– MDC in the IT group was lower than that in IC ($p = 0.026$) group (**Figure 1E**), PDC and CD16+ MDC have no difference among each stage (data not shown).

As another important role of APC, we also compared the mean fluorescence intensity (MFI) of HLA-DR expression in monocyte cells. There was no difference found between CHB and the normal group. However, in different stages of CHB, results show significantly lower MFI expression in IT than that in IC ($p = 0.019$) and RA ($p = 0.002$) stage (**Figure 1F**).

Peripheral Blood Breg Elevated in CHB Patient

We compared B cell subsets in the peripheral blood of healthy control and CHB patients. We circled the plots of highly expressed CD38 and CD24 in CD19+ cells, which we considered as regulatory B cells (Breg) (**Figure 2A**). Our data revealed that the number of Breg in CHB was higher than that in the healthy group ($p < 0.001$). The CHB patients with abnormal ALT were higher than normal ones ($p = 0.040$) (**Figure 2C**). In addition, Breg cells were positively correlated with the concentration of ALT and HBV DNA in CHB ($p = 0.017$ and $p = 0.031$, respectively), and negatively correlated with IL-17 and IFN-γ that was secreted by CD4+ T cells ($p = 0.041$ and $p = 0.011$, respectively) (**Figure 2D**). In comparison to other subclasses, we found that the proportion of CD27– Naïve B cells decreased in CHB ($p = 0.005$), on the contrary, the Memory B cells increased ($p = 0.005$). There was no significant difference in plasma blast between healthy and CHB groups (**Figure 2B**).

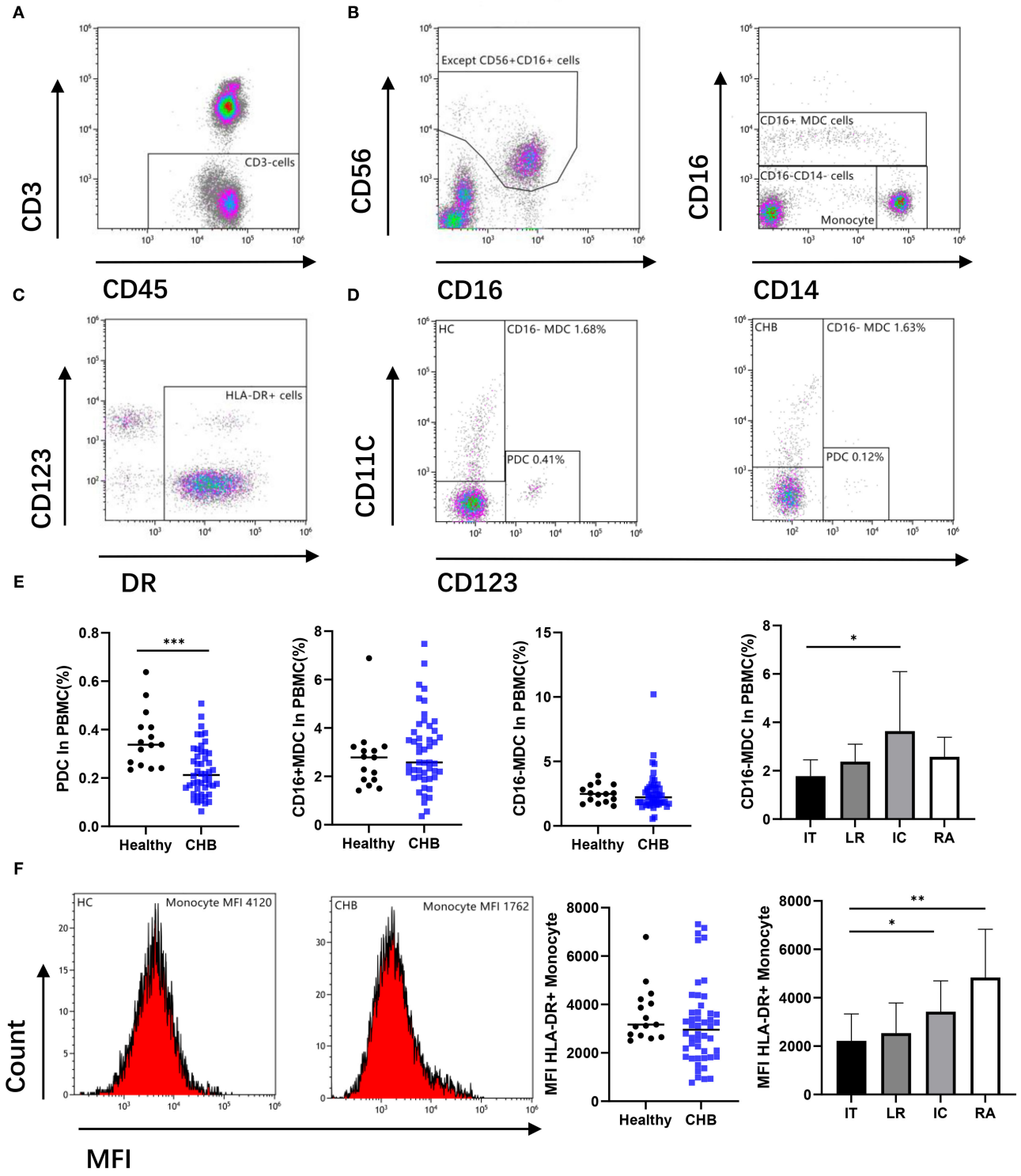

FIGURE 1 | Antigen-presenting cells (APCs) were altered during chronic hepatitis B virus (HBV) infection. **(A,B)** Remove the CD56 positive cell from the CD3 negative gate, and the remaining parts were divided into CD16+ myeloid dendritic cell (MDC), Monocyte, and CD16–CD14– cell. **(C,D)** Circle the DR positive cells from CD16 and CD14 double negative cells, there are CD123+ (plasma dendritic cell, PDC) and CD11c+ (CD16– MDC) cells among them. Representative flow cytometry plot indicates the percent frequencies of PDC as well as CD16– MDC in healthy ($n = 15$) and CHB patients ($n = 48$) respectively. **(E)** According to different alanine aminotransferase (ALT) and HBV DNA levels, all chronic hepatitis B (CHB) were divided into immune tolerant (IT; $n = 13$), lower replicative (LR; $n = 13$), immune clearance (IC; $n = 12$) and reactivation (RA; $n = 10$). The percentages of PDC and MDC in different groups were compared. **(F)** Mean fluorescence intensity (MFI) of DR in CD14+CD16– Monocyte cell was compared. Each data point represents an individual subject. Horizontal lines show the median. Statistically significant differences are indicated by $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

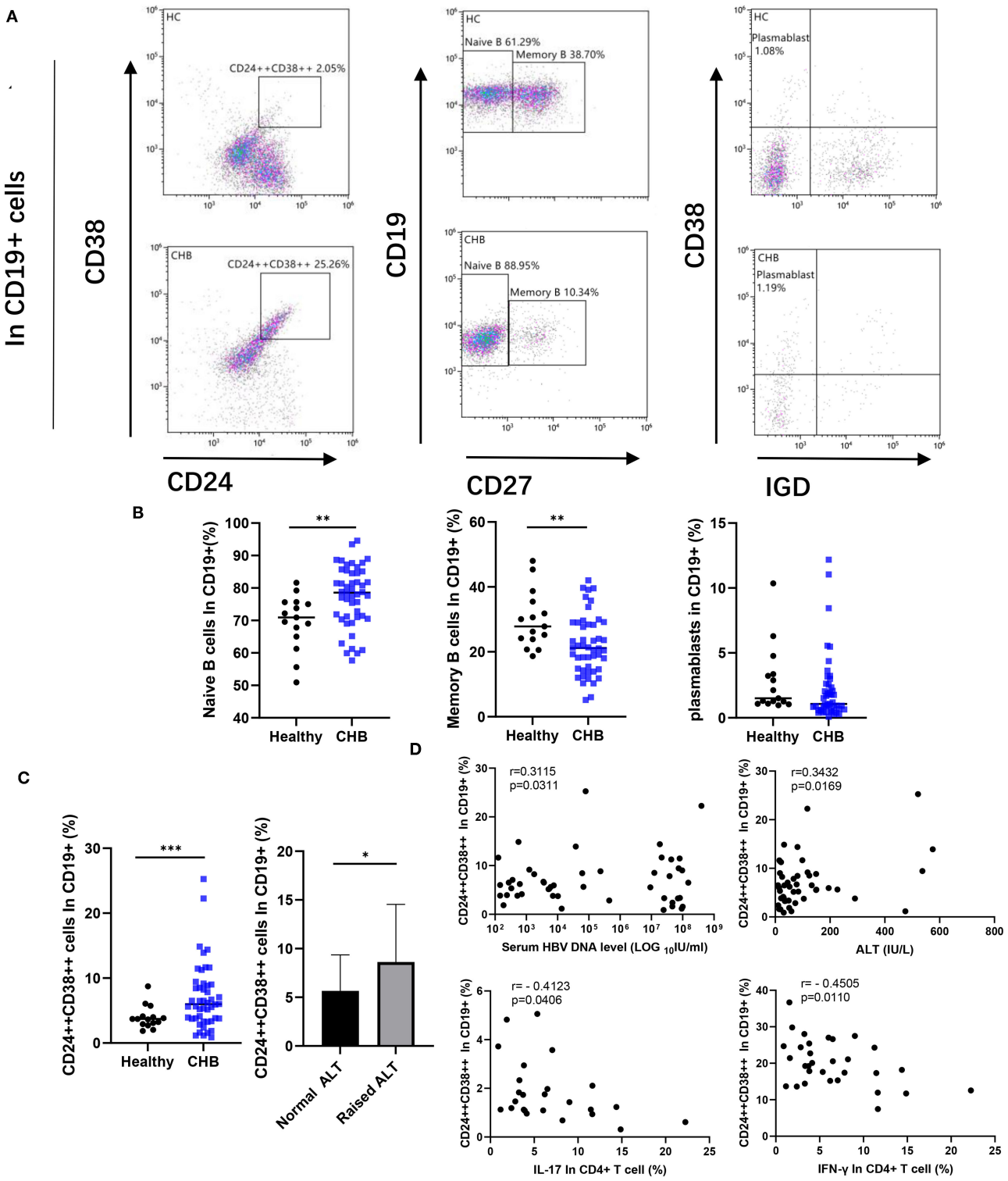

FIGURE 2 | Comparative analysis of peripheral B cell subsets. **(A)** A representative dot plot of B cell subsets in healthy and chronic hepatitis B (CHB). The cells strongly expressed CD24 and CD38 were defined as Regulatory B cells (Breg). We circled CD19+CD27− subset as Naïve B cell, and CD19+CD27+ cells as Memory B cell. The CD19+CD27+ cells further divided into Plasmablast (CD38+IgD−) according to the expression of surface marker CD38 and IgD. **(B)** Percentage of each subset among CD19+ B cells in healthy controls ($n = 15$), and CHB ($n = 48$) were compared. **(C)** Percentage of Breg in CHB was compared with the healthy controls, meanwhile the difference between normal ($n = 26$) and abnormal alanine aminotransferase (ALT; $n = 22$) in CHB patients was also showed. **(D)** The correlation among the Breg, the concentration of ALT, HBV DNA in serum of CHB patient ($n = 48$), and cytokines secreted by CD4+ T cells ($n = 31$) as shown in the figure. Statistically significant differences are indicated by $^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

Circulating T Follicular Helper Cell Increased in CHB, and Treg in Peripheral Blood Did Not Have a Difference in Healthy Control

The CD4+Th subsets were further analyzed. The CD127–CD25+ cells were defined as regulatory T cells (Treg). The cells with high expression of CXCR5 and PD-1 were regarded as circulating T follicular helper cells (cTfh) (**Figures 3A,B**). Our results revealed that there was no significant difference of Treg in peripheral blood between CHB and healthy controls or between CHB patients with abnormal and normal ALT (**Figure 3D**). In the analysis of cTfh, we found that the ratio in CHB was higher than in healthy patients ($p = 0.035$). Similarly, in a different stage of CHB, cTfh in IC was higher than in IT patients ($p = 0.043$) (**Figure 3E**).

Increased PD-1 Expression in T Cells

We examined the expression of programmed cell death protein 1 (PD-1) in T cells (**Figure 3C**). The results indicate that PD-1 expression on both CD4+ and CD8+ T cells was significantly higher in CHB patients than in healthy controls ($p = 0.019$ and $p < 0.001$, respectively), suggesting increased T cell exhaustion in CHB patients. Interestingly, we did not find differences in PD-1 expression in the comparison of different stages of CHB (**Figure 3E**).

NK Cells Functionality Impaired in CHB and Displayed an Altered Phenotype

We measured the percentage of subsets of immune cells defined by CD56 expression levels. We observed that there was an increase in the proportion of NK cells in the group of CD56 positive in CHB patients ($p = 0.038$), while the number of CD56 dim subsets did not change (**Figure 4B**). The CD56 dim NK cell effector capacities were then analyzed with two different functions: cytotoxicity and the production of cytokines. Our results showed that the MFI of granzyme and perforin, which represent the cytotoxicity function of NK cells, was not significantly different in CHB and healthy controls. But the function of IFN-γ secretion by NK cells was decreased in CHB ($p < 0.001$) (**Figures 4A,C**). In order to determine whether the poor functionality could be explained by the altered expression of certain receptors, we characterized the phenotype of NK cells in CHB patients compared to healthy controls. The results of the study revealed that the inhibitory receptor NKG2A is elevated ($p = 0.025$) and, in contrast, the activating receptor NKP30 is decreased ($p = 0.009$) in CHB patients (**Figures 4A,B**).

Enhanced T-Cell Cytotoxicity and Impaired Cytokine Secretion in CHB Patients

To investigate the alterations of T cells functions in CHB disease. We also analyzed the two main functions, cytotoxic and cytokine secretion of T cells. As shown in **Figures 5A–C**, T cells from CHB patients presented higher expression of granzyme and perforin than controls ($p = 0.038$ and $p = 0.024$, respectively). This result suggested enhanced cytotoxic function of T cells in CHB. In contrast, cytokine secretion was impaired in CHB patients, and our results showed that the IFN-γ positivity in CD8+ T cells and the proportion of IFN-γ, IL-21, IL-17 in CD4+ T cells were all significantly lower than those in healthy controls ($p = 0.038$, $p = 0.014$, $p = 0.024$, and $p = 0.006$, respectively) (**Figure 5D**).

IL-2 and IL-6 in Plasma Increased in CHB

The concentrations of cytokine in plasma were measured by Cytometric bead array (CBA). The capture antibodies for IL-2, IL-6, and IL-10 were coated on 5 um microspheres differentiated by three different levels of APC fluorescence intensity, respectively. IFN-γ, IL-17, IL-4, IL-12, and TNF-α were coated on 7 um microspheres of five APC fluorescence intensity levels in turn, and the PE channel was used for color development before being converted to cytokine concentration on the standard curve (**Figure 6A**). We found increased concentrations of IL-2 and IL-6 in the plasma of CHB patients, that were significantly different in the comparison of data from healthy controls (**Figure 6B**). We further compared the IL-2 and IL-6 in different immune phases, and the results showed that IL-2 in the IT group was lower than that in IC ($p = 0.043$), no significant differences were found in IL-6 across the four immune phases (data not shown).

DISCUSSION

Natural Killer cells account for ∼5–15% of peripheral blood lymphocytes (13). It is the main immune cell of the body to deal with a viral infection. They can directly attack the infected cells through the cytotoxic functions of intercellular contents, and they can secrete multiple cytokines, such as interferon-γ (IFN-γ). CD3–CD56 + NK cells identified by flow cytometry can be further subdivided into NK cells of CD56dim and CD56 positive (14). The former is the main group that makes up NK cells, expressing CD16 (Fc receptor) and KIR receptors, playing a cytotoxic role, and the latter main function is the secretion of cytokines (15). Although CD56brightNK cells account for very little in the blood, this distribution can be significantly altered by persistent viral infection (16). Our study showed increased CD56brightNK cells in CHB patients, while CD56dimNK cells did not differ from normal, which was consistent with some of the previous literature reports (17).

Natural Killer cells are activated during acute HBV infection before the onset of adaptive immunity, and the effective immune response of NK cells leads to initial control of the acute infection in the early stages (18). However, when the disease enters a chronic stage, NK cells do not necessarily undergo physical loss but remain in a state of "exhaustion" with poor or no function (19). In the peripheral blood NK cell function test in the disease group, we found that the cytotoxicity of NK cells and the mean fluorescence intensity of protein in CHB patients were not significantly different from those in the healthy controls, while the function of IFN-γ secretion was significantly decreased, indicating impaired NK cell function. The phenomenon that the cytotoxic effect remains unchanged or even increases while the cytokine production ability decreases is called the "functional dichotomy" (16) by some scholars, and this functional defect may be more conducive to the survival of the virus. It is unfortunate

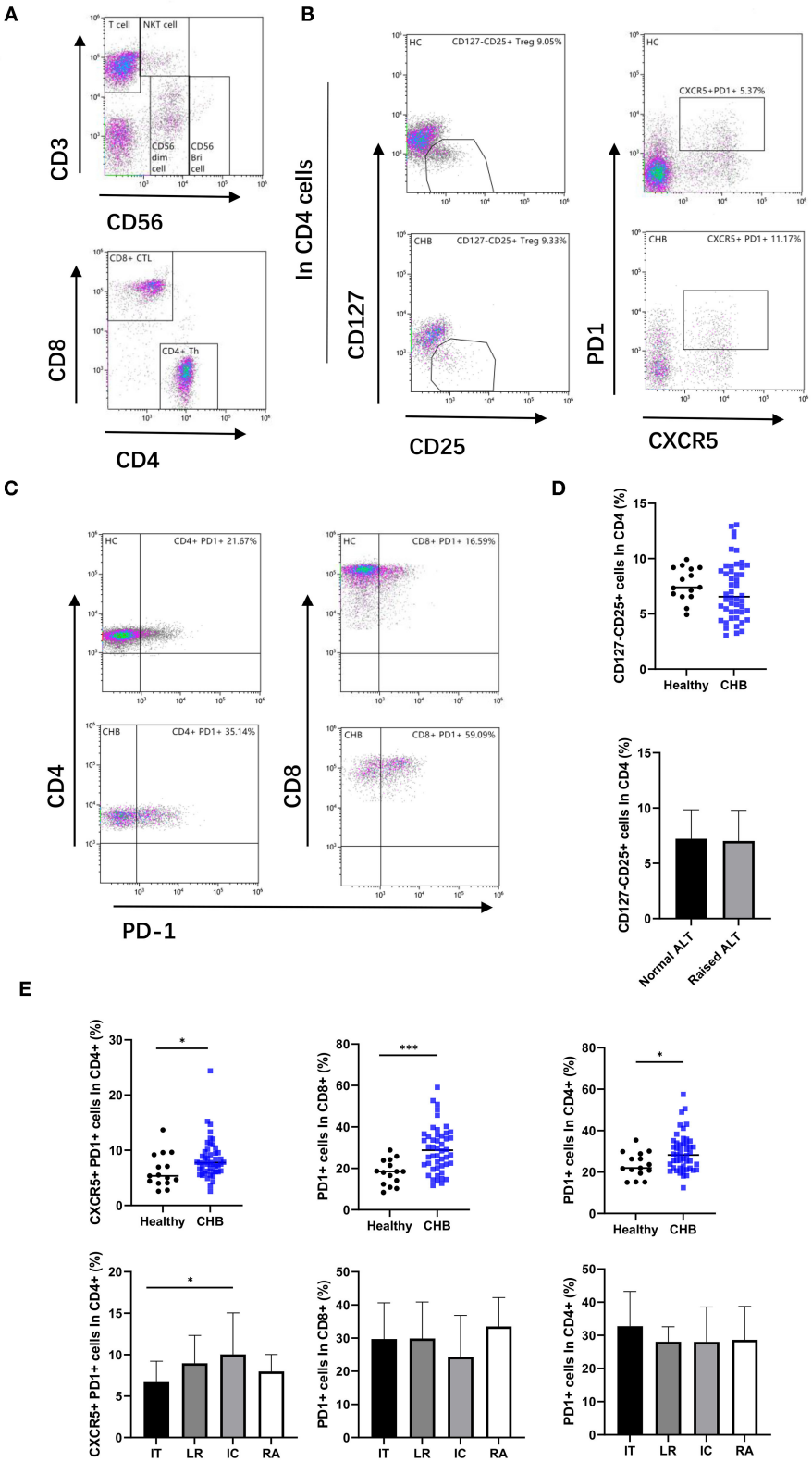

FIGURE 3 | Alter T cell subsets in CHB patient. **(A)** According to the expression of CD3 and CD56, lymphocytes were divided into T cell, CD56 bright NK cell, CD56 dim NK cell, and NKT cell, and then, the T cells were divided into CD4+ Th cell and CD8+ CTL cell. **(B)** The representative flowcytometry plot indicates the percent frequencies of Treg and cTFH cells in healthy controls and CHB patients, respectively. The gating strategy for the analysis of Treg is CD4+CD127–CD25+, and CD4+PD1+CXCR5+ plots defined as the cTFH. **(C)** Representative plot of PD-1 expression on CD4 and CD8 T cells in different groups. **(D)** Comparison frequencies

(Continued)

FIGURE 3 | of Treg between healthy controls ($n = 15$) and CHB patients ($n = 48$), and that in ALT normal ($n = 26$) and abnormal patients ($n = 22$). **(E)** The percentage of cTFH and PD-1 positive cells in different groups were shown. Immune tolerant (IT; $n = 13$), lower replicative (LR; $n = 13$), immune clearance (IC; $n = 12$) and reactivation (RA; $n = 10$). Statistically significant differences are indicated by $^{*}p < 0.05$; $^{***}p < 0.001$.

that in the study of the cytokine secretion function, we only performed experiments on 31 specimens and did not discuss more detail in different immune phases of CHB, which is a limitation of this study, and we will do further analysis in subsequent research.

The function of NK cells is closely regulated by activating and inhibiting receptors. The interactions between NK cell receptors and corresponding ligands determine the state of NK cells (20). In a chronic viral infection, the function of NK cells may be impaired by changes in their receptors (21). To determine whether poor NK cell function in CHB patients can be explained by altered expression of activated or inhibitory receptors, we examined NK cell membrane surface receptors. We found that the expression of the NK cell-activating receptor NKp30 was significantly decreased compared with normal controls, while the expression of the inhibitory receptor NKG2A was increased in CHB patients.

Interestingly, it was found that T cells also had "exhaustion" in CHB patients. On one hand, the inhibitory receptor PD-1 of both CD4+ and CD8+ T cells was significantly increased, and on the other hand, the ability of each T cell subpopulation to secrete cytokines was decreased to varying degrees. More importantly, we also found a phenomenon similar to NK cells "functional dichotomy" in T cells, and our results showed the higher cytotoxic function of CTL than healthy controls. Studies have shown that dysfunctional NK CHB cells may exhibit the same molecular characteristics at both transcriptional and protein levels, and it is a kind of signal involved in calcium balance characteristics (22), which may also explain the consistency of "exhaustion" of NK cells and T cells in our results.

Immunoinhibitory cytokines and regulatory cells are also involved in the lymphocyte "exhaustion" process of CHB, and negative regulation is an important factor in the induction of CD8+ T and NK cell exhaustion (23–25). For example, it has been found that NKG2A + NK cell dysfunction comes from Tregs derived IL-10. Blockade of IL-10 leads to the reduction of NKG2A + NK cells and increases IFN-γ + NK cells (26). Our study defines the CD4 + CD25 + CD127-phenotype as Treg cells while CD19 + CD24+ + CD38+ + is the Breg cells. After comparing with the two major regulatory cells of peripheral blood, we found no significant changes in Treg in patients with CHB. There is a consistent view in these articles that Treg cells in the liver of CHB patients are significantly increased and are positively correlated with the poor prognosis of the disease, while there are different reports on the change of the number of Treg in the peripheral blood (27–29). At the same time, Treg cells belong to a category with high heterogeneity. Current studies suggest that there are also subsets of immune cells that promote inflammation in Treg cells. Hence, simple CD4+CD25+CD127– cannot well identify the truly negatively regulated cell populations in Treg. Therefore, the role of Treg in CHB still needs further exploration (30).

On the other hand, in Breg analysis, we found that Breg was significantly increased in patients with CHB, which was positively correlated with ALT and DNA levels, and negatively correlated with cytokine secretion of T cells. In previous studies on CHB, studies on B cells mainly focused on antibody production, while other functions such as antigen presentation and immune regulation, which are closely related to immune tolerance and liver injury, were ignored (31). We speculated that long-term high viral load leads to an inflammatory response in the body, and in order to avoid the damage of inflammatory response to organs, the production of Breg cells is increased. The Breg can inhibit the inflammatory response and induce immune cell exhaustion, resulting in immune escape of the virus. It has been reported that Breg can inhibit IFN-γ production through IL-35 secretion (32), which is consistent with the view of this study.

We also compared the concentrations of plasma cytokines in CHB patients. Among the detected cytokines, the levels of IL-2 and IL-6 in CHB patients were increased, while the widely reported inhibitory factor, IL-10, was not significantly changed (19, 33), which may be limited by methodology. Different from ELISA used in some previous studies, this experiment adopted the liquid flow CBA method, which showed good linearity in the detection of high values of cytokines, while for low values, the fitting curve may not well reflect the true results. At the same time, there were also several cases of significantly increased IL-10 in the disease group, but the data distribution was skewed, and no statistically significant difference was found. As a common pro-inflammatory factor, the increased level of IL-6 has been reported in various infections and tumors, and there have also been studies on the antiviral mechanism of IL-6 in hepatitis B (34, 35). In general, IL-6 has high sensitivity and poor specificity. IL-2 plays an important role in the cytotoxic role of CD8+ T cells, which can bind to IL-2R to directly kill the infected liver cells (36). Our results showed an increase in IL-2 in CHB patients, particularly in the IC phase, suggesting that the pathway of T-cells killing viruses through IL-2 may exist in patients with chronic infection.

We found no difference in levels of IFN-γ in serum between healthy and CHB, which does not contradict previous studies showing a decrease in the proportion of IFN-γ positive T cells in CHB patients. IFN-γ is low in healthy serum and needs to be stimulated for the cells to secrete it in large quantities, and the results of previous experiments can only suggest an impaired function of cytokine secretion by T cells in CHB patients. Perhaps there is a decrease in the concentration of IFN-γ in inflammatory local tissue when there is a CHB infection compared to the acute infection period, but this needs further experimental.

