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Serum HBV DNA plus RNA shows superiority in reflecting the activity of intrahepatic cccDNA in treatment-naïve HBV-infected individuals

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ABSTRACT

Background: Both serum hepatitis B virus (HBV) DNA and RNA can reflect intrahepatic covalently closed circular DNA (cccDNA) activity. However, correlations among viral markers haven't been fully explored.
Objectives: Here we investigated the correlations between serum HBV RNA and other viral markers in acute hepatitis B patients and treatment-naïve chronic HBV-infected individuals.
Study design: The serum viral markers of 19 acute hepatitis B patients and 84 treatment-naïve chronic HBV-infected individuals at different infection stages were quantified. Correlations among viral markers were analyzed by Pearson's or Spearman's correlation analysis.
Results: Serum viral markers and intrahepatic cccDNA levels were lower in acute hepatitis B patients than in treatment-naïve chronic HBV-infected individuals. Serum HBV RNA levels were positively correlated with serum HBV DNA, HBsAg and intrahepatic cccDNA levels in HBeAg-positive chronic HBV-infected individuals. Total serum HBV nucleic acids (HBV DNA plus RNA) showed superiority in reflecting intrahepatic cccDNA activity. Stratified analysis revealed that such correlations were only found in HBeAg-positive chronic hepatitis B phase. Moreover, high-frequency R193M and P196A mutations were found in the RT region of HBV polymerase leading to lower serum HBV DNA and higher serum HBV RNA levels in HBeAg-negative chronic HBV infection phase.
Conclusions: HBV replication capability was lower in acute hepatitis B patients than in chronic HBV-infected individuals. In treatment-naïve HBeAg-positive chronic HBV-infected individuals, serum HBV DNA plus RNA showed superiority in reflecting intrahepatic cccDNA activity than each alone. Moreover, mutated RT region of HBV polymerase might lead to the attenuated reverse transcriptional activity of HBV polymerase in HBeAg-negative chronic HBV infection phase.

1. Background

Hepatitis B virus (HBV) infection is a major pathogenic factor leading to chronic hepatitis B (CHB), cirrhosis, liver failure and hepatocellular carcinoma (HCC) [1]. According to the reports of WHO, more than 2 billion people have been infected with HBV and approximately 10% of them finally become chronic HBV infection [2]. Almost 650,000 people die of liver failure, cirrhosis and HCC caused by HBV infection annually [3]. The key step within HBV life cycle is the formation of covalently closed circular DNA (cccDNA), which could serve as the template to produce HBV genome and viral proteins [4–6]. It has been suggested that a complete cure of chronic hepatitis B means the clearance of cccDNA within all of the infected hepatocytes, while a functional cure implicates a status of hepatitis B surface antigen (HBsAg) loss or even seroconversion, accompanied with undetectable of serum HBV DNA and the persisted transcriptionally inactivation of the intrahepatic cccDNA [7]. Unfortunately, although the currently used antiviral agents can efficiently inhibit HBV replication, neither the nucleos(t)ides analogues nor the type-1 interferon is able to eliminate cccDNA [8–10]. Consequently, viral rebound frequently occurs once the

Abbreviations: cccDNA, covalently closed circular DNA; HBV, hepatitis B virus; CHB, chronic hepatitis B; HCC, hepatocellular carcinoma; NAs, nucleos(t)ide analogues; IFN, interferon; HBsAg, hepatitis B surface antigen; HBeAg, hepatitis B envelope antigen; pgRNA, pregenomic RNA

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Table 1
Clinical details of the studied subjects

	Phase I (n = 9)	Phase II (n = 53)	Phase III (n = 6)	Phase IV (n = 16)	*P* value
Gender(M/F)	6/3	46/7	4/2	12/4	0.327
Age (years)	18 (10–34)	29 (14–43)	29.5(11.25–46.2)	35 (7–41)	0.587
ALT (IU/L)	34 (17–39.5)	94 (44–247.5)	24 (18–34.75)	184(53.75–445.75)	0.078
AST (IU/L)	31 (25–42)	72(35.5–195.5)	26 (25–33.25)	77 (51.5–243.5)	0.154
HBV DNA	8.729	7.069	3.364	6.664	
(log copies/mL)	(8.024 − 9.044)	(5.444 − 8.469)	(2.569 − 3.832)	(5.312 − 8.489)	< 0.001
HBV Genotype (B/C)	4/5	12/41	3/3	7/9	0.193
HBeAg status	positive	positive	negative	negative	

