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ORIGINAL PAPER

Serum HBV RNA levels among untreated adults with chronic hepatitis B in distinct immune phases and liver histopathology statuses

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Abstract
HBV RNA is a novel serum biomarker that reflects intrahepatic HBV covalently closed circular DNA (cccDNA) transcription activity. Serum HBV RNA levels among treatment-naïve adults during the natural history of chronic hepatitis B (CHB) and distinct liver histopathology statuses remain elusive. In our study, we include a total of 411 treatment-naïve CHB patients, among which 43 patients were HBeAg-positive immune-tolerant [IT(e^+)], 84 patients were HBeAg-positive immune active [IA(e^+)], 65 patients in HBeAg-negative immune active phases [IA(e^-)], 149 patients were HBeAg-negative inactive phases [IC(e^-)], and 70 patients were in Gray Zone (GZ). HBV RNA was measured in this cohort and its potential correlation with traditional serological markers and liver histopathology were analyzed. Our data showed that HBV RNA was strongly correlated with HBV DNA, HBeAg, HBsAg and ALT. Further subgroup analysis revealed a close correlation between HBV RNA and HBV DNA in patients in the IA (e^+) and IA (e^-) phases, but neither in IT(e^+) nor IC(e^-) phase. HBV RNA levels were consistently increased with the advanced degrees of hepatic inflammation, but not hepatic fibrosis. Of note, HBV RNA from HBeAg-positive patients negatively correlated with liver fibrosis, whereas HBV RNA from HBeAg-negative patients was weakly associated with liver inflammation. To sum up, serum HBV RNA shows a distinct profile among CHB patients in different immune statuses and hepatic histopathology stages/grades. Simultaneous testing of HBV RNA and traditional indicators might provide a comprehensive clinical assessment of CHB patients.

Keywords HBV RNA · Liver histopathology · Hepatitis B virus · Chronic hepatitis B

Introduction

It is estimated by World Health Organization (WHO) that about 296 million people are suffering from chronic hepatitis B virus (HBV) infection globally. Chronic HBV infection has continuously become a huge public health problem, posing great threat to worldwide healthcare (World Health

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Organization 2021). Chronic hepatitis B (CHB) is a chronic necroinflammatory liver disease caused by persistent hepatitis B virus infection (presence of HBsAg and/or HBV DNA for at least 6 months) (Lok and McMahon 2007; Terrault et al. 2018). According to natural history in adults, CHB patients can be classically divided into four immune phases depending on the levels of alanine aminotransferase (ALT) and HBV DNA: immune-tolerant CHB [IT(e^+)], hepatitis B e antigen (HBeAg)-positive immune active CHB [IA(e^+)], HBeAg-negative immune active CHB [IA(e^-)] and inactive CHB [IC(e^-)] (Terrault et al. 2016, 2018). Meanwhile, there are still some CHB patients who do not fit into these determined phases, known as indetermined "Gray Zone (GZ)" patients (Bonacci et al. 2018; Ren et al. 2022). In our previous studies, GZ patients were classified into four subgroups from GZ-A to GZ-D in one-to-one correspondence to the four immune phases (Yao et al. 2021; Wang et al. 2023). Our previous study also showed that more than one-quarter of CHB patients are in GZ, and a relatively small but non-negligible proportion of CHB patients in GZ are at risk for advanced liver fibrosis or cirrhosis.

It is widely promoted that "clinical cure of CHB" has been a realizable goal of treatment, with increasing CHB patients receiving anti-HBV therapy including nucleos(t)ide analogues (NAs) or interferon (IFN) (Tang et al. 2018; Nguyen et al. 2020). According to the 2018 American Association for the Study of Liver Diseases (AASLD) guidance and the 2019 Chinese guidelines for CHB management, CHB patients with only mild intrahepatic inflammation and fibrosis are not recommended to initiate anti-HBV treatment immediately (Terrault et al. 2018; Chinese Society of Infectious Diseases, Chinese Medical Association 2019). It is also remained unclear whether GZ patients should receive anti-HBV therapy. In clinical practice with a CHB patient, the timing of treatment initiation is primarily decided by his/her serum ALT level, HBV DNA and hepatitis B surface antigen (HBsAg) level, which could not fully reflect intrahepatic HBV replication (Höner zu Siederdissen et al. 2017; Zhou et al. 2021). It is the gold standard to have liver biopsy to evaluate the hepatic immune status, which is both costly and invasive (Russo et al. 2018). Thus, solid and reliable serum indicators are in urgent need to help determine the histopathological status without invasive liver biopsy, assess the intrahepatic virus activity and evaluate the timing of anti-HBV therapy.

Recently, novel serum surrogates for covalently closed circular DNA (cccDNA), particularly HBV RNA, have attracted research interest (Köck et al. 1996; Coffin et al. 2019; Liu et al. 2019). HBV RNA is in essence the HBV pregenomic RNA (pgRNA) in the viral-like particle (Shen et al. 2020). It is originated from the transcription of cccDNA, and then released into blood directly without the reverse transcription. An important reason why intrahepatic HBV is so hard to eliminate is the presence of cccDNA within hepatocytes (Nassal 2015; Allweiss and Dandri 2017). Since HBV RNA is less affected by the presence of immune complexes of HBsAg and HBsAb, serum HBV RNA is considered to be a novel HBV biomarker with great clinical potential (Coffin et al. 2019). Emerging evidence has demonstrated that serum HBV RNA reflects intrahepatic cccDNA level and its transcriptional activity (Giersch et al. 2017; Wang et al. 2021). In CHB patients after long-term NAs treatment, cccDNA is also associated with HBV RNA level (van Bömmel et al. 2018; Carey et al. 2020; Liu et al. 2020). Furthermore, serum HBV RNA shows superiority in monitoring the sustained viral respond (SVR) and the exhaustion of the intrahepatic cccDNA in comparison with HBV DNA in CHB patients after NAs treatment (Tsuge et al. 2013). Opinion has risen that HBV RNA might be a reliable indicator to reflect the activity of HBV in hepatocytes and guide the initiation of anti-HBV treatment (Fanning et al. 2019; Mak et al. 2021). We aimed to investigate the distribution of HBV RNA over the natural course of treatment-naïve CHB patients and the correlation of HBV RNA with serological indicators and liver histopathology.

Methods

Objects

In this study, 498 treatment-naïve CHB patients hospitalized at Nanjing Drum Tower Hospital (Nanjing, China) from October 2017 to December 2021 were recruited. All the patients were HBsAg-positive for at least 6 months. Eighty-seven of these patients were excluded from this study with the following conditions: (1) co-infection with any other virus including hepatitis C virus (HCV), hepatitis D virus (HDV) and human immunodeficiency virus (HIV); (2) co-existence with decompensated liver cirrhosis, hepatocelluar carcinoma or other malignancies; (3) concurrent with other liver diseases, including primary biliary cirrhosis, autoimmune hepatitis, alcoholic liver disease, nonalcoholic fatty liver disease; (4) had received liver transplantation before enrollment. This study was approved by the Ethics Committee of Nanjing Drum Tower Hospital.

Definitions of CHB immune phases and Gray Zone subgroups

The immune phases of CHB patients were defined according to AASLD 2016 CHB guidance (Supplemental Table 1). Recently, CHB patients who do not meet the criteria above were categorized as indetermined "Gray Zone (GZ)" (Yao et al. 2021; Wang et al. 2023). We further classified these CHB patients in GZ status into

four subgroups in one-to-one correspondence to the four immune phases as following: (1) GZ-A: HBeAg-positive, normal ALT levels, serum HBV DNA $\leq 10^6$ IU/mL; (2) GZ-B: HBeAg-positive, elevated ALT levels, serum HBV DNA $\leq 2 \times 10^4$ IU/mL; (3) GZ-C: HBeAg-negative, normal ALT levels, serum HBV DNA $\geq 2 \times 10^3$ IU/mL; (4) GZ-D: HBeAg-negative, elevated ALT levels, serum HBV DNA $\geq 2 \times 10^3$ IU/mL.