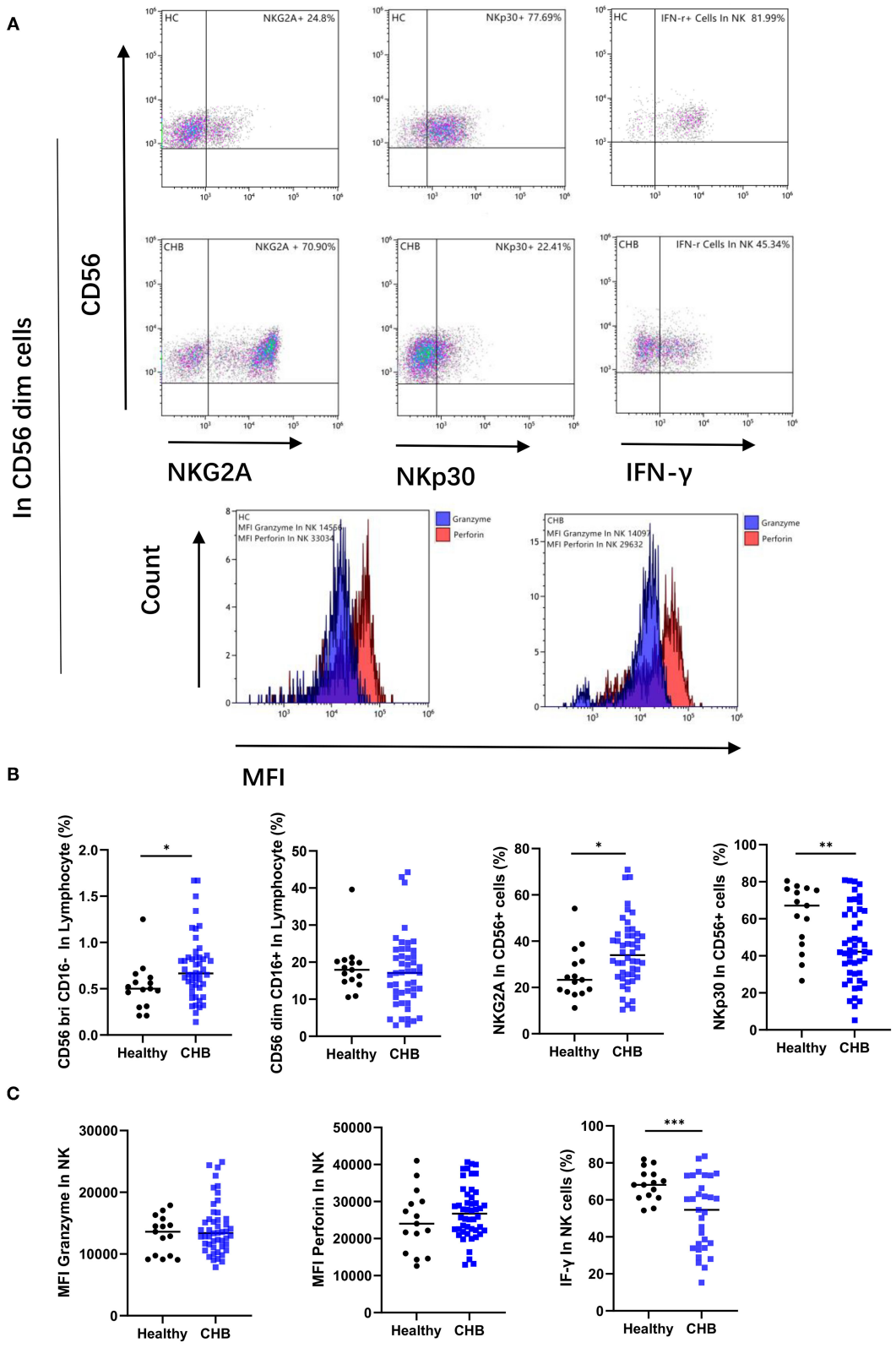

FIGURE 4 | Study of the phenotype and function of natural killer (NK) cells in chronic hepatitis B (CHB) patients. **(A)** Typical results showing, the expression of the NK receptor, and the secretion of cytokine by NK cells, as well as the Mean fluorescence intensity (MFI) of the granzyme (blue) and perforin (red) in NK cells. **(B)** Comparison of NK cell numbers and phenotypes in CHB patients ($n = 48$) and healthy controls ($n = 15$), including the numbers of two subpopulations with high and low CD56 expression, and the expression of NK inhibitory receptor NKG2A and activating receptor NKp30. **(C)** Comparison of NK cell granzyme, perforin ($n = 48$), and IFN-γ secretion functions in CHB ($n = 31$) and healthy control groups ($n = 15$).

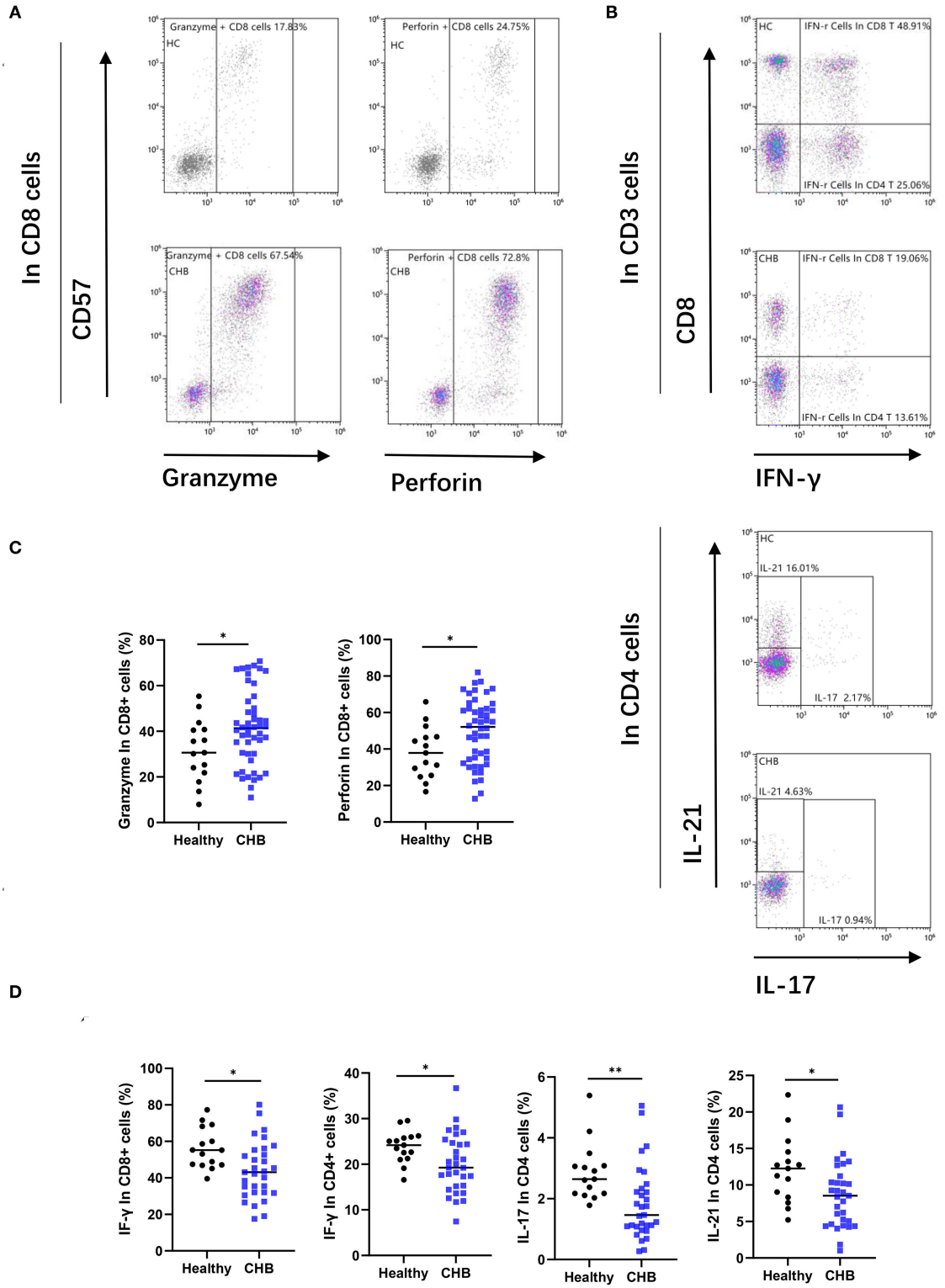

FIGURE 5 | Chronic hepatitis B (CHB) patients show functional alteration in T cells. **(A)** Representative flow-cytometry plots, as well as the proportion of granzyme and perforin in CD8+T cells, are shown. **(B)** Peripheral blood mononuclear cells (PBMCs) from healthy controls and CHB patients were incubated for 3 h after stimulating by specialized tubes containing stimulating agents (PMA) and ionomycin. The proportion of CD8+ CTL cells positive for IFN-r and the gating strategy for analyzing the cytokine secretion capacity of CD4+ TH cells are shown in representative flow-cytometry plots. **(C)** Comparison of granzyme and perforin expression in CD8+ cells in healthy controls ($n = 15$) and CHB patients ($n = 48$). **(D)** Comparison of the functions of various types of cytokine secretion by T cells subset between healthy controls ($n = 15$) and CHB patients ($n = 31$).

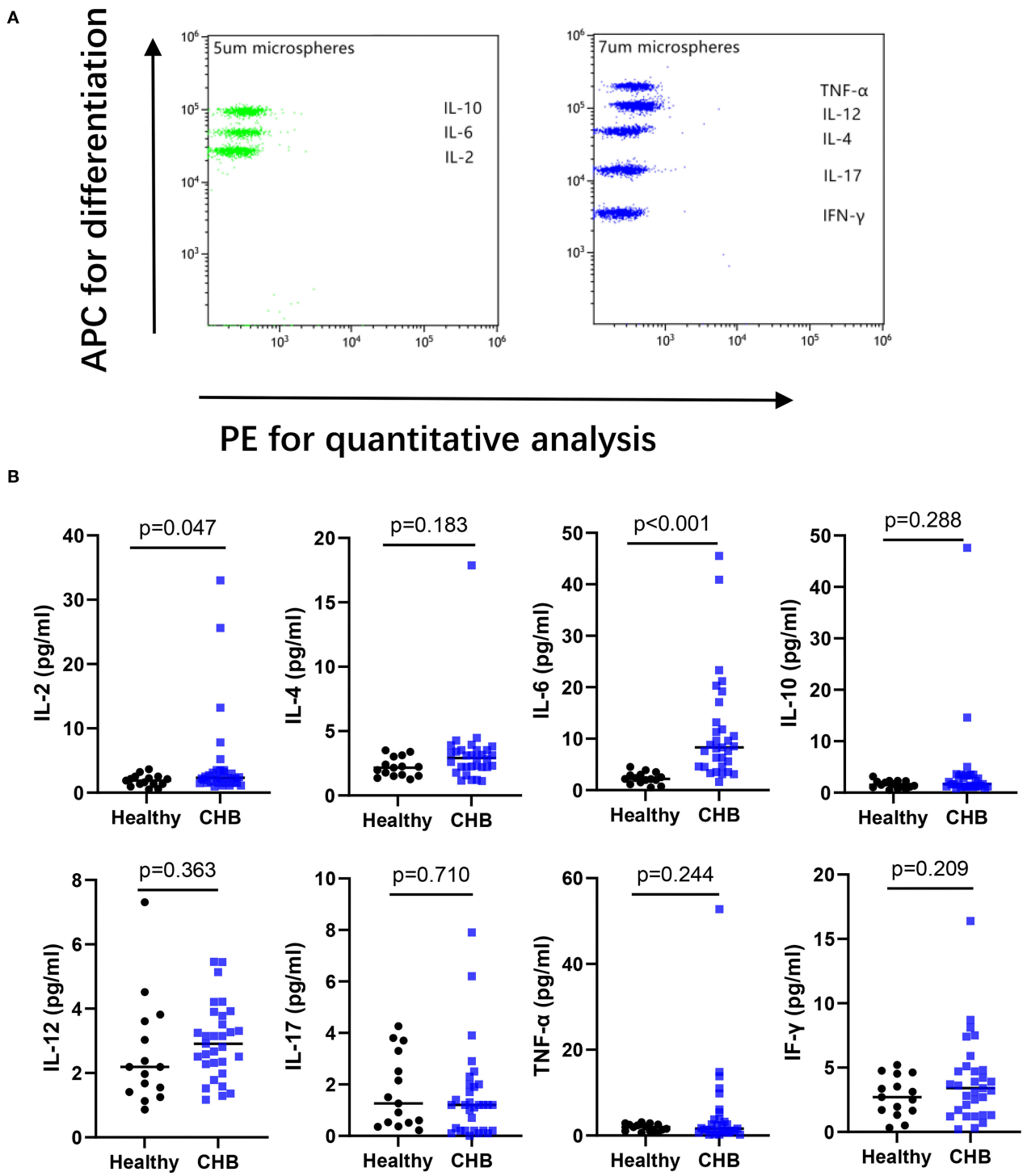

FIGURE 6 | Analyzing the cytokines in plasma by flow cytometry. **(A)** An example of cytokine test by Cytometric Bead Array from healthy controls. **(B)** The concentration of IL-17, IFN-γ, IL-2, IL-6, TNF-α, IL-12, IL-10, IL-4 between healthy ($n = 15$), and CHB patients ($n = 31$) were compared.

A dendritic cell is the most common antigen presenting cell (APC). Dendritic cells (DCs) are divided into myeloid dendritic cells (myeloid dendritic cell, MDC) and serplike dendritic cells (plasmacytoid dendritic cell, PDC). DCs play important roles in the initiation and maintenance of immune cell function (37). However, the number and function of

DCs change significantly in chronic hepatitis B infection (38). Our results showed that the proportion of PDC in peripheral blood of CHB patients was significantly reduced. The MDC of CD16– was not significantly different between CHB and healthy subjects, but it was different at different stages of chronic infection, suggesting that MDC showed more obvious immune deficiency during the immune tolerance period of CHB. Evidence has shown that the function of NK cells depends on their interaction with DC. On one hand, DC cells inhibit their ligand NKG2A by expressing gradually weakened HLA-E, thus activating NK cells (39). On the other hand, activated NK cells promote the development and maturation of MDC cells by secreting cytokines such as IFN-γ. Our results show that CHB patients have high expression of NKG2A and impaired NK cell IFN-γ secretion, which also supports the above argument.

Interestingly, IL-21 is the main effector factor of cTfh cells, which has a good positive correlation with the number of cTfh, and cTfh cells can achieve the regulation of B cell development through IL-21 (40). However, our study showed increased cTfh cells in CHB patients, while IL-21 secretion is decreased, suggesting that cTfh cells in CHB patients may achieve support for B cell function through other mechanisms. Studies have found that the ability of cTfh cells to produce IL-21 in response to hepatitis B surface antigen (HBsAg) during chronic hepatitis B virus infection is defective. However, cTfh cells can fully support B cells' response by producing interleukin-27 (IL-27), no matter how low IL-21 is (41). This result may provide new ideas for hepatitis B immunotherapy, and the mechanism is worth further exploration.

In summary, we found disorders in the immune cells of peripheral blood in CHB patients, especially NK cells and T cells. This phenomenon may be related to the increase of regulatory B cells and the decrease of DC cells in peripheral blood.

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 studies involving human participants were reviewed and approved by the Ethics Committees of the Tongde Hospital of Zhejiang Province (Identification No. 2019KY048). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

XJ and Z-hY contributed to study concept and design, acquisition of data, analysis and interpretation of data, and drafting of the manuscript. LL contributed to statistical analysis. SL contributed to samples collections. GZ and WL contributed to study concept and design, study supervision, and critical revision of the manuscript. All authors have read and approved 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/fmed.2021.759292/full#supplementary-material

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ORIGINAL RESEARCH
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Prognostic Value of Serum Exosomal AHCY Expression in Hepatitis B-Induced Liver Cirrhosis

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Objective: We aimed to investigate serum exosomal adenosylhomocysteinase (AHCY) expression in hepatitis B-induced liver cirrhosis (HBV-LC) patients and to determine the prognostic value of serum exosomal AHCY.

Methods: We collected serum samples from 100 patients with chronic hepatitis B (CHB) and from 114 HBV-LC patients to test serum exosomal AHCY expression using ELISA.

Results: Compared with the CHB and Grade A and B HBV-LC groups, the level of exosomal AHCY expression was significantly higher in the HBV-LC group [376.62 (291.50–448.02) vs. 248.12 (189.28–324.63), $P > 0.001$] and the Grade C HBV-LC group [408.70 (365.63–465.76) vs. 279.76 (215.16–336.07), $P > 0.001$], respectively. Serum exosomal AHCY expression and MELD score had a significant positive correlation ($r = 0.844$, $P < 0.001$). Survival curve analysis showed that patients with low exosomal AHCY expression had significantly longer survival than patients with high exosomal AHCY expression ($P = 0.0038$). The receiver operating characteristics (ROC) curve showed that the area under the curve (AUC) value for the mortality prediction ability of serum exosomal AHCY in HBV-LC patients was 0.921, which was higher than the values for the MELD score (AUC 0.815) and Child-Pugh classification (AUC 0.832), with a sensitivity and specificity of 93.41 and 76.00%, respectively.

Conclusions: The serum exosomal AHCY level is a novel potential prognostic biomarker in HBV-LC patients, which may be great significance for the prognosis of HBV-LC patients.

Keywords: adenosylhomocysteinase (AHCY), exosomes, hepatitis B-related cirrhosis (HBV-LC), prognosis predictor, chronic hepatitis B (CHB)

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INTRODUCTION

Hepatitis B virus (HBV) is a pathogen that causes a generalized epidemic that constitutes a global problem (1). Worldwide, approximately 2 billion people have been infected by HBV, nearly 400 million people carry the HBV, and almost 20 million individuals have chronic hepatitis B (CHB). In hepatitis B-induced liver cirrhosis (HBV-LC) following chronic HBV infection, hepatocytes gradually become necrotic; moreover, this necrosis leads to the fibronodular proliferation of

hepatocytes and hepatic tissues, and the normal liver lobules are replaced by pseudobullets (2, 3). In cirrhosis, the recurrent and continuous progression of hepatic fibrosis and inflammation can lead to hepatic dysfunction, ascites, esophagogastric varices and variceal bleeding, portal hypertension, acute kidney injury, and hepatic encephalopathy; with disease progression, cirrhotic patients can develop life-threatening hepatocellular carcinoma (HCC) (4, 5). In 2015 in China, there were 460,000 and 420,000 primary HCC cases and HCC-related deaths, respectively, which constituted more than 50% of the total global incidence and mortality of HCC; thus, HCC has become a public health problem that endangers human health (6, 7). The early diagnosis and treatment of cirrhosis are of great importance for the prognosis of patients with HCC. At present, imaging, histopathological examination, and serum index assessment are routinely used as the main diagnostic modalities to assess the stage of cirrhosis (8).

Originally identified from the supernatant of cultured sheep reticulocytes, exosomes are vesicles (diameter 30–150 nm; density 1.10–1.18 g/ml) (9) that contain various proteins, mRNA, and miRNA lipids; thus, exosomes can be used as carriers for information transfer and modulate various *in vivo* biological activities and thereby provide a novel route for cell communication (10, 11). Exosomes facilitate the study of the pathophysiology, diagnosis, treatment, and prognosis of many diseases because of their unique lipid bilayer membrane structure that protects their biological properties and helps to maintain their stability at extremely low temperatures and for long durations (12–14). The liver is one of the most important organs in the human body, and many liver cells can either secrete exosomes or are exosome-target cells, such as hepatocytes, bile duct epithelial cells, hepatic stellate cells (HSC), mononuclear macrophages, natural killer T lymphocytes, lymphocytes, etc. (15, 16). Furthermore, exosomes secreted by different cells have different functions. Exosomes are involved in the pathogenesis of HCC, viral hepatitis, liver fibrosis, and alcoholic and non-alcoholic fatty liver disease, and, increasingly, exosomal proteins and miRNAs have been identified as potential biomarkers of various diseases (17, 18).

S-adenosyl-L-homocysteine hydrolase (SAHase), a highly conserved enzyme, catalyzes the reversible hydrolysis of S-adenosyl-L-homocysteine (SAH) to homocysteine (Hcy) and adenosine (Ado) (19) and thereby regulates the intracellular adenosylhomocysteinase (AHCY) concentration, which is considered important for transmethylation reactions. In a mouse model of liver injury for screening a novel serum marker, Vazquez et al. found that the AHCY concentration significantly increased with the increasing severity of liver injury (20).

This study was performed to determine the expression of the prognostic assessment value of serum exosomal AHCY in HBV-LC patients.

Abbreviations: HBV, hepatitis B virus; CHB, chronic hepatitis B; HBV-LC, hepatitis B-induced liver cirrhosis; HSC, hepatic stellate cells; AHCY, adenosylhomocysteinase; NTA, nanoparticle tracking analysis; TEM, transmission electron microscopy.

MATERIALS AND METHODS

Patients

We retrospectively collected serum samples from 100 CHB and 114 HBV-LC patients who were admitted to the First Hospital of Zhejiang University School of Medicine, the Second People's Hospital of Yancheng City, and the Fifth People's Hospital of Wuxi from August 2019 to August 2020, with a follow-up duration of 3 months.

The CHB case was defined as: there is a history of hepatitis B or HBsAg positive for more than 6 months, and HBsAg and/or HBV DNA are still positive. The diagnostic criteria for cirrhosis was as follows:

Diagnostic Basis of Compensated Cirrhosis (One Out of Four Required)

(1) Histologically consistent with the diagnosis of cirrhosis. (2) Endoscopy demonstrates esophagogastric varices or ectopic varices of digestive tract, except for non-cirrhotic portal hypertension. (3) Imaging examinations such as B-super, LSM, or CT indicate the characteristics of cirrhosis or portal hypertension. For example, splenomegas and portal veins $\geq$1.3 cm, LSM assays meet the diagnostic boundaries of cirrhosis for different etiology. (4) For those without histology, endoscopy, or imaging examination, the following inspection indicators indicate the presence of cirrhosis (two out of four required).

(1) PLT $< 100\times109$/L, and there is no other reason to explain; (2) Serum albumin $<$ 35g/L, excluding other causes such as malnutrition or kidney disease; (3) INR $>$ 1.3 or PT extension (deactivation of thrombosis or anticoagulants for over 7 days); (4) AST/PLT Ratio Index (APRI): adult APRI score $>$2. Attention should be paid to the effects of factors such as antisense drugs on APRI.

Diagnostic Basis of Decompensated Cirrhosis

On the basis of cirrhosis, complications of portal hypertension and/or impaired liver function occur. (1) Have the diagnostic basis of cirrhosis; (2) Portal hypertension related complications occur, such as ascites, esophageal varicose vein rupture bleeding, sepsis, hepatoencephalopathy, liver and kidney syndrome, and so on.

Re-compensated Cirrhosis and Reversal of Cirrhosis

Clinical studies have shown that patients with decompensated HBV and HCV-related cirrhosis can significantly improve liver function through effective antiviral treatment, including improving liver compensatory function, reducing portal hypertension related complications, and ultimately avoiding liver transplantation, similar to compensated cirrhosis. Liver function re-compensation during antiviral therapy is more common in patients with HBV-associated cirrhosis than in patients with HCV-associated cirrhosis. At present, the definition of re-compensation for decompensated cirrhosis is still unclear and controversial. In short, patients with decompensated cirrhosis, due to effective control of etiology, effective treatment of complications or prevention, will no longer appear decompensated cirrhosis events (ascites, gastrointestinal bleeding, hepatic encephalopathy) in a longer period of

time (at least 1 year), but still exist compensated cirrhosis clinical and laboratory characteristics, which is considered "re-compensated cirrhosis."

We excluded patients with: (1) non-HBV-related cirrhosis; (2) a history of relevant drug treatment within the last month; and (3) severe heart, brain, kidney disease, thrombocytopenia, and so on.

The Child-Pugh grading system comprises five items (21)—ascites, albumin, total bilirubin, hepatic encephalopathy, and prothrombin time—that constitute a total score in the range of 5–15 points, based on which it is categorized as grades A, B, and C (total score: 5–8, 9–11, and 12–15 points, respectively).

The MELD score includes three objective indices (22): serum bilirubin concentration, serum creatinine concentration, and INR, as well as the etiology of liver cirrhosis. The MELD score is calculated as follows:

MELD score = 9.57 × In (serum creatinine) + 3.78 × In (serum bilirubin) + l1.2 × In (INR) + 6.43 × (The etiology of liver cirrhosis, wherein alcoholic and cholestatic etiologies were assigned 0 points, and the rest were scored 1 point).

The study protocol was approved by the Ethics Committee of the First Hospital of Zhejiang University School of Medicine (No. 2017003), and informed consent from each patient was obtained.

Serum Exosome Separation

Before the serum exosome separation, we washed the qEV separation column (qEV original 35 and 70 nm, Izon Christchurch, New Zealand) with at least 10 ml PBS (1×), which we then extracted from the top of the sieve plate with a pipette before removing the bottom sliding cap from the column. Then, we added 500 μl sample to the top of the sieve plate and immediately replaced the bottom sliding cap, and collecting 0.5 ml of the fraction. When the final sample is placed inside the top sieve plate of the column (at the same level), add 2.5 ml PBS (1×) to obtain a final void volume of 3 ml. The 1.5 ml liquid, which is the higher purity exosome solution, is retained after the void volume is collected in a 1.5-ml EP tube.

Transmission Electron Microscopy

For transmission electron microscopy (TEM), we diluted the exosome and filtered the 5 μl-sample by adding it drop by drop onto the copper net and then incubated it for 5 min at room temperature. Thereafter, we used blotting paper on one side to absorb the excess liquid, added a drop of 2% uranium peroxide acetate to the copper net, and re-incubated the net for 1 min at room temperature. Following this step, we used blotting paper on one side of the net to absorb the excess liquid, allowed the net to dry for approximately 20 min at room temperature, and then observed and photographed the shape of the exosome under the electron microscope.

Nanoparticle Tracking Analysis

For the nanoparticle tracking analysis (NTA), the frozen samples were thawed in a water bath at 25°C and placed on ice. The exosomes were diluted with PBS (1×) and used directly for the NTA (ZetaVIEW S/N 17-310) assay. The NTA software (ZetaView 8.04.02) was used to analyze the movement of particles and to calculate the number of exosomes.

Western Blotting

For the gel preparation, we used a 1.5-mm glass plate with a 15-well sample comb to prepare a 12% isolate gel and a 5% concentrate gel according to the molecular weight of the target protein. Electrophoresis was carried out at a steady pressure difference of 80 V until the loading buffer (indicator) entered the separation gel and then changed to a steady pressure of 120 V, which was maintained until the loading buffer reached the bottom of the gel to terminate electrophoresis. We selected a PVDF membrane that had a pore size of 0.22 μm and maintained a constant current of 200 mA for a transfer time of 90 min with 5% skimmed milk powder diluted in PBST, closed for 1 h. The PVDF membrane was washed three times, for 10 min each, using PBST and then placed in the hybridization cassette. The corresponding antibody was added, and the membrane was placed on a decolorization shaker overnight at 4°C. Then, the mix was shaken slowly to bring it to room temperature. The primary antibody [Annexin V (sc-393669, 1:1,000), CD9 (sc-13118, 1:2,000), Tsg101 (sc-7964, 1:2,000), and CD63 (sc-5275, 1:1,000); Santa Cruz Biotechnology, Inc., Texas, USA] was removed and the membrane was washed three times using PBST for 10 min each time; the secondary antibody was added into the hybridization cassette along with the membrane, which was then placed on a shaker and slowly shaken and incubated at room temperature for 1 h. Then, the secondary antibody was removed and the membrane was washed three times using PBST, for 10 min in each wash cycle. We added the appropriate amount of ECL luminescent solution and used program one in the digital imaging system to continuously photograph the membrane for a maximum duration of 1 min.

ELISA

For the ELISA < the residual cells were removed from the plasma samples and the cell fragments were diluted with 1× PBS (1:500 diluted) and the exosomes were precipitated with 100 ml RIPA lysis buffer on ice for 30 min. The samples were diluted with PBS (1:3 diluted) after shaking and mixing. The ELISA plate that was previously coated with the AHCY antibody was taken out, and we added blank control solution to one well and solutions of gradient concentration to seven wells. The diluted exosome samples constituted a 100-μl solution. After incubation at 37°C for 60 min, the liquid in the well is discarded, and the plate is rotated and dried. Then, we added 100 μl Solution A to the plate, covered it with the film, incubated the plate in an oven at 37°C for 1 h, and then washed the plate three times. We added 100 μl Solution B to the plate, covered the plate with film, baked the plate in the oven at 37°C for half an hour, and then washed the plate five times. Next, we added 100 μl TMB substrate solution and colored it in the dark at 37°C for 20 min, and 50 μl of the termination reaction solution was added to the plate. The absorbance value was detected using the microplate reader at 450 nm wavelength.