M: Male; F: Female; Phase I: HBeAg-positive chronic HBV infection phase; Phase II: HBeAg-positive chronic hepatitis B phase; Phase III: HBeAg-negative chronic HBV infection phase; Phase IV: HBeAg-negative chronic hepatitis B phase.

anti-viral treatments are discontinued [11,12]. Since cccDNA is responsible for HBV persistent infection and is the main barrier for eradicating the virus [13], to monitor the level of intrahepatic cccDNA is of clinical significance to evaluate the therapeutic efficacy and estimate the treatment endpoint. Intrahepatic cccDNA monitoring relies on liver biopsy, however, several obstacles including liver invasion, inadequate specimen size, inter-observer variability and potential complications prevent the widespread use of liver biopsy [14–18]. Therefore, serum biomarkers which can reflect the status of intracellular cccDNA are required.

Previous studies have indicated that the levels of serum HBV DNA, HBV RNA and HBsAg were positively correlated with the amount of intrahepatic cccDNA in chronic HBV-infected individuals [19]. Although serum HBV DNA can be efficiently inhibited to an undetectable level in a considerable number of CHB patients after receiving NAs treatment, loss of serum HBsAg is rarely to achieve [20,21]. In addition to cccDNA, the integrated HBV DNA fragments could also encode HBsAg [22–27], which may bring some problems to the relationship between positive serum HBsAg and intrahepatic cccDNA activity under the long-term NAs treatment [28]. Therefore, under the long-term NAs treatment, a more sensitive serum marker that could reflect the activity of intrahepatic cccDNA is needed.

It has been reported that, except for HBV DNA, HBV RNA is also present in the serum of HBV-infected individuals, and can predict the efficacy of NAs treatment [29–32]. We and other groups have recently confirmed that the serum HBV RNA is the encapsidated pregenomic RNA (pgRNA) present in virus-like particles [31,33]. Moreover, based on the persistent loss of serum HBV RNA, we proposed the "para-functional cure" of chronic hepatitis B which was different from the "functional cure" characterized by serum HBsAg loss [28].

The natural course of chronic HBV infections can be typically divided into four distinct disease phases: HBeAg-positive chronic HBV infection phase characterized with normal alanine aminotransferase levels and high levels of serum HBV DNA and HBeAg; This phase may last for decades and eventually transit into HBeAg-positive chronic hepatitis B phase characterized with elevated alanine aminotransferase levels and fluctuating serum HBV DNA levels; HBeAg-negative chronic HBV infection phase characterized with normal alanine aminotransferase levels, low HBV DNA levels (usually less than 2000 IU/ml), negative HBeAg and/or positive anti-HBe; HBeAg-negative chronic hepatitis B phase occurred in a subset of patients who develops recurrent necroinflammatory liver disease with negative HBeAg, but with elevated alanine aminotransferase levels and increased HBV DNA levels [34–36].

In this study, the potential clinical significance of serum HBV RNA in reflecting the activity of intrahepatic cccDNA was explored, and the relationships among the levels of serum viral markers and intrahepatic cccDNA were analyzed in acute hepatitis B patients and treatment-naïve chronic HBV-infected individuals in four phases.

2. Objectives

Here we explored the potential use of serum HBV DNA plus RNA to reflect the intrahepatic cccDNA activity.

3. Study design

3.1. Study subjects

The study population consisted of 19 acute hepatitis B (AHB) patients and 84 treatment-naïve chronic HBV-infected individuals who were admitted to Beijing 302 Hospital from January 2008 to December 2012. For chronic HBV-infected individuals, including 62 HBeAg-positive ones and 22 HBeAg-negative ones, could be divided into four phases according to 2017 EASL clinical practices guideline on the management of hepatitis B virus infection [36]. Briefly, natural history of chronic HBV infection has been schematically divided into four phases, taking into account the presence of HBeAg, HBV DNA levels, alanine aminotransferase (ALT) values and eventually the presence or absence of liver inflammation, which are HBeAg-positive chronic HBV infection phase, HBeAg-positive chronic hepatitis B phase, HBeAg-negative chronic HBV infection phase and HBeAg-negative chronic hepatitis B phase. Here in our study, there were 9 individuals in HBeAg-positive chronic HBV infection phase, 53 in HBeAg-positive chronic hepatitis B phase, 6 in HBeAg-negative chronic HBV infection phase and 16 in HBeAg-negative chronic hepatitis B phase. The diagnosis criteria for chronic hepatitis B and the definitions of four natural infection processes were mentioned in our previous study [19]. The clinical characteristics of the studied subjects were shown in Table 1. The vast majority individuals were infected with HBV genotype C, with the percentage of 69%. The remains were infected with HBV genotype B. The experimental protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki. This study was approved by the Ethics Committee of Peking University Health Science Center. Written informed consent was obtained from each patient.