Quantification of serum HBV RNA

Serum HBV RNA was determined using RNA simultaneous amplification testing method (HBV-SAT) assay (Rendu biotechnology, Shanghai, China) which provides direct detection of HBV RNA by a capture probe method, with a lower limit of quantification (LLOQ) of 50 copies/mL (i.e., 1.70 $\log_{10}$ copies/mL). The linear range of assay was from 1×10^2copies/mL to 1×10^8copies/mL. When the results were in the range of $(0–5) \times 10^1$copies/mL, the assay was repeated to calculate the mean value for statistical purposes. Undetectable results were regarded as negative set to 0 $\log_{10}$ copies/mL.

Clinical data collection

Demographic and laboratory parameters including age, gender, ALT, aspartate transaminase (AST), alkaline phosphatase (ALP), glutamyl transpeptidase (GGT), albumin (ALB), total bilirubin (TBIL), HBsAg, hepatitis B surface antibody (HBsAb), HBeAg, hepatitis B e antibody (HBeAb), hepatitis B core antibody (HBcAb), serum HBV DNA were retrospectively collected from electronic medical records (EMR) of all the patients. The lower limits of quantification and detection for HBV DNA were 500 and 100 copies/mL, respectively. Values below these thresholds were randomly estimated using a uniform distribution [(1.70–2.70) and (0.01–1.70) $\log_{10}$ copies/mL, respectively].

Assessment of liver histology

Liver biopsy was performed in standard procedures. Liver histopathology was evaluated by two independent pathologists in a double-blind fashion. Scheuer scoring system (Schcuer 1991) was used for liver inflammation grades (G0–4) and fibrosis stages (S0–4) (Rockey et al. 2009). If the results of the two pathologists were inconsistent, the final outcome will be evaluated by a third independent pathology expert. Significant fibrosis was defined as a fibrosis stage of S2-4, severe fibrosis as S3-4 and cirrhosis as S4. Significant inflammation was defined as an inflammation grade of G2-4.

Statistical analysis

Data are presented as mean ± standard deviation (SD) or median (interquartile range, IQR) or count (percentage) as appropriate. Kruskal–Wallis test and Fisher's exact test were performed for comparisons. Spearman rank correlation coefficient (ρ) was used to assess the correlations between serum biomarkers. Statistical analysis were performed in Graphpad prism 9 (version 9.0; GraphPad software, San Diego, California, USA) and R4.1.3 (R Foundation for Statistical Computing, Vienna, Austria). P value < 0.05 was considered statistically significant.

Results

Baseline characteristics of CHB patients

A total of 498 treatment-naïve CHB patients were recruited from October 2017 to December 2021. Among them, 411 patients were finally enrolled in this study, and 87 patients were excluded (Supplemental Fig. 1). The baseline characteristics of CHB patients are summarized in Table 1. Among the enrolled patients, 140 were HBeAg-positive and 271 were HBeAg-negative, while 234 received liver biopsy. Forty-three patients (10.46%) with immune tolerance CHB [IT(e^+)], 84 (20.44%) with HBeAg-positive immune active CHB [IA(e^+)], 65 (15.82%) with HBeAg-negative immune active CHB [IA(e^-)], and 149 (36.25%) are inactive carriers [IC(e^-)]. The median age of the participants was 41 (IQR 33–52) years, and 40.10% were female. The median HBV DNA was 4.03 (IQR 2.73–7.34) $\log_{10}$ copies/mL, and the median HBV RNA was 3.31 (IQR 1.89–6.90) $\log_{10}$ copies/mL. Among the four immune phases and the GZ group, we identified distinct profile of age, biochemical markers (ALT, AST, ALB, GGT and ALP), virological markers (HBsAg, HBsAb, HBeAg, HBeAb and HBcAb), HBV DNA load and serum HBV RNA levels (all $P < 0.001$).

Clinical characteristics of different GZ subgroups and patients who received liver biopsy

In our study, seventy CHB patients included were classified into the GZ group, in which 6 (8.57%) in GZ-A, 8 (11.43%) in GZ-B, 12 (17.14%) in GZ-C, and 44 (62.86%) in GZ-D. The clinical characteristics of the GZ group patients are shown in Supplemental Table 2. The majority (80.00%) of GZ patients were HBeAg-negative. Patients in the GZ-A and GZ-B subgroups were slightly younger than the other two subgroups ($P < 0.05$). There was no difference in gender among the four subgroups ($P = 0.822$). The laboratory parameters including ALB, ALP, TBIL, HBsAg, HBsAb, HBcAb were comparable among four groups of patients in

Table 1 Baseline characteristics of all CHB patients

	Total	HBeAg -positive		HBeAg -negative		GZ	P value
		IT (e^+)	IA (e^+)	IA (e^-)	IC (e^-)		
	N = 411	N = 43, 12[a]	N = 84, 49[a]	N = 65, 26[a]	N = 149, 77[a]	N = 70[a]	
Age	41.0 [33.0;52.0]	31.0 [28.5;37.0]	34.0 [31.0;39.0]	51.0 [38.0;61.0]	45.0 [38.0;57.0]	41.0 [33.2;51.0]	< 0.001
Gender							0.9
Female	165 (40.1%)	19 (44.2%)	30 (35.7%)	26 (40.0%)	61 (40.9%)	29 (41.4%)	
Male	246 (59.9%)	24 (55.8%)	54 (64.3%)	39 (60.0%)	88 (59.1%)	41 (58.6%)	
ALT (U/L)	24.4 [20.0;34.8]	24.2 [21.5;29.9]	62.3 [44.3;124]	54.4 [42.8;92.9]	20.5 [17.5;22.9]	40.2 [30.1;59.9]	< 0.001
AST (U/L)	31.7 [20.9;55.7]	20.8 [19.1;24.3]	36.4 [27.3;69.0]	38.4 [28.0;60.6]	19.0 [14.2;24.9]	25.8 [20.8;35.9]	< 0.001
ALB (g/L)	43.1 [40.7;45.1]	43.1 [41.7;44.8]	42.6 [39.5;44.4]	42.7 [40.3;44.6]	44.2 [42.0;45.9]	41.5 [37.5;43.1]	< 0.001
GGT (U/L)	21.6 [15.7;39.5]	16.0 [13.3;19.6]	31.0 [18.7;46.8]	33.7 [21.6;45.0]	17.7 [13.3;22.0]	32.1 [20.6;44.7]	< 0.001
ALP (U/L)	63.0 [50.7;77.4]	59.9 [50.9;72.2]	65.6 [54.9;81.1]	67.6 [53.5;88.6]	61.5 [51.6;72.2]	60.2 [41.4;73.1]	0.009
TBIL (umol/L)	11.5 [8.90;15.6]	10.5 [8.40;14.1]	11.9 [9.30;15.5]	12.1 [10.0;17.0]	11.4 [8.90;15.7]	11.9 [7.98;15.5]	0.286
Serum HBV RNA (Log_{10}copies/mL)	3.31 [1.89;6.90]	7.19 [6.94;7.45]	7.29 [6.91;7.63]	3.40 [2.60;4.44]	1.89 [0.00;2.61]	2.98 [0.00;3.82]	< 0.001
Serum HBV DNA (Log_{10}copies/mL)	4.03 [2.73;7.34]	7.80 [7.61;8.13]	7.83 [7.31;8.11]	4.93 [4.24;5.68]	2.34 [1.30;3.12]	3.16 [2.73;4.11]	< 0.001
HBsAg (Log_{10}IU/mL)	3.43 [2.59;4.26]	4.70 [4.60;4.88]	4.45 [3.94;4.76]	3.18 [2.90;3.67]	2.70 [1.58;3.36]	3.42 [2.75;3.72]	< 0.001
HBsAb (mIU/mL)	0.31 [0.00;0.98]	0.10 [0.00;0.58]	0.21 [0.00;0.83]	0.28 [0.00;1.27]	0.49 [0.10;1.55]	0.31 [0.02;0.88]	0.002
HBeAg (S/CO)(among HBeAg positive)	3.12 [2.82;3.17]	3.15 [3.12;3.19]	3.11 [2.71;3.17]	N/A	N/A	0.00 [0.00;0.00]	< 0.001
HBeAb (S/CO)	0.02 [0.01;39.2]	56.6 [51.7;64.3]	49.4 [28.9;57.7]	0.02 [0.01;0.02]	0.02 [0.01;0.02]	0.02 [0.01;0.18]	< 0.001
HBcAb (S/CO)	9.19 [8.11;10.2]	7.57 [6.12;8.64]	8.55 [7.42;9.74]	9.74 [9.20;10.6]	9.23 [8.43;10.3]	9.46 [8.74;10.2]	< 0.001