Statistical Analysis

Data were statistically analyzed using SPSS 22.0, and the measurement data are expressed as ($x \pm s$). One-way ANOVA and the SNK-q test for two-way comparison were used to

determine the intergroup differences. Pearson linear correlation analysis and logistic one-way risk factor analysis were used to evaluate the predictive ability of plasma exosome-derived AHCY. We calculated the survival rate with the Kaplan Meier method. ROC curve analysis was used to assess the prognostic value of exosomal AHCY levels in HBV-LC patients. Differences were considered statistically significant at $P < 0.05$.

RESULTS

Characteristics of Exosomes

The results of the TEM observation are shown in **Figure 1A**. Against a clear background, aggregates of exosomes were distributed while connected. The exosomes had a diameter of 100–200 nm and were shaped like a double disk-like vesicular structure that was completely covered with a lipid envelope. The results of exosome particle size detected by NTA are shown in **Figure 1B**. The median value of the overall particle size was approximately 100 nm, and particle size was mainly distributed between 50 and 200 nm. Western blotting showed the positive expression of the exosome marker proteins Annexin V, CD9, Tsg101, and CD63 in the exosome group (**Figure 1C**).

Level of Exosomal AHCY Expression in HBV-LC Patients

We evaluated the level of exosomal AHCY expression in HBV-LC patients ($n = 141$) and compared the expression levels with those in the CHB ($n = 100$) group. **Table 1** shows the baseline characteristics of the CHB and HBV-LC groups. In the CHB group (age, mean ± SD: 53.43 ± 10.88 years), there were 61 male and 39 female participants; in the HBV-LC group (age, mean ± SD: 54.06 ± 11.99 years), there were 79 male and 62 female participants. The intergroup differences in age and sex were not statistically significant ($P > 0.05$). Compared with the CHB group, the level of exosomal AHCY expression was significantly higher in the HBV-LC group [376.62 (291.50–448.02) vs. 248.12 (189.28–324.63), $P < 0.001$; **Figure 2**].

TABLE 1 | Baseline characteristics of CHB and HBV-LC groups.

Variables	CHB ($n = 100$)	HBV-LC ($n = 141$)	*P*
Age (years)	53.43 ± 10.88	54.06 ± 11.99	0.678
Gender (M/F)	61/39	79/62	0.441
WBC (10^9/L)	4.2 (3.0–5.5)	7.0 (4.9–11.1)	<0.001
HB (g/L)	127 (112–141)	110 (89–126)	<0.001
PLT ($\times 10^9$/L)	84 (52–132)	70 (42–114)	0.014
ALT (U/L)	31 (18–68)	79 (40–164)	<0.001
AST (U/L)	38 (26–87)	103 (56–177)	<0.001
TBIL (μmol/L)	20.6 (14.7–40.9)	178.0 (50.5–388.0)	<0.001
DBIL (μmol/L)	9.7 (6.3–19.3)	190.9 (53.8–299.5)	<0.001
ALB (g/L)	35.12 ± 7.09	30.00 ± 5.44	<0.001
BUN (mmol/L)	5.20 (4.28–7.66)	10.40 (5.56–18.16)	<0.001

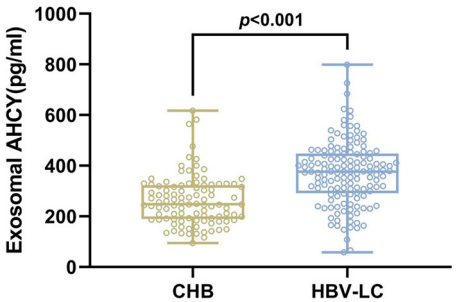

FIGURE 2 | Expression level of exosomal AHCY in HBV-LC patients.

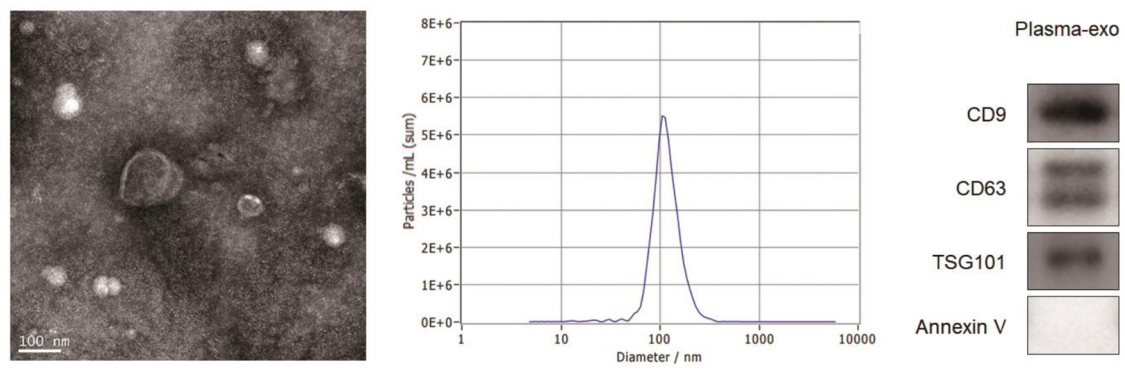

FIGURE 1 | Exosome characterization. **(A)** TEM images showed that aggregates of exosomes were distributed while connected. The exosomes had a diameter of 100–200 nm and were shaped like a double disk-like vesicular structure that was completely covered with a lipid envelope. **(B)** The results of exosome particle size detected by NTA showed that the median value of the overall particle size was approximately 100 nm, and particle size was mainly distributed between 50 and 200 nm. **(C)** Western blotting showed the positive expression of the exosome marker proteins Annexin V, CD9, Tsg101, and CD63 in the exosome group.

Correlation Between the Serum Exosomal AHCY Expression and the Child-Pugh Class and MELD Score in the HBV-LC Group

Following the Child-Pugh classification, the HBV-LC patients were assigned to the Grade A, B, and C groups. Compared with the Grade A and B HBV-LC groups, the level of exosomal AHCY expression was significantly higher in the Grade C HBV-LC group [408.70 (365.63–465.76) vs. 279.76 (215.16–336.07), $P < 0.001$; **Figure 3A**]. In addition, we found a significant positive correlation between the serum exosomal AHCY level and the MELD score ($r = 0.844$, $P < 0.001$; **Figure 3B**).

Correlation Between the Serum Exosomal AHCY Level and Clinical Parameters in the HBV-LC Group

Using the mean exosomal AHCY expression level (370.21) in the HBV-LC group as a cutoff point, we assigned the HBV-LC patients into the high expression and low expression groups based on exosomal AHCY expression. **Table 2** shows the baseline characteristics of the two subgroups. The levels of WBC, HB, ALT, AST, TBIL, DBIL; the INR and PT; and the MELD score and Child-Pugh grade of the patients in the high expression group were significantly higher than those in patients in the low expression group; however, the PLT count was significantly lower in patients in the high expression than in the low expression group. The survival curve analysis showed a significantly longer survival time for patients in the low expression group than for patients in the high expression group ($P = 0.0038$; **Figure 4**).

Prognosis Predictive Ability Based on the Serum Exosomal AHCY Level in HBV-LC Patients

Finally, we evaluated the prognosis prediction ability based on the serum exosomal AHCY expression in HBV-LC patients. The receiver operating characteristics (ROC) curve showed an area under the curve (AUC) value of 0.921 for serum exosomal AHCY expression in predicting the mortality risk of HBV-LC patients, which was higher than the AUC of the MELD score (0.815) and the Child-Pugh classification (0.832) (**Figure 5**). The sensitivity and specificity of serum exosomal AHCY expression in prognosis prediction were 93.41 and 76.00%, respectively.

DISCUSSION

Cirrhosis following an HBV infection is a chronic disease wherein the body, after HBV invasion, undergoes gradual liver cell necrosis that results in the fibronodular hyperplasia of liver tissue and the replacement of normal liver lobes by false lobes (23). In cirrhosis, repeated and continuously progressive liver fibrosis and inflammation induce loss of liver function, which results in decompensated liver function, ascites, esophageal gastric varices and variceal bleeding, portal hypertension, acute kidney injury, hepatic encephalopathy, and even HCC as well as several serious complications. In China, there were 460,000 primary liver cancer cases and 420,000 liver cancer-related deaths in 2015. The incidence of primary liver cancer in China in 2015 exceeded 50% of the total global incidence of primary liver cancer, which has emerged as a public health problem that endangers human health. Thus, the early diagnosis and treatment of cirrhosis are of great significance for the prognosis of patients.

In mammals, AHCY is the only enzyme that mediates the reversible catalysis of SAH to Ado and cysteine (24). The earliest manifestation of alcohol-related liver disease is steatosis, which is characterized by the accumulation of lipid droplets in liver cells. Arumugam et al. (25) demonstrated that many pathological changes, including steatosis, are associated with the alcohol-induced increase in SAH hepatocytes. A study that investigated the impaired Hcy metabolism in patients with alcohol-related liver disease from Taiwan (26) showed that impaired Hcy metabolism may disrupt antioxidant status. The role of alcohol-induced endoplasmic mesh stress in SREBP regulation and fatty

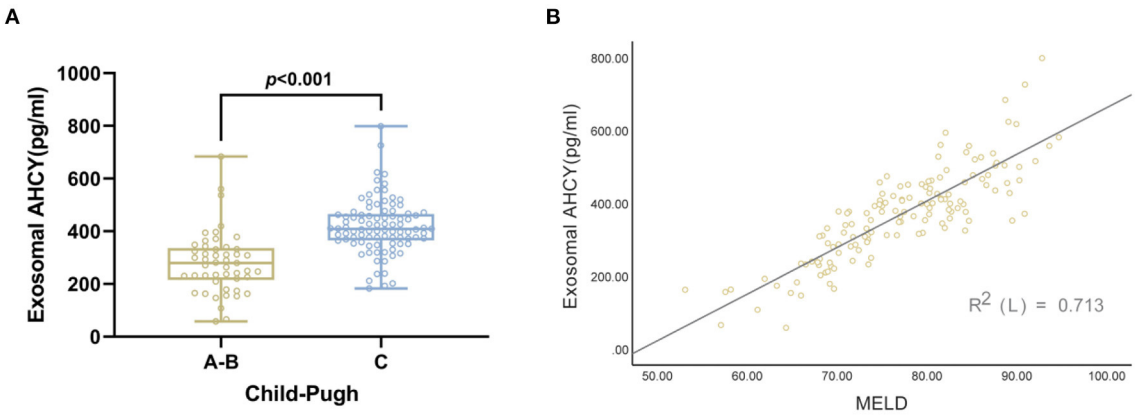

FIGURE 3 | Correlation between serum exosomal AHCY level and child Pugh and MELD score in HBV-LC patients group. **(A)** The level of serum exosomal AHCY in HBV-LC patient with grade C group was significantly higher than that in HBV-LC patient with Grade A, B group [408.70 (365.63–465.76) vs. 279.76 (215.16–336.07), $P > 0.001$]; **(B)** there was a significant positive correlation between serum exosomal AHCY levels and MELD score ($r = 0.844$, $P < 0.001$).

TABLE 2 | Baseline characteristics of exosomal AHCY high and low expression in HBV-LC group.

Variables	Total ($n = 141$)	Exosomal AHCY high expression ($n = 76$)	Exosomal AHCY low expression ($n = 65$)	*P*
Age (years)	54.06 ± 11.99	53.29 ± 11.56	54.95 ± 12.50	0.413
Gender (M/F)	79/62	40/36	39/26	0.380
WBC (10^9/L)	7.0 (4.9–11.1)	8.0 (5.7–11.4)	6.6 (3.6–10.3)	0.038
Hb (g/L)	110 (89–126)	116 (95–133)	107 (80–123)	0.044
PLT ($\times 10^9$/L)	70 (42–114)	62 (35–104)	80 (53–120)	0.044
ALT (U/L)	79 (40–164)	103 (54–178)	62 (28–127)	0.004
AST (U/L)	103 (56–177)	118 (72–231)	72 (32–152)	<0.001
TBIL (μmol/L)	178.0 (50.5–388.0)	296.0 (146.8–472.8)	52.0 (19.0–201.5)	<0.001
DBIL (μmol/L)	190.9 (53.8–299.5)	253.5 (143.4–354.6)	55.0 (17.5–216.5)	<0.001
ALB (g/L)	30.00 ± 5.44	29.31 ± 5.38	30.81 ± 5.43	0.104
BUN (mmol/L)	10.40 (5.56–18.16)	11.82 (7.29–18.75)	8.60 (5.05–15.85)	0.072
Cr (μmol/L)	103 (72–182)	119 (76–194)	94 (70–128)	0.062
UA (μmol/L)	283 (174–468)	289 (182–491)	273 (164–446)	0.604
INR	1.84 (1.41–2.43)	2.18 (1.83–2.64)	1.46 (1.22–1.84)	<0.001
PT (s)	21.6 (16.8–27.8)	25.3 (21.5–31.8)	17.1 (14.5–21.1)	<0.001
BA	50 (28–77)	51 (28–80)	46 (28–74)	0.457
AFP (ng/ml)	69.5 (12.0–161.8)	73.0 (18.2–170.4)	65.6 (5.6–153.1)	0.375
PCT	1.29 (0.58–2.44)	1.16 (0.69–2.16)	1.57 (0.42–2.63)	0.791
ESR	14 (6–29)	13 (5–29)	17 (7–29)	0.618
CRP (mg/L)	21.5 (11.2–69.2)	21.6 (13.2–64.7)	19.3 (9.0–78.8)	0.513
MELD score	77.18 ± 8.33	82.23 ± 5.77	71.28 ± 6.88	<0.001
Child-Pugh	10 (9–11)	11 (10–12)	9 (7–10)	<0.001

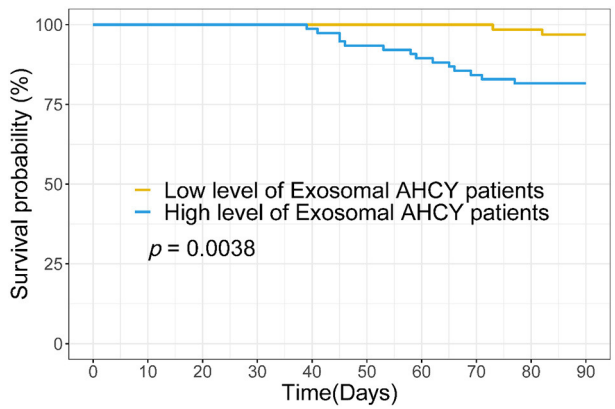

FIGURE 4 | Survival curve analysis the relationship between serum exosomal AHCY level and survival time for HBV-LC patients.

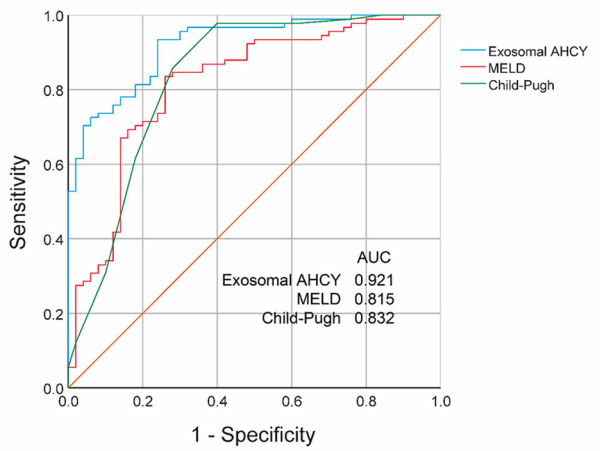

FIGURE 5 | Prognosis predictive ability based on the serum exosomal AHCY level in HBV-LC patients.

liver, as well as the exact mechanism of beetroot protection, include decreased Hcy and SAH levels or increased S-adenosine methionine concentrations. Stender et al. (27) found that AHCY deficiency is associated with early-onset HCC. Vazquez et al. (20) screened novel serum markers in a mouse model of liver injury and demonstrated that AHCY expression significantly increased with the increase in the degree of liver injury. However, there are no studies of the role of exosomal AHCY in HBV-LC.

In this study, exosomes in the serum samples from patients with CHB and HBV-LC were separated, and TEM, NTA, and Western blotting were used to identify the characteristics of the exosomes. Transmission electron microscopy showed that exosomes had an interconnected aggregated distribution in a clear background, a diameter of 100–200 nm, and a

complete lipid envelope, and were shaped as a double-disk vesicle. Nanoparticle tracking analysis showed an overall median exosome particle size of approximately 100 nm (range 50–200 nm). Western blotting revealed the positive expression of Annexin V, CD9, Tsg101, and CD63 in the exosome group. The exosomal AHCY levels of patients with HBV-LC were significantly higher than those of patients with CHB.

The Child-Pugh classification and the MELD score are important evaluation criteria in patients with severe liver disease. Therefore, we separately analyzed the correlation between the serum exosomal AHCY level and the abovementioned two assessment systems. The HBV-LC patients with Grade C cirrhosis had a significantly higher serum exosomal AHCY level than the HBV-LC patients with Grade A and B cirrhosis. In addition, we found a significant positive correlation between the serum exosomal AHCY level and the MELD score The above-described results indicated that the serum exosomal AHCY level has a good correlation with the two classic scoring models. Furthermore, we studied the correlation between the serum exosomal AHCY level and clinical parameters in the patients in the HBV-LC group and in the high expression and low expression subgroups that were based on exosomal AHCY expression. The levels of WBC, HB, ALT, AST, TBIL, and DBIL; the INR and PT; and the MELD score and Child-Pugh grade were significantly higher in patients with high expression of exosomal AHCY; however, the PLT count was significantly lower in patients with high exosomal AHCY expression than the PLT count in patients with low exosomal AHCY expression. Survival curve analysis showed that the survival time of patients with low exosomal AHCY expression was significantly longer. Finally, we evaluated the prognosis prediction ability of exosomal AHCY in HBV-LC patients. The ROC curve showed that the AUC value of serum exosomal AHCY for predicting the mortality risk of HBV-LC patients was higher than the AUC values of the MELD score and the Child-Pugh; moreover, exosomal AHCY expression showed good sensitivity and specificity in predicting the prognosis of HBV-LC patients.

This study has some limitations. First, the sample size of the study patients was not large; therefore, the predictive value of serum exosomal AHCY expression requires evaluation in multicenter, large studies. Second, this study lacked a control group for a controlled evaluation to verify whether the absence of exosomal AHCY expression is specific for the prognosis prediction in CHB or HBV-LC patients. Thirdly, our study did not involve the relevant mechanism research of AHCY involved in the development of disease.

In summary, our study showed that serum exosomal AHCY expression in HBV-LC patients is significantly upregulated and shows a good correlation with the Child-Pugh classification and the MELD score for prognosis prediction. HBV-LF patients with low exosomal AHCY expression had longer survival. The AUC value of serum exosomal AHCY in predicting the mortality risk of HBV-LC patients was higher than the AUCs of the MELD score and the Child-Pugh classification, which may facilitate the establishment of serum exosomal AHCY expression as a novel prognostic biomarker in HBV-LC.

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 studies involving human participants were reviewed and approved by the Ethics Committee of the First Hospital of Zhejiang University School of Medicine (No. 2017003). 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

LT directed and supervised the study and revised the manuscript. CY and MW designed and performed most of the experiments. JY and HW participated in some experiments, analyzed the data, and completed the figures. LT and YW wrote the manuscript. All authors have read and approved the final manuscript.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2021.777452/full#supplementary-material

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Incidence and Influencing Factors of New Hepatitis B Infections and Spontaneous Clearance: A Large-Scale, Community-Based Study in China

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Background: Hepatitis B surface antigen (HBsAg) is widely used in hepatitis B screening, and HBsAg seroclearance indicates hepatitis B eradication. Few studies have explored the incidence of and determinants for spontaneous seroclearance using a long-term follow-up cohort study. Our research aimed to examine the incidence of and influencing factors for hepatitis B virus infection and spontaneous clearance of HBsAg from a large-scale cohort in China.

Methods: A total of 151,926 resident individuals in Tongxiang underwent HBsAg screening at least thrice in a 7-year period. Serum samples collected at baseline and follow-up examinations were tested for HBsAg. Cox proportional hazard models were used to analyze determinants of HBsAg seroclearance and persistent HBsAg presence.

Results: Among the 151,926 participants, new hepatitis B infections occurred in 4,497 participants, yielding an incidence rate of 571.38 per 100,000 person-years. The incidence rate for males was higher than that for females. In the multivariate Cox regression analysis, female gender, alcohol drinking history, hepatitis family history and middle-age group were predictors for persistent positive HBsAg status.

Conclusions: The incidence rate of new hepatitis B infections was 571.38 per 100,000 person-years. Male and aged people in this community cohort have a higher infection rate. Alcohol drinking and hepatitis family history were risk factor leading to chronic infection. Female and middle-aged people were prone to persistent positive HBsAg status.

Keywords: hepatitis B surface antigen, hepatitis B virus, natural history, incidence rate, influencing factor

INTRODUCTION

Hepatitis B is an infectious disease induced by hepatitis B virus (HBV) that mainly invades the liver and can induce a variety of liver diseases, such as acute or chronic hepatitis, hepatic failure, liver cirrhosis and hepatic carcinoma (1, 2). In 2015, the WHO estimated that 257 million persons (3.5% of the global population) were living with chronic HBV infection (3). Most of them were adults who had no hepatitis B vaccine immunization in infancy. These individuals suffer from HBV

infection and are also the source of infection for other non-vaccinated people. After infection with HBV, 1–2% of cases evolve into fulminant hepatic failure, and 5–10% of adult cases evolve into chronic infection (1). In addition, others typically have no symptoms and progress to spontaneous clearance of HBV within ∼3 months (4–6). Since its discovery in the 1960s, hepatitis B surface antigen (HBsAg, also commonly known as Australia antigen) has become an important serological marker for screening for HBV infection (7). HBsAg is a distinctive surface antigen of HBV with an envelope protein and excess coat particles. HBsAg is positive in serological testing in acute and chronic hepatitis B infections (8) and indicates a current HBV infection. For most people, HBsAg spontaneously vanishes via seroconversion to anti-HBs antibodies (an antibody for the hepatitis B surface antigen) in a few months, indicating viral clearance. For cases evolving into chronic HBV infection, HBsAg remains positive with other serologic marker alterations. Individuals with chronic HBV infection may not develop clinical symptoms for as long as 30 years before apparent hepatic impairment, and a patient will not be aware of his or her disease (9). Therefore, for early diagnosis and early intervention, HBV serological marker screening remains important in regions with a high prevalence of HBV infection for early diagnosis and early intervention (1, 2, 10).

In China, the prevalence of HBV infection was estimated to be 9.75% in 1992, as determined by HBsAg testing. Due to the Expanded Program on Immunization (EPI) for infants established in 1992, the prevalence declined to 7.18% in 2006, according to a national epidemiological survey (11, 12). Previous studies have reported the incidence rate of spontaneous HBsAg seroclearance in chronic infections to be between 0.5 and 1.4% annually. Statistical indicators including but not limited to increasing age, male sex, HBV genotype B, and low initial HBV-DNA levels indicated high rate of spontaneous HBsAg seroclearance in these cohort studies (13–17). However, long-term, community-based studies of a large cohort of HBV carriers to examine the full range of factors leading to HBsAg seroclearance are still lacking. Since 2010, we have conducted a population-based infectious disease cohort study in Zhejiang Province, China. This research is based on a Demonstration Area Construction project aimed at whole-population infectious disease screening, intervention and follow-up, and it has enrolled over 200 thousand participants in the county of Tongxiang, representing approximately one-quarter of the local population (815.8 thousand), for HBsAg screening. In the following 7 years, all participants were invited to receive a free HBsAg examination every year. All the adult members in this cohort have no HBV vaccination history during the neonatal period and underwent no additional HBV vaccine administration. Therefore, this study provides an incidence rate of HBV infection and elucidates the factors influencing spontaneous HBsAg seroclearance in an adult population based on a remarkably large-scale, prospective cohort data set.

Abbreviations: HBV, hepatitis B virus; HBsAg, hepatitis B virus surface antigen; anti-HBs antibodies, hepatitis B virus surface antibody; WHO, World Health Organization; HR, hazard ratio.

METHODS

Study Cohort

This research began in October 2010 and ended in December 2020. The study cohort was enrolled before October 2010 in the county of Tongxiang, Zhejiang Province, China. All cohort participants born after 1992 received routine administration of HBV vaccination during infancy and then underwent no additional HBV vaccine administration. The baseline survey was conducted with the help of the Mega-Project for National Science and Technology Development for the "13th Five-Year Plan of China" and the Health Commission of Zhejiang Province. After receiving appropriate training by the lead researchers, the physicians of each participating hospital began to conduct medical examinations, interviews, and laboratory tests on subjects who volunteered for free medical and health examinations. Approximately 700 physicians from 50 hospitals in Tongxiang were invited by the Department of Health of Zhejiang Province to participate.

In total, 208,175 residents in Tongxiang, representing approximately one-quarter of the whole resident population (815.8 thousand), voluntarily received a free HBsAg baseline survey before October 2010. Individuals who had lived in the county for more than 24 months were invited to participate in the study cohort. We invited every participant to receive an annual health examination in the local hospitals. During each visit, medical staff working for our research team inquired about symptoms and performed a physical examination accompanied by HBsAg testing for every participant. A total of 151,926 participants were HBsAg negative at the baseline survey and received at least 3 HBsAg screenings during the research period (**Figure 1**).

All cohort participants provided informed written consent, and informed written consent was obtained from guardians on behalf of minors. The study was approved by the Ethics Committee of The First Affiliated Hospital at the School of Medicine of Zhejiang University. All data were analyzed anonymously. The information collected consisted of basal demographics (sex, age, education level, marital status, medical insurance, family history, smoking history and alcohol drinking history) and laboratory tests (HBsAg).

