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Analysis of serum hepatitis B virus RNA levels among HBsAg and HBsAb copositive patients and its correlation with HBV DNA

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Abstract

There are approximately 2 billion HBV-infected individuals worldwide, and approximately 1.87% to 7% of these individuals are copositive for HBsAg and HBsAb.

Our study detected hepatitis B virus pgRNA (HBV RNA) levels in HBsAg and HBsAb copositive patients and then analyzed the correlation with HBV DNA, HBsAg, ALT, and AST levels. A total of 149 HBsAg and HBsAb copositive patients were identified from 66,617 outpatients.

HBV RNA, HBV DNA, HBsAg, ALT, and AST serum levels were significantly different in different natural phases of HBV infection (immune tolerance phase, immune clearance phase, low replication phase, and reactivation phase) with statistical significance ($P < .01$). HBV RNA levels were positively correlated with HBV DNA, HBsAg, ALT, and AST levels. HBV RNA and HBV DNA levels were significantly increased in the HBeAg-positive group (66 patients) compared with the HBeAg-negative group (83 patients) ($P < .01$). In the HBeAg-positive group, HBV RNA levels were positively correlated with HBV DNA and HBsAg levels. In the HBeAg-negative group, HBV RNA levels were positively correlated with HBV DNA. Serum HBV RNA levels were positively correlated with HBV DNA, HBsAg, ALT, and AST levels.

HBV RNA could be used as a virological indicator for antiviral therapy in HBsAg and HBsAb copositive hepatitis B patients.

Abbreviations: cccDNA = covalently closed DNA, HBV RNA = hepatitis B virus pgRNA, NAs = nucleot(s)ide and its analogs, pgRNA = pregenomic RNA, VR = virological response.

Keywords: copositive patients, HBV DNA, HBV RNA, hepatitis B virus

1. Introduction

Hepatitis B virus (HBV) infection is a serious public health problem, and there are approximately 2 billion HBV-infected individuals worldwide. It is estimated that approximately 786,000 people die from chronic HBV infection-associated cirrhosis or hepatocellular carcinoma every year.[1–4] In recent years, HBV serological patterns have changed due to mutations in the HBV gene, optimization of detection reagents, improvements in test methods, and drug resistance caused by long-term medication. As a special serological patterns of HBV infection, the rate of HBsAg and HBsAb copositive is increasing. It has been reported that HBsAg and HBsAb copositive appear in approximately 1.87% to 7% of HBV-infected patients[5–7] and many studies showed that this type of HBV-infection have some special features.[8,9]

In the *Guidelines of Prevention and Treatment for Chronic Hepatitis B* (2015) issued by the Chinese Society of Hepatology and Chinese Society of Infectious Diseases, it is stated that if a sustained off-treatment response cannot be obtained at the basic endpoint of CHB treatment, the long-term virological response (VR) should be maintained with antiviral therapy to continue suppression so that HBV DNA cannot be detected.[10] However, this situation cannot be achieved with the use of existing reagents for HBV DNA. HBV covalently closed DNA (cccDNA) only exists in the nucleus of infected hepatocytes and cannot be damaged by existing antiviral drugs, and a high proportion of virological rebound and disease relapse often occur after drug withdrawal.[11] Studies have shown that it is difficult to clinically

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cure CHB due to the existence of cccDNA.[12,13] However, the detection of cccDNA requires liver biopsy, which is invasive and not applicable in clinical practice. The disappearance of HBV DNA only indicates that the reverse transcription of the virus is effectively inhibited and cannot reflect the status of transcriptional activity of cccDNA. Therefore, it is urgent to identify a new serological marker to replace cccDNA in the clinic. In recent years, it has been reported that HBV pregenomic RNA (HBV RNA), which is produced from the transcription of cccDNA in the nucleus of infected hepatocytes, exists in the serum or plasma of HBV-infected patients. The envelope of nucleocapsid-encapsulated HBV RNA was obtained in the absence of reverse transcription, which was then released from the infected hepatocytes into serum or plasma where it was detected. HBV RNA levels in serum reflect the expression levels of cccDNA and its transcriptional activity.[14–16]

Above all, in this study, we selected the samples which HBsAg and HBsAb were copositive. The levels of HBV RNA, HBV DNA, HBsAg, ALT and AST in different natural phases of disease were compared. The correlations between HBV RNA and HBV DNA, HBsAg, ALT, and AST were analyzed as well as the influence of HBeAg expression on HBV RNA, providing new virological indicators for antiviral therapy in patients with hepatitis B.