IT immune tolerant, *IA* immune active, *IC* Inactive carrier, *GZ* Gray Zone, *ALT* alanine aminotransferase, *AST* aspartate transaminase, *ALB* albumin, *GGT* glutamyl transpeptidase, *ALP* alkaline phosphatase, *TBIL* total bilirubin, *HBsAg* hepatitis B surface antigen, *HBsAb* hepatitis B surface antibody, *HBeAg* hepatitis B e antigen, *HBeAb* hepatitis B e antibody, *HBcAb* hepatitis B core antibody, *N/A* Not applicable

[a]Number of liver biopsy specimen

the gray zone (P > 0.05). AST, ALT and GGT levels were similar in GZ-A and GZ-C groups, but were significantly lower than those in GZ-B and GZ-D groups (P < 0.05). HBV DNA and HBV RNA levels from patients in the GZ-D group were significantly lower than other groups (P < 0.05).

The clinical characteristics of CHB patients who received liver biopsy are summarized in Supplemental Table 3. Among these patients, 149 (63.70%) had no significant or mild inflammation was defined as G0-1, 58 (24.80%) had significant inflammation as G2, 27 (11.50%) had severe inflammation as G3-4. Meanwhile, 164 (70.09%) had none to mild fibrosis as S0-1, 53 (22.65%) had significant fibrosis as S2, and 17 (7.26%) had severe fibrosis or cirrhosis as S3-4.

Comparison of HBV RNA, HBV DNA and HBsAg in CHB patients in different immune phases

Serum HBV RNA and HBV DNA were detected in all HBeAg-positive patients. Seventy-three percent of HBeAg-negative patients had positive HBV RNA and 63.12% were HBV DNA positive. HBV DNA, HBsAg and HBV RNA levels were strongly associated with each other (Fig. 1a–c). The median level of HBV RNA were different in every phase of CHB (P < 0.001): IT(e^+) phase 7.19 (IQR 6.94–7.45) log_{10} copies/mL, IA(e^+) phase 7.29 (IQR 6.91–7.63) log_{10} copies/mL, IA(e^-) phase 3.40 (IQR 2.60–4.44) log_{10} copies/mL and IC(e^-) group 1.89 (IQR 0–2.61) log_{10} copies/mL, the highest HBV RNA was in the IA(e^+) phase, and the lowest in the IC(e^-) phase. GZ patients had 2.98 (IQR 0–3.82) log_{10} copies/mL of HBV RNA (Fig. 1b). ALT level was different in the GZ group compared to all other groups (P < 0.001) (Fig. 1d). The distribution of HBV RNA and other serological markers in the GZ subgroups was summarized in Fig. 1e–h. HBsAg levels were comparable among four GZ subgroups, whereas HBV RNA and HBV DNA levels from GZ-D subgroup were lower than that of all the other subgroups. The median ALT levels were significantly higher in the GZ-D subgroup [52.1 (IQR 40.1–62.4) U/L] than other GZ subgroup (Fig. 1h).

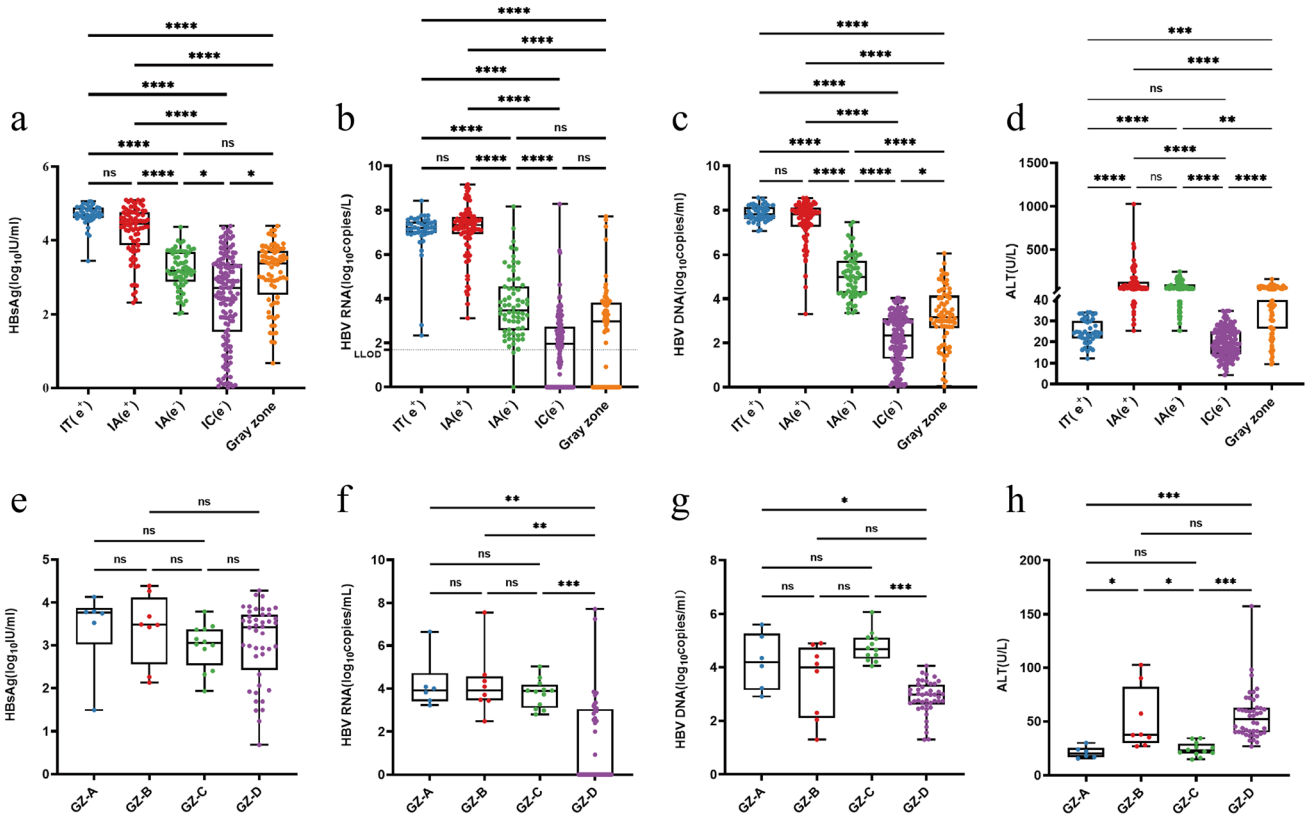

Fig. 1 Serological and viral markers by CHB immune phases and GZ subgroups. **a** Serum HBsAg by CHB immune phases. **b** Serum HBV RNA by CHB immune phases. **c** Serum HBV DNA by CHB immune phases. **d** Serum ALT level by CHB immune phases. **e** Serum HBsAg by GZ subgroups. **f** Serum HBV RNA by GZ subgroups. **g** Serum HBV DNA by GZ subgroups. **h** Serum ALT level by CHB GZ subgroups