Serological Testing

A 5-mL venous blood sample was collected during the annual health examination and kept in a low temperature container (controlled from 4 to 10 μC) and delivered to Adicon Clinical Laboratories (Hangzhou, China) on the same day for sample processing and serological testing. Commercially available enzyme immunoassay kits (Acon Biotech Co., Hangzhou, China) were used for the HBsAg measurements. Verification of elevated test results was performed by retesting the samples using the same kits. Only samples that were positive on both tests were considered true positives. For the purpose of analysis,

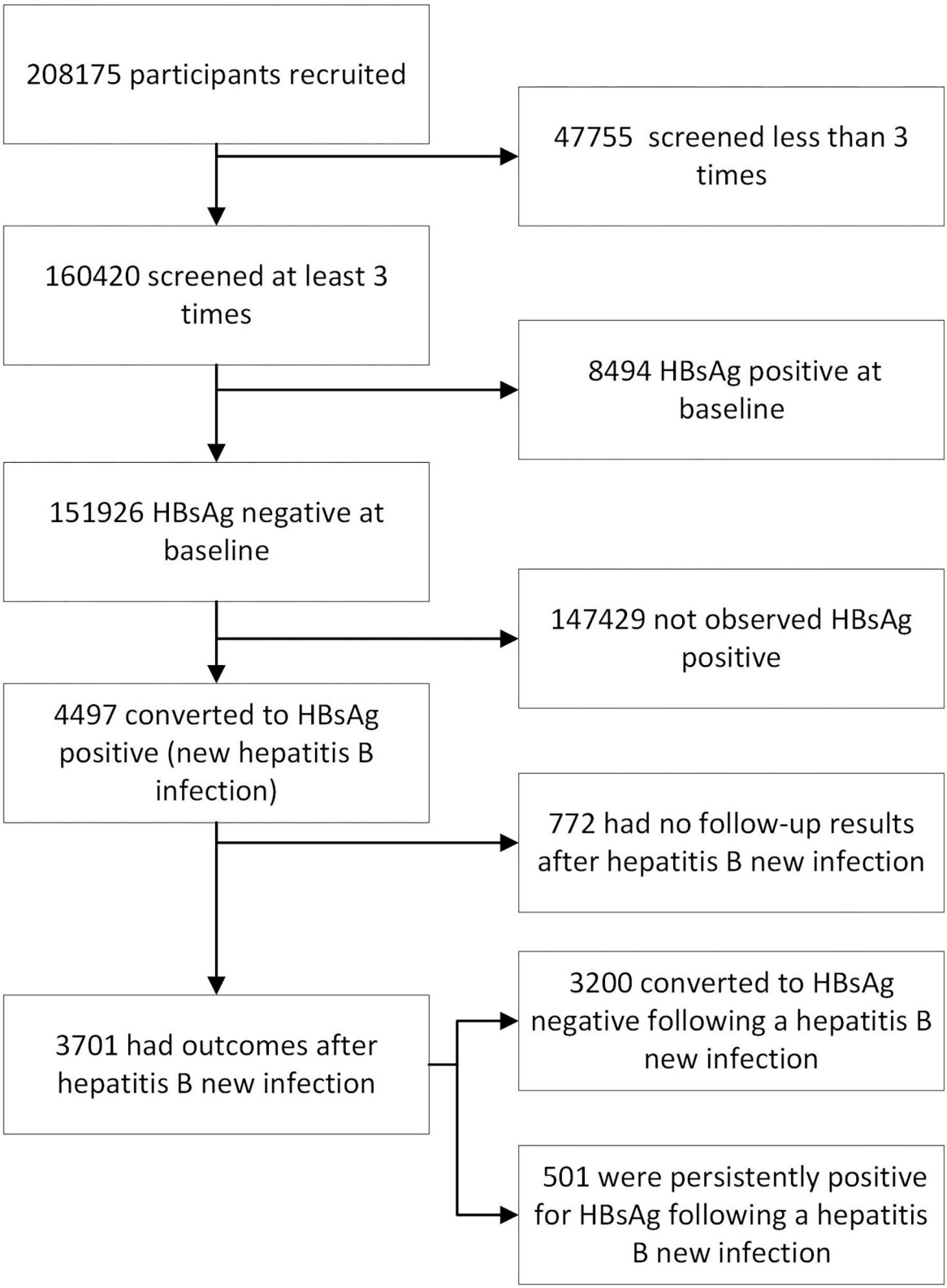

FIGURE 1 | The flowchart for this cohort study (n = 208,175) of HBsAg status from 2010 to 2016 in Zhejiang Province, China.

HBsAg positivity was considered indicative of current hepatitis B infection.

Statistical Analysis

The person-years of follow-up was calculated from the date of enrolment to the date of the last blood test and the onset of the specific outcomes of interest. The time from HBV infection to HBsAg seroclearance was artificially defined as the midpoint of two adjacently continuous tests with different results (18). Participants without HBV infection or HBsAg seroclearance were defined as censored for specific study outcomes. Data were managed and analyzed using SPSS software version 25.0 (SPSS, Inc., Chicago, IL, USA). Categorical data, such as incidence rates of different sexes or age groups, were compared using the chi-square test, and *p*-values were adopted for pairwise comparisons. The Kaplan–Meier method was used to examine the cumulative probability of HBV infection, HBsAg seroclearance, and annual incidence rate, and univariate analysis of each variable was also performed. Annual incidence rates were calculated directly from the survival table, and a chi-square trend test was then performed to evaluate the pattern of change. The results of different levels of each variable were compared with log-rank tests. Cox proportional hazards models were used to analyze both the univariate and multivariate-adjusted rate ratios (with 95% confidence intervals) of HBsAg seroclearance/persistent positive HBsAg associated with various determinants. Variables significant in the univariate analyses were included in multivariate analyses. Statistical significance was determined by 2-tailed tests ($p < 0.05$).

RESULTS

Basic Characteristics of Participants

We analyzed a total of 151,926 participants with more than 3 consecutive HBsAg tests. In total, 57,089 of participants were male, and 94,837 were female. The average age was 66.59 ± 11.68 years with a minimum age of 9 years and a maximum age of 106 years. Among all the participants, 4,497 were found to experience a HBsAg positive conversion (as shown in **Table 1**), indicating a new HBV infection. Another 147,429 participants were consistently observed to be HBsAg negative.

Incidence of New Hepatitis B Infection

After 5.18 person-years of follow-up, new hepatitis B infections occurred in 4,497 participants, yielding an incidence rate of 571.38 per 100,000 person-years in average. The incidence rate of new hepatitis B infection for males was significantly higher than that for females (684.88 vs. 536.68 per 100,000 person-years, respectively, $p < 0.001$), and different age groups also had different incidence rates ($p < 0.001$). Incidence of new hepatitis B infection in 30–60 years age groups are comparatively lower than that of 20–30 and aged (60 years and above) groups. The new infection rate increased distinctly with age in the 60 years and above groups. The 11–20 years age group have an extremely high new infection incidence. They were not took into the saliency analysis due to the insufficient sample size. The cumulative incidence of new hepatitis B infections by 3, 5, and 10 years was 0.018, 0.028 and 0.047, respectively (**Figure 2A**). The annual new infection incidence rate exhibited a decreasing tendency (**Supplementary Table 1**). Overall infection rate of this cohort tend to a platform of 5 percent.

TABLE 1 | Demographic characteristics of the 151,926 participants who were HBsAg negative at baseline and who experienced HBsAg positive conversion.

Variables	HBsAg positive conversion	Total	Person-years of follow-up	Annual incidence rate (per 100,000)	*p*
Gender					<0.001
Female	2,647	92,190	5.35	536.68	
Male	1,850	55,239	4.89	684.88	
Age group					<0.001
0–10	0	1	1.25	0.00	NA
11–20	6	60	3.92	2550.70	NA
21–30	13	561	3.57	648.38	a,b,c
31–40	52	2,722	4.45	428.90	a,b
41–50	199	10,878	4.78	382.51	a
51–60	527	24,778	5.15	413.06	a,b
61–70	1,825	56,658	5.46	590.17	c,d
71–80	1,264	38,369	5.29	622.61	c,d
81–90	561	16,384	4.59	745.76	c,d
91 and above	50	1,515	3.69	895.14	b,d
Total	4,497	151,926	5.18	571.38	

a, b, c, d: each letter denotes a subset of age group categories whose incidence rates do not differ significantly from each other. The age groups of 0–10 and 11–20 years were not applicable in the statistics due to the insufficient sample size.

Incidence and Influencing Factors of New Hepatitis B Infection Outcomes

A total of 4,497 participants were observed to have new hepatitis B infections; however, 796 of these participants were excluded from further study because of missing data. Among the remaining 3,701 participants, after 5.40 ± 1.85 person-years of follow-up (1.41 years after HBV infection), 3,200 (86.46%) were found to have a HBsAg seroclearance, while the other 501 (13.54%) were persistently HBsAg positive (**Table 2**). The cumulative incidence of HBsAg seroclearance at 1, 2, and 5 years was 0.573, 0.802 and 0.861, respectively (**Figure 2B**). Seroclearance was normally completed in the first 3 years, after which it reached a plateau. The annual incidence rate of HBsAg seroclearance was significantly different between the first 2 years and the following years (**Supplementary Table 2**). Noteworthy, spontaneous HBsAg seroclearance hardly occurred since the 5th year after infection. Overall seroclearance rate of this cohort tend to a platform of 86 percent.

Kaplan-Meier survival curves illustrated that females were more vulnerable to persistent positive HBsAg than males (**Figure 3A**). Among various age groups (every 10 years was divided into a group), we observed the younger (21–30-, 31–40-, 41–50- and 51–60 year) groups were more likely to be

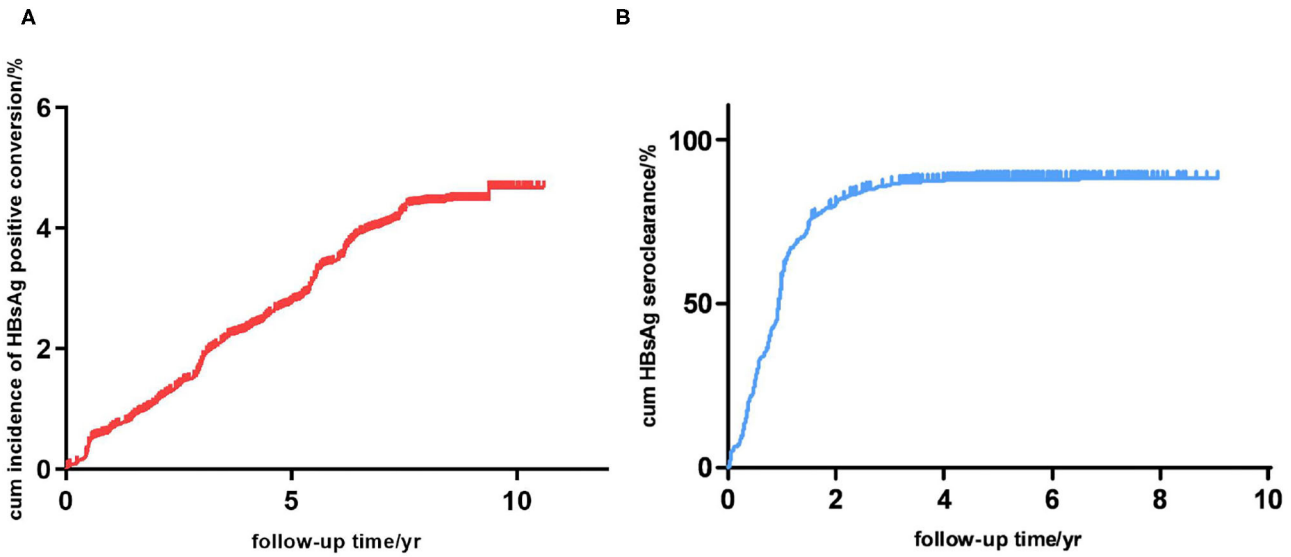

FIGURE 2 | (A) Cumulative incidence of new hepatitis B infection in this study cohort ($n = 151,926$). **(B)** Cumulative incidence of HBsAg seroclearance in participants with hepatitis B infection ($n = 4,497$).

persistently HBsAg positive than the elderly (61 years and above) groups in a univariate Cox regression (**Figure 3B** and **Table 2**). The univariate Cox regression study also demonstrated that female gender, alcohol drinking history and Hepatitis B Family history were potential risk factors for persistent HBsAg positivity. Smoking history don't seem to have a significant impact on the spontaneous clearance rate.

In the following multivariate Cox regression analysis, female gender, middle-age, alcohol drinking history and Hepatitis B Family history were still statistically significant predictors of persistent infection (**Table 3**). People of female gender were found to have a slightly higher risk for persistently HBsAg positivity with a multivariate-adjusted rate ratio of 1.31 (95% CI, 1.02–1.68). Furthermore, the age stratification analysis found that middle-age populations, especially in the 31- to 40-, 41- to 50-, and 51- to 60 year groups, were prone to being persistently HBsAg positive with hazard ratios of 2.77 (95% CI, 1.57–4.88), 2.76 (95% CI, 1.83–4.17), and 2.63 (95% CI, 1.79–3.86), respectively. Individuals with a history of drinking process a relatively higher risk (hazard ratios of 1.56) contrast to the no-drinking group. Family history also affected the spontaneous clearance rate distinctly. Person with a family history of hepatitis B have approximately twice the risk of chronic infection compared with people from normal family.

DISCUSSION

Our study focused on the fluctuation of HBsAg in a large-scale community susceptible population with >140 thousand residents. China government started the national infant hepatitis B vaccination project in 1992 and have not carried out any national adult hepatitis B vaccination policy. So no adult members in this cohort have HBV vaccination history. The annual HBV infection rate was 571.38 per 100,000 person-years, and considering the HBsAg seroclearance rate (86.46%), these results approximate the incidence of hepatitis B (68.58–81.54 per 100,000 person-years) during 2010–2016 from the Chinese public health science data center (19). Still the practical HBV infection rate in this community cohort may be underestimated and be higher than 571.38 per 100,000 person-years on account of some research limitations. For the HBsAg screening interval is 12 months in our research, new-infected people who experienced spontaneous seroclearance within 1 year may be omitted in annual screening. Data reported from national public health organizations were also doomed to underestimate the actual number of cases for the same reason, which was obtained by small-scale a cross-sectional survey (19).

The cohort members of this study are mainly susceptible adult. So the transmission routes could only be unsafe sexual behavior, blood contact and syringe-sharing of drug-addict. Considering the good performance of local government in blood products safety controlling and drug-fighting, most new infections in this study should be sexually transmitted and accidentally blood contact (20). Our survey showed that the incidence rate of new hepatitis B infection for males was significantly higher than that for females (684.88 vs. 536.68 per 100,000 person-years, $p < 0.001$), and 31–40, 41–50, 51–60 age groups had lower new infection rate than younger and elder age groups (**Table 1**). The phenomenon that males were more likely to have been infected with HBV than females reported by many previous studies could mainly be explained by behavioral differences (11, 21–27). In this research it may relate to the relatively conservative ideology in this county. Female and middle-aged male bearing stable marital status tend to have few extramarital unsafe sexual behavior and accidental blood contact in conservative ideological county, leading to few chance

TABLE 2 | Outcomes and univariate analysis of HBsAg positive conversion.

Variables	Negative conversion (1)	Persistent infection (2)	Total number	*p*
Total	3,200 (86.46%)	501 (13.54%)	3,701	
Gender				<0.001
Male	1,384 (88.32%)	183 (11.68%)	1,567	
Female	1,816 (85.10%)	318 (14.90%)	2,134	
Age group				<0.001
11–20	0	0	0	
21–30	8 (80.00%)	2 (20.00%)	10	
31–40	26 (66.67%)	13 (33.33%)	39	
41–50	98 (65.77%)	51 (34.23%)	149	
51–60	227 (69.85%)	98 (30.15%)	325	
61–70	1,341 (87.36%)	194 (12.64%)	1,535	
71–80	1,033 (90.93%)	103 (9.07%)	1,136	
81–90	435 (92.16%)	37 (7.84%)	472	
91 and above	32 (91.43%)	3 (8.57%)	35	
Hepatitis B family history	Unavailable for 5 (0.14%) participants			<0.001
0	2,840 (88.09%)	384 (11.91%)	3,224	
1	358 (75.85%)	114 (24.15%)	472	
Smoking history	Unavailable for 170 (4.59%) participants			0.405
0	2,537 (85.85%)	418 (14.15%)	2,955	
1	493 (85.59%)	83 (14.41%)	576	
Alcohol drinking history	Unavailable for 76 (2.05%) participants			0.002
0	2,906 (86.87%)	443 (13.13%)	3,375	
1	221 (80.07%)	55 (19.93%)	276	

of infection. In this study we found a relatively high rate of new infection in older people. The aging people of this cohort should be speculated to experience more unsafe sex and blood exposure than other age group, contrary to traditional ideas. So it is an unfortunate misunderstanding of our culture that older people are asexual. In this fixed cohort in open community, susceptible people who have common unsafe sexual behavior roughly maintained at a fixed ratio. They were estimated to be all infected (approximately 5 percent of this cohort) in no-intervention state within 10 years (**Figure 2**). This could also explain the decreasing tendency of annual new infection rate (**Supplementary Table 1**). At the same time the HBsAg prevalence in young people who have been vaccinated is <1.0% (12). This showed the importance and necessity of vaccine intervention in adult people in a hepatitis B high burden country without adult vaccination coverage.

We also found that 86.46% of patients were observed to have HBsAg seroclearance, furthermore females and individuals between 31 and 60 years were inclined to develop chronicity. This finding is consistent with a recent Chinese community-based study indicating 8.50% chronicity (28). While androgen was widely recognized as a poor prognostic marker for the chronic patient (29–31), in the early stage of infection, serum testosterone may protect the male from chronic hepatitis B infection on account of higher SRD5A2 enzyme activity (32). This may explain the higher risk of persistent infection in female. Seroclearance rates of young and middle age groups in our study approach the result of some previous publications (24, 33, 34). The result that young people under 30 had slightly higher seroclearance rate than middle age group may attribute to their better immuno-competence. However it's really a puzzled phenomenon that age group elder than 60 have distinctly high spontaneous seroclearance rate (**Table 2**). In theory aging is speculated as negative influencing factor for spontaneous HBsAg seroclearance because aged people usually have lower sex hormone level and immunity. Some researches revealed that aged people have roughly similar or lower spontaneous seroclearance rate contrast to the middle-age group (24, 35). However large-scale real world data of spontaneous HBsAg seroclearance in aging people were few in previous publications. Some other studies appealed opposite findings. For instance a Hong Kong study of 4,568 cohort members and a research of 148 cohort members in Netherlands also found middle-age was associated with developing chronic infection and low HBsAg seroclearance rate compared with aging people (25, 26). The confusing result has not been explained clearly and could partly be due to the increasingly complex innate and adaptive immune responses in elder people (27). High interferon level associated with high viral load in aged individuals may promote the clearance of virus in the early stage (27, 36). Another explanation may be more occult infection occurred in aging people. Occult hepatitis B infection is defined by the persistence of viral genome in the liver in individuals who are tested negative for HBsAg (37). The prevalence data are quite difficult to obtain. In this research the occult infection cannot be simply detected by HBsAg screening. A series of studies found that occult hepatitis B infection prevalence seems to be higher among individuals at high risk of HBV infection and relatively weak baseline liver function status (37–39). So the aging group of our research are more susceptible to occult infection. In spite of the high vanishment rate of HBsAg, resident aging people should receive more liver function monitoring.

We also observed the influence of alcohol drinking history, smoking history and hepatitis family history. It has been reported that alcohol drinking could increase susceptibility to HBV infection and has negative impact at the onset of chronic infection (40). Our research revealed a obviously harmful effect of drinking in new infection individual. They process 1.56 times risk of persistent infection contrast to no-drinking people. Prohibition is a useful suggestion to suppress chronic infections for these susceptible adult community population. Smoking could affect NK cell related antiviral immunity and promote HBV infection progression (41). In this study we found a tiny adverse impact of smoking with no significant statistic difference (**Table 2**). Smoking may not have a major impact on the early stages of infection. Nonetheless cigarette-controlling

is still recommended with other hepatitis health intervention considering the chronic damage of smoking leading to liver dysfunction, cirrhosis and hepatocellular carcinoma (41). People who have hepatitis family history may have more chance of virus exposure and carry more susceptibility genes. A series of SNPs or haplotypes in cytokines, MHC class II and a number of chemokines genes have been found to relate with HBV infection and clearance (42). Our statistics showed that possessing Hepatitis B family history definitely reduced spontaneous clearance. Susceptibility gene screening may be costly in hepatitis B high endemic countries. But appropriate health education could benefit hepatitis families by controlling virus exposure and practicing regular hepatitis screening. In summary more attention should be paid to these people who possess risk factors of chronic infection such as female, middle age, alcohol drinking, and hepatitis B family history (**Table 3**).

Previous studies proposed that both viral and host factors were determinants of seroclearance; however, this proposal has still not been completely elucidated (4). Infants were believed to have 80–90% chronicity (7, 43–46), and 23–46% of children ≤6 years old were chronic carriers of HBsAg in a Taiwanese/Chinese population (47–49). Immune competent adults were the least likely to develop chronic infection with an infection rate of 5–10% (4–6, 28, 50). HBV genotype C2 is more prone to chronic progression than B2 (28).

In conclusion, the results of this study have offered a large-scale real world data of HBV new infection and spontaneous seroclearance in vulnerable adult community people. In this community cohort, the overall infection rate and spontaneous clearance incidence are ∼5 and 86%, respectively. Considering the relatively high new infection incidence rate, adult hepatitis B vaccination policy should be considered in hepatitis B high-burden countries. The incidence rate for males was significantly higher than that for females. Female gender, middle-age, alcohol drinking and hepatitis family history were predictors for persistent positive HBsAg status. Continuous follow-up for individuals with the above risk factors are warranted.

A few limitations of this research should be mentioned. Most HBsAg seroclearance cases in our study occurred in the first 2 years, although natural seroclearance of HBV in acute infection is defined as HBsAg clearance in <6 months. It is apparently some HBsAg seroclearance cases have not been monitored. Thus, statistical analysis and study design methods determined that the HBsAg seroclearance interval time should be ∼1 year, ideally considering an arbitrarily defined midpoint screening and a 1 year screening. For example, with "negative-positive-negative" consecutive screening results, the time of new hepatitis B infection would be the midpoint of the first and second screenings, and the time of HBsAg seroclearance would be the midpoint of the second and third screenings, making the HBsAg seroclearance interval time exactly 1 year. However, in practice, the time interval between screenings would fluctuate, resulting in HBsAg seroclearance interval times ranging from <1 to 2

TABLE 3 | Multi-variate analysis of HBsAg persist positive rate with 95% CI for each predictor.

	aHR	95% CI-low	95% CI-high	*p*
Gender				0.033
Male		Reference		
Female	1.31	1.02	1.68	
Age group			<0.001	
91	Reference			
21–30	1.55	0.65	3.73	0.327
31–40	2.77	1.57	4.88	<0.001
41–50	2.76	1.83	4.17	<0.001
51–60	2.63	1.79	3.86	<0.001
61–70	1.14	0.8	1.64	0.468
71–80	1	0.69	1.43	0.988
81–90	1.03	0.71	1.49	0.885
Alcohol drinking history	1.56	1.06	2.29	0.025
Hepatitis B Family history	2.28	1.71	3.04	<0.001

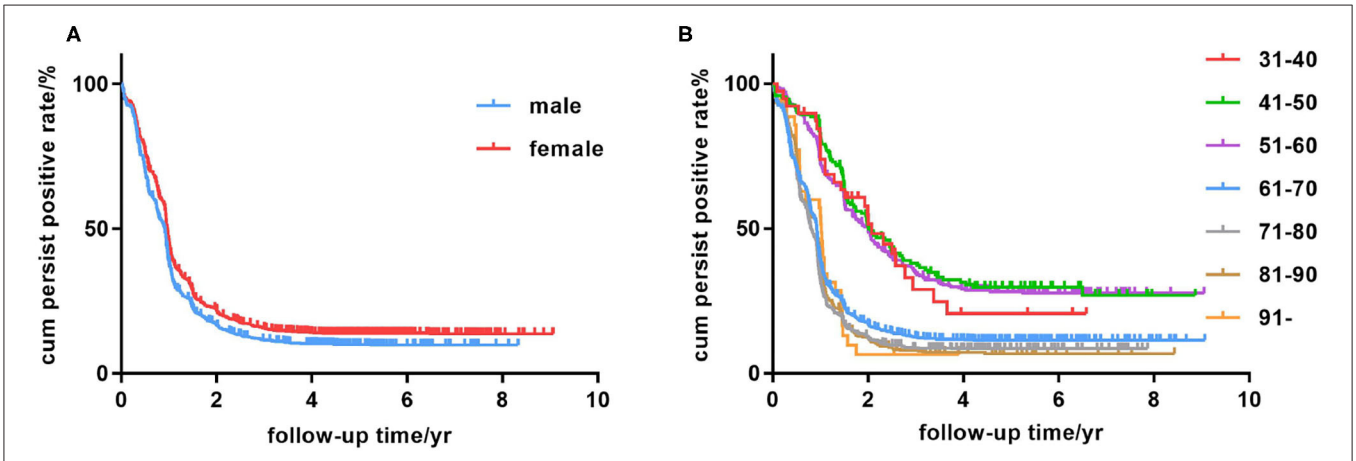

FIGURE 3 | Kaplan-Meier analysis of age group, sex, education level and medical insurance condition. **(A)** Persistent HBsAg positive rate in the male and female groups. **(B)** Persistent HBsAg positive rate in the 31- to 40-, 41- to 50-, 51- to 60-, 61- to 70-, 71- to 80-, 81- to 90-year, and 91-year-and-above age groups.

years or more. In our large-scale screening we only tested HBsAg as symbol of new infection. This would have omitted a series of occult infection cases. Moreover, elder cohort members were more than young people due to the better compliance. Imbalance of age distribution may lead to bias of total new infection rate and seroclearance rate.

DATA AVAILABILITY STATEMENT

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

AUTHOR CONTRIBUTIONS

LL, KX, and HH designed the study. HH, YS, MH, and YZ collected the data. HH and YS analyzed the data and interpreted the results. HH, YS, and MH wrote the manuscript. LL and KX revised the manuscript from the preliminary draft to submission. LL supervised the whole study. 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/fmed.2021.717667/full#supplementary-material

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REVIEW
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The Role of Exosomes in Viral Hepatitis and Its Associated Liver Diseases

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Exosomes, the important carriers between cells, can carry proteins, micro ribonucleic acids (miRNAs), long non-coding RNAs (lncRNAs) and other molecules to mediate cellular information transduction. They also play an important role in the pathogenesis, prognosis and treatment of viral hepatitis and its associated liver diseases. Several studies have reported that viral hepatitis and its associated liver diseases, including hepatitis A, B, C and E; hepatic fibrosis and hepatocellular carcinoma, were closely associated with exosomes. Exploring the role of exosomes in viral hepatitis and associated liver diseases will enhance our understanding of these diseases. Therefore, this review mainly summarised the role of exosomes in viral hepatitis and its associated liver diseases to identify new strategies for liver diseases in clinical practise.

Keywords: biomarkers, exosomes, fibrosis, hepatocellular carcinoma (HCC), therapeutic targets, viral hepatitis

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INTRODUCTION

Exosomes, firstly discovered in 1980, are circular or elliptical membrane vesicles of endocytic origin with a diameter of ∼30–150 nm, which are released into the extracellular environment after the fusion of the polycystins and plasma membranes (1). Exosomes are secreted by different body cells, including fat, dendritic, T, B, stem and tumour cells, which could be found in blood, urine and cerebrospinal fluid (2–5). Exosomes are produced by plasmacytoid dendritic cells (pDCs) and released after the fusion of multivesicular bodies (MVB) with the plasma membrane. They are composed of proteins, peptides, lipids, messenger ribonucleic acids (mRNAs), microRNAs (miRNAs), deoxyribonucleic acid (DNA) and other components (6) and can be transported to adjacent or distant organs and tissues through blood circulation (7). They can participate in various important physiological and pathological processes of the human body and affect disease development, which plays an important role in cell communication, migration, angiogenesis, immune response and tumour cell growth (8, 9).

Viral hepatitis occurs worldwide, including the following five viruses as the main clinical manifestations: hepatitis A, B, C, D and E. By regulating host immune response and mediating hepatitis virus replication, exosomes could influence the pathogenesis of hepatitis virus. The exosomes released from cells infected with hepatitis virus can carry nucleic and protein components, which would help hepatitis virus participate in immune escape. Meanwhile, exosomes derived from immune cells help eliminate viruses and antiviral immune defence. Exosomes released or received by the liver cells can be used for cell-to-cell communication between healthy and damaged livers (10). Moreover, exosomes produced by hepatocytes infected with the hepatitis

virus spread the infection and disrupt the innate immune response of chronic viral hepatitis (11). Similarly, they can also activate the body's immune response to hepatitis infection (12). As important carriers between cells, exosomes are involved in virus transmission, immune regulation, antiviral response and viral microenvironment and play an important role in the pathogenesis, prognosis and treatment of viral hepatitis and its associated liver diseases (**Figure 1**). Therefore, this review mainly summarised the role of exosomes in viral hepatitis and its associated liver diseases, aiming at providing new strategies for the clinical treatment of liver diseases.