2. Materials and methods

2.1. Patients and subjects

The results of HBV serological marker tests (HBsAg, HBsAb, HBeAg, HBeAb, and HBcAb) in 66,617 outpatients from the First Affiliated Hospital of Chongqing Medical University from May 2017 to May 2018 were analyzed. A total of 149 HBsAg and HBsAb copositive patients (**without virus treatment**) aged 22 to 62 years (median, 32) were identified and divided into an HBeAg-positive group (66 patients) and an HBeAg-negative group (83 patients) based on HBeAg detection results. Serum HBV RNA, HBV DNA, ALT, and AST levels were analyzed in these patients. Only 90 of the 149 HBsAg and HBsAb copositive patients could be assigned to different phases (11 patients were in the immune tolerance phase, 31 patients in the immune clearance phase, 15 patients in the low replication phase and 33 patients in the reactive phase). Clinical data on all subjects were collected by questionnaires and by reviewing medical records, and informed consent was obtained from all subjects. All procedures of this study were approved by the Ethics Committee of the First Affiliated Hospital of Chongqing Medical University.

Patients were eligible for the study if they were diagnosed with CHB infection based on the diagnostic criteria in accordance with the *Guidelines of Prevention and Treatment for Chronic Hepatitis B* (2015) issued by the Chinese Society of Hepatology and Chinese Society of Infectious Diseases; did not have other hepatitis virus disease and HIV infections; did not have other liver diseases (such as autoimmune hepatitis and alcoholic hepatitis, etc); and had not recently received immunosuppressants.

2.2. Detection of indicators

HBV serological markers (HBsAg, HBsAb, HBeAg, HBeAb, and HBcAb) were detected by chemiluminescence microparticle immunoassay using an ARCHITECT i2000SR Automatic Chemiluminescence Immunoanalyzer (Abbott, USA), and the HBsAg and HBsAb copositive samples were identified. The following criteria for reactivity were employed: HBsAg > 0.04 IU/mL, HBsAb > 10.00 mIU/mL, HBeAg > 1 S/CO, HBeAb < 1 S/CO and HBcAb > 1 S/CO.

Serum ALT and AST levels were quantitatively detected by the rate method using a Hitachi RL7600 automatic biochemical analyzer with the following reference intervals: ALT, 7 to 40 U/L; AST, 13 to 40 U/L.

HBV B, C, and D genotypes were detected by Cobas 480 real-time fluorescent PCR (Roche, USA) using an HBV genotyping kit (PCR-fluorescent probe method, Triplex International Biosciences Co., Ltd., China).

HBV DNA load was detected by Cobas 480 real-time fluorescent PCR (Roche, USA) using a Hepatitis B Virus Nucleic Acid Kit (PCR-fluorescent probe method, Sansure Biotech Co., Ltd., China) with a lower limit of detection of 1.0×10^2 IU/mL.

HBV RNA load was detected by Cobas 480 real-time fluorescent PCR (Roche, USA) using a Hhepatitis B virus pgRNA (HBV RNA) Kit (PCR-fluorescent probe method, Hotgen Biotech Co., Ltd., China) with a lower limit of detection of 3.0×10^2 copies/mL.