Correlations between serum HBV RNA, HBV DNA and HBV biomarkers

The potential correlations between serum HBV RNA, HBV DNA and different HBV biomarkers were determined. As shown in Fig. 2, HBV RNA correlated with all the biomarkers except TBIL and ALB in all patients (Fig. 2a). HBV RNA was strongly positive correlated with HBV DNA ($\rho = 0.77$, $P < 0.001$), HBeAg ($\rho = 0.76$, $P < 0.001$), HBsAg ($\rho = 0.64$, $P < 0.001$), while moderately positive correlated with ALT ($\rho = 0.41$, $P < 0.001$) and AST ($\rho = 0.35$, $P < 0.001$). Among HBeAg-positive patients (Fig. 2b), HBV RNA was moderately correlated with HBV DNA ($\rho = 0.48$, $P < 0.01$) and HBsAg ($\rho = 0.49$, $P < 0.001$). Among HBeAg-negative patient (Fig. 2c), HBV RNA had a moderate correlation with serum HBV DNA ($\rho = 0.44$, $P < 0.01$). In the GZ group, HBV RNA had a moderate correlation with HBV DNA ($\rho = 0.46$, $P < 0.01$) and HBeAg ($\rho = 0.44$, $P < 0.01$) and no correlation with other serum markers. Further subgroup analysis revealed a close correlation between HBV RNA and HBV DNA in patients in the IA (e^+) phase ($\rho = 0.52$, $P < 0.001$; Fig. 2d), IA (e^-) phase ($\rho = 0.64$, $P < 0.01$; Fig. 2e) and GZ ($\rho = 0.46$, $P < 0.01$; Fig. 2f) but neither in IT (e^-) nor IC (e^-) phase (Fig. 2g, h).

HBV RNA level with liver inflammation grade and liver fibrosis stage

Since HBV RNA is a novel CHB virological biomarker which represent the hepatic cccDNA level, we further determined whether HBV RNA was associated with the grades of hepatic inflammation and fibrosis. HBV RNA levels varied among different inflammation grades, with the lowest median level in G0-1 [3.01 (0–3.96) $\log_{10}$copies/mL], intermediate level in G2 [3.69 (2.11–6.58) $\log_{10}$copies/mL], and the highest level in G3-4 [4.86 (3.72–7.23) $\log_{10}$copies/mL] (Fig. 3a). HBV DNA showed a similar trend as HBV RNA (Fig. 3b), but there was no difference of HBV DNA between G2 and G3-4 stages. Both HBV RNA and HBV DNA were not comparable among CHB patients with different liver fibrosis stages (Fig. 3c, d).

The correlations of HBV RNA with liver inflammation grades and fibrosis stages were then further analyzed. HBV RNA levels were weakly correlated with liver inflammation

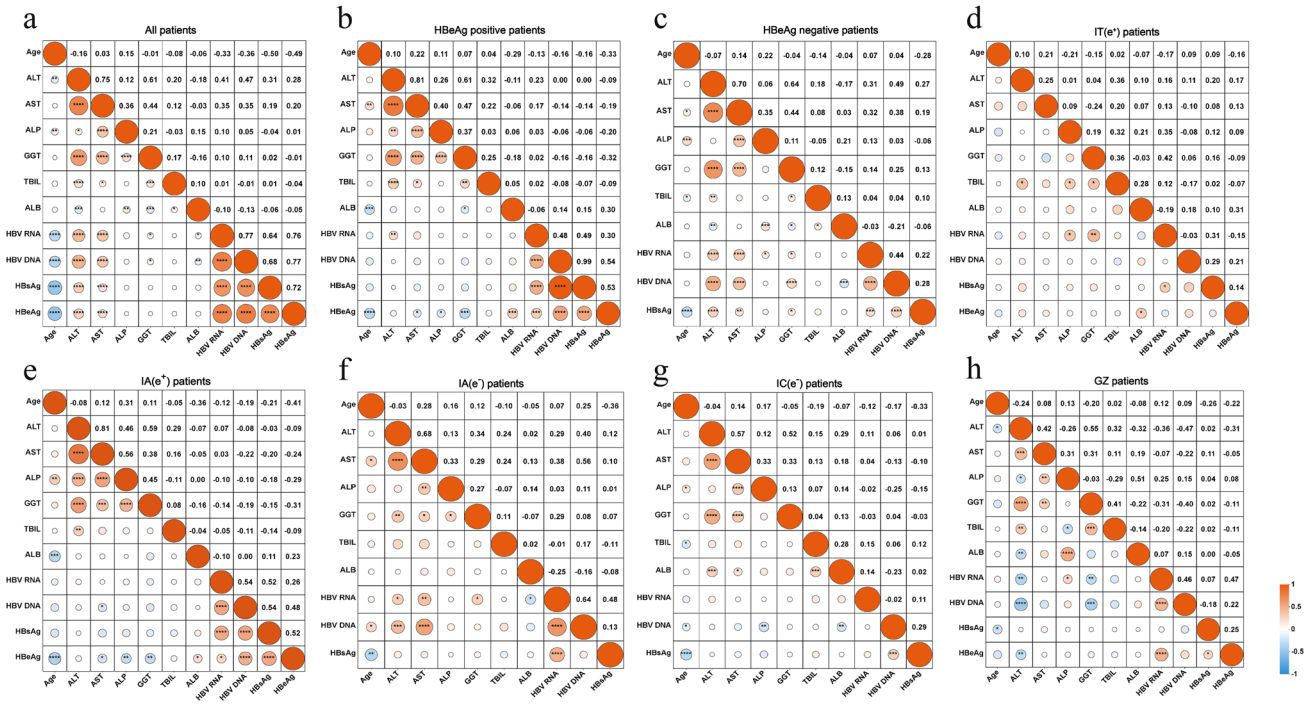

Fig. 2 Heatmap of pairwise correlations between different serological and viral biomarkers. The correlations of each serum HBV markers in the overall patients (**a**), HBeAg-positive patients (**b**), HBeAg-negative patients (**c**), IT(e+) patients (**d**), IA(e^+) patients (**e**), IA(e^-) patients (**f**), IC(e^-) patients (**g**), and GZ patients (**h**). Orange fields indicate positive correlations. The numbers in the fields represent Spearman's correlation coefficient (ρ) and associated P-values. *HBV* hepatitis B virus, *TBIL* total bilirubin, *ALT* alanine aminotransferase, *AST* aspartate transaminase, *HBeAg* hepatitis B e antigen, *HBsAg* hepatitis B surface antigen

grade (ρ = 0.28, P < 0.01, Fig. 3e) and not correlated with liver fibrosis stage (ρ = 0.07, P = 0.32; Fig. 3g); Similarly, HBV DNA levels were weakly correlated with liver inflammation grade (ρ = 0.21, P < 0.01; Fig. 3f) and not with liver fibrosis stage (ρ = 0.01, P = 0.86; Fig. 3h). Our data suggested that serum HBV RNA and HBV DNA both correlated weakly with liver inflammation grades and not with fibrosis stages. However, serum HBV RNA (ρ = 0.28, P < 0.01) had a slightly higher correlation with inflammation grade than HBV DNA (ρ = 0.21, P < 0.01).

HBV RNA level with liver inflammation grade and liver fibrosis stage in subgroups

We further analyzed the correlation of HBV RNA with hepatic inflammation and fibrosis in either HBeAg-positive patients or HBeAg-negative patients. Among HBeAg-positive patients, HBV RNA level had no statistical difference across inflammatory grades (Fig. 4a), and had no correlation with the grade of inflammation (Fig. 4b). HBV DNA level was the highest in G0-1 patients (Fig. 4c), with a weak negative correlation with inflammation grade (ρ = − 0.37, P < 0.01; Fig. 4d). HBV RNA level was the lowest in S3-4 patients (Fig. 4e), which had a negative correlation with the stage of liver fibrosis (ρ = − 0.4, P < 0.01; Fig. 4f). Patients with S3-4 fibrosis had the lowest HBV DNA level (Fig. 4g), which had a negative correlation with degree of liver fibrosis (ρ = − 0.44, P < 0.01; Fig. 4h).