The Role of Exosomes in Hepatitis A

Hepatitis A is caused by the hepatitis A virus (HAV) and is mainly transmitted through the faecal–oral route. HAV is a hepatophilic positive-chain RNA virus (13). Its worldwide spread is episodic and can cause acute liver disease but does not establish a persistent infection. Infected human cells can produce two types of HAV particles: non- and quasi-enveloped. Non-enveloped virus particles are stable in the faeces of infected people, whereas quasi-enveloped virus particles are present in the blood of infected individuals. The presence of quasi-envelopes protects the virus from immune response. Therefore, quasi-enveloped virus particles can spread to the liver (14).

Quasi-enveloped HAV (eHAV) was reportedly responsible for viral transmission and pDC activation (15). eHAV can be germinated from the endosomes of the HAV capsid into the MVB through the exosomes (16). HAV cell receptor 1 and cholesterol transporter NPC1 participate in the transport of exosomes from HAV-infected cells through mesh protein-mediated endogenous action, thereby promoting HAV infection (17). Furthermore, Costafreda et al. demonstrated that exosomes and HAV have similar fusion mechanisms independent of envelope glycoproteins (18). Jiang et al. confirmed that HAV structural protein pX could interact with apoptosis-associated gene 2-interacting protein X to promote virion and exogenous protein secretions through exosome-like vesicles (19).

Exosomes can protect virions from antibody-mediated neutralisation in HAV-infected cells. The presence of these exosomes can also prevent the detection of HAV by the host immune system and facilitate the spread of HAV in the liver. However, HAV virions coated with exosomes may limit replication after an eHAV infection, slowing the spread of HAV in the cells (15).

Nowadays, the diagnosis and therapeutic effects of exosomes in HAV infection have not been thoroughly explored. These results suggest that exosomes make a great difference in HAV transmission and protect HAV from the detection of the host immune system. Therefore, future studies should focus on the mechanism of action of exosomes in innate immunity and immune evasion to advance the exosomal diagnostic process and HAV infection treatment.

Abbreviations: HAV, Hepatitis A virus; HBV, Hepatitis B virus; HCV, Hepatitis C virus; HEV, Hepatitis E virus; HCC, Hepatocellular carcinoma; CHB, Chronic hepatitis B; pDCs, Plasmacytoid dendritic cells; lncRNAs, Long non-coding RNAs; miRNAs, micro ribonucleic acids; ACLF, acute-on-chronic liver failure; HSCs, hepatic stellate cells; HF, Hepatic fibrosis.

The Role of Exosomes in Hepatitis B

Several studies have reported on the role of exosomes in hepatitis B (**Table 1**). Hepatitis B virus (HBV) is a common liver-specific enveloped DNA virus that can cause chronic hepatitis B (CHB). CHB is a global epidemic infecting ∼2 billion people, of which 240 million had chronic infection (30). Approximately 650,000 people die from HBV infection and liver diseases caused by HBV infection annually (31). In recent years, several studies have demonstrated that exosomes can play a role in and influence the replication, transmission, diagnosis and treatment of HBV by regulating HBV replication and transmission. A previous study showed that immune-related miRNAs could be involved in inflammatory and immune responses (32). Zhang et al. reported that miR-199a-3p and miR-210 effectively reduced the expression of hepatitis B surface antigens (HBsAg), thus inhibiting HBV replication (20). Ninomiya et al. reported that exosome-associated tetramine CD63 contributes to the efficient assembly of HBV and its infectivity (21).

Similarly, exosomes can regulate immune response, revealing the underlying mechanisms of immune escape. Yang et al. analysed the serum samples of patients with CHB and found that serum exosomes contained HBV components. These exosomes can induce active HBV infection in the primitive liver cells, inhibit the lethality of natural killer (NK) cells and destroy the body's immune response, thereby promoting HBV replication and transmission (22). Kapoor et al. also found that transcription and translation products of the HBx gene in HBV can be transported to the recipient cells through exosomes and promote HBV transmission by improving the liver microenvironment (23).

Exosomes are important in the predictive diagnosis of HBV infection. Zhao et al. compared the protein composition of Huh7 cell exosomes infected with HBx and that of the control group and confirmed the presence of liver cancer-related proteins, indicating that specific proteins of serum exosomes can be considered as HBV and HBV-related liver cancer markers (24). Jiao et al. also demonstrated that exosomes with albumin and vascular endothelial growth factor (VEGF) may be more accurate and specific biomarkers for assessing liver regeneration and prognosis in patients with acute-on-chronic liver failure (ACLF), whereas exosomes with CD63 and albumin may be early warning markers for patients with ACLF (25). The serum exosomal long-chain non-coding RNA nuclear-rich transcript 1 was reported to predict the 90-day mortality in patients with ACLF (26).

Exosomes also have antiviral activity. Kwon et al. demonstrated that type I interferon-alpha (IFN-α) can be an effective treatment for HBV infection (33). Interferon can inhibit the covalent closure of circular DNAs through HBsAg and HBV, which exhibit antiviral activity and effectively inhibit HBV replication (27). Li et al. also demonstrated that antiviral response induced by IFN-α can be transported from the liver non-parenchymal cells to HBV-infected cells through exosomes, leading to the storage of immune memory and exerting antiviral functions (28). Macrophage-derived exosomes transfer IFN-α-associated miRNA from the macrophages to HBV-infected hepatocytes through endocytosis and macropinocytosis and have antiviral activity against HBV replication and expression (29).

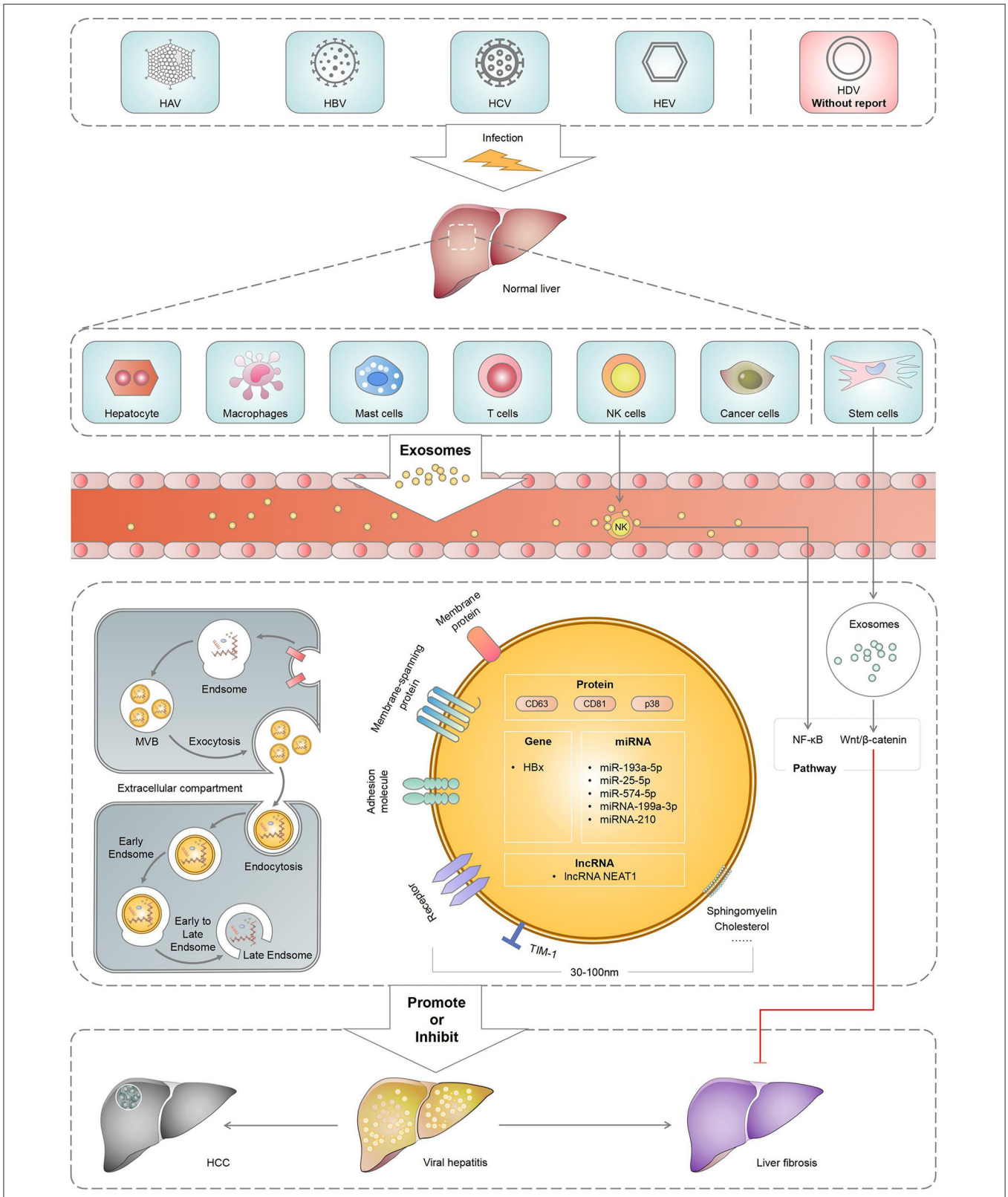

FIGURE 1 | The role of Exosomes in the occurrence, development and metastasis of HCC.

TABLE 1 | The role of exosomes in hepatitis B.

Exosome and related molecules	Role	References
MiR-199a-3p and miR-210	The up-regulation of miR-199a-3p and miR-210 may play a role in regulating HBV replication.	(20)
Exosome-associated tetramine CD63	CD63 contributes to the efficient assembly of HBV and its infectiousness.	(21)
Exosomes derived from serum samples in CHB patients	Exosomes can induce active HBV infection and inhibit the lethality of NK cells.	(22)
Neutral sphingomyelinase2, CD9 and CD81	The HBx gene of hepatitis B virus can influence hepatic microenvironment via exosomes.	(23)
Exosomes purified from HBV-infected patients' sera	Specific proteins of serum exosomes can be used as markers of HBV and HBV-related liver cancer.	(24)
Exosomes with CD63 and albumin	Exosomes with CD63 and albumin may be early warning markers for ACLF patients.	(25)
Serum exosomal lncRNA NEAT1	The serum exosomal lncRNA NEAT1 might be a prognostic biomarker for 90-day mortality of ACLF.	(26)
Macrophage-derived exosomes	Exosomes can transfer IFN-α-related miRNAs, which can inhibit HBV replication and expression.	(27)
Exosomes from LNPCs	Exosomes can mediate the cell-to-cell transmission of IFN-α-induced antiviral activities.	(28)
LNPC-derived exosomes	Exosomes could transmit IFN-α-induced antiviral activity to HBV replicating hepatocytes	(29)

miR, microRNA; HBV, hepatitis B virus; CHB, chronic hepatitis B; NK, natural killer; ACLF, acute-on-chronic liver failure; IFN, type I interferon; NEAT1, nuclear-enriched abundant transcript 1; LNPC, liver nonparenchymal cells.

The Role of Exosomes in Hepatitis C

Hepatitis C virus (HCV) is a positive-chain RNA virus transmitted through the blood, affecting >71 million people worldwide (34). HCV infection is an important cause of end-stage liver disease. Therefore, the presence of exosomes is considered to play an important role in HCV replication and transmission. Masciopinto et al. reported the presence of HCV RNA after isolating exosomes from hepatocytes of patients with hepatitis C (35). Furthermore, Ramakrishnaiah et al. confirmed that HCV is transmitted by transporting exosomes between liver cells (36). HCV RNA in exosomes was also found to form protein complexes with Ago2, HSP90 and miR-122 to enhance stability and infectivity and promote replication and transmission (37).

HCV-related exosomes can play a role in the immune evasion process, and HCV can hijack exosomes released by the cells and evade the host immunity. HCV-related exosomes can also induce RUNXOR and RUNX1 expressions via the STAT3-miR124 axis, and RUNXOR and RUNX1 up-regulation may promote myeloid suppressor cell differentiation and host immune response inhibition, thereby evading host immunity (38). Ji et al. concluded that HCV can facilitate galectin-9 secretion in monocytes, which inhibit T-cell-mediated-specific immune response after interacting with T-cell Ig and mucin domain protein-3 (39).

The inhibitory effect of exosomes on viruses is considered a potential treatment for HCV infection. Exosomes can be the medium of HCV RNA transportation to pDCs (40). HCV RNAs were found to act on toll-like receptor 7 to activate pDC, thus promoting IFN synthesis and release and inhibiting HCV replication and transmission (41). Giugliano et al. found that human liver sinus endotho-othesotropic cells (HLSECs) can internalise HCV virus particles through intercellular contact, act on the conformation recognition receptor, which can up-regulate IFN gene expression, increase type I and III interferon levels, stimulate HLSECs to secrete exosomes and eventually inhibit HCV virus replication (42). Aydin et al. showed that blocking the release of extracellular vesicles and exosomes can significantly affect viral replication without affecting the host cell viability. Therefore, they suggested that inhibiting the extracellular vesicle release could be a potential antiviral strategy for the treatment of HCV and other emerging RNA viruses (43).

HCV-associated exosomes can affect virus replication and transmission and mediate immune evasion (**Table 2**). Further efforts are needed to explore the role of exosomes in HCV infection and to provide new ideas for the diagnosis and treatment of HCV infection.

The Role of Exosomes in Other Hepatitis Infections

To date, the role of exosomes in hepatitis D virus has not been systematically reported. However, as an intestinally transmitted and liver-obsessed virus, the effects of exosomes on hepatitis E virus (HEV) and its scoring model have been mostly investigated (44). Exosomes participate in the immune escape of HEV, enrich the cholesterol and phosphatidylserine levels, increase the HEV intake in the liver cells and promote HEV replication and transmission (45). Nagashima et al. found that HEV was transferred and released through MVB. The mechanisms underlying HEV infection in rats may be similar to those in humans (46). Primadharsini et al. confirmed that HEV in rats is released through MVB screening and that HEV release in rats requires a pathway associated with exosomes (47).

Non-enveloped HEV and eHEV enter the cells through different mechanisms. The main route of eHEV entry into

TABLE 2 | The role of exosomes in hepatitis C.

Exosome and related molecules	Role	References
HCV-CD81	HCV-CD81 complex may leave cell in the form of exosomes.	(35)
Hepatocyte-derived exosomes	Hepatic exosomes would transmit productive HCV infection *in vitro*.	(36)
Exosomes isolated from HCV-infected individuals or Huh7.5 cell supernatants	HCV RNA in exosomes can form protein complexes to and promote its replication and transmission.	(37)
HCV-related exosomes	HCV-related exosomes can induce RUNXOR and RUNX1 expression via the STAT3-miR124 axis.	(38)
HCV-RNA-containing exosomes	HCV RNA can be transported to plasmacytoid dendritic cells through exosomes.	(40)
Exosomes derived from HLSECs	HLSECs induce the release of antiviral exosomes to inhibit HCV replication.	(42)
Extracellular vesicles	The inhibition of extracellular vesicle release may be a potential antiviral strategy for the treatment of HCV.	(43)

HCV, hepatitis C virus; RUNXOR, RUNX1 overlapping RNA; RUNX1, runt-related transcription factor 1; STAT3, signal transducer and activator of transcription 3; HLSEC, human liver sinusoidal endothelial cells.

the cells is through the mesh protein-mediated endophagus. Compared with non-enveloped HEV, eHEV binds to the cells much less efficiently and requires longer inoculation time to achieve its maximum infectiousness (48). Degradation of the eHEV membrane in the lysosomes to achieve membrane removal may greatly increase its infectiousness. Non-competitive neutral phospholipase inhibitors GW4869 or silent Rab27A/Hrs gene expression can inhibit the secretion of exosomes, resulting in a significant reduction in HEV release, providing a new treatment strategy for hepatitis E (46).

The Role of Exosomes in Hepatitis-Associated Hepatic Fibrosis

Hepatic fibrosis (HF) is caused by excessive production and accumulation of insoluble collagen and extracellular matrix components after sustaining chronic liver damage. Various chronic liver diseases can lead to HF and even liver cirrhosis. Activation of hepatic stellate cells (HSCs) is a primary event that results in HF development (49).

Exosomes can promote HF development. Exosomes from damaged liver cells are rich in cytochrome P450, and the reactive oxygen produced by cytochrome P450 2E1 (CYP2E1) can produce superoxide anion free radicals, hydrogen peroxide and strong oxidants, and increased CYP2E1 levels under various pathophysiological conditions can lead to hepatocellular apoptosis through the oxidative stress mechanism (50). Exosomes from damaged hepatocytes containing cytochrome P450 are speculated to be involved in the development of fatty degeneration by increasing the expression of fibrin and hepatocyte apoptosis (51). Hepatocyte lipotoxic fatty acid damage produces exosomes rich in miR17-92 clusters, which can be absorbed by HSCs, resulting in fibrotic activation (52). Exosomes released from the epithelial cells can activate fibroblasts to trigger fibrosis. Furthermore, exosomes produced by damaged epithelial cells are absorbed by adjacent fibroblasts, resulting in increased production of α-smooth muscle actin and type I collagen to drive HF (53). Exosomes from CCL4-processed hepatocytes include different types of self-RNA and toll-like receptor 3, which can increase IL-17 production in the liver γδT cells. Increased inflammatory cytokine levels were closely associated with HSC activation (54). T cells produced by IL-17 can regulate TGF-β1 in the Kupffer cells and directly activate HSCs (55).

The expression pattern of miRNAs in the serum rich in exosomes is a highly potential biomarker for diagnosing the grade and stage of liver diseases. Niu et al. analysed the serum exosomes of patients and rats with HF and found that exosome miR-155 can serve as a non-invasive biomarker for the diagnosis and progression of HF (56). Chen et al. also showed that miR-103-3p in the serum exosomes of patients with HF may be an HF biomarker (57). Exosomes can also be used for the treatment of HF. Exosomes from healthy humans may be beneficial to patients with HF, and the primary mechanism for repairing damaged liver may be the release of paracrine factors (58). Existing reports demonstrated that exosomes are the source of umbilical cord-filled, fat-filled and bone marrow interstitial stem cells for the possible treatment of HF (59–61).

HF formation is closely associated with HSC activation. Exosomes can regulate HSC activation and have an anti-fibrosis effect. Chen et al. found that serum exosomes from healthy donors have anti-fibrosis properties, partly owing to specific miR components with therapeutic effects on activated HSCs or damaged liver cells. Serum exosomes in healthy individuals have anti-fibrosis effects. MiR-34c, miR-151-3p, miR-483-5P, miR-532-5P and miR-687 expressions were higher in healthy mice than those in mice with fibrosis, and these miRNAs can inhibit the expression of fibrogenic genes in activated HSCs (62). Exosomes derived from the human bone mesenchymal stem cells were reported to reduce HF by inhibiting Wnt/β-catenin signalling to prevent HSC activation (61). Autophagy in HSCs was reported to reduce HF by inhibiting the release of fibrotic exosomes, indicating that exosomes can serve as potential new anti-fibrosis biological agents and have a positive therapeutic effect against fibrosis and important transformational significance for the treatment of fibrosis-related diseases (63).

Exosomes can promote and antagonise HF. Activated HSCs can also release fibrin-rich exosomes, suggesting that they

are new biomarkers of potential pathological conditions and play a key role in the identification and treatment of HF-related diseases.

The Role of Exosomes in Hepatitis-Associated Hepatocellular Carcinoma

Hepatocellular carcinoma (HCC) is a highly life-threatening cancer and the leading cause of death in patients with cirrhosis. Its incidence in China accounts for 50% of global cases and deaths (64). Researchers have been focusing on the early diagnosis and treatment of HCC (65). Exosomes can functionally carry active proteins, RNA and other types of molecules that are associated with the cancer pathology (66). Therefore, investigating the role of exosomes may promote HCC diagnosis and treatment.

Exosomes can be involved in the occurrence, development and metastasis of HCC mainly through RNA transport and protein-mediated cellular communication. Kogure et al. found that exosomes in HCC cells contain varied miRNAs and can significantly promote the non-adhesive growth of liver cancer cell strains to promote tumour progression by regulating the transformational growth factor in their receptor cells to activate the kinase-1 (TAK1) signalling pathway (67). Li et al. reported that exosomes can transfer long-chain non-coding RNA FAL1 into HCC cells to promote cell growth, proliferation, migration and invasion (68). Exosomes derived from HCC cells (HepG2) were reported to be actively internalised by adipocytes, causing significant transcriptomic changes. Adipocytes treated by tumour exosomes could promote tumour growth, enhance angiogenesis and recruit more macrophages in a mouse model (69). Chen et al. also demonstrated that exosomes from highly metastatic MHCC97H cells can be ingested by the less metastatic HCC cells and subsequently promote malignant behaviours of the recipient cells. Exosomes derived from tumours may promote epithelial-to-mesenchymal transformation through signal transduction, further promoting HCC invasion and metastasis (70). Furthermore, Wei et al. found that Vps4A can regulate the secretion and ingestion of exosomes containing oncogenic and tumour suppressor miRNAs, and its down-regulated expression in HCC tissues can promote HCC development and metastasis (71). Recently, the loss of miR-320a was found to inhibit the miR-320a-PBX3-MAPK signalling pathway, induce epithelial–mesenchymal transformation and cyclin-dependent kinase-2 and MMP-2 expressions to promote the HCC development and metastasis (72).

The exchange of RNA and protein through exosomes not only plays a key role in the HCC pathogenesis and progression but also identifies specific and sensitive biomarkers for HCC recurrence and prognosis as potential non-invasive biomarkers and therapeutic targets. Examination of exosomes is conducive to promptly reflect the severity and possible progression of the disease and to control the development of the disease in the high-risk population. Serum exosomes hsa-circ-0028861 and hsa-circ-0070396 can serve as new biomarkers of HCC caused by HBV (73, 74). MiR-125b-5p and miR-223-3p can also be used as novel non-invasive biomarkers for HBV-positive HCC at an early CHB stage (75). Circulating exosome differentiation of antagonistic non-protein-coded RNAs is highly correlated with disease progression of HCV-associated HCC and may be a non-invasive prognostic biomarker for HCV-associated HCC (76).

Exosomes for the treatment of HCC are receiving increasing attention, including the adipose mesenchymal hepatocyte-, hepatocyte- and dendritic-cell-derived exosomes. Lou et al. transfected AMSC with miR-122, and the extracted adipose mesenchymal hepatocyte-derived exosomes changed the miR-122 target gene expression so that cancer cells could be sensitised to chemotherapeutic drugs. Intratumoral injection of exosomes could significantly improve the anti-tumour effects of sorafenib on HCC *in vivo* and enhance the chemotherapeutic sensitivity of HCC (77). Cheng et al. demonstrated that hepatocyte-derived exosomes could inhibit the HCC cell progression through the STAT3 pathway (78). A study that injected dendritic-cell-derived exosomes expressing AFP into the HCC mouse model found that DEXAFP was thought to induce a strong antigen-specific immune response, which significantly inhibited the HCC occurrence in mice (79). Currently, the application of exosomes in the treatment of HCC is limited to basic experiments, and further studies are required to explore the applications of exosomes in clinical practise.

CONCLUSION

Exosomes can carry proteins, miRNAs, lncRNAs and other molecules to mediate cellular information transduction, which plays a bidirectional role in viral hepatitis and its associated liver diseases. They can also encapsulate and transport the hepatitis virus, promote viral replication and transmission, mediate the antiviral response and serve as the target of immunotherapy. Furthermore, exosomes can reverse fibrosis and become the key mediators of fibrosis formation. Cell communication between exosomes can promote HCC development and metastasis. However, they can also inhibit the occurrence of HCC as an immunosuppressor. The mechanisms of exosome communication will enhance our understanding of liver pathophysiology, indicating their great potential as molecular biomarkers for the diagnosis and prognosis of liver diseases and as new therapeutic methods. Although studies on exosomes have made great progress in recent years, further efforts are required to use exosomes as biomarkers for the treatment of liver diseases in clinical practise.

AUTHOR CONTRIBUTIONS

HZ and NJ had the idea for the article. YY and Z-hY performed the literature search and data analysis. HZ and CX drafted and critically revised the work. All authors contributed to the article and approved the submitted version.

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REVIEW
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Suppression of Interferon-α Treatment Response by Host Negative Factors in Hepatitis B Virus Infection

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Chronic hepatitis B virus (CHB) infection remains a major global public health issue for which there is still lacking effective curative treatment. Interferon-α (IFN-α) and its pegylated form have been approved as an anti-HBV drug with the advantage of antiviral activity and host immunity against HBV infection enhancement, however, IFN-α treatment failure in CHB patients is a challenging obstacle with 70% of CHB patients respond poorly to exogenous IFN-α treatment. The IFN-α treatment response is negatively regulated by both viral and host factors, and the role of viral factors has been extensively illustrated, while much less attention has been paid to host negative factors. Here, we summarized evidence of host negative regulators and parameters involved in IFN-α therapy failure, review the mechanisms responsible for these effects, and discuss the possible improvement of IFN-based therapy and the rationale of combining the inhibitors of negative regulators in achieving an HBV cure.

Keywords: chronic hepatitis B, interferon-α, negative regulators, host factors, non-response

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INTRODUCTION

Chronic hepatitis B virus (CHB) infection affects more than 290 million people worldwide, with 1.5 million new infections each year, estimated by World Health Organization (1). The lifetime risk of developing hepatocellular carcinoma (HCC) and liver cirrhosis among HBV carriers ranges from 10 to 25% (2) and from 15 to 40% (3), respectively. HBV is a partially double-stranded circular DNA virus belonging to the Hepadnaviridae family (4), first discovered in an Australian aborigine (5). HBV has 10 genotypes (A-J) with nearly 40 sub-genotypes distributed in distinct areas, while the infectious in East Asia are most commonly HBV genotype B and C (3, 6). Current HBV treatment strategies can be categorized into 2 groups: interferons (IFN) and nucleos (t)ide analogs (NAs).

IFNs are a group of signaling proteins released by host cells in response to various pathogens, including viruses, bacteria, and parasites. IFN-α belongs to type I IFNs and has been approved as an anti-HBV therapy. Although with multiple adverse effects and inconvenient administration, IFN-α has the strength of a relatively short interval of treatment, without risk of drug resistance, higher rate of hepatitis B e antigen (HBeAg) seroconversion, and particularly hepatitis B surface antigen (HBsAg) seroclearance, which is unable to reach by current NAs administration (7, 8). Therefore, it is important to identify CHB patients who will benefit from treatment before the start of IFN-α-based therapy. Besides, IFN's efficacy is far less than satisfactory,

with only one-third of HBeAg positive CHB patients achieved HBeAg seroconversion after the IFN-α therapy, and even less efficacy was observed in HBeAg negative patients (9). The molecular mechanisms responsible for the failure of IFN-α treatment are not well-understood, but evidence shows that both viral and host factors are involved. Viral factors include HBV genotype, mutations within the HBV genome, and baseline level of viral load. Individuals infected with HBV-C and D (comparing to HBV-A and B) (6, 10), HBV has mutations within the HBV pre-core and/or basal core promoter (PC and/or BCP) region (6, 11–13), and high baseline viral load are tend to be more resistance to IFN-α therapy. The molecular mechanisms underlying the virally mediated resistance to IFN-α have been summarized in another review (14). In addition to the viral factors, host factors play an equally important role in modulating the effectiveness of IFN-α therapy for CHB patients' treatment. Some host molecules function as negative regulators of IFN therapy by inhibiting IFN production or signaling pathways, while several host parameters provide essential information of liver function and are able to predict the efficacy of IFN-α therapy. Therefore, a thorough understanding of the mechanism responsible for host factor-mediated inhibition of IFN therapy is needed for providing therapeutic targets to improve the efficacy of IFN-α treatment in terms of HBV infection. In this review, we summarize host negative regulators that impair IFN-α therapy of HBV infection and host parameters that can predict the IFN-α therapy efficacy, review the underlying mechanisms, as well as discuss the potential therapeutic approaches for controlling HBV infection.