2.3. Assignment of HBV-infected patients to different phases

According to the *Guidelines of Prevention and Treatment for Chronic Hepatitis B* (2015), the natural history of HBV infection can be divided into 4 phases: immune tolerance phase, immune clearance phase, inactive or low replication phase, and viral reactive phase. The immune tolerance phase is characterized positive HBsAg, positive HBeAg, HBV DNA $> 1 \times 10^6$ IU/mL, and normal ALT levels; the immune clearance phase is characterized by positive HBsAg, positive HBeAg, HBV DNA $> 2 \times 10^3$ IU/mL, and continuous or intermittent increases in ALT levels; the inactive or low replication phase is characterized by negative HBeAg, positive anti-HBe, HBV DNA $< 1 \times 10^3$ IU/mL, and normal ALT levels; and the viral reactive phase is characterized by negative HBeAg, positive anti-HBe, HBV DNA $> 1 \times 10^3$ IU/mL, and continuous or repeated increases in ALT levels.

2.4. Statistical methods

Data in the study were analyzed with SPSS 17.0 software. The X^2 test was used for enumeration data that did not conform to a normal distribution. The results are described statistically by the median (interquartile range) [M (P25–P75)]. The Mann–Whitney *U* test was used for between-group comparisons, and the Kruskal–Wallis H test was used for multigroup comparisons. Spearman correlation analysis was also used with a *P*-value <.05 being statistically significant.

3. Results

3.1. Description of common clinical characteristics

Among 149 HBsAg and HBsAb copositive patients, the levels of HBsAg, HBV RNA, HBV DNA, ALT, and AST are expressed as the median (interquartile range) [M (P25–P75)]. In total, 149 HBsAg and HBsAb copositive patients were divided into the HBeAg-positive group (66 patients, 44.30%) and HBeAg-negative group (83 patients, 55.70%) based on the results of HBeAg detection, as shown in Table 1.

Table 1

Description of common clinical characteristics of the HBsAg and HBsAb co-positive patients.

Clinical characteristics	Results
n	149
Sex	
Male n (%)	86 (57.72%)
Female n (%)	63 (42.28%)
Age (yr)	32 (22–62)
HBsAg (IU/mL)	193.78 (39.56,1730.65)
HBV DNA (LOG IU/mL)	4.72 (3.38,5.92)
HBV RNA (LOG copies/mL)	2.24 (0.00,3.28)
ALT (U/L)	34 (21,66)
AST (U/L)	32 (22,62)
HBeAg	
Positive n (%)	66 (44.30%)
Negative n (%)	83 (55.70%)

3.2. Analysis of HBV genotype

Among the 149 HBsAg and HBsAb copositive patients, 141 (94.63%) were genotype B, and 7 (5.37%) were genotype C. In the HBeAg-positive group and HBeAg-negative group, a significant difference in genotype distribution ($X^2=6.395$, $P<.01$) was noted, as shown in Table 2.

3.3. Analysis of the effect of HBeAg on HBV RNA

Among the 149 HBsAg and HBsAb copositive patients, HBV RNA and HBV DNA levels were higher in the HBeAg-positive group than in the HBeAg-negative group (HBV RNA: U= 1161.50, $P<0.01$; HBV DNA: U=1080.00, $P<0.01$) with statistically significant differences. However, the differences in HBsAg, ALT, and AST expression levels were not statistically significant ($P>.05$), as shown in Table 3.

3.4. Natural courses of HBsAg and HBsAb copositive patients

Only 90 of the 149 HBsAg and HBsAb copositive patients could be assigned to different phases. Among them, 11 patients (12.22%) were in the immune tolerance phase, 31 (34.44%) in the immune clearance phase, 15 (16.67%) in the low replication phase and 33 (36.67%) in the reactive phase. HBV RNA, HBV DNA, HBsAg, ALT and AST levels differed among the different phases of the HBV infection course (H=35.73, $P<.01$; H= 52.43, $P<.01$; H=11.71, $P<.01$; H=39.21, $P<.01$; H=40.46, $P<.01$) with statistical significance, as shown in Table 4. HBV RNA, HBV DNA, and HBsAg levels in the immune tolerance phase were increased compared with those in immune clearance phase, low replication phase and reactive phase with statistically significant differences (HBV RNA: U=35.00, 1.00, 12.00, $P<.01$; HBV DNA: U=31.00, 1.00, 22.50, $P<.01$: HBsAg:

Table 2

Analysis of HBV genotype.