In HBeAg-negative patients, the highest HBV RNA level was in G3-4 patients (Fig. 5a), and there was a weak correlation with liver inflammation (ρ = 0.21, P < 0.01; Fig. 5b). HBV RNA level had no statistical difference across different hepatic fibrosis stages (Fig. 5e), and had no correlation with the stage of liver fibrosis (ρ = 0.11, P = 0.23; Fig. 5f). HBV DNA level was not significantly altered among patients with different inflammation grades and fibrosis stages (Fig. 5c and g). Consistently, HBV DNA was not correlated with the degree of inflammation (ρ = 0.11, P = 0.18; Fig. 5d) and liver fibrosis (ρ = − 0.01, P = 0.95; Fig. 5h).

Discussion

In this study, serum HBV RNA of 411 CHB patients who had never been treated with anti-HBV drugs were analyzed. There were statistic differences in serum HBV RNA level among treatment-naïve CHB patients who were in different immune phases, as well as those defined as GZ phase. Meanwhile, liver histopathology was evaluated among 234 of these participants. In all cases, we did not observe the

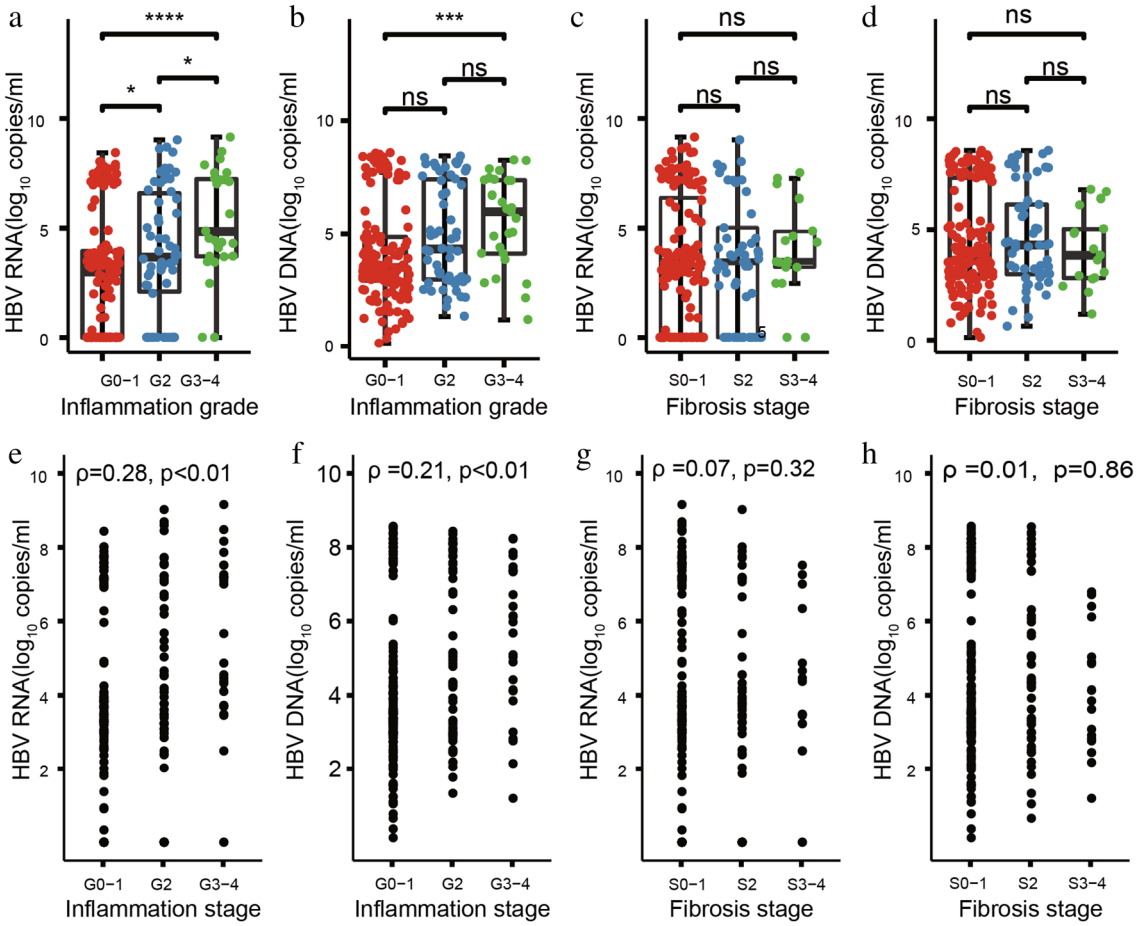

Fig. 3 Distribution and correlations between serum HBV RNA and HBV DNA levels in CHB patients with different histological inflammation grades and fibrosis stages. Serum HBV RNA levels in CHB patients with different inflammation grades (**a**), Serum HBV DNA levels in CHB patients with different inflammation grades (**b**), Serum HBV RNA levels in CHB patients with different fibrosis stage (**c**), Serum HBV DNA levels in CHB patients with different fibrosis stage (**d**). Correlations between serum HBV RNA and inflammation grades in patients with chronic HBV infection (**e**), Correlations between serum HBV DNA and inflammation grades in patients with chronic HBV infection (**f**), Correlations between serum HBV RNA and fibrosis stages in patients with chronic HBV infection (**g**), Correlations between serum HBV DNA and fibrosis stages in patients with chronic HBV infection (**h**)

correlation of HBV RNA with inflammation grade and liver fibrosis stage, which is different from previous reports (Wang et al. 2018a; Huang et al. 2020). In addition, the correlations between HBV RNA and liver inflammation grade and fibrosis stage are different in patients with different HBeAg status. Therefore, HBV RNA is more likely to be an effective indicator for CHB monitoring.

Consistent with previous studies (Butler et al. 2018; Mak et al. 2021; Ghany et al. 2021), serum HBV RNA and HBV DNA levels were comparable among different immune phases of CHB, while HBV RNA was 1–2 logs lower than HBV DNA. HBV RNA level was relatively higher in the IT(e^+) and IA(e^+) phases, intermediate in patients in the IA(e^-) and GZ phases, and the lowest in the IC(e^-) phase. These findings suggest that baseline serum HBV RNA level varies over the course of chronic HBV infection, but is not as effective as serum HBV DNA and ALT when utilized for distinguishing between different immune phases.

Consistent with previous reports (Ghany et al. 2021; Liao et al. 2022), Our study also revealed a good correlation between serum HBV RNA and HBV DNA, higher than the correlations with all other traditional HBV biomarkers. HBV RNA and HBV DNA were moderately correlated in IA(e^+), IA(e^-) and GZ phases, while in IT(e^+) and IC(e^-) phases they were not correlated. HBV RNA and HBsAg were moderately correlated in IA(e^+) and IA(e^-) phases, but not in other immune phases or GZ phases. HBV RNA and ALT were positively correlated in the IA(e^-) phase, negatively correlated in GZ phases, and not correlated in other phases. In contrast to the overall natural history, the correlation between serum HBV RNA and HBV DNA was reduced or vanished at different immune phases. These results differed