CLASSICAL IFN AND ISGs PRODUCTION PATHWAYS IN RESPONSE TO HBV INFECTION AND ANTIVIRAL ACTIVITY OF IFN-α

Interferon (IFN) was originally discovered in 1957 by Isaacs and Lindenmann, and was named for their ability to interfere with viral replication (15, 16). Three interferon families are discovered—type I, II, and III. The type I IFN family encodes 13 partially homologous IFN-α subtypes in humans, IFN-β and several single gene products (IFN-ε, IFN-τ, IFN-κ, IFN-ω, IFN-δ, and IFN-ζ). The type IFN II family comprises a single gene product, IFN-γ, while the type III IFN family consists of IFN-λ1, IFN-λ2, IFN-λ3 (also known as IL-29, IL-28A and IL-28B, respectively), and IFN- λ4 (17, 18). Type I IFNs are secreted by almost all virus-infected cells including hepatocytes and by specialized blood lymphocytes, while the production of IFN-γ is restricted to immune cells, including natural killer (NK) cells, macrophages, and T cells. IFN-α and pegylated IFN-α have been approved for the treatment of chronic hepatitis B. IFN- α binds to its receptor (IFN-α/β-receptor, IFNAR) leading to the downstream signaling pathway and result in the expression of various IFN-stimulated genes (ISGs), which have multiple functions including anti-viral, anti-proliferation, anti-tumor, and immunomodulation. Some of these directly inhibit virus transcription and translation, others function as host immune modulators by NK cells activation, Dendritic cells (DCs) maturation, CD8+ T-cell augmentation, and B cell response (19). The antiviral effect against HBV infection of IFN was first known in 1976 by giving human fibroblast IFN to patients with HBsAg-positive chronic aggressive hepatitis (20).

The IFN response is initially induced by the recognition of HBV components. Multiple forms of nucleic acid are generated during the HBV life cycle, including double-strand relaxed circular DNA (rcDNA) and covalently closed circular DNA (cccDNA), single-strand RNAs, as well as double-strand RNAs, all of which could stimulate pattern recognition receptors (PRRs) on virus infection. Studies suggested that in the early phase of infection, HBV could activate some PRRs, which in turn stimulate the innate immune response to limit viral replication and clearance (21). For instance, Viral DNA is sensed by DEAD-box protein 41 (DDX41), cyclic GMP-AMP synthase (cGAS), and γ-IFN-inducible protein 16 (IFIT16), leading to the activation of stimulator of IFN genes (STING). RNA is recognized by either Toll-like receptor 3 (TLR3) or cytoplasmic sensors such as retinoic acid-inducible gene-I (RIG-I) and melanoma differentiation-associated gene 5 (MDA5), leading to its association with the mitochondrial antiviral signaling protein (MAVS). Both MAVS recruitment and STING activation lead to TANK-binding kinase 1 (TBK1) phosphorylation, which can activate IFN regulatory factor 3 (IRF3) and IRF7, two important transcriptional factors required for induction of type I IFN (22). In the HBV setting, Kupffer cells and liver parenchymal cells can recognize HBV components through intracellular PRRs such as TLRs and RIG-1-like receptors (RLRs), which induce the production of type I IFN (23). Several studies have also suggested TLR, RIG-I, STING, and myeloid differentiation primary response 88 (MyD88)-dependent pathway may participate in HBV-mediated IFN-induction in human cells (24, 25) and the pathways are meanwhile blocked by HBV polymerase in infected cells (26–28). In the MYD88-dependent pathway, MYD88 recruits a set of signal cascades such as MAPK and NF-κB through receptor-interacting serine/threonine protein kinase (RIPK/RIP) (29). After IFN-α was secreted by infected cells and blood cells, it will recruit in a pathway to induce ISGs expression.

The IFN-α signaling cascade is initiated through interactions with a multisubunit cell surface receptor consisting of two distinct receptor subunits, IFN-α receptor 1 (IFNAR1) and IFNAR2, leading to the activation of IFNAR-associated tyrosine kinases, Janus kinases 1 (JAK1) and tyrosine kinase 2 (Tyk2), which phosphorylate both IFNAR1 and IFNAR2 subunits, followed by the activation and phosphorylation of signal transducer and activator of transcription factors (STATs) in the canonical IFN signaling. STAT1 and STAT2 then form heterodimers and are joined by an Interferon regulatory factor 9 (IRF9) to form an active transcription factor complex known as IFN-stimulated gene factor 3 (ISGF3). ISGF3 translocates into the nucleus and binds to the IFN-stimulated response element (ISRE) to initiate transcription of ISGs to affect HBV replication or modulate host immune response (30). IFN-α exerts antiviral activity against HBV by both inducing antiviral gene products that inhibit viral replication in hepatocytes and by modulating the host immune system. Many ISGs are

known to inhibit HBV replication at different steps, including inhibiting cccDNA transcription and HBV nucleocapsid formation, suppressing the activity of HBV enhancers, as well as control the HBV replication at the post-transcriptional level (31).

The other complex formed by STAT1 homodimers binds to the GAS motif and mainly active pro-inflammatory gene expression. After STAT1 homodimers bind to the GAS enhancer elements in the promoters of IFN-stimulate genes, genes encoding pro-inflammatory cytokines and apoptotic factors are induced (32). Type I IFN can also activate STAT3 homodimers and result in the production of both pro-inflammatory cytokines and anti-inflammatory cytokines (such as interleukin-10 (IL-10). In the non-canonical IFN signaling, IFN can induce a set of genes independent of STATs, including MAPKs and PI3K, to initiate ISGs transcription (33). In a STAT-independent manner, type I IFNs activate both p38, which is an upstream activator of several genes regulated by ISREs and GAS elements, and mammalian target of rapamycin (mTOR), which regulates mRNA translation (34).

Type I IFN can activate other important members of innate immunity, including natural killer (NK) cells and natural killer T (NKT) cells, recruiting them to the infected tissues and recognize infected hepatocytes. These immune cells secrete cytokines that promote intracellular HBV clearance and induce apoptosis. Pegylated IFN-α treatment enhanced recovery of memory T cells in CHB patients by down-regulating inhibitory receptors and up-regulating effector molecules (35). The antiviral effect against HBV of IFN and its immunomodulatory function have been finely summarized in several reviews (22, 31, 34).

NEGATIVE REGULATORS IN IFN INDUCTION AND SIGNALING PATHWAY

Viruses could disrupt IFN responses by co-opting negative regulatory systems or use the antiviral system to their advantage (36), for example, HBV could hijack several ISGs either to facilitate its replication or impede innate immune response to HBV. Although viral component recognition by PRRs is essential for effective antiviral immune responses, the inflammatory immune response, including type I IFN production, has to be tightly regulated to prevent advert immune-related pathologies. Because activation of those inhibitory processes can cause IFN-α therapy suppression, understanding the molecular basis and identify related host negative regulators could provide targets for improving IFN-α treatment efficacy.

Studies showed that IFNAR1 and IFNAR2 in peripheral blood mononuclear cells and lymphocytes increased in CHB patients, but their level had a positive correlation with HBV-DNA in liver tissue (37). Several negative regulators mainly functioning to suppress IFNs expression or disturb the function or expression of IFNAR, thereby inhibit the JAK-STAT pathway and ISGs production (**Figure 1**). The related negative regulators are summarized in **Table 1**.

p38 MAPKs

Mammalian p38 mitogen-activated kinases (MAPKs) are activated by cellular stresses and inflammatory cytokines and are critical for normal immune and inflammatory response (63). Several studies showed HBV infection could activate mitogen-activated protein kinases (MAPKs), including p38 MAPK kinases, c-Jun N-terminal kinase (JNK), and extracellular signal-regulated kinases (ERKs) (64–67). p38 MAPK, but not JNK or ERK1/2, was significantly phosphorylated in Huh7 cells after HBV infection and thereby promote intracellular HBV replication (68) with the involvement of STAT3 (67). p38 MAKP also acts as a negative regulator of type I IFN pathway through unfolded protein response (UPR)-stimulated priming phosphorylation of IFNAR1, ensuring IFNAR1 down-regulation and type I IFN signaling attenuation (38). Given this knowledge as well as the important role of HBV in p38 MAPKs production, we hypothesized that negative regulation of p38 MAPKs should stabilize IFNAR1 and improve IFN therapy efficacy against chronic HBV infection, which requires further investigation.

MMP-9

Matrix metalloproteinase 9 (MMP-9) is expressed in normal leukocyte and transformed cells, and is associated with IFNAR1 degradation (39). IFNAR1 degradation can be promoted by either ligand binding and cellular signaling induced ubiquitination-dependent pathway, or Ser532 phosphorylation and p38 kinase activity requiring ligand-independent way (69). MMP-9 can be activated by HBV in CHB patients (70, 71) and in turn facilitates HBV replication through attenuating IFN/JAK/STAT signaling, interacting with IFNAR1 to promoting its phosphorylation, ubiquitination, subcellular distribution, and degradation of IFNAR1 through lysosome pathways in Ser532 phosphorylation and p38 independent manner, as well as blocking its binding to IFN-α (39). However, a recent study found type I IFN strongly promotes the clearance of MMPs (72), thus, further study is needed to investigate the role of MMPs in the IFN-α therapy mediated antiviral efficacy against HBV infection.

IFITMs

Interferon-induced transmembrane proteins (IFITMs) are a family of small proteins that localize in the plasma and endolysosomal membranes (73) and were originally described based on their expression after IFN therapy (74). The human IFITM family comprises five members, including immune-related IFITM1, IFITM2, and IFITM3, as well as IFITM5 and IFITM10 with an unknown role in immunity (75). Brass et al. first described IFITM proteins as antiviral factors that target the early life cycle steps of several viruses (76). HCV infection could be restricted by IFITM proteins by targeting the endocytosed HCV virion for lysosomal degradation (77). However, our previous study indicated IFITM2 was shuttled by exosomes to DCs and inhibit IFN-α synthesis *via* inhibiting phosphorylation of extracellular signal-regulated kinase (ERK), TANK-binding kinase 1 (TBK1), and interferon regulatory factor 3 (IRF3), thereby blocking anti-HBV efficacy of exogenous IFN-α in chronic HBV infection (40). Thus, down-regulating IFITM2

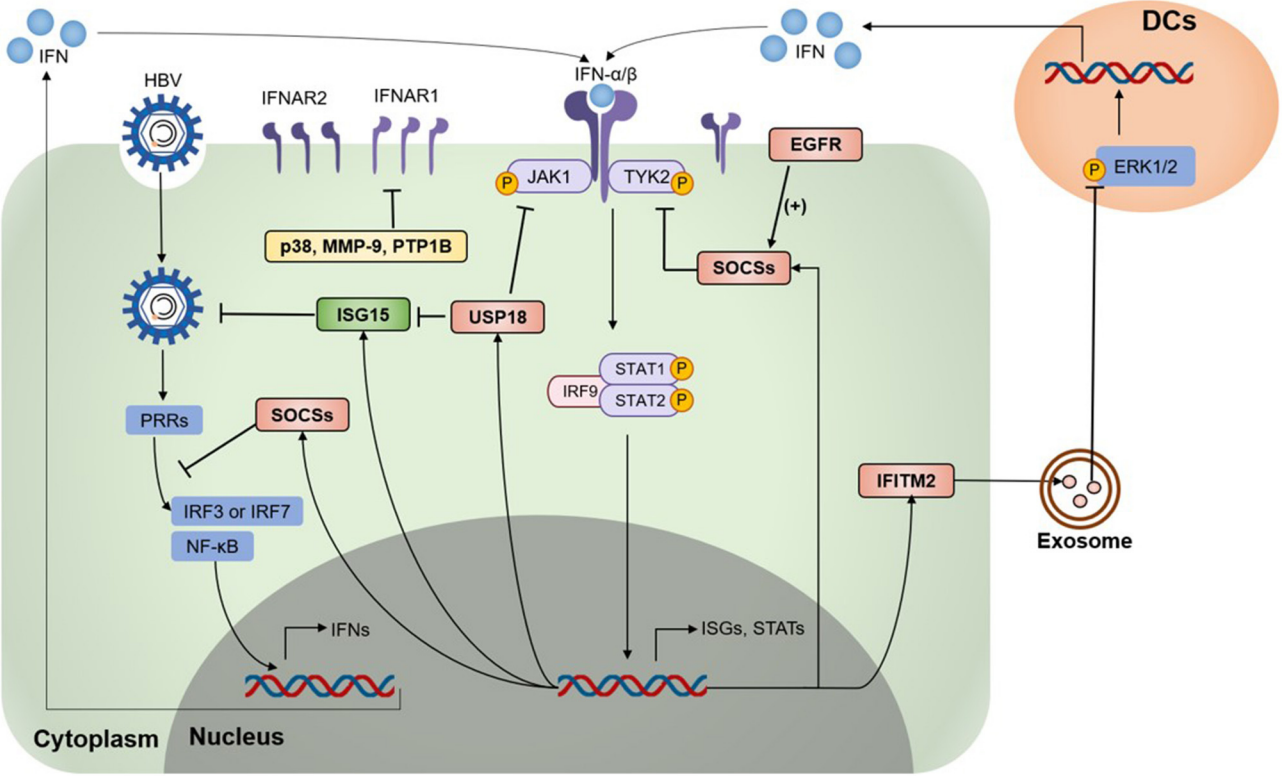

FIGURE 1 | Type I IFN signaling is down-regulated by negative regulators. Various negative regulators cross-regulate the type I interferon (IFN) response, which modulates the expression levels and activation states of IFN signaling components. On HBV infection and invasion, pattern-recognition receptors (PRRs) recognize HBV and activate the downstream pathway to induce IFN expression. IFNAR1/2 recognize type I IFN and activate Janus kinases 1 (JAK1) and tyrosine kinase 2 (Tyk2), followed by the activation and phosphorylation of signal transducer and activator of transcription factors (STATs), leading to the expression of various IFN-stimulated genes (ISGs). Several ISGs were identified as negative regulators of the IFN signaling pathway, including the suppressor of cytokine signaling (SOCS) proteins, ubiquitin-specific protease 18 (USP18), Interferon-induced transmembrane protein 2 (IFITM2), p38 mitogen-activated kinases (p38 MAPKs), matrix metalloproteinase 9 (MMP-9), epidermal growth factor receptor (EGFR), protein tyrosine phosphatases 1 B (PTP1B), and some regulators awaiting exact mechanisms. HBV, hepatitis B virus; IRF3, IFN regulatory factor 3; IRF7, IFN regulatory factor 7; ERK1/2, extracellular signal-regulated kinase 1/2; DCs, dendritic cells.

may be a potential strategy to improve the therapeutic response to IFN-α treatment.

Most negative regulators function in the various biological processes in IFN production and signaling pathways, including inhibit IFN expression, disturb IFNAR function, and suppress the expression of ISGs which serve as anti-HBV molecules (**Table 1**).

USP18 and ISG15

Ubiquitin-specific protease 18 (USP18, also known as UBP43) and Interferon stimulated gene 15 (ISG15) have been studied extensively for their negative regulation of type I IFN signaling. Various studies showed that ISG15/USP18 signaling activation is involved in IFN therapy non-response in both HBV and HCV patients (78, 79). Responding to viral infection or IFN stimulation, both USP18 and ISG15 are strongly upregulated (80). USP18 belongs to the ubiquitin protease family, which is responsible for removing ubiquitin or ubiquitin-like proteins from their conjugated substrates (81). ISG15 is a ubiquitin-like modifier that binds other proteins in a process called ISGylation, and its antiviral activity was first observed using a recombinant chimeric Sindbis virus and IFNAR$^{-/-}$ mice model (82). ISG15 conjugated to various cellular substrates *via* an enzymatic cascade: E1 activating enzyme UbE1L (83), E2-conjugating enzyme UbCH8 (84, 85), and various E3 ligase (86, 87). ISG15 targets host and viral proteins and impacts diverse cellular pathways, including RNA splicing, chromatin remodeling/polymerase II transcription, cytoskeleton organization and regulation, stress responses, and translation mainly in a conjugation-dependent manner (88, 89). By deISGylating ISG15, USP18 was stabilized by unconjugated ISG15 *via* preventing its S-phase kinase-associated protein (SKP2)-induced ubiquitylation, therefore mediates downregulation of type I IFN signaling during HBV infection (41). Independent of its deconjugating activity, USP18 also competes with JAK1 for binding to the IFNAR2 and therefore impede IFN signaling (42). Because USP18 and ISG15 are inducible by viral factors, these inhibitory proteins may be important mediators in reducing IFN therapy efficacy by negatively regulating IFN-α signaling and host immune against chronic HBV infection.

TABLE 1 | Involvement and the possible underlying mechanisms of host negative regulators in the inhibition of IFN-α therapy.

Host negative regulators	Possible underlying mechanisms
Negative regulators in IFN signaling	
p38 MAPKs	Attenuate IFN-α through down-regulating IFNAR1 by UPR-stimulated priming phosphorylation of IFNAR1 (38).
MMP-9	Inhibit IFN-α signaling *via* the promotion of IFNAR1 degradation through lysosome pathways (39).
IFITMs	IFITM2 inhibits the antiviral activity through inhibiting IFN-α synthesis by inhibiting phosphorylation of ERK, TBK1, and IRF3 (40).
USP18	USP18 attenuates IFN-α signaling *via* both ISGylating ISG15 (41) and compete with JAK1 for binding to the IFNAR2 (42).
SOCSs (SOCS 1-3 and CIS)	SOCS 1-3 and CIS suppress the efficacy of IFN-α therapy by suppress IFN-α production (43, 44) and inhibiting JAK-STAT pathway to reduce the duration of antiviral genes expression (45, 46).
EGFR	EGFR affects IFN-α therapy *via* the induction of SOCS3 (47).
SHP2	SHP2 inhibits IFN-α signaling by a PKCβ-dependent pathway (48, 49).
PTP1B	PTP1B may inhibit IFN-α signaling *via* binding and dephosphorylating IFNAR1 (50).
NT5C3	NT5C3 may inhibit the IFN-α therapeutic effect by binding and sequestering miR-122, which is an anti-HBV molecule (51).
Cytokines, chemokines and non-coding RNAs	
Peripheral IFNAR	The mechanisms are not clear and may related to oxidative stress and the disturbance of IFNAR function (37, 52).
TNF-α	TNF-α may inhibit IFN-α therapy efficacy *via* the induction of SOCS3 and SHP2 (53).
IL-10	IL-10 inhibits IFN-α signaling *via* the induction of SOCS (54); IL-10 suppresses IFN-α and IFN-γ production (55, 56).
IL-8	IL-8 suppresses the antiviral efficacy and signaling of IFN-α (57).
IL-28B genetic polymorphisms	IL-28B rs12979860 CC genotype and rs8099917 TT genotype indicate better treatment response, mechanisms are undiscovered (58).
miR-146a	miR-146a inhibits IFN-α *via* suppression of STAT1 and attenuation of ISGs production (59, 60).
miR-3613-3p	miR-3613-3p decreases the expressions of IFN-α and IFN-β through targeting CMPK1 (61).
miR-21	miR-21 promotes an anti-inflammatory response by increasing IL-10 (61).
circRNA hsa-circ-0004812	circRNA hsa-circ-0004812 impairs IFN-induced immune response by regulating FSTL1 (62).

MAPKs, mitogen-activated kinases; IFNAR, interferon-α/β-receptor; UPR, unfolded protein response; MMP-9, Matrix metalloproteinase 9; IFITMs, Interferon-induced transmembrane proteins; ERK, extracellular signal-regulated kinase; TBK1, TANK-binding kinase 1; IRF3, interferon regulatory gactor 3; USP18, ubiquitin-specific protease 18; SOCS, suppressor of cytokine signaling; CIS, cytokine-inducible SH2; EGFR, epidermal growth factor receptor; PKCβ, protein kinase Cβ; PTPs, Protein tyrosine phosphatases; STAT1, signal transducer and activator of transcription factor 1; ISGs, IFN-stimulated genes; CMPK1, cytidine monophosphate kinase 1; FSTL1, Follistatin-related protein 1.

SOCSs

In addition to USP18 and ISG15, the suppressor of cytokine signaling (SOCS) proteins and cytokine-inducible SH2 (CIS) also play important roles in downregulating IFN-α signaling, particularly the JAK-STAT signaling pathway (90), and suppress type I IFN production. To date, SOCS/CIS family includes eight proteins (SOCS 1-7 and CIS) which sharing similar structures: a central Src-homology 2 (SH2) domain, a C-terminal SOCS box, and a highly variable N-terminal region (91). The SOCS box functions to recruit an E3 ubiquitin ligase complex, so the SOCS box-containing proteins can act as substrate-recognition modules to mediate the polyubiquitination and subsequent degradation of substrate proteins by the 26S proteasome (92). Responding to IFN signaling and cytokine stimulation, SOCS 1-3 and CIS proteins are induced and negatively regulate the JAK-STAT-mediated cytokine signal responsible for its production in a classic negative feedback loop (30, 45), while SOCS4-7 primarily focus on regulating the growth factor receptor signal (93, 94). SOCS1 and SOCS3, the most widely studied proteins in this family, possess a kinase inhibitory region (KIR) at the N-terminal region that inhibits JAK tyrosine kinase activity by acting as a pseudo-substrate (95). At the same time, SOCS1 is related to IFNAR1 specific signals, thereby abrogating tyrosine phosphorylation of STAT1 and reducing the duration of antiviral genes expression (46). Apart from regulating the IFN-α signaling pathway, SOCS proteins can suppress type I IFN production by binding and degrading key molecules in the TLR signaling pathway, including myeloid differentiation factor 88 (MYD88)-adaptor-like (Mal) protein (43) and IRF7 (44). HBV increased SOCS1 and SOCS3 expression and resulting in sustained STAT3 activation (96, 97). Thus, the SOCS family, especially SOCS1 and SOCS3, inhibit both type I IFN production and IFN-α signaling pathway in HBV infection and provides targets for improving IFN-α-mediated antiviral effect.

EGFR

Epidermal growth factor (EGF) receptor (EGFR) is a transmembrane protein that belongs to the ErbB family of receptors and is well-studied for its association with a number of cancers. EGFR has recently been shown to be a host entry cofactor triggering HBV internalization (98). Inhibition of EGFR activity enhances the antiviral efficacy of IFN-α by both inducing SOCS3 expression to reduce IFN-α-induced STAT3 activity and enhancing STAT1-mediated antiviral response. Moreover, EGFR inhibitors inhibited the replication, antigen syntheses, and cccDNA reservoir of HBV (47). Thus, EGFR inhibitors could be an important candidate that facilitates IFN-α therapy against HBV infection.

PTPs

Protein tyrosine phosphatases, known as PTPs, is a large family that encodes enzymes that are divided into the classical, phosphotyrosine (pTyr)-specific phosphatases and the dual specificity phosphatases (DSPs) (99), regulating the phosphorylation state of many important signaling molecules. The overexpression of HBx or NF-κB led to increased SHP2 expression, which is a ubiquitously expressed PTP, *via* NF-κB binding to the *Shp2* promoter (100). SHP2, encoded by Ptpn11, containing two SH2 domains and acts as a negative regulator of IFN-induced STAT activation in a protein kinase Cβ (PKCβ)-dependent pathway (48, 49). PTP1B is another PTP protein and is capable of binding and dephosphorylating IFNAR1 and thereby suppress type I IFN signaling in human cells. PTP1B inhibitors robustly augmented the type I IFN-mediated antiviral effects against HCV (50). However, the knowledge on PTP1B expression and its function needs further study in chronic HBV infection. Currently, it is estimated that as many as 125 PTP genes are in the human genome, and most of them have not been identified (101). Thus, additional studies are required to identify PTP proteins that suppress the IFN-α signal pathway and therefore attenuate therapy in chronic HBV infection.

Other Molecules

Apart from the above-mentioned molecules, other molecules involved in the down-regulation of IFN therapy efficacy include NEIL3, TDG, and NT5C3, but the exact biological mechanisms underlying the influence of these molecules in IFN-α treatment response remain to be determined. It is reported that positive response to IFN-α treatment in CHB patients is associated with a lower level of NEIL3 and thymine DNA glycosylase (TDG), two intra-hepatic base excision repair (BER) genes (102), but the underlying mechanism is unknown. MiR-122, a miRNA that has been demonstrated to inhibit HBV replication by directly targeting the HBV pre-genomic RNA sequence or by indirectly modulating HO-1 and CCNG1/p53 pathways (103), could be sequestered by an ISG, NT5C3 (51). Therefore, NT5C3 could efficiently inhibit miR-122 and suppress IFN therapeutic efficacy. However, the function of NT5C3 and miR-122 need further confirmation in clinical research. In addition, numerous molecules have been shown to negatively regulate type I IFN signaling, including DCST1, PIAS1, and so on (32, 104), although further studies are needed to clarify their function in HBV infection.

ROLE OF CYTOKINES, CHEMOKINES, AND miRNAs IN NEGATIVE REGULATION OF IFN-α THERAPY IN CHB INFECTION

Peripheral IFNAR

In general, the anti-HBV activity of type I IFN is mediated through binding to their unique receptors, following by JAK-STAT pathway activation and ultimately ISGs production. Cell-surface IFNAR consists of 2 major subunits: IFNAR1 and IFNAR2. However, soluble IFNAR was reported as an inhibitory factor of IFN therapy in both HBV and HCV infection (52, 105). The expression of IFNAR1 and IFNAR2 in lymphocytes and monocytes was increased in CHB patients which was negatively correlated to plasma glutathione S-transferase (GST), a class of cytosolic enzyme participated in the protection of cell from reactive oxygen species (ROS), suggesting that oxidative stress play an important role in the upregulation of IFNAR in CHB patients (37, 52). Oxidative stress could modulate protein misfolding and then disrupt the biological protein conformation. Therefore, oxidative stress may influence IFNAR function, further studies are required to find out the mechanism.

TNF-α

TNF is a pleiotropic cytokine that exerts homeostatic and pathogenic bioactivities (106). Elevated levels of TNF-α are observed in serum and hepatocytes of patients with acute or chronic hepatitis B (107), possibly through ERKs and NF-κB pathway (108). TNF-α may play a role in downregulating HBV replication in hepatocytes by non-cytopathic mechanisms and synergizing with IFN in suppressing viral replication (109, 110). On the other hand, TNF-α was reported as a negative regulator during IFN-α production determined by HBsAg (55). It is also reported that the injections of synthetic TNF-α could inhibit IFN-α-induced signals in the liver and both SOCS3 and SHP2 contributes to the inhibitory effect (53). However, anti-TNF-α-agents may lead to enhanced HBV replication and cause reactivation of HBV infection in HBsAg carrier and Occult carrier (anti-HBc+) patients (111). Consequently, the ultimate impact of TNFα-mediated effects on IFN therapy against HBV is a question that remains to be resolved.

IL-10

Interleukin 10 (IL-10), also known as cytokine synthesis inhibitory factor (CSIF), is a pleiotropic cytokine with anti-inflammatory and immunosuppressive activities. HBcAg stimulated IL-10 production by CD4+ and CD8+ cells, as well as by monocytes from CHB patients, which serves as a viral strategy to downregulate the host immune response and allow viral persistence in the host (112). HBsAg inhibited the production of IFN-α by pDCs through the induction of monocytes that secreted IL-10 (55). IL-10 also suppresses NK cell IFN-γ production without altering cytotoxicity or death-ligand expression (56). In addition, SOCS1 and SOCS3 are both produced in response to IL-10, and both function as negative regulators during JAK-STAT signaling (54). On the other hand, evidence suggests that heterogeneity in the promoter region of the IL-10 gene has a role in determining the initial response of HBV infection to IFN-α therapy. IL-10 variants were more frequent among virologically sustained response (SR) compared with non-responders (NR) to IFN-α-2b. Carriage of the−592A allele,−592A/A genotype, and−1082/1819/-592 ATA haplotype was associated with SR (113).