HBV genotypes	n	HBeAg positive group	HBeAg negative group	X^2	*P*-value*
Genotype B	141 (94.63%)	59 (89.39%)	82 (98.79%)	6.395	<.01
Genotype C	8 (5.37%)	7 (1.61%)	1 (1.21%)		
In total	149	66	83	–	–

* Categorical variables: chi-squared test, genotype distribution $P<.05$.

Table 3

Analysis of the effect of HBeAg on HBV RNA.

Indicators	HBeAg positive group	HBeAg negative group	U/X^2	*P*-value†
Number of patients n	66 (44.30%)	83 (55.70%)	–	–
Age (yr)	42.09±16.15	52.88±14.03	73.06	.043
Male n (%)	40 (60.6%)	46 (55.42%)	0.41	.525
Female n (%)	26 (39.4%)	37 (44.58%)		
HBV RNA (LOG IU/mL)	3.272.24, (4.34)	1.17 (0.00, 2.80)	1161.50	.000*
HBV DNA (LOG IU/mL)	5.61 (4.88, 6.69)	3.75 (2.98, 5.00)	1080.00	.000**
HBsAg (IU/mL)	217.07 (43.72, 8038.54)	160.41 (18.34, 1444.51)	2288.00	.085
ALT (U/L)	39.50 (21.00, 72.00)	30.00 (20.00, 59.00)	2233.00	.053
AST (U/L)	35.00 (21.75, 72.50)	30.00 (23.00, 60.00)	2382.00	.172

† Categorical variables: chi-squared test; continuous variables: *U* test. HBV RNA: *$P<.01$, HBV DNA, **$P<.01$).

Table 4

Analysis of different natural phases of HBsAg and HBsAb co-positive patients.

Clinical Phases	n	HBV RNA (LOG copies/ml)	HBV DNA (LOG IU/ml)	HBsAg (IU/ml)	ALT (U/L)	AST (U/L)
Immune tolerance phase	11	5.04 (4.61, 5.65)	7.78 (6.27, 8.23)	12567.24 (193.27, 25,000.00)	28 (18, 35)	21 (20, 31)
Immune clearance phase	31	2.92 (1.99, 3.84)	5.58 (5.09, 6.45)	187.42 (40.49, 1454.56)	67 (41, 152)	71 (41, 141)
Low replication phase	15	0.00 (0.00, 1.38)	2.57 (2.34, 2.86)	62.13 (2.18, 998.41)	23 (16, 30)	25 (23, 27)
Viral reactive phase	33	2.68 (0.00, 3.11)	5.26 (4.10, 6.00)	687.65 (51.07, 2204.77)	62 (37, 90)	60 (37, 83)
H	–	35.73	52.43	11.71	39.21	40.46
P-value†	–	.00*	.00**	.01***	.00****	.00*****

† Continuous variables: H test, HBV RNA:*$P<.01$; HBV DNA:**$P<.01$; HBsAg: ***$P<.01$; ALT: ****$P<.01$; AST *****$P<.01$.

Table 5

Correlation analysis between HBV RNA and HBV DNA, HBsAg, ALT, AST.

Indicators	Spearman correlation coefficient r	*P*-value*
HBV DNA (LOG IU/mL)	0.667	.000*
HBsAg (IU/mL)	0.330	.000**
ALT (U/L)	0.263	.001***
AST (U/L)	0.218	.007****

Spearman correlation test HBV DNA: *$P < .01$; HBsAg: **$P < .01$; ALT: ***$P < .01$; AST ****$P < .01$.

U = 83.50, 25.00, 86.00, $P < .01$). Fifty-nine of the 149 HBsAg and HBsAb copositive patients were unclassified. HBV RNA, HBV DNA, HBsAg, ALT, and AST levels were 1.68 (0.00, 2.93), 3.85 (3.14, 4.98), 147.43 (25.75, 1516.12), 23 (17.32), and 24 (19.32.5), respectively.