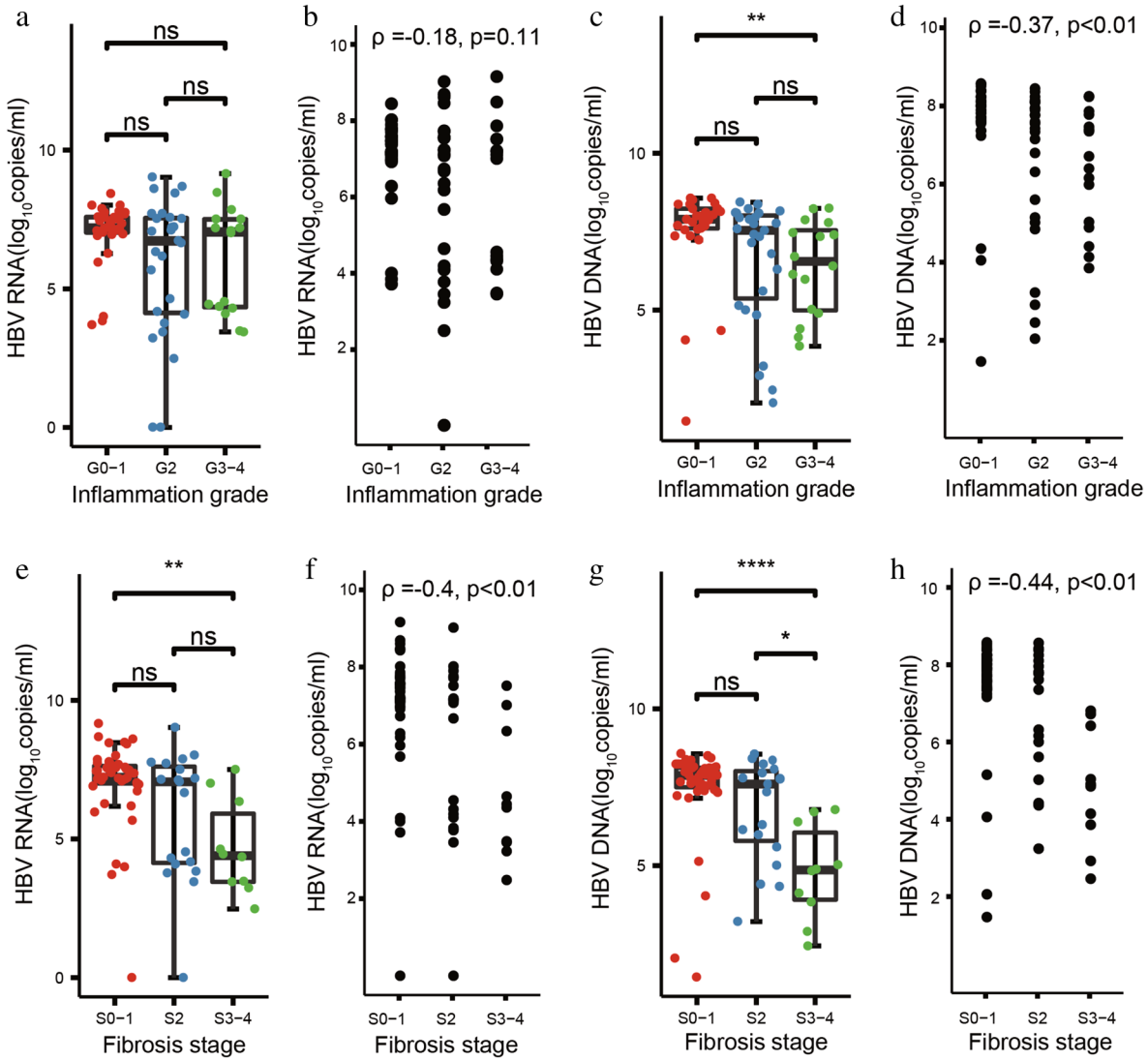

Fig. 4 Distribution and correlations between serum HBV RNA and HBV DNA with different histological inflammation grades and fibrosis stages in HBeAg-positive CHB patients. Serum HBV RNA levels at different inflammation grades in HBeAg-positive CHB patients (**a**), Serum HBV DNA levels at different inflammation grades in HBeAg-positive CHB patients (**b**), Serum HBV RNA levels at different fibrosis stages in HBeAg-positive CHB patients (**c**), Serum HBV DNA levels at different fibrosis stages in HBeAg-positive CHB patients (**d**); Correlations between serum HBV RNA levels and inflammation grades in HBeAg-positive CHB patients (**e**), Correlations between serum HBV DNA levels and inflammation grades in HBeAg-positive CHB patients (**f**), Correlations between serum HBV RNA levels and fibrosis stages in HBeAg-positive CHB patients (**g**), Correlations between serum HBV DNA levels and fibrosis stages in HBeAg-positive CHB patients (**h**)

to some extent from previous reports (Wang et al. 2018a; Liao et al. 2022).

According to current guidelines (Terrault et al. 2018), CHB patients with elevated levels of HBV DNA, ALT and AST should initiate anti-HBV treatment, as these are the populations with the greatest risk of advanced hepatic fibrosis, liver cirrhosis and hepatocellular carcinoma (Zhou et al. 2021). Many studies have elucidated that HBV RNA is specifically transcribed from intrahepatic cccDNA, thus it can directly reflect the transcriptional activity of intrahepatic cccDNA and has been considered as a surrogate biomarker for the transcriptional activity (Giersch et al. 2017; Huang et al. 2018; Wang et al. 2021). Serum HBV RNA may play a good complementary role in guiding clinical treatment.

We found small but meaningful differences in HBV RNA level in GZ patients among different subgroups (GZ-A to GZ-D). Compared with the lower levels in the GZ-D subgroup some of which were below the detection limit. It is possible that the intrahepatic cccDNA was cleared or in a state of transcriptional silence (Huang et al. 2018), or the detection sensitivity of the kit needs to be further improved. Our previous study has found that GZ-D patients had the