3.2. Viral nucleic acid extraction and reverse transcription

Total viral nucleic acid mixture was extracted from 200 μL serum of chronic HBV-infected individuals or 200 μL HuH-7 cell supernatant by EasyPure Viral RNA Kit (TransGen Biotech, Beijing, China) according to the manufacture's instruction. The reverse transcription was carried out using Thermo Scientific RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham, MA, USA). The primer sequence for reverse transcription was 5′ TCTCACACCGTAACACACGACACAGG CGAGGGAGTTCTTCTTCTA 3′.

3.3. Quantitation of serum HBV RNA and HBV DNA by quantitative PCR

The levels of serum HBV RNA and HBV DNA were detected by quantitative polymerase chain reaction (qPCR) in Applied Biosystems

StepOne plus Real-Time PCR Systems (Applied Biosystems, Mannheim, USA). The levels of serum HBV RNA were detected as previously described [33]. Briefly, HBV RNA was isolated using the EasyPure Viral RNA Kit (TransGen Biotech, Beijing, China) according to the manufacturer's instructions and treated with DNase I (Thermo Fisher Scientific, Waltham, MA, USA). For DNase I treatment, every reaction mixture was comprised of 1.1 μL 10 × DNase I Buffer, 0.5 μL RNase-Inhibitor (50 U/μL), and 8.4 μL total nucleic acids. The reaction was performed at 37 °C for 30 min, then added 1 μL EDTA and 1 μL RT primer to each reaction mixture, incubated at 65 °C for 10 min to inactivate DNase I. Finally, the tube was put on the ice for 2 min. The DNase I treated HBV RNA was reverse transcribed using RevertAid First Strand DNA Synthesis Kit (Thermo Fisher Scientific, Waltham, MA, USA). The qPCR reaction mixture (30 μL) contained 15 μL 2 × mix (LightCycler®480 Probes Master, Roche, Mannheim, Germany), 1 μL forward primer (10 μM), 1 μL reverse primer (10 μM), 1 μL TaqMan probe (10 μM), 3 μL cDNA template and 9 μL double distilled water. The reaction mixture was denatured at 95 °C for 5 min, followed by 40 cycles at 95 °C for 20 s, 60 °C for 40 s. Moreover, to ensure the reliability of HBV RNA level, the levels of serum HBV RNA were also detected by HBV pgRNA One-Step RT-qPCR Kit (kindly provided by Beijing Hotgen Biotech. Co. Ltd) following manufacture's instruction.

The level of HBV DNA was quantified with Hepatitis B viral DNA Quantitative Fluorescence Diagnostic Kit (Sansure Biotech, Changsha, China) following the manufacture's instruction.

3.4. Quantification of serum HBsAg

The levels of serum HBsAg were detected as previously described [19,37]. Briefly, the titer of serum HBsAg was quantitated by a chemiluminescence assay using the Architect i2000SR platform and Abbott Architect HBsAg reagents (Abbott Laboratories, Chicago, IL). The lower limit of detection was 0.05 IU/mL. If the initial test value was higher than the upper limit of detection (250 IU/mL), the samples were diluted (1:500) and reassessed.

3.5. Quantification of intrahepatic cccDNA

Intrahepatic HBV cccDNA was extracted and quantitated as previously described [19,37]. Briefly, about 30 mm formalin fixed paraffin-embedded (FFPE) liver biopsy tissue was sectioned to 6 mm each for DNA extraction. The DNA was extracted using QIAamp FFPE DNA Mini Kit (QIAGEN, GmbH, Hilden, Germany). PSAD (Epicentre, Madison, WI, USA) was used to digest HBV rcDNA, replicative dsDNA and ssDNA. Afterwards, rolling Circle Amplification (RCA) was conducted to selectively amplify circular cccDNA. Using the RCA products as template, HBV cccDNA was further quantified by TaqMan qPCR mediated with a pair of primers and a probe that targets the gap region of HBV genome. To quantitate cell numbers, a set of primers and a probe for quantitatively detecting human beta-actin gene were also used in qPCR process. Based on an estimation of 6.667 pg/hgDNA per cell, cell number was calculated [19].