IL-8

Interleukin 8 (IL-8), or chemokine (C-X-C motif) ligand 8 (CXCL8) is a pro-inflammatory chemokine produced by various cell types to recruit leukocytes to the sites of infection or

tissue injury (114). It has been demonstrated that serum IL-8 amounts is elevated during HBV reactivation, and HBeAg-negative patients had significantly higher levels of IL-8 transcripts in the liver than HBeAg-positive patients (115). Accumulating evidence indicates that IL-8 may contribute to counteracting IFN-α antiviral action (116, 117). During the IFN challenge, IL-8 expression induced by HBV can impair the ability of endogenous IFN-α to inhibit early stages of viral replication, thus facilitate viral persistence, and can also contribute to the poor response to IFN-α treatment (57). IL-8 is induced by HBV through various mechanisms, but the mechanism underlying the IL-8 affecting the anti-HBV function of IFN-α therapy still needs further discussion.

IL-28B

Interleukin-28B (IL-28B), also known as IFN-λ3, has been reported by numerous studies that its genetic polymorphisms are related to the therapeutic efficacy of PEG-IFN-α. Sonneveld et al. reported the proportions of IL-28B genotypes were 77, 19, and 5% for AA/AG/GG at rs12980275 and also for CC/CT/TT at rs12979860, respectively (118). rs12980275 genotype AA and rs12979860 genotype CC were associated with a higher probability of HBeAg seroconversion (118). A meta-analysis summarized that IL-28B rs12979860 CC genotype and rs8099917 TT genotype indicated a better treatment response than non-CC and non-TT genotypes for PEG-IFN-α in patients with CHB (58).

miRNAs

MicroRNAs (miRNAs) are a class of non-coding small RNAs that play important roles in modulating gene expression mainly by base-pairing to the 3' translational region (3' UTR) of mRNAs. miRNAs have been reported to be widely involved in antiviral immunity and some studies show miRNA plays a role in HBV-host interaction. HBV could change the expression profiles of cellular miRNA and escape host immune clearance (119). It was shown that nearly 90% of the total miRNAs in the liver are comprised of ∼10 miRNAs and miR-122 is the major contribution with 50–70% of the miRNA profile (120). Zhang et al. identified 11 response-related miRNAs, including let-7a, miR-30a, miR-1290, miR-106b, miR-1224-5p, miR-939, miR-1281, miR-198, let-7f, miR-22, and miR-638, by performing miRNA microarray analysis of plasma samples and liver biopsy samples from CHB patients who received IFN therapy and using the OneR feature ranking and incremental feather selection method to ultimately establish a prediction model for predicting the initial response of HBV patients receiving IFN injections (121). Not all of the 11 miRNAs in the predicted model is negatively associated with the IFN-α therapy efficacy. Results obtained by Tan et al. suggest that the aberrant expression of miRNAs was associated with different therapy outcomes, and monitoring the fluctuation patterns of miRNAs was important for predicting the IFN-α therapeutic effects. They also identified 11 miRNAs to be associated with the effect of HBV therapy, namely, let-7d-5p, let-7f-5p, let-7i-5p, miR-122-5p, miR-126-3p, miR-1307-3p, miR-181a-5p, miR-21-5p, miR-425-5p, miR-652-3p, and miR-320a-3p, with 9 miRNAs significantly declined after therapy in the responder group: let-7d-5p, let-7f-5p, let-7i-5p, miR-126-3p, miR-1307-3p, miR-181a-5p, miR-21-5p, miR-425-5p, and miR-652-3p (122).

miRNAs could modulate the IFN-α therapy effect in multiple processes, including type I IFN production, IFN signaling pathway, as well as ISGs transcription and translation. The effect of miRNA on IFN and its signaling pathway has been well-illustrated in many reviews (123, 124), indicating that miR-130a, let-7b, miR-26a, miR-24, miR-146a, and many other miRNAs could down-regulate the effect of IFN therapy. However, only a few miRNAs are studied extensively in CHB patients. HBV infection promotes miR-146a transcription, which suppressed STAT1 and ultimately attenuated the production of type I IFN-induced antiviral factors, as well as impairing T cell function, resulting in IFN resistance and immune defects during chronic HBV infection (59, 60). miR-3613-3p was upregulated in the CHB patients suppressed IFN-induced anti-HBV immune response by targeting cytidine monophosphate kinase 1 (CMPK1) (61), a kinase responsible for the metabolism of CMP, UMP, and deoxycytidine analogs (125), and ultimately decrease the relative expressions of IFN-α and IFN-β. miR-21 has been reported to be associated with the effect of HBV therapy (122) and promote an anti-inflammatory response by increasing IL-10 production through down-regulating programmed cell death 4 (PDCD4) (126). But the detailed mechanism of miR-21 affecting the anti-HBV effect of IFN-α therapy still needs further investigation.

Some miRNAs exhibit an immune-modulating or IFN therapy enhancing effect in HBV infection. HBeAg could elevate miR-212-3p expression in macrophages, and thereby decrease the production of cytokines through targeting MAPK1 (127). Higher pretreatment plasma miR-301a-3p and miR-145-5p levels were observed in CHB patients who achieved combined response or HBsAg loss following peg-IFN/NA therapy compared to non-responders (120). miR-155 enhances innate antiviral immunity against HBV through promoting JAK/STAT signaling pathway by targeting SOCS1 and mildly inhibits HBV infection in human hepatoma cells (128). In conclusion, miRNAs play an important role in regulating the efficiency of IFN therapy in CHB patients, and could act as predictive factors of IFN therapy outcome, or be used for discovery for better treatment strategies. But the mechanism underlying these miRNAs calls for more attention.

LncRNAs and circRNAs

Long non-coding RNAs (lncRNAs) are a subset of non-coding RNAs >200 nucleotides in length and have recently been suggested to act as both positive and negative regulators in viral infections utilizing various mechanisms, mediated by their specific sequences or structural motifs that bind DNA, RNA, or protein (129). Recent study suggests that HBx-LINE1, a hybrid RNA transcript of the human LINE1 and the HBV-encoded X gene can serve as a sponge for sequestering miR-122 and may therefore promote HBV expression and replication, but whether HBx-LINE1 function during IFN-α treatment is still unknown (130). On the other hand, lncRNA#32 has been reported to be positively associated with type I IFN signaling in HBV infection by regulating APOBEC3A and APOBEC3G expression (131). Given the important and complicated roles of lncRNAs in IFN

anti-HBV treatment, more extensive studies are required to develop a more comprehensive profile of lncRNAs in HBV infection and IFN therapy.

Circular RNAs (circRNAs) are closed long non-coding RNAs and can bind miRNA and act as a miRNA sponge to regulate gene expression, they may also play a role in negatively regulating IFN therapy outcomes. Among top ten circRNAs significantly up-regulated in CHB compared to normal controls, circRNA has-circ-0004812 impairs IFN-induced immune response by sponging miR-1287-5p to regulate Follistatin-related protein (FSTL) 1 in chronic hepatitis B (62, 132). Several circRNAs have been demonstrated to function in HBV infection, including hsa_circ_0005389, hsa_circ_0000038, hsa_circ_0000650, hsa_circ_4099, hsa_circ_0000976, hsa_circ_0007750, hsa_circ_0027089, and hsa_circ_100338, but it is still unknown whether they function in IFN-α therapy of HBV infection (133, 134). Therefore, due to presently poor understanding of circRNAs expression, function and regulation, further studies are desired to identify the mechanisms behind different circRNA regulation patterns associated with IFN-α treatment in HBV infection.

HOST CLINICAL PARAMETERS IN NEGATIVELY REGULATION OF IFN-α THERAPY

Patients' clinical characteristics are parameters that provide direct information of liver function and have been used for predicting the efficacy of IFN-α therapy. The appearance or upregulation of several parameters is associated with a lower response rate in IFN-α treatment (**Table 2**). Published data have also demonstrated that HBV serum markers, including HBsAg, HBeAg, and HBV DNA, are baseline predictors of IFN therapy response, which has been summarized in several reviews (153, 154).

Age

Patient age is a host factor that is related to IFN-α therapy responsiveness in Chronic HBV infection. Generally, it is believed that younger individuals are more likely to develop a sustained virological response to IFN-α treatment than older individuals. Bonino et al. reported that patients under 40 years of age had higher combined response rates than patients over 40 years of age, regardless of which treatment they received (with or without lamivudine) ($p < 0.024$; OR: 1.3 per 10-year decrease; 95% CI: 1.0–1.7), indicating that aging imparts a negative influence in the IFN-α treatment efficacy (135). Similar results were showed in other clinical researches (136). Aging enhances the vulnerability to liver injury mainly by increasing oxidative stress and inflammatory response, accelerating cellular senescence, as well as suppressing mitochondrial function (137). A retrospective study showed that age parameters were also associated with significant histological abnormalities in patients with persistently normal ALT levels (PNALT) (155). Moreover, impairments of cellular, humoral, and innate immunity in the elderly may be another explanation responsible for decreased virological response to IFN-α therapy in older patients (138).

Gender and Hormone

Gender is another important host factor associated with IFN-mediated antiviral efficacy against HBV infection. It is summarized that females are more likely to achieve HBeAg seroconversion after IFN therapy comparing to males (10, 139), which may be an effect of sex hormones. Androgen-androgen receptor (AR) complex activates HBV RNA transcription through binding to the 2 response elements inside Enh I, while estrogen and/or its receptor suppressed HBV replication (156). The expression level of estrogen receptors (ESRs) in the cytosol of peripheral blood mononuclear cells (PBMCs) was significantly lower in patients with CHB than in healthy controls, and ESR1 mRNA expression at week 4 of PEG IFN-α treatment was a better indicator for treatment response although the underlying mechanism is still unknown (140). Further studies are required to clarify the effect of sex hormones in IFN-α therapy.

ALT Levels

Alanine transaminase (ALT) is a transaminase enzyme and is clinically measured as a biomarker of liver injury. ALT is associated with significant histological characteristics to some extent in HBeAg-positive and -negative patients and its normalization are related to favorable long-term CHB infection treatment outcomes. Several studies indicated that a high baseline ALT level can be a predictor of sustained treatment response (10, 139, 141).

Bile Acids

Bile acids are steroid acids and were found to be related to the IFN-α treatment. Elevated hydrophobic bile acid concentration impairs IFN-α treatment in patients with CHB by inhibiting Jak1- and Tyk2-phosphorylation, resulting in a decreased mRNA and protein expression of ISGs, including myxovirus resistance protein A (MxA) and dsRNA-activated protein kinase (PKR) (142). The uptake of bile acid into hepatocytes is induced by Na^+-taurocholate cotransporting polypeptide (NTCP), which also plays an essential role in HBV entry into the target cells. HBV infection may promote the expression of genes involved in lipid and bile acid metabolism by binding to NTCP and limits its function, thus promoting compensatory BA synthesis (157).

Obesity and Hepatic Steatosis

High body mass index (BMI) or obese status is related to the presence of steatosis in CHB patients. Clinical researches showed that the occurrence of hepatic steatosis is significantly high in CHB patients, decreasing the sustained viral response (SVR) rates to PEG-IFN (143–145). Recent studies indicated that obesity attenuates and prolongs the type I IFN response that shows antiviral inefficacy. Obese individuals have a high level of blood leptin, resulting in the upregulation of the SOCS3. The elevated leptin and SOCS3 levels could reduce type I IFN response and cause other immune dysfunction associated with T cells and B cells in people with obesity (146).

TABLE 2 | Involvement of host parameters in reflecting the IFN-α therapy efficacy in CHB patients.

Host parameters	Modulation of IFN-α therapy	Possible underlying mechanisms
Age	Older individuals have lower response to IFN-α treatment than younger individuals (135, 136).	Impairments of host immunity and more advanced liver disease in the elderly may be responsible for a poor response in older individuals (137, 138).
Gender	Females are more likely to have a sustained response to IFN-α (10, 139).	Estrogen may enhance the efficacy of IFN-α therapy (140).
ALT*	High baseline level of ALT may indicate sustained treatment response (10, 139, 141).	ALT is associated with liver injury.
Bile acids	Individuals who have elevated hydrophobic bile acid concentration may have lower response to IFN-α therapy (142).	Bile acids impairs IFN-α treatment by inhibiting Jak1- and Tyk2-phosphorylation and ISGs expression (142).
Obesity	High BMI* and hepatic steatosis are related to a decreased sustained viral response rate to PEG-IFN (143–145).	Reduce type I IFN response through upregulating SOCS3 and leptin; cause other immune dysfunction associated with T cells and B cells (146).
Insulin resistance	Insulin resistance was associated with virological response to HBeAg-positive immune-reactive CHB patients' therapy with IFN-α (147).	Insulin resistance state affect IFN-α efficacy mainly through downregulating IFN-γ, TNF-α and multiple cytokines (147, 148).
Alcohol	IFN-α therapy is ineffective in those who have alcohol abuse.	Alcohol decrease T cells activation and function and impairs IFN production (149); inhibit IFN-γ-signaling through JAK-STAT1 pathway (149).
Anti-IFN antibodies	Anti-IFN antibodies negatively influence the antiviral effect at early stages of the IFN-α therapy (150–152).	Anti-IFN antibodies may attenuate IFN-α therapy *via* neutralizing effect (150–152).

**ALT, alanine transaminase; BMI, body mass index.*

Insulin Resistance

The relationship between HBV infection and insulin resistance (IR) has not been completely elucidated, but it is reported that HBV infection was associated with increased IR (158). Researches illustrated that IR was associated with virological response to HBeAg-positive immune-reactive CHB patients' therapy with IFN-α mainly through downregulation of IFN-γ, TNF-α and multiple cytokines (147, 148). On the other hand, IFN-α was reported to be a catastrophic feature of the diabetic islet microenvironment, although the conclusion is controversial (159, 160). Therefore, the relationship of diabetes and IFN-α treatment is complicated and need further study.

Alcohol

Alcohol abuse causes rapid progression of liver disease in HBV infected patients and allows HBV to persist chronically mainly by increasing HBV replication, inducing oxidative stress, and suppressing the immune response. As reported, alcohol reduces the number of T-cells and changes the ratio of T-cell types, resulting in decreased T-cell activation and function, thereby impair IFN production (149). On the other hand, the combination of HBV and ethanol metabolites impairs IFN-γ-signaling through the JAK-STAT1 pathway (149). Moreover, alcohol and HBV synergistically promote hepatic steatosis (161), which has been reported as another negative host factor of IFN therapy against HBV infection.

Anti-IFN Antibodies

Endogenous anti-IFN antibodies have been reported to be stimulated after the treatment with IFN. Multiple studies indicated that anti-IFN antibodies may negate the antiviral effects of IFN-α by neutralizing the effect (150–152). Anti-IFN was significantly more likely to develop in patients who received lower doses (2.5 or 5 MU/m^2) of IFN than in those who received a higher dose (10 MU/m^2) (151). Another study showed that the anti-IFN activity may negatively influence the effect of the IFN therapy on CHB patients at the early stages of the therapy, but the appearance of the anti-IFN activity at the end of a long-term IFN therapy has no significant influence on the treatment outcome (152).

CONCLUDING REMARKS

Improving the efficacy of IFN-α for chronic HBV infection as well as understanding the mechanisms underlying HBV- and host-mediated resistance to IFN-α therapy remains a challenge. Identifying and inhibiting the pathways or molecules that negatively regulate IFN-α signaling and therapy, as well as finding host parameters that predict the response of patients with CHB to antiviral therapy are obvious ways to improve the efficacy of IFN-α. Diversity negative factors function at many levels of IFN signaling: the recognition of HBV by the host immune system, the production of IFNs, the recognition of IFNs by their receptors, and the JAK-STAT signaling pathway.

IFN-α therapy prescript with inhibitors of negative regulators is a potential therapeutic strategy for achieving a clinical cure. However, the concentration, duration, and tissue specificity of the negative regulators are important parameters in their synergistic effect, and we need to know more. We may need further investigation to identify an appropriate level and ratio among the inhibitors of different regulators to suitably inhibit the negative regulators, limit the toxicity, and enable a return to homeostasis in a majority of CHB patients. For negative regulators with different duration of effects, for example, before, during, and after

the IFN-α therapy, the inhibition strategy may be appropriate and desirable to be flexible. Inhibitors are better to function at the same biological procedure during the IFN-α therapy, so that enhancing its anti-HBV effect accurately. For negative regulators mainly expressed in the liver tissue, the effect of inhibition should be limited in the liver to minimum side effects.

Negative host factors also serve as predictive factors for predicting the IFN-α therapy treatment outcome of CHB patients before and during the therapy. Host parameters including age, gender, ALT level, and BMI have been recommended in guidelines and widely used in deciding whether to administer IFN-α therapy for CHB patients. miRNA and cytokines are fluctuating factors important for predicting the IFN-α therapeutic effects that worth and easy to be monitored. Some negative factors only saw upregulation in liver cells with the procedure of liver biopsy, thus, these molecules should be put last on the list of predictors.

In addition to the host negative factors mentioned above, host genetic factors can provide unexpected regulators of IFN-α therapy and influence the therapeutic effects in complicated manners, which has been well-summarized in other reviews (162, 163). Single nucleotide polymorphisms (SNPs) could also serve as potential markers that predict hepatitis B patient response (164). When combined with basic clinical parameters and other genetic and epigenetic markers, more reliable treatment indexes can be developed and ultimately applied to the clinic.

Thus, as discussed above, IFN therapy needs more novel and reliable biomarkers to improve management, and novel combination strategies to substantially increase the efficacy of treatment of CHB patients.

AUTHOR CONTRIBUTIONS

JW, LD, and HT contributed to the design of the review. JW and LD searched the database and wrote the first draft of the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

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Changes and Clinical Significance of PIVKA-II in Hepatitis E Patients

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Increased protein induced by vitamin K absence or antagonist-II (PIVKA-II) levels had been widely reported in patients with hepatocellular carcinoma (HCC) and chronic hepatitis. However, the role of PIVKA-II in hepatitis E is unclear. The aim of this study was to clarify the changes related with PIVKA-II and its clinical significance in hepatitis E. We enrolled 84 patients with hepatitis E hospitalized in two hospitals from December 2019 to June 2021. The levels of serum PIVKA-II and related serological indicators in the patients were determined to elucidate the role of PIVKA-II in hepatitis E. We observed that 59.51% (50/84) of patients showed an increase in PIVKA-II levels. Compared with the normal PIVKA-II group (<32 mAU/L), patients in the elevated PIVKA-II group (>32 mAU/L) had much higher serum total bilirubin (TBIL), direct bilirubin (DBIL), indirect bilirubin (IBIL), and total bile acid (TBA) levels ($p < 0.05$ for each). Compared with the slightly elevated PIVKA-II group (32–125 mAU/L), patients in the significantly elevated PIVKA-II group (>125 mAU/L) had much lower serum albumin, alanine aminotransferase (ALT), aspartate transaminase (AST) levels, and longer days for the hospital stay ($p < 0.05$ for each). The association of PIVKA-II with TBIL and DBIL was an inverted U-shaped curve with an inflection point at 199.1 mAU/L). The association of PIVKA-II with IBIL was a U-shaped curve with an inflection point at 18.6 mAU/L while the association of PIVKA-II with albumin was an inverted U-shaped curve with an inflection point at 18.6 mAU/L. With the improvement of the disease, PIVKA-II levels were gradually decreased and finally returned to normal. This trend was consistent with that of bilirubin, and a peak appeared in the third week. Therefore, findings from our study show that the increase in PIVKA-II levels can be related to the degree of hepatic insufficiency in patients with hepatitis E, wherein PIVKA-II levels are transiently increased, and the trend of change can be related to the disease course.

Keywords: hepatitis E, PIVKA-II, liver function, liver insufficiency, acute hepatitis

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INTRODUCTION

Hepatitis E is an acute self-limiting disease caused by the hepatitis E virus (HEV) and is mainly transmitted through the fecal-oral route (1). According to statistics from the WHO, approximately 20 million people worldwide are infected with HEV each year, with more than 3 million cases of acute hepatitis E and 70,000 patient deaths (2). Thus, hepatitis E has become one of the most important public health problems worldwide.

Protein induced by vitamin K absence or antagonist-II (PIVKA-II), or des-gamma-carboxy prothrombin (DCP), is produced when the body is deficient in vitamin K and prothrombin cannot be shuttled into prothrombin or prothrombin is not fully shuttled, increasing the prothrombin levels and, consequently, PIVKA-II levels (3, 4). PIVKA-II is often used for the auxiliary detection of patients with hepatocellular carcinoma (HCC) accompanied with Alpha-fetoprotein (AFP) (5). Large scales of studies had analyzed PIVKA-II for patients with HCC using patients with chronic hepatitis as the control group and found slightly elevated PIVKA-II in patients with chronic hepatitis (6). We speculated that there might also be an increase of PIVKA-II in acute hepatitis, and the increase might be related to the degree of liver damage. Moreover, the role of PIVKA-II in hepatitis E is still unclear. Thus, we aimed to explore the relationship between PIVKA-II levels and HEV infection in this study, to obtain a better understanding of the role of PIVKA-II in hepatitis E.

MATERIALS AND METHODS

Patients

We enrolled 84 patients with hepatitis E in this study, including 69 patients who were referred to the First Affiliated Hospital of Wenzhou Medical University from December 2019 to June 2021 and 15 patients who were admitted to Taizhou Hospital of Zhejiang Province from December 2019 to June 2021. Inclusion criteria were as followed: HEV infection was diagnosed by testing for anti-HEV immunoglobulin M (IgM) using a serum enzyme-linked immunosorbent assay (ELISA) test. Hepatitis E cases were defined based on positive serum anti-HEV IgM, combined with clinical presentation of acute hepatitis (e.g., elevated liver enzymes and/or jaundice and/or non-specific symptoms, such as fatigue, itching, and nausea). The following exclusion criteria were established as previously reported (7): (1) use of antibiotics during the previous month; (2) current bacterial or fungal infections; (3) co-infection with hepatitis A virus, hepatitis B virus, or hepatitis C virus, or the presence of alcoholic fatty liver disease; (4) drug-induced liver disease; (5) autoimmune liver disease; (6) liver cancer, reproductive embryonic cancer, and/or female pregnancy; (7) co-infection with cytomegalovirus or Epstein–Barr virus; (8) presence of metabolic associated fatty liver disease; (9) approval for liver transplantation; and (10) incomplete data.

Studies involving human participants were reviewed and approved by the ethics committee of the First Affiliated Hospital of Wenzhou Medical University and Taizhou Hospital of Zhejiang Province (approval number: no. 2021-zz-162). Due to the retrospective study design involving electronic health records and no additional interventions, written informed consent was waived from the patients or their relatives.

Measurement of the PIVKA-II and Other Serological Indicator Levels

Serum levels of PIVKA-II were detected using an Abbott I 1000 automatic immunoassay analyzer. Peripheral blood was obtained from each patient, no matter fasting or not. The serum was obtained by centrifuging for 5 min at 3,000 rpm and stored at −80°C until testing. All serum samples were kept in duplicate. All the operational processes regarding the measurement of PIVKA-II were blind to measurers. Detection of HEV IgM *via* ELISA was performed by Shanghai Kehua Biological Engineering Co., Ltd. Alanine aminotransferase (ALT), aspartate transaminase (AST), alkaline phosphatase (ALP), γ-glutamyl transpeptidase (GGT), total bilirubin (TBIL), direct bilirubin (DBIL), indirect bilirubin (IBIL), total bile acid (TBA), and albumin levels were analyzed using a Beckman AU5800 automatic biochemical detector. AFP was analyzed using a Beckman DXI800. The prothrombin time (PT) was analyzed using the Stago R Max. The above reagents were all original kits, and the standard operating procedure was strictly followed during the test.

Statistical Analysis

All statistical analyses were performed using SPSS version 26.0, EmpowerStats (http://www. empowerstats.com), and package R (http://www.Rproject.org). For continuous data, the data were displayed as median (minimum–maximum) or as the actual value of the classification data. Number (%) was for categorical variables. Baseline features were summarized using descriptive statistics. Groups were compared using chi-square tests for categorical variables, Mann-Whitney U tests were used for continuous variables for comparing two independent groups. A $p < 0.05$ was considered to be statistically different.

RESULTS

Baseline Characteristics Based on Normal or Elevated PIVKA-II

The flow chart for screening the patients with hepatitis E is shown in **Figure 1**. A total of 84 patients with hepatitis E aged 25–77 years were included in our study, with the baseline characteristics according to normal PIVKA-II or elevated PIVKA-II are presented in **Table 1** and **Figure 2**. Of them, 67 were men and 17 were women. The median age was 53 years old, ranging from 25 to 77. Since the range of reference value for PIVKA-II is 11–32 mAU/L, we adopted 32 mAU/L as the cut-off value to divide the 84 patients into the normal group (n = 34) with PIVKA-II ≤32 mAU/L and elevated group (n = 50) with PIVKA-II >32 mAU/L. The median level of PIVKA-II in normal group was 24.6 mAU/L range from 10.6 to 31.4 mAU/L, while their counterpart in the elevated group was much higher as 53.8 mAU/L with significant statistical differences ($p < 0.001$). Compared with the normal PIVKA-II group, patients in the elevated PIVKA-II group had much higher serum TBIL, DBIL, IBIL, and TBA levels ($p < 0.05$ for each). There were no significant differences in the distribution of age and gender between the two groups. Moreover, no significant differences were found in levels of albumin, ALT, AST, ALP, GGT, AFP, PT, and days for the hospital stay.

Association Between Clinical Biochemical Indexes and Degree of Elevated PIVKA-II

Following Marrero (8), we used 125 mAU/ml as the grouping cut-off value. Further, we divided the above 50 patients with elevated PIVKA-II levels into two groups according to the degree

of elevation: the slightly elevated group with PIVKA-II 33–125 mAU/ml and the significantly elevated group with PIVKA-II >125 mAU/L. The median level of PIVKA-II in the significantly elevated group was 318.1 mAU/ml, much higher than their counterpart of 48.8 mAU/ml in the slightly elevated group ($p < 0.001$). Compared with the slightly elevated PIVKA-II group, patients in the significantly elevated PIVKA-II group had much lower serum albumin, ALT, AST levels, and longer days for the hospital stay ($p < 0.05$ for each). However, there were no significant differences in the serum TBIL, DBIL, IBIL, ALP, GGT, and TBA levels between the two groups. The detailed information is shown in **Table 2** and **Figure 2**.

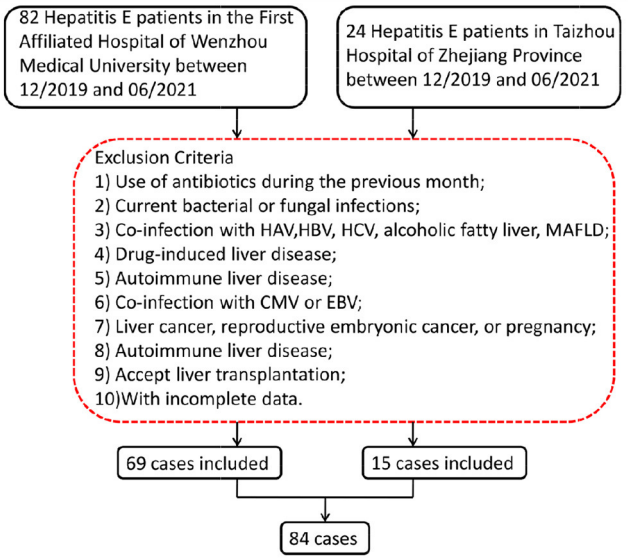

FIGURE 1 | Screening flow chart for patients with hepatitis E. HAV, hepatitis A virus; HBV, hepatitis B virus; HCV, hepatitis C virus; MAFLD, metabolic associated fatty liver disease; CMV, cytomegalovirus; EBV, Epstein–Barr virus.