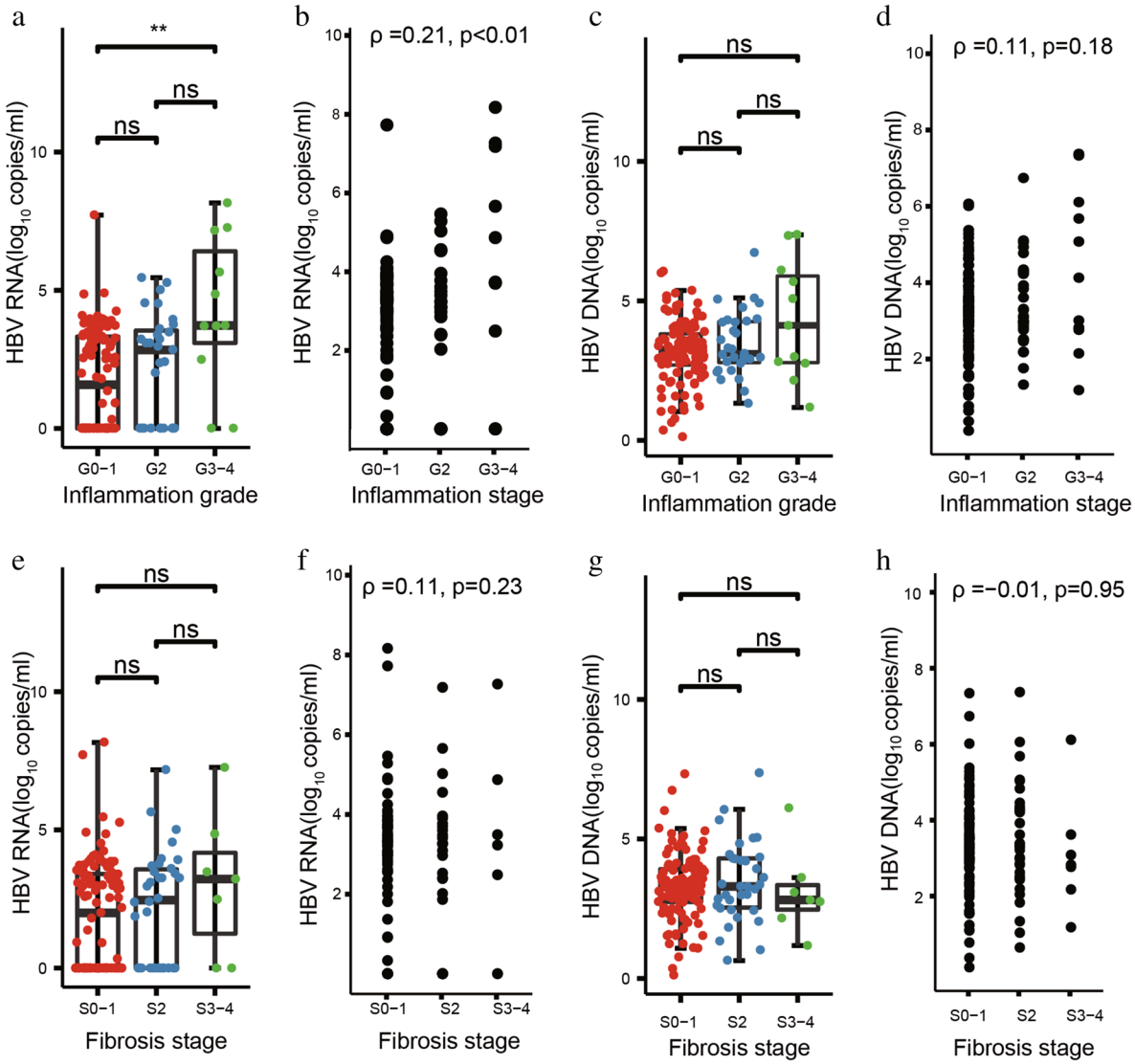

Fig. 5 Distribution and correlations between serum HBV RNA and HBV DNA with different histological inflammation grades and fibrosis stages in HBeAg-negative CHB patients. Serum HBV RNA levels at different inflammation grades in HBeAg-negative CHB patients (**a**), Serum HBV DNA levels at different inflammation grades in HBeAg-negative CHB patients (**b**), Serum HBV RNA levels at different fibrosis stages in HBeAg-negative CHB patients (**c**), Serum HBV DNA levels at different fibrosis stages in HBeAg-negative CHB patients (**d**); Correlations between serum HBV RNA levels and inflammation grades in HBeAg-negative CHB patients (**e**), Correlations between serum HBV DNA levels and inflammation grades in HBeAg-negative CHB patients (**f**), Correlations between serum HBV RNA levels and fibrosis stages in HBeAg-negative CHB patients (**g**), Correlations between serum HBV DNA levels and fibrosis stages in HBeAg-negative CHB patients (**h**)

lowest proportion of advanced liver fibrosis and cirrhosis (Yao et al. 2021; Wang et al. 2023). In addition, compared with the other three subgroups of GZ, the levels of HBV RNA and ALT in patients in the GZ-B subgroup were relatively higher, suggesting that patients in the GZ-B subgroup had higher activity of cccDNA transcription and viral replication (Mak et al. 2021; Wang et al. 2021). Serum ALT level has always been considered as a sensitive traditional hepatitis biomarker (Kim et al. 2008). With positive HBeAg, elevated ALT and HBV RNA levels, we speculated that CHB patients in the GZ-B subgroup may have a higher risk of further development of liver disease compared with other GZ patients.

HBV RNA levels could reflect dynamic changes of in CHB patients who had been treated with NAs (Lai et al. 2017; Wang et al. 2018b, 2021; Huang et al. 2018). Our data showed that distinct profile of HBV RNA for patients with different liver inflammation grades. HBV RNA level was the highest in patients with severe liver inflammation (G3-4), followed by those with moderate liver

inflammation (G2). Patients without or with only mild liver inflammation (G0-1) had the lowest HBV RNA level. HBV RNA was weakly correlated with liver inflammation grade, indicating that the level of serum HBV RNA may reflect liver inflammation in CHB patients. Nevertheless, we did not find a significant correlation between serum HBV RNA and the severity of liver fibrosis. Our data was different from a recent study (Huang et al. 2020), which might be due to different methods of HBV RNA in these two studies. In addition, our study had relative fewer patients with S3-4 liver fibrosis in this study, which may had caused selection bias.

HBV RNA was negatively correlated with fibrosis stage in HBeAg-positive CHB patients, and the lowest level was in patients with a liver fibrosis stage of S3-4, suggesting that serum HBV RNA levels decreased with the progression of liver inflammation and fibrosis in HBeAg-positive patients. Patients with positive HBeAg and high HBV viral load were mostly in the IT(e^+) phase or IA (e^+), with mild liver inflammation and low stage of fibrosis. Patients with repeated inflammation and necrosis of liver cells had high liver fibrosis stage, due to liver tissue damage (Bataller and Brenner 2005; Henderson et al. 2020). Severe liver tissue damage leads to the reduction of HBV RNA and HBV DNA particles released from liver cells into the blood (Nguyen et al. 2020). In HBeAg-negative patients, there were differences in the levels of serum HBV RNA among different liver inflammation grades. Serum HBV RNA level was significantly higher in patients with an inflammation grade of G3-4 than in patients with G0-1 and G2. Serum HBV RNA was weakly correlated with liver inflammation grade, and there was no statistic difference in the distribution of serum HBV DNA levels. HBV RNA and HBV DNA levels were neither statistically different nor correlated in different liver fibrosis stages.

There were several limitations in this study. Firstly, this study only recruited treatment-naive CHB patients. Whether our finding could be generalized to the treated CHB patients needs further verification. Secondly, the correlations between HBV RNA and liver histological severity needs further validation with larger sample size. Thirdly, due to technical reasons, cccDNA levels in situ liver tissue was not detected in this study, thus the correlations between cccDNA and other indicators were not investigated.

Serum HBV RNA varies in the natural course of chronic hepatitis B infection, but its role in differentiating immune stages of CHB is limited. In HBeAg-positive patients, HBV RNA correlates with the degree of liver fibrosis, these findings could help to improve understanding of the clinical value of HBV RNA. Simultaneous surveillance of HBV RNA and other serological indicators may be of benefit for physicians in the management of patients with CHB.

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Declarations

Competing interests The authors declare no competing interests.

References

Allweiss L, Dandri M (2017) The role of cccDNA in HBV maintenance. Viruses 9:156. https://doi.org/10.3390/v9060156

Bataller R, Brenner DA (2005) Liver fibrosis. J Clin Invest 115:209–218. https://doi.org/10.1172/JCI24282

Bonacci M, Lens S, Mariño Z et al (2018) Anti-viral therapy can be delayed or avoided in a significant proportion of HBeAg-negative Caucasian patients in the Grey Zone. Aliment Pharmacol Ther 47:1397–1408. https://doi.org/10.1111/apt.14613

Butler EK, Gersch J, McNamara A et al (2018) Hepatitis B virus serum DNA and RNA levels in Nucleos(t)ide analog-treated or untreated patients during chronic and acute infection. Hepatology 68:2106–2117. https://doi.org/10.1002/hep.30082

Carey I, Gersch J, Wang B et al (2020) Pregenomic HBV RNA and hepatitis B core-related antigen predict outcomes in hepatitis B e antigen-negative chronic hepatitis B patients suppressed on nucleos(T)ide analogue therapy. Hepatology 72:42–57. https://doi.org/10.1002/hep.31026

Chinese Society of Hepatology, Chinese Medical Association, Chinese Society of Infectious Diseases, Chinese Medical Association (2019) The guidelines of prevention and treatment for chronic hepatitis B (2019 version). Chin J Hepatol 35:2468–2669. https://doi.org/10.3760/cma.j.issn.1007-3418.2015.12.002

Coffin CS, Zhou K, Terrault NA (2019) New and old biomarkers for diagnosis and management of chronic hepatitis B virus infection. Gastroenterology 156:355-368.e3. https://doi.org/10.1053/j.gastro.2018.11.037