3.6. HBV RT region amplification by nested PCR

The amplification of HBV RT region was performed by nested PCR with the outer primers: forward: 5′ CCTACTGTTCAAGCCTCCAAGC 3′ (nt 1856–1877), reverse: 5′ GTTCCCAAGAATATGGTGACCC 3′ (nt 2820–2841), and the inner primers: forward: 5′ ACTGTTCAAGCCTCC AAGCTGT 3′ (nt 1859–1880), reverse: 5′ GGTTAGGATAGAACCTAGC AGG 3′ (nt 2640–2661). PCR products were purified by gel extraction, then were sent to company for sequencing (Sangon Biological Engineering Technology Company, Shanghai, China).

3.7. Plasmid construction

R193M and P196A mutant 1.2 × HBV plasmid were constructed on the basis of 1.2 × HBV wild type plasmid by the site directed rapid mutagenesis method with the primers as follows. For R193M, the primers were forward: 5′ CCATTTGTTCAGTGGTTCGTATGGCTTTCCCCC ACTGTTTGGC 3′, reverse 5′ GCCAAACAGTGGGGGAAAGCCATACGAA CCACTGAACAAATGG 3′. For P196A, the primers were forward: 5′ CAGTGGTTCGTAGGGCTTTCGCCCACTGTTTGGCTTTCAGTTA 3′, reverse 5′ TAACTGAAAGCCAAACAGTGGGCGAAAGCCCTACGAACCA CTG 3′.

3.8. Cell culture

The human liver cancer cell line HuH-7 [38] were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (Gibco, Carlsbad, Calif, USA).

3.9. Transfection

HuH-7 cells were seeded into 6-well plates at a density of 5×10^5 cells per well and cultured for 18 h. Each well was transfected with 3 μg wild-type, R193M or P196A mutant 1.2 × HBV plasmid using Lipofectamine 2000 (Invitrogen, Carlsbad, Calif, USA). Six hours after transfection, medium was replaced with fresh DMEM without penicillin/streptomycin. 48 h later, the culture supernatant was collected and each well was washed by PBS for 6 times and then added 3 mL fresh DMEM to each well and collected 1 mL culture supernatant used to guide the baseline level of HBV RNA and HBV DNA. Another 48 h later, the culture supernatant was collected.

3.10. Statistical analysis

Data were presented as median and inter-quartile range. Differences between variables was tested by Student's *t*-test or one-way ANOVA test where it was appropriate. Regression and Pearson's correlation analysis were applied to compare the correlations among viral markers. A *P*-value (2-tailed) of 0.05 was considered statistically significant. All statistical analyses were carried out in Statistical Program for Social Sciences (SPSS 21.0 for Windows; SPSS Inc., Chicago, IL).

4. Results

4.1. The levels of serum viral markers and intrahepatic cccDNA in acute hepatitis B patients and treatment-naïve chronic HBV infected individuals

Serum HBV RNA levels in 19 acute hepatitis B (AHB) patients were significantly lower than those of treatment-naïve chronic HBV-infected individuals. Consistently, the levels of serum HBV DNA, HBsAg and intrahepatic cccDNA were all lower than those in chronic HBV-infected individuals, indicating that active anti-HBV immune response in AHB patients could effectively eliminate the infected hepatocytes as well as the intrahepatic cccDNA (Fig. 1A). However, since the levels of serum HBV RNA were undetectable (below the lower limit of detection) in 10 of 19 AHB patients, the correlations between viral markers were not further analyzed in AHB patients.

For 84 chronic HBV-infected individuals, there are 62 HBeAg-positive and 22 HBeAg-negative individuals. Compared to that in HBeAg-negative individuals, significantly higher levels of serum HBV RNA ($P = .002$), HBV DNA ($P = .023$), and HBV DNA plus RNA ($P = .005$) were detected in those HBeAg-positive individuals, the later ones even showed marginal significant higher levels of serum HBsAg ($P = .053$) and intrahepatic cccDNA ($P = .061$) (Fig. 1B).