3.5. Correlation analysis between HBV RNA and HBV DNA, HBsAg, ALT, and AST

Correlation analysis between HBV RNA and HBV DNA, HBsAg, ALT, and AST in the 149 HBsAg and HBsAb copositive patients: HBV RNA levels in serum were positively correlated with HBV DNA (correlation efficient r = 0.667, $P = .000$), HBsAg (correlation efficient r = 0.330, $P = .000$), ALT (correlation efficient r = 0.263, $P = .001$) and AST levels (correlation efficient r = 0.218, $P = .007$), as shown in Table 5 and Figure 1A–D. In the HBeAg-positive group, HBV RNA levels were positively correlated with HBV DNA (correlation coefficient r = 0.595, $P = .000$) and HBsAg levels (correlation coefficient r = 0.508, $P = .000$), as shown in Table 6. In the HBeAg-negative group, HBV RNA levels were positively correlated with HBV DNA levels (correlation coefficient r = 0.530, $P = .000$), as shown in Table 7.

4. Discussion

The coexistence of HBsAg and anti-HBs in patients with CHB infection has been reported. Various factors can lead to the coexistence of HBsAgs and anti-HBs[17–20]: genetic mutation in the S or pre-S region of HBV causes a change in the immunogenicity of the "a" determinant of surface antigen; infection of the mutant HBV strain or double infection or successive infection of different subtypes of HBV; and genetic mutation induced by long-term medication. A total of 66,617 samples of HBV serological markers were screened in this study, and 149 that were HBsAg and HBsAb copositive were selected for analysis.

Current guidelines recommend that the existing virological indicators (HBV DNA, HBeAg status, HBV genotypes) and clinical "variables" (ALT, liver histology or noninvasive tests) can be used to determine the necessity of antiviral drugs and to assess the progression of the disease.[16,21,22] Of the 149 HBsAg and HBsAb copositive patients, 141 (94.63%) were genotype B, and 7 (5.73%) were genotype C, indicating that B is the dominant genotype. A significant difference in the distribution of genotypes