Changes of PIVKA-II and Clinical Biochemical Indicators During Hospitalization

During hospitalization, the serological indicators and PIVKA-II levels of the patients were monitored. As the disease gradually improved, the trend in the PIVKA-II and TB levels was similar; that is, both had peaks that appeared in the third week, which was 2 weeks later than that of transaminase. The peak of AFP appeared was 1 week later than that of PIVKA-II in the fourth week is shown in **Table 3**.

The Correlation Between PIVKA-II With Bilirubin and Albumin Levels

The scatter diagram and smooth curve fittings used to characterize the non-linear relationship between PIVKA-II with TBIL, DBIL, IBIL, and albumin levels are shown in **Figures 3**, **4**. PIVKA-II is positively correlated with total bilirubin ($r = 0.563$, $p = 0.00$), positively correlated with direct bilirubin ($r = 0.556$, $p = 0.00$), positively correlated with indirect bilirubin ($r = 0.357$, $p = 0.00$), and negatively correlated with albumin ($r = -0.264$, $p = 0.006$).

The association between PIVKA-II with TBIL and DBIL was an inverted U-shaped curve, with the point of inflection

TABLE 1 | Baseline characteristics of patients with hepatitis E.

Parameter	Total($n = 84$)	Normal group ($n = 34$)	Elevated group($n = 50$)	*P* value
Age (years)	53 (25–77)	52 (29–77)	52 (25–74)	0.616
Gender				0.084
Male	67 (79.8%)	24 (70.6%)	43 (86.0%)	
Female	17 (20.2%)	10 (29.4%)	7 (14.0%)	
TBIL (μmol/L)	115.5 (7–460.3)	42.0 (7.0–236.0)	120.5 (12.0–460.3)	<0.001
DBIL (μmol/L)	94.6 (2–396)	27.8 (2.0–232.0)	95.5 (6.0–396.0)	<0.001
IBIL (μmol/L)	21.0 (4–164.7)	10.6 (4.0–77.9)	16.0 (4.0–164.7)	0.015
Albumin (g/L)	35.8 (23.9–46.4)	36.2 (23.9–46.4)	35.5 (27.0–45.6)	0.512
ALT (U/L)	1,405.14 (25–5,406)	724.0 (25–5,406)	1,217.0 (32.0–4,228.0)	0.219
AST (U/L)	772.7 (22–6,436)	166.0 (22–6,436)	382.5 (33.0–5,720.0)	0.111
ALP (U/L)	208.8 (61–475)	174.0 (61.0–404.0)	201.0 (84.0–475.0)	0.155
GGT (U/L)	276.4 (31–903)	232.5 (34.0–640.0)	209.5 (31.0–903.0)	0.781
TBA (μmol/L)	168.1 (2–477)	104.1 (2.0–477.0)	238.5 (2.4–450.0)	0.039
AFP (ng/mL)	46.4 (1.22–474.0)	33.2 (1.2–340.1)	20.4 (1.5–474.0)	0.830
PT (s)	21.5 (10–337)	14.0 (12.2–337.0)	13.5 (10.0–19.8)	0.247
PIVIK-II (mAU/L)	66.7 (10.6–546.1)	24.6 (10.6–31.4)	53.8 (33.0–9,235.0)	<0.001
Hospital days	13.7 (4–36)	10.5 (4.0–33.0)	13.0 (7.0–36.0)	0.085

Median (min–max) for continuous variables: the p was calculated by the linear regression model. N (%) for categorical variables: the p was calculated by the chi-square test. $p < 0.05$ was considered statistically significant. TBIL, total bilirubin; DBIL, direct bilirubin; IBIL, indirect bilirubin; TBA, total bile acid; ALT, alanine aminotransferase; AST, aspartate transaminase; GGT, γ-glutamyl transpeptidase; TBA, total bile acid; AFP, Alpha-fetoprotein.

identified using a two piecewise linear regression model, at 199.1 mAU/ml (**Table 4**). For a PIVKA-II <199.1 mAU/ml, every 1 mAU/ml increase in PIVKA-II was associated with a 1.6 μmol/L greater TBIL (95% CI: 1.1–2.0); by comparison, for individuals with a PIVKA-II >199.1 mAU/ml, a 1 mAU/ml increase in PIVKA-II was associated with a 0.7 μmol/L decrease in TBIL (95% CI: −1.1 to −0.2). Similarly, for a PIVKA-II <199.1 mAU/ml, every 1 mAU/ml increase in PIVKA-II was associated with a 1.4 μmol/L greater DBIL (95% CI: 1.0–1.8); by comparison, for individuals with a PIVKA-II >199.1 mAU/ml, a 1 mAU/ml increase in PIVKA-II was associated with a 0.6 μmol/L decrease in DBIL (95% CI: −1.0 to −0.3).

The association between PIVKA-II with IBIL was a U-shaped curve, with the point of inflection identified using a two piecewise linear regression model, at 18.6 mAU/ml (**Table 4**). For a PIVKA-II <18.6 mAU/ml, every 1 mAU/ml increase in PIVKA-II was associated with a 5.4 μmol/L lower IBIL (95% CI: −9.8 to −0.9); by comparison, for individuals with a PIVKA-II >18.6 mAU/ml, a 1 mAU/ml increase in PIVKA-II was associated with a 0.1 μmol/L increase in IBIL (95% CI: 0.0–0.1). However, the association between PIVKA-II with albumin was an inverted U-shaped curve, with the point of inflection identified using a two piecewise linear regression model, at 18.6 mAU/ml (**Table 4**). For a PIVKA-II <18.6 mAU/ml, every 1 mAU/ml increase in PIVKA-II was associated with a 1.1 μmol/L greater albumin (95% CI: 0.2–2.0).

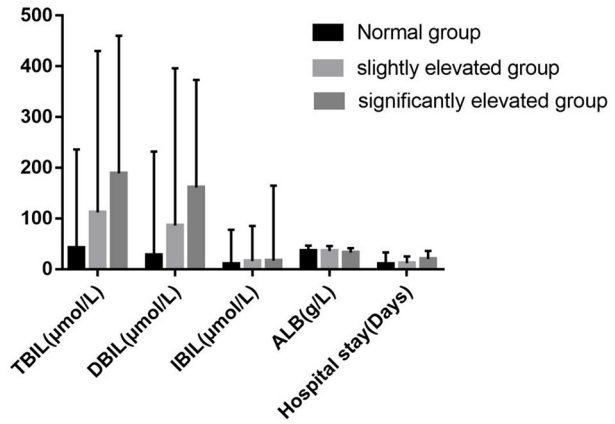

FIGURE 2 | Levels of clinical biochemical indexes in different groups of serum PIVKA-II levels. PIVKA, protein induced by vitamin K absence or antagonist-II.

DISCUSSION

Hepatitis E is distributed worldwide and is prevalent in many developing countries in Africa and Asia (9). In fact, the WHO has considered hepatitis E as an important public health problem in developing countries (2). In the recent years, with the improvement of the living standards of the Chinese population, the hepatitis E epidemic has been more controlled than that before, but there are still sporadic cases, and small-scale hepatitis E outbreaks have been reported from time to time (10–12). In addition, the age of onset has become a new feature in the epidemiology of hepatitis E in the country. An epidemiological survey on hepatitis E in China showed that the number of reported cases aged 45–69 years accounted for more than half of all reported cases (10). Moreover, Li et al. reported that the prevalence of anti-HEV antibodies had increased with age (13). Most cases of hepatitis E are self-limiting, and progression to acute liver failure is rare. However, older and diabetic patients are more likely to develop acute liver failure and die when compared with younger and non-diabetic patients (14). Pregnant women, especially those in their last trimester, had a case fatality

TABLE 2 | Association between clinical biochemical indexes and degree of elevated PIVKA-II.

Parameter	slightly elevated (<125 mAU/L)	significantly elevated (>125 mAU/L)	*P* value
PIVKA-II (mAU/L)	48.8 (33.0–124.6)	318.1 (127.2–9,235.0)	<0.001
TBIL (μmol/L)	112.0 (12.0–430.0)	189.0 (45.0–460.3)	0.050
DBIL (μmol/L)	86.0 (6.0–396.0)	161.0 (37.0–373.0)	0.037
IBIL (μmol/L)	16.0 (4.0–85.3)	17.0 (8.0–164.7)	0.390
Albumin (g/L)	36.2 (27.6–45.6)	32.5 (27.0–41.3)	0.018
ALT (U/L)	1,461.0 (97.0–4,228.0)	237.0 (32.0–3,203.0)	0.002
AST (U/L)	564.0 (44.0–5,720.0)	89.0 (33.0–1,294.0)	0.008
ALP (U/L)	200.0 (84.0–466.0)	208.5 (115.0–475.0)	0.921
GGT (U/L)	219.0 (36.0–840.0)	150.0 (31.0–903.0)	0.181
TBA (μmol/L)	241.0 (2.4–450.0)	202.7 (17.0–264.0)	0.431
AFP (ng/mL)	15.9 (1.5–474.0)	28.4 (4.2–455.2)	0.383
PT (s)	13.4 (1.0–18.0)	15.0 (12.3–19.8)	0.165
Hospital stay (Days)	12.0 (7.0–25.0)	20.0 (10.0–36.0)	0.005

Median (min–max) for continuous variables. $p < 0.05$ was considered statistically significant. PIVKA, protein induced by vitamin K absence or antagonist-II; TBIL, total bilirubin; DBIL, direct bilirubin; IBIL, indirect bilirubin; TBA, total bile acid; ALT, alanine aminotransferase; AST, aspartate transaminase; GGT, γ-glutamyl transpeptidase; TBA, total bile acid; AFP, Alpha-fetoprotein.

TABLE 3 | Changes in PIVKA-II and clinical biochemical indicators during hospitalization.

Time (weeks)	< 1	1–2	2–3	3–4	> 4
PIVKA-II (mAu/mL)	45.8 (38.7–369.8)	74.7 (31.7–534.7)	1,194.3 (616.6–2,622.1)	41.1 (20.0–885.3)	36.2 (29.2–116.0)
TBIL (μmol/L)	135.3 (7.0–460.3)	160.2 (16.1–242)	245.3 (160–357)	245.0 (41.7–425)	37.8 (21–50.7)
DBIL (μmol/L)	107.8 (2.0–396.0)	130.2 (7–223)	216.5 (141–318)	212.3 (23.7–363)	21.8 (10.7–31.1)
IBIL (μmol/L)	27.5 (2–164.7)	12.4 (4–19)	28.8 (19–81)	32.8 (11–62)	15.9 (10.3–19.6)
ALP (U/L)	232.8 (61–475)	104.5 (95–123)	141.3 (120–166)	117.0 (109–143)	123.0 (95–131)
ALT (U/L)	798 (243.5–1,542.3)	89.0 (55.5–134.5)	87.0 (43.1–241.0)	33.0 (28.5–88.5)	21.5 (20.0–35.5)
AST (U/L)	296.0 (77.0–878.0)	42.0 (33.0–71.0)	89.0 (59.0–16.0)	41.0 (30.5–65.0)	41.0 (34.0–55.0)
GGT (U/L)	150.0 (91.3–443.3)	104.0 (72.0–160.5)	66.5 (51.7–88.5)	71.0 (66.5–143.5)	31.5 (21.0–45.5)
TBA (μmol/L)	210.4 (2–477)	142.6 (12.1–273)	239.7 (177.4–363)	41.7 (41.7–41.7)	8.8 (4.5–13.2)
AFP (ng/ml)	7.1 (1.8–27.8)	6.5 (4.1–1,032.1)	132.6 (41.8–396.4)	148.6 (8.1–119.1)	88.7 (7.8–98.0)
PT (s)	15.7 (1.0–337)	14.1 (13.5–14.9)	13.8 (13.4–14.3)	17.1 (13.1–20.7)	13.1 (13.1–13.1)
Albumin (g/L)	34.4 (31.9–38.5)	33.3 (28.9–36.3)	30.8 (30.3–34.0)	36.0 (34.3–41.1)	36.8 (36.5–38.4)

TBIL, total bilirubin; DBIL, direct bilirubin; IBIL, indirect bilirubin; TBA, total bile acid; ALT, alanine aminotransferase; AST, aspartate transaminase; GGT, γ-glutamyl transpeptidase; TBA, total bile acid; AFP, Alpha-fetoprotein.

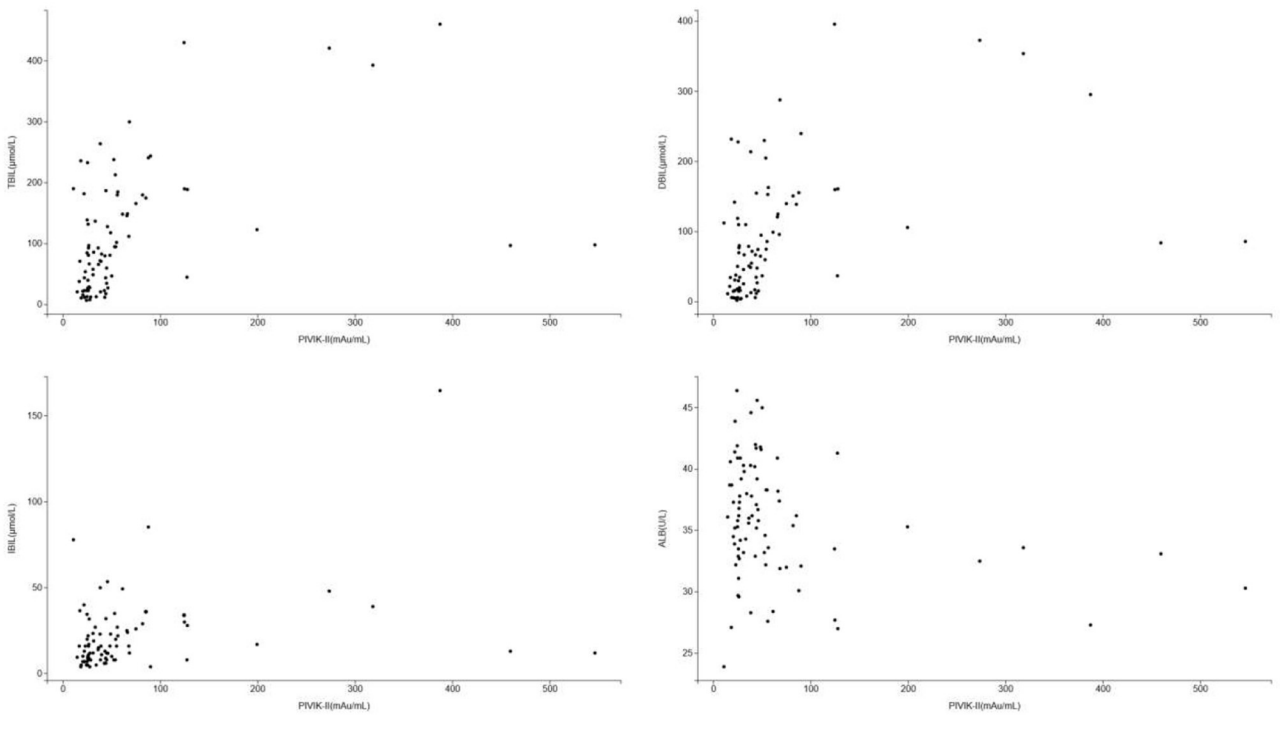

FIGURE 3 | Scatter diagram for the correlation between PIVKA-II and bilirubin and albumin. Each black point represents a sample. PIVKA, protein induced by vitamin K absence or antagonist-II. PIVKA, protein induced by vitamin K absence or antagonist-II.

rate of more than 10% (15, 16). In our current study, these patients had a median value of 53 years old ranged from 25 to 77. There were no significant differences in age distribution between the normal PIVKA-II group and the elevated PIVKA-II group. Pregnant patients were not included in our study and none of the 84 patients progressed to acute liver failure during hospitalization.

Protein induced by vitamin K absence or antagonist-II -II is a protein produced due to vitamin K deficiency or antagonists and is mainly composed of liver cell particles. Carboxylase system and epoxide reductase work together to produce PIVKA-II. The glutamic acid residues in the γ-carboxyglutamate structure are not fully carboxylated to γ-carboxyglutamate and lose their normal activity (17, 18). PIVKA-II has been recently found to

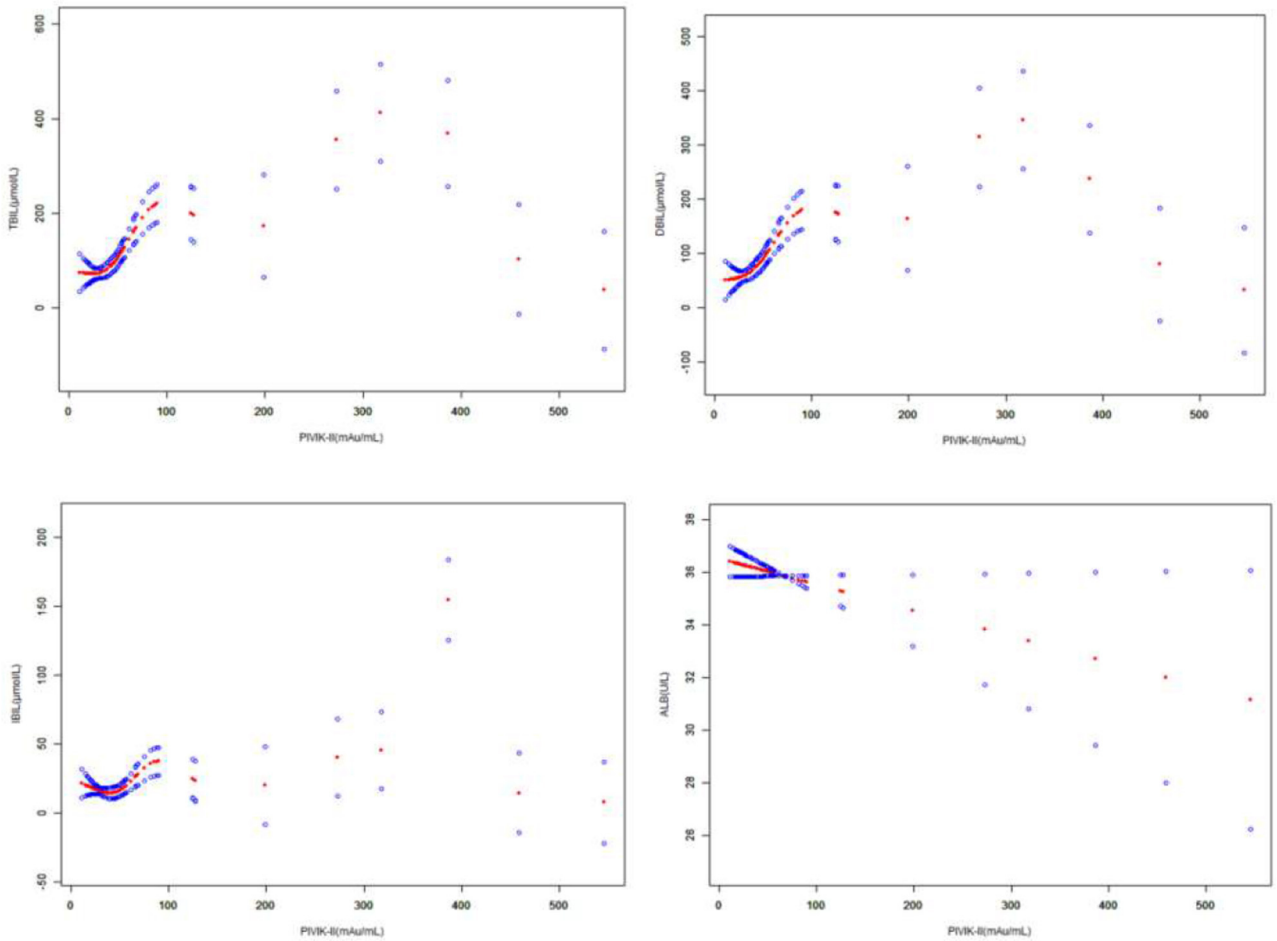

FIGURE 4 | The correlation between PIVKA-II and bilirubin and albumin. The solid red line represents the smooth curve fit between variables. Blue bands represent the 95% CI from the fit. Gender and age were adjusted. PIVKA, protein induced by vitamin K absence or antagonist-II.

have a good diagnostic value for early liver tumors, especially HCC (19). However, PIVKA-II elevation is not specific for HCC diagnosis, reported in many other disease situations. Moreover, Wu et al. reported that PIVKA-II levels in non-cirrhotic chronic hepatitis B patients were higher than that in the healthy control group (20). Other factors, such as vitamin K deficiency, taking warfarin, primary gastric adenocarcinoma, transplant rejection, lack of nutrition, intestinal flora imbalance, renal failure, inflammatory bowel disease, and alcoholic liver disease, led to increased serum PIVKA-II levels in non-HCC patients (6). Besides, Takikawa reported that PIVKA-II helps to monitor the severity of acute liver injury (21). In this study, we included 84 cases of patients with hepatitis E and measured the levels of the PIVKA-II and various serological indicators, and monitored the indicators during hospitalization. The PIVKA-II levels increased in 59.51% (50/84) of patients with hepatitis E, four cases had PIVKA-II levels >1,000 mAU/ml, the highest of which was 9,235 mAU/ml. We classified the degree of increase in the PIVKA-II levels into two groups. Compared with the slightly elevated PIVKA-II group, patients in the significantly elevated PIVKA-II group had much lower serum albumin, ALT, AST levels, and longer days for the hospital stay. An elevated PIVKA-II correlated with a greater TBIL, DBIL, IBIL, and lower albumin. Moreover, we identified a non-linear relationship between PIVKA-II with TBIL and DBIL with a point of inflection at 199.1 mAU/ml and a non-linear relationship between PIVKA-II with IBIL and albumin with a point of inflection at 18.6 mAU/ml. These results indicated that PIVKA-II levels might be related with the severity of hepatitis E.

An increase in prothrombin levels also leads to the production of abnormal prothrombin (18). Therefore, we believe that PIVKA-II may be associated with the severity of acute liver damage. Actually, the underlying mechanism behind the effect of HEV infection on serum PIVKA-II levels remains unclear. We believe that this may be related to liver cell damage and metabolic disorders in patients with hepatitis E, which decreased the ability of liver cells to synthesize protein and may weaken the function of vitamin K-dependent shuttling

TABLE 4 | Threshold effect analysis of PIVKA-II on TBIL, DBIL, IBIL, and albumin using the two-piecewise linear regression model.

Parameters	Adjustedβ (95% CI), *P*-value
TBIL	
Fitting by the standard linear model	0.4 (0.2, 0.6), <0.001
Fitting by the two-piecewise linear model	
Inflection point	**199.1**
PIVKA-II < 199.1 (mAU/mL)	1.6 (1.1, 2.0), <0.001
PIVKA-II >199.1 (mAU/mL)	−0.7 (−1.1, −0.2), 0.003
Log likelihood ratio	<0.001
DBIL	
Fitting by the standard linear model	0.3 (0.1, 0.5) 0.002
Fitting by the two-piecewise linear model	
Inflection point	**199.1**
PIVKA-II < 199.1 (mAU/mL)	1.4 (1.0, 1.8) <0.001
PIVKA-II >199.1 (mAU/mL)	−0.6 (−1.0, −0.3) 0.001
Log likelihood ratio	<0.001
IBIL	
Fitting by the standard linear model	(0.0, 0.1) 0.006
Fitting by the two-piecewise linear model	
Inflection point	**18.6**
PIVKA-II< 18.6 (mAU/mL)	−5.4 (−9.8, −0.9) 0.020
PIVKA-II>18.6 (mAU/mL)	0.1 (0.0, 0.1) 0.002 0.014
Log likelihood ratio	
Albumin	
Fitting by the standard linear model	
Fitting by the two-piecewise linear model	−0.0 (−0.0, 0.0) 0.064
Inflection point	**18.6**
PIVKA-II < 18.6 (mAU/mL)	1.1 (0.2, 2.0) 0.017
PIVKA-II >18.6 (mAU/mL)	−0.0 (−0.0, −0.0) 0.026 0.013
Log likelihood ratio	

Gender and age were adjusted. PIVKA, protein induced by vitamin K absence or antagonist-II.

enzymes, causing the metabolic utilization of vitamin K. Besides, elevated PIVKA-II levels in hepatitis E may also be related with Vitamin K absence. Regretfully, the relationship between serum vitamin K concentration and serum PIVKA-II levels was not explored in our current study because data on serum vitamin K levels are unavailable. Moreover, no related pieces of literature were previously published about the relationship between Vitamin K absence and HEV infection. Therefore, whether Vitamin K absence existed in patients with HEV infection was still unclear and the effect of HEV infection on serum PIVKA-II levels needs to be clarified in future research.

With the gradual recovery of patients with hepatitis E, PIVKA-II levels also gradually decreased, and the peak was delayed by 2 weeks compared with the peak of transaminase, which was roughly similar to that of bilirubin. The peak of AFP was 1 week later than that of PIVKA-II. Many studies believe that AFP exists in the cytoplasm of oval cells or hyperproliferative cells (22, 23), which indicates the proliferation of liver cells after injury, and the new liver cells may synthesize AFP briefly in the early stage. We believe that PIVKA-II is related to liver cell damage, so there may be a certain node in the abnormal metabolism and hyperplasia of liver cell necrosis, which is a turning point in the disease of the patients with hepatitis E. However, we did not have enough cases in this study, and the sample size needs to be further expanded.

Based on the above information, it could be seen that more than half of patients with hepatitis E had elevated PIVKA-II levels, which were all transiently elevated, and the patients gradually recovered after a few weeks. With the recovery of patients from hepatitis E, none of the cases continued to progress. Therefore, PIVKA-II could be a significant reference for the course of hepatitis E disease. Moreover, we can refer to the PIVKA-II level of patients with hepatitis E to assess the severity of the patient's condition and the trend of the outcome to assist in the diagnosis and treatment of the disease further. If the patient's PIVKA-II continues to rise, further examinations should be undertaken, and the possibility of HCC should be ruled out. The association between PIVKA-II with TBIL, DBIL, IBIL, and albumin was a U-shaped or inverted U-shaped curve with an obvious point of inflection. However, this study had some limitations. First, this was a retrospective study. The data collection time was relatively short. The study population was recruited from only two centers. We need to conduct more cases and perform long-term follow-up evaluations. Second, we excluded individuals with HCC from our study sample as HCC may have a significant influence on PIVKA-II. Third, there remains the possibility of bias caused by other potential confounding factors that we did not adjust for.

DATA AVAILABILITY STATEMENT

The research involves patient private information, 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 Ethics Committee of the First Affiliated Hospital of Wenzhou Medical University and Taizhou Hospital of Zhejiang Province (approval number: No. 2021-zz-162). Due to the retrospective study design involving electronic health records and no additional interventions, written informed consent was waived from the patients or their relatives.

AUTHOR CONTRIBUTIONS

YC, YY, NY, and MLu contributed to the conception and design of the study. YC organized the database. YY performed the statistical analysis. YC wrote the first draft of the manuscript. SL, MLi, XX, HS, YJ, SZ, and HS wrote sections of the manuscript. All the authors contributed to manuscript revision, read, and approved the submitted version.

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