EASL's new guideline classifies the chronic HBV-infected individuals into four subgroups [36]. First of all, the levels of serum HBV RNA and DNA in these phases were compared. As shown in Fig. 1C, the levels of

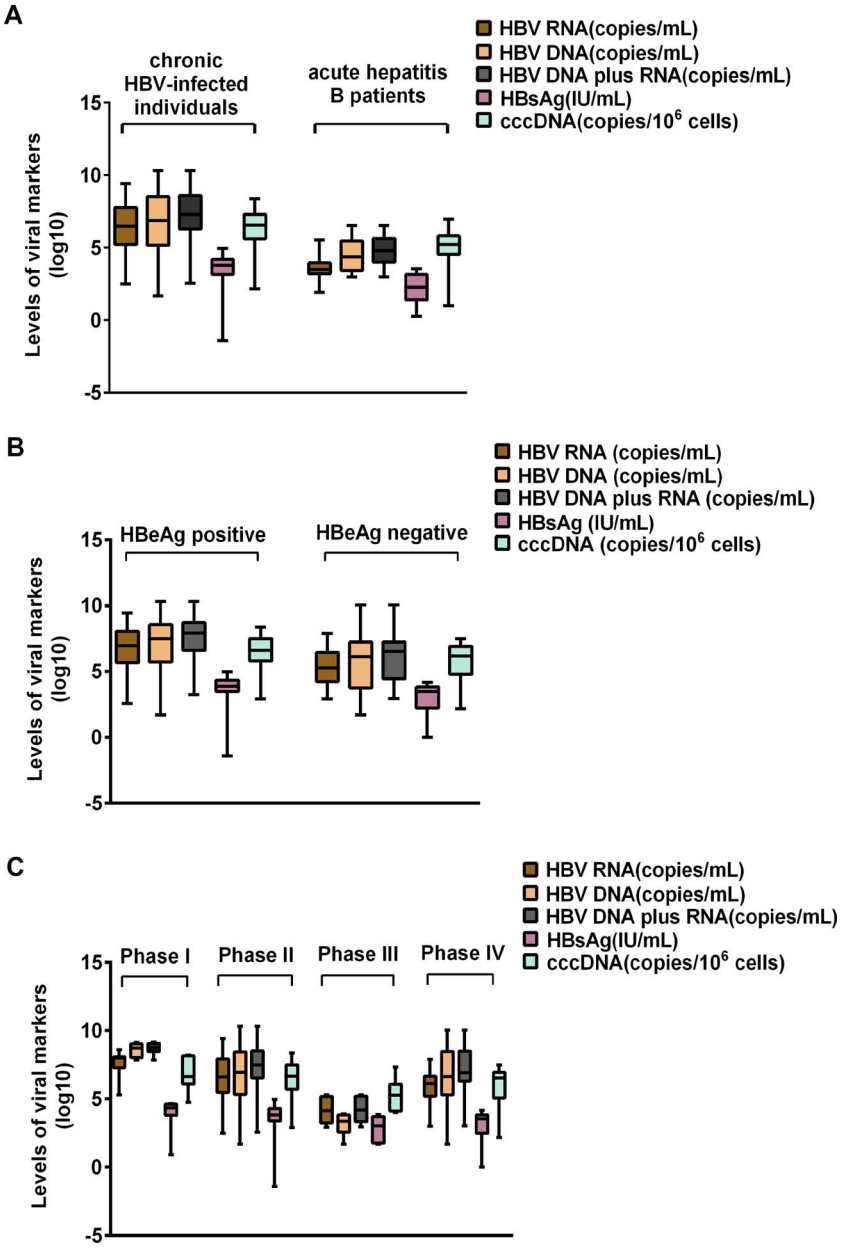

Fig. 1. Comparison of serum viral biomarkers and intrahepatic cccDNA levels in acute hepatitis B patients and treatment-naïve chronic HBV-infected individuals. (A) Comparison of viral biomarkers between chronic HBV-infected individuals and acute hepatitis B patients. (B) Comparison of viral biomarkers between HBeAg-positive and HBeAg-negative chronic HBV-infected individuals. (C) Comparison of viral biomarkers among four different natural phases of chronic HBV infection. Phase I: HBeAg-positive chronic HBV infection phase; Phase II: HBeAg-positive chronic hepatitis B phase; Phase III: HBeAg-negative chronic HBV infection phase; Phase IV: HBeAg-negative chronic hepatitis B phase.

Table 2
Correlations between serum HBV RNA and HBV DNA or HBsAg in HBeAg-positive and −negative chronic HBV-infected individuals.