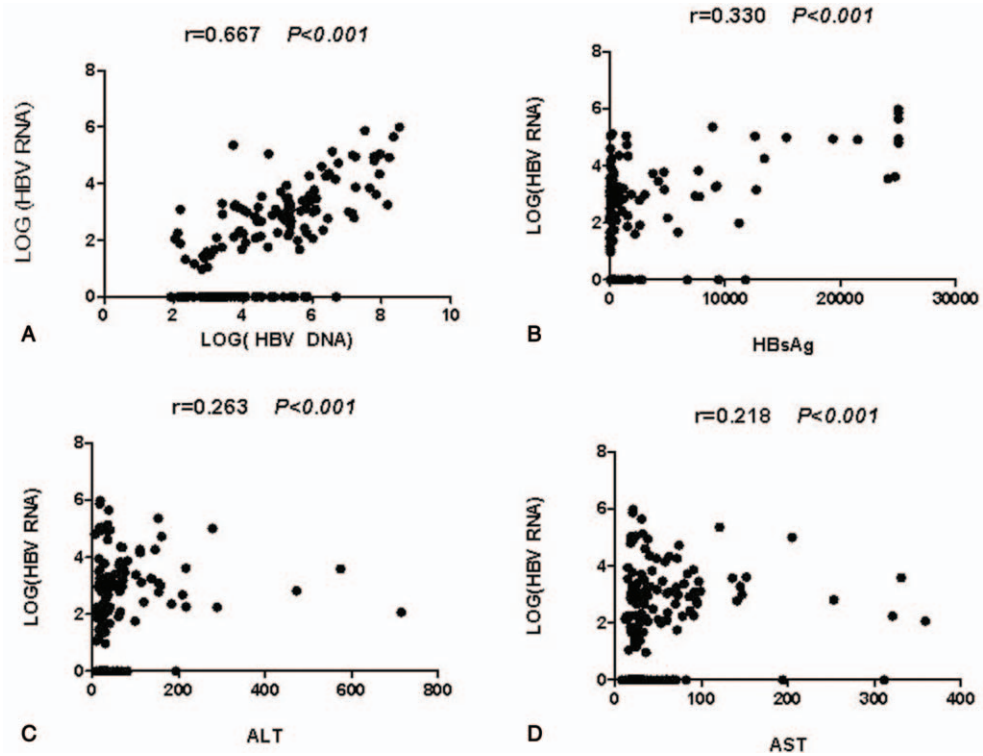

Figure 1. A–D Correlation of HBV RNA, HBV DNA, HBsAg, ALT, and AST levels. A, HBV RNA levels were positively correlated with HBV DNA (correlation efficient r = 0.667, $P = .000$). B, HBsAg (correlation efficient r = 0.330, $P = .000$). C, ALT (correlation efficient r = 0.263, $P = .001$). D, AST (correlation efficient r = 0.218, $P = .007$).

Table 6

Correlation between HBV RNA and HBV DNA, HBsAg, ALT, AST in HBeAg positive group.

Indicators	Spearman correlation coefficient r	*P*-value*
HBV DNA (LOG IU/mL)	0.595	.000*
HBsAg (IU/mL)	0.508	.000**
ALT (U/L)	−0.051	.687
AST (U/L)	−0.128	.304

Spearman correlation test HBV DNA:* $P<.01$; HBsAg: ** $P<.01$.

Table 7

Correlation between HBV RNA and HBV DNA, HBsAg, ALT, AST in HBeAg negative group.

Indicators	Spearman correlation coefficient r	*P*-value*
HBV DNA (LOG IU/mL)	0.530	.000*
HBsAg (IU/mL)	0.117	.294
ALT (U/L)	0.433	.000**
AST (U/L)	0.446	.000***

Spearman correlation test HBV RNA:* $P<.01$; ALT: ** $P<.01$; AST *** $P<.01$.

($X^2=6.395$, $P<.01$) was noted in both the HBeAg-positive and HBeAg-negative groups.

Currently, nucleot(s)ide and its analogs (NAs) are widely used in the antiviral therapy of CHB. Mechanistically, these agents control the reverse transcription of HBV (inhibiting viral DNA polymerase proteins with reverse transcription activity) and inhibit the synthesis of DNA to play an antiviral role. However, NAs cannot eradicate cccDNA in the nucleus of hepatocytes, but cccDNA levels were evident. Its transcriptional activity is the main factor that impedes clinical cure in CHB patients through antiviral therapy. The guidelines define HBV DNA levels below the lower limit of detection in serum as VR and use it as one of the treatment endpoints for drug withdrawal.[23–26] Virological rebound occurs in many CHB patients after drug withdrawal for half a year or more when HBV DNA levels are below the lower limit of detection, leading to disease recurrence.[11] The disappearance of HBV DNA only indicates that the reverse transcription of HBV was effectively inhibited and does not reflect the transcription status of cccDNA.

Therefore, it is urgent to identify a new HBV virological marker to evaluate the efficacy of antiviral therapy in CHB patients.

In 1996, German scholars found the presence of HBV RNA in the serum of chronic HBV-infected patients.[27] HBV RNA in serum is pregenomic RNA (pgRNA) that has not undergone reverse transcription. These pgRNAs exist in the nucleocapsid of mature viral particles and are called "HBV RNA virus-like particles".[16,28] Detection of HBV RNA levels in serum or plasma is of great significance to the auxiliary diagnosis of HBV infection, the monitoring of therapeutic effects of NAs on CHB patients and the prediction of drug withdrawal.