Fanning GC, Zoulim F, Hou J, Bertoletti A (2019) Therapeutic strategies for hepatitis B virus infection: towards a cure. Nat Rev Drug Discov 18:827–844. https://doi.org/10.1038/s41573-019-0037-0

Ghany MG, King WC, Lisker-Melman M et al (2021) Comparison of HBV RNA and hepatitis B core related antigen with conventional HBV markers among untreated adults with chronic hepatitis B in North America. Hepatology 74:2395–2409. https://doi.org/10.1002/hep.32018

Giersch K, Allweiss L, Volz T et al (2017) Serum HBV pgRNA as a clinical marker for cccDNA activity. J Hepatol 66:460–462. https://doi.org/10.1016/j.jhep.2016.09.028

Henderson NC, Rieder F, Wynn TA (2020) Fibrosis: from mechanisms to medicines. Nature 587:555–566.https://doi.org/10.1038/s41586-020-2938-9

Höner zu Siederdissen C, Maasoumy B, Cornberg M (2017) What is new on HBsAg and other diagnostic markers in HBV infection. Best Pract Res Clin Gastroenterol 31:281–289. https://doi.org/10.1016/j.bpg.2017.04.009

Huang H, Wang J, Li W et al (2018) Serum HBV DNA plus RNA shows superiority in reflecting the activity of intrahepatic cccDNA in treatment-naïve HBV-infected individuals. J Clin Virol 99–100:71–78. https://doi.org/10.1016/j.jcv.2017.12.016

Huang C, Li Q, Xu W et al (2020) Serum HBV RNA levels predict significant liver fibrosis in patients with chronic HBV infection. Discov Med 29:119–128

Kim WR, Flamm SL, Di Bisceglie AM, Bodenheimer HC (2008) Serum activity of alanine aminotransferase (ALT) as an indicator of health and disease. Hepatology 47:1363–1370. https://doi.org/10.1002/hep.22109

Köck J, Theilmann L, Galle P, Schlicht H (1996) Hepatitis B virus nucleic acids associated with human peripheral blood mononuclear cells do not originate from replicating virus: hepatitis B virus nucleic acids associated with human peripheral blood mononuclear cells do not originate from replicating virus. Hepatology 23:405–413. https://doi.org/10.1002/hep.510230303

Lai C-L, Wong D, Ip P et al (2017) Reduction of covalently closed circular DNA with long-term nucleos(t)ide analogue treatment in chronic hepatitis B. J Hepatol 66:275–281. https://doi.org/10.1016/j.jhep.2016.08.022

Liao H, Li L, Zheng WV et al (2022) Characteristics of HBV novel serum markers across distinct phases in treatment-naïve chronic HBV-infected patients. Dis Markers 2022:1–13. https://doi.org/10.1155/2022/4133283

Liu S, Zhou B, Valdes JD et al (2019) Serum hepatitis B virus RNA: a new potential biomarker for chronic hepatitis B virus infection. Hepatology 69:1816–1827. https://doi.org/10.1002/hep.30325

Liu S, Liu Z, Li W et al (2020) Factors associated with the biphasic kinetics of serum HBV RNA in patients with HBeAg-positive chronic hepatitis B treated with nucleos(t)ide analogues. Aliment Pharmacol Ther 52:692–700. https://doi.org/10.1111/apt.15890

Lok ASF, McMahon BJ (2007) Chronic hepatitis B. Hepatology 45:507–539. https://doi.org/10.1002/hep.21513

Mak L, Cloherty G, Wong DK et al (2021) HBV RNA profiles in patients with chronic hepatitis B under different disease phases and antiviral therapy. Hepatology 73:2167–2179. https://doi.org/10.1002/hep.31616

Nassal M (2015) HBV cccDNA: viral persistence reservoir and key obstacle for a cure of chronic hepatitis B. Gut 64:1972–1984. https://doi.org/10.1136/gutjnl-2015-309809

Nguyen MH, Wong G, Gane E et al (2020) Hepatitis B virus: advances in prevention, diagnosis, and therapy. Clin Microbiol Rev 33:e00046-e119. https://doi.org/10.1128/CMR.00046-19

Ren S, Wang W, Lu J et al (2022) Effect of the change in antiviral therapy indication on identifying significant liver injury among chronic hepatitis B virus infections in the grey zone. Front Immunol 13:1035923. https://doi.org/10.3389/fimmu.2022.1035923

Rockey DC, Caldwell SH, Goodman ZD et al (2009) Liver biopsy. Hepatology 49:1017–1044. https://doi.org/10.1002/hep.22742

Russo FP, Imondi A, Lynch EN, Farinati F (2018) When and how should we perform a biopsy for HCC in patients with liver cirrhosis in 2018? A review. Dig Liver Dis 50:640–646. https://doi.org/10.1016/j.dld.2018.03.014

Schcuer J (1991) Classification of chronic viral hepatitis: a need for reassessment. J Hepatol 13:372–374. https://doi.org/10.1016/0168-8278(91)90084-o

Shen S, Xie Z, Cai D et al (2020) Biogenesis and molecular characteristics of serum hepatitis B virus RNA. PLoS Pathog 16:e1008945. https://doi.org/10.1371/journal.ppat.1008945

Tang LSY, Covert E, Wilson E, Kottilil S (2018) Chronic hepatitis B infection: a review. JAMA 319:1802. https://doi.org/10.1001/jama.2018.3795

Terrault NA, Bzowej NH, Chang K-M et al (2016) AASLD guidelines for treatment of chronic hepatitis B: hepatology, month 2015. Hepatology 63:261–283. https://doi.org/10.1002/hep.28156

Terrault N, Lok A, McMahon B et al (2018) Update on prevention, diagnosis, and treatment and of chronic hepatitis B: AASLD 2018 hepatitis B guidance. Hepatology 67:1560–1599. https://doi.org/10.1002/hep.29800

Tsuge M, Murakami E, Imamura M et al (2013) Serum HBV RNA and HBeAg are useful markers for the safe discontinuation of nucleotide analogue treatments in chronic hepatitis B patients. J Gastroenterol 48:1188–1204. https://doi.org/10.1007/s00535-012-0737-2

van Bömmel F, van Bömmel A, Krauel A et al (2018) Serum HBV RNA as a predictor of peginterferon Alfa-2a response in patients with HBeAg-positive chronic hepatitis B. J Infect Dis 218:1066–1074. https://doi.org/10.1093/infdis/jiy270

Wang J, Yu Y, Li G et al (2018a) Natural history of serum HBV-RNA in chronic HBV infection. J Viral Hepat 25:1038–1047. https://doi.org/10.1111/jvh.12908

Wang J, Yu Y, Li G et al (2018b) Relationship between serum HBV-RNA levels and intrahepatic viral as well as histologic activity markers in entecavir-treated patients. J Hepatol 68:16–24. https://doi.org/10.1016/j.jhep.2017.08.021

Wang X, Chi X, Wu R et al (2021) Serum HBV RNA correlated with intrahepatic cccDNA more strongly than other HBV markers during peg-interferon treatment. Virol J 18:4. https://doi.org/10.1186/s12985-020-01471-2

Wang J, Yan X, Zhu L et al (2023) Significant histological disease of patients with chronic hepatitis B virus infection in the grey zone. Aliment Pharmacol Ther 57:464–474. https://doi.org/10.1111/apt.17272

World Health Organization (2021) World health statistics 2021: monitoring health for the SDGs, sustainable development goals. World Health Organization, Geneva

Yao K, Liu J, Wang J et al (2021) Distribution and clinical characteristics of patients with chronic hepatitis B virus infection in the grey zone. J Viral Hepat 28:1025–1033. https://doi.org/10.1111/jvh.13511

Zhou J, Wang F-D, Wang M-L et al (2021) Antiviral therapy for chronic HBV infection with persistently normal alanine aminotransferase: controversy and consensus. Front Med (lausanne) 8:717125. https://doi.org/10.3389/fmed.2021.717125