	HBV RNA		
	Total (n = 84)	HBeAg positive (n = 62)	HBeAg negative (n = 22)
HBV DNA	r = 0.574, $P < .001$	r = 0.532,$P < .001$	r = 0.741, $P < .001$
HBsAg	r = 0.545, $P < .001$	r = 0.537, $P < .001$	r = 0.151, $P = .503$

serum HBV RNA, HBV DNA and the sum of them were found highest in HBeAg-positive chronic HBV infection phase, and lowest in HBeAg-negative chronic HBV infection phase. While, the intrahepatic cccDNA levels remained relatively stable among four phases (Fig. 1C). Moreover, no significant difference of the serum HBsAg levels were observed among four phases.

4.2. Serum HBV RNA was positively correlated with serum HBV DNA and HBsAg in chronic HBV-infected individuals

In general, the level of serum HBV RNA was positively correlated with serum HBV DNA (Pearson r = 0.574, $P < .001$) and HBsAg (Spearman r = 0.545, $P < .001$) in the 84 chronic HBV-infected individuals. Stratified analysis revealed that such correlation of serum HBV RNA with serum HBV DNA (Pearson r = 0.532, $P < .001$) or with HBsAg (Spearman r = 0.537, $P < .001$) remained in HBeAg-positive individuals. However, serum HBV RNA was just positively correlated with serum HBV DNA (Pearson r = 0.741, $P < .001$), but not with serum HBsAg (Spearman r = 0.151, $P = 0.503$) in HBeAg-negative individuals (Table 2). Moreover, the positive correlation of serum HBV RNA with serum HBV DNA (Pearson r = 0.421, $P = .002$) and HBsAg (Spearman r = 0.549, $P < .001$) were also present among the CHB patients in HBeAg-positive chronic hepatitis B phase. In contrast, serum HBV RNA was found only positively correlated with serum HBV DNA in individuals in HBeAg-negative chronic HBV infection phase (Pearson r = 0.906, $P = .013$), and in HBeAg-negative chronic hepatitis B phase

Table 3
Correlations between intrahepatic cccDNA and serum HBV RNA, HBV DNA, as well as HBV DNA plus RNA, respectively.

	Intrahepatic cccDNA		
	Total (n = 84)	HBeAg positive (n = 62)	HBeAg negative (n = 22)
HBV RNA	r = 0.363, $P < .001$	r = 0.390, $P = .002$	r = 0.100, $P = .654$
HBV DNA	r = 0.367, $P < .001$	r = 0.329, $P = .009$	r = 0.333, $P < .001$
HBV DNA plus RNA	r = 0.412, $P < .001$	r = 0.431, $P < .001$	r = 0.258, $P = .246$

(Pearson r = 0.6, $P = .014$). Serum HBV RNA was not correlated with the level of serum HBsAg in HBeAg-negative individuals.

4.3. Serum HBV DNA plus RNA could better reflect the activity of intrahepatic cccDNA than each alone in chronic HBV-infected individuals

The correlations between intrahepatic cccDNA and serum HBV DNA or HBsAg have been previously proved in HBeAg-positive chronic HBV-infected individuals [19]. Also, serum HBV RNA had been suggested as a good viral marker in reflecting the activity of intrahepatic cccDNA in HBeAg-positive chronic HBV-infected individuals [28]. Here, we further demonstrated that when taking serum HBV DNA together with serum HBV RNA, in sum their correlation with the level of intrahepatic cccDNA (Pearson r = 0.412, $P < .001$) was relatively better than either HBV RNA (Pearson r = 0.363, $P < .001$) or HBV DNA (Pearson r = 0.367, $P < .001$) alone in chronic HBV-infected individuals. Noticeably, the correlation between serum HBV DNA plus RNA and intrahepatic cccDNA (Pearson r = 0.431, $P < .001$) was present only in HBeAg-positive chronic HBV-infected individuals, but not in HBeAg-negative chronic HBV-infected individuals (Pearson r = 0.258, $P = .246$) (Table 3).

Further stratified analysis revealed that in HBeAg-positive but not in HBeAg-negative chronic HBV-infected individuals, the serum HBV DNA plus RNA (Pearson r = 0.438, $P = .001$) remained a better correlation with the level of cccDNA, as compare to serum HBV RNA (Pearson r = 0.407, $P = .003$) or HBV DNA (Pearson r = 0.317, $P = .021$) alone. Above results suggested that serum HBV DNA plus RNA showed superiority in reflecting the activity of intrahepatic cccDNA in HBeAg-positive chronic HBV-infected individuals.

Since the levels of cccDNA were relatively stable (Fig. 1), the significant differences of the levels of serum HBV DNA and RNA among the four phases of chronic HBV infection might due to the differences of viral replication efficacy in different phases. It is reasonable to postulate that the more HBV DNA each cccDNA produces, the higher replication efficiency of the virus will have. Therefore, the ratio of serum HBV DNA to intrahepatic cccDNA would likely reflect the HBV replication efficiency to some extent [19]. As shown in Fig. 2A, the ratio of serum HBV DNA to intrahepatic cccDNA was significantly lower in HBeAg-negative chronic HBV infection phase, as compare to that in other three phases. Though the same tendency was observed in the ratio of serum HBV RNA with cccDNA, the lower value in HBeAg-negative HBV infection phase did not reach a statistical difference (Fig. 2B), Noticeably, such tendency reversed when compared the serum HBsAg level with the amount of intrahepatic cccDNA (Fig. 2C). This phenomenon could indicate that in HBeAg-negative HBV infection phase, the serum HBsAg could be largely originated from the integrated HBV DNA fragments, as some reports suggested.

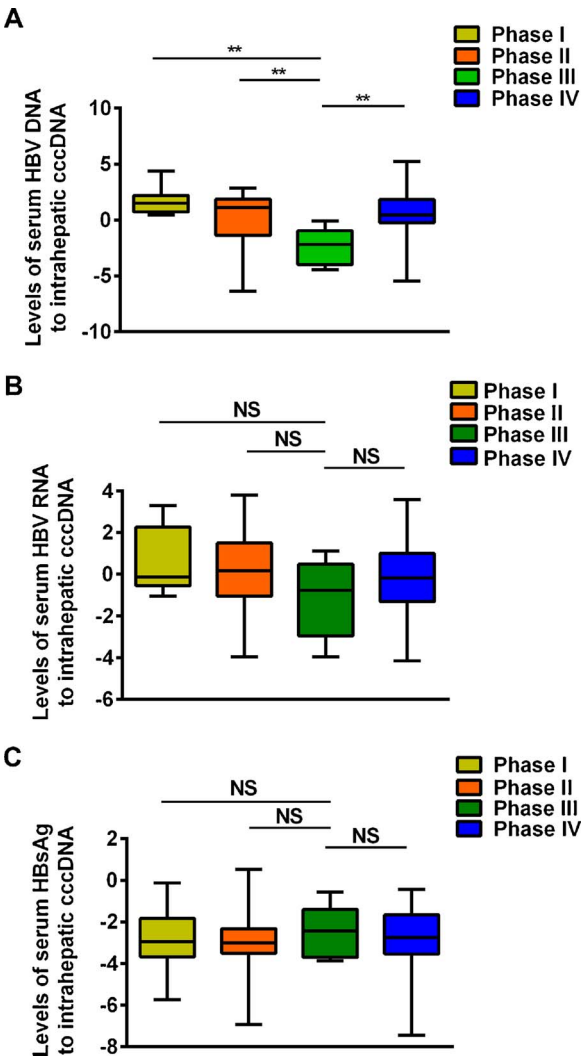

Fig. 2. Comparison of the HBV replication and transcription efficiencies in four phases of chronic HBV infection. (A) The HBV replication efficiencies were assessed by comparing the ratio of serum HBV DNA to intrahepatic cccDNA in four phases of chronic HBV infection. The HBV transcription efficiencies were assessed by comparing the ratio of serum HBV RNA to intrahepatic cccDNA (B), or the ratio of serum HBsAg to intrahepatic cccDNA (C), in four phases of chronic HBV infection. Phase I: HBeAg-positive chronic HBV infection phase; Phase II: HBeAg-positive chronic hepatitis B phase; Phase III: HBeAg-negative chronic HBV infection phase; Phase IV: HBeAg-negative chronic hepatitis B phase.

4.4. The mutations of HBV RT region led to the heterogeneity of HBV polymerase activity

It has been reported that the ratio of serum HBV RNA to HBV DNA can reflect the reverse transcription efficiency of pgRNA, the higher the ratio of serum HBV RNA to HBV DNA, the lower the reverse transcription efficiency of pgRNA [37]. As shown in Fig. 3A, the ratio of serum HBV RNA to HBV DNA in HBeAg-negative chronic HBV infection phase was higher than those in other three phases, suggesting that the reverse transcription efficiency of pgRNA was lowest in HBeAg-negative chronic HBV infection phase. Given that the chronic HBV-infected individuals in HBeAg-negative chronic HBV infection phase usually had longer infection history [39], and taken into consideration the fact that the reverse transcriptase lack of 3′ to 5′ exonuclease activity, it is reasonable to suspect that it might attributed to the mutations in the RT region of HBV polymerase, which accumulated during the disease progression. Indeed, PCR-based direct sequencing of HBV DNA derived from individuals of HBeAg-negative chronic HBV infection phase revealed mutations in RT region. We sequenced all six samples in HBeAg-

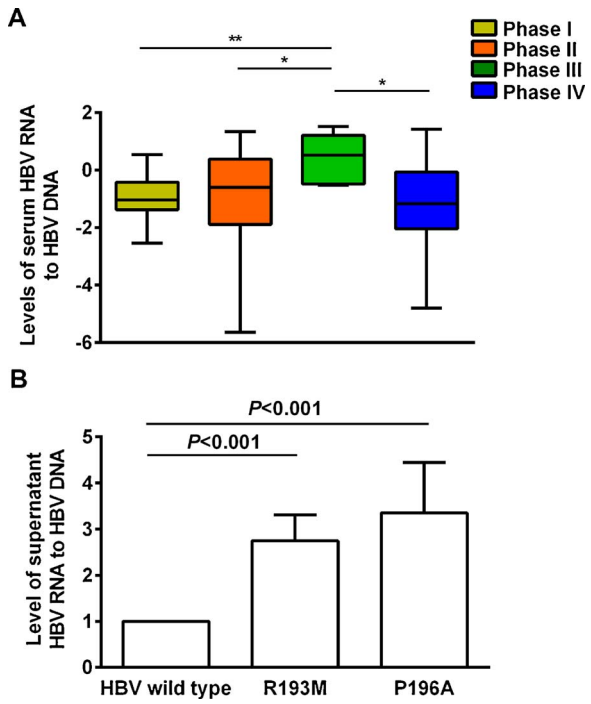

Fig. 3. Analyze the effect of HBV polymerase RT region on HBV reverse transcription efficiency. (A) The ratio of serum HBV RNA to HBV DNA in four different phases of chronic HBV-infected individuals. (B) The ratio of HBV RNA to HBV DNA in the culture supernatant of wild type, R193M or P196A mutated 1.2 × HBV infected HuH-7 cells. Phase I: HBeAg-positive chronic HBV infection phase; Phase II: HBeAg-positive chronic hepatitis B phase; Phase III: HBeAg-negative chronic HBV infection phase; Phase IV: HBeAg-negative chronic hepatitis B phase.

negative chronic HBV infection phase and found that five in six individuals possessed mutations in RT region and the rest one presented the wild type sequence. The most frequently occurred mutations were R193M and P196A, which was not previously reported. Such mutations were found in four of five individuals who possessed mutations in RT region. All detected mutations were listed in Table 4. Further, the reverse transcription efficiency of R193M and P196A mutated HBV were detected *in vitro*. The result revealed that the ratios of serum HBV RNA to serum HBV DNA of R193M or P196A mutated HBV were higher than that of wild type HBV, implicating less pgRNAs were reversely transcribed into viral DNA. This result suggested that both mutated HBV possessed lower reverse transcription efficiencies (Fig. 3B).

5. Discussion

Just like serum HBV DNA and HBsAg the widely used viral biomarkers, serum HBV RNA could also be used to reflect the intrahepatic cccDNA activity [28,40]. More and more evidences showed that serum HBsAg could also be produced from the HBV DNA fragments integrated into human genome [27,41,42]. Therefore, serum HBsAg may not an optimal viral marker in reflecting the activity of intrahepatic cccDNA in chronic hepatitis B patients after receiving a long-term antiviral therapy. Though both serum HBV DNA and HBV RNA can only be produced from cccDNA, HBV DNA may not truly reflect the activity of intrahepatic cccDNA once the reverse transcription of pgRNA is blocked by NAs therapy. In such case, serum HBV RNA or HBV DNA plus RNA shows superiority in reflecting cccDNA activity. In this study, we found that serum HBV DNA plus RNA could better reflect intrahepatic cccDNA activity than either serum HBV RNA or DNA alone in HBeAg-positive chronic HBV-infected individuals. However, such correlation disappeared in HBeAg-negative chronic HBV-infected individuals, which