Only 90 of the 149 HBsAg and HBsAb copositive patients could be assigned to different phases according to their natural course of disease. Among them, 11 patients (12.22%) were in the immune tolerance phase, 31 (34.44%) were in the immune clearance phase, 15 (16.67%) were in the low replication phase and 33 (36.67%) were in the viral reactive phase. HBV RNA, HBV DNA, HBsAg, ALT and AST levels were significantly different in different natural phases of HBV infection ($H=35.73$, $P<.01$; $H=52.43$, $P<.01$; $H=11.71$, $P<.01$; $H=39.21$, $P<.01$; $H=40.46$, $P<.01$). HBV RNA, HBV DNA, and HBsAg levels in the immune tolerance phase were significantly increased compared with those in the immune clearance phase, low replication phase and viral reactive phase ($P<.01$). HBsAg and HBsAb copositive patients who were in the viral reactive phase were the most prevalent, accounting for 36.67%. This finding suggests that new HBV virological markers are in great need to monitor antiviral therapy in HBsAg and HBsAb copositive patients. In addition, with the advancement of different natural phases, HBV RNA levels gradually decreased with decreasing HBV DNA levels during immune tolerance, immune clearance and the low replication phase. Therefore, the combined detection of HBV RNA, HBV DNA and other indicators has important clinical significance in the assignment of CHB patients to different phases, and the judgment of their condition and can provide a more reliable clinical basis for antiviral therapy in CHB patients.

In this study, the following correlation analysis results between HBV RNA levels and HBV DNA, HBsAg, ALT, and AST levels in the 149 HBsAg and HBsAb copositive patients were noted: serum HBV RNA levels were all positively correlated with HBV DNA (correlation efficient $r=0.667$, $P=.000$), HBsAg (correlation efficient $r=0.330$, $P=.000$), ALT (correlation efficient $r=0.263$, $P=.001$) and AST levels (correlation efficient $r=0.218$, $P=.007$). The best correlation was noted between HBV RNA levels and HBV DNA levels, which is consistent with previous studies.[29–30] Therefore, HBV RNA can be used as a potential virological marker to assess the efficacy of antiviral therapy in CHB patients. A recent study indicated that HBV RNA may exhibit a fast and significant decline that correlates with treatment response and HBsAg loss at long-term follow-up during PEG-IFN treatment for HBeAg-negative CHB.[31] In addition, HBeAg expression levels impacted HBV RNA and HBV DNA expression levels; in other words, HBV RNA and HBV DNA expression levels were higher in the HBeAg-positive group than in the HBeAg-negative group with a statistically significant difference (HBV RNA: $U=1161.50$, $P<.01$; HBV DNA: $U=1080.00$, $P<.01$). In the HBeAg-positive group, HBV RNA levels were positively correlated with HBV DNA (correlation coefficient $r=0.595$, $P=0.000$) and HBsAg (correlation coefficient $r=0.508$, $P=.000$) levels. In the HBeAg-negative group, HBV RNA levels were positively correlated with HBV DNA levels (correlation coefficient $r=0.530$, $P=.000$). Studies have shown that HBV RNA levels are independently associated with HBeAg status, ALT levels, HBV genotype and basal core promoter mutations, especially the status of HBeAg, which is the most relevant factor in HBV RNA levels (probably in high transcriptional activity).[29] Therefore, HBeAg status affects HBV RNA expression. In our study, in the HBeAg-negative group, ALT and AST with HBV RNA below LLD are normal, ALT and AST with higher HBV RNA are $>2\times$ ULN, this is why HBV RNA levels were positively correlated with AST/ALT. This result was similar with another study.[32] In the future, we will increase the sample capacity for follow-up research and explore the potential mechanism.

HBV RNA could be used as a virological indicator for antiviral therapy in HBsAg and HBsAb copositive patients with hepatitis B; HBeAg expression impacted the expression of HBV RNA. We believe that the real VR should be the absence of DNA viruses and RNA viruses. Especially when receiving NAs, the criterion for judging VR should be that both HBV DNA and RNA levels in serum are below the lower limit of detection. To correctly assess the therapeutic effect of antiviral therapy on HBsAg and HBsAb

copositive patients and their disease progression, quantitative detection of HBV RNA levels combined with the simultaneous detection of HBV DNA and HBsAg levels and HBV genotypes is recommended as the clinical basis for the assessment. Using the method, we provide better ideas for the clinical treatment of CHB and a more effective clinical basis.

Author contributions

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Writing – original draft: Yu Xiang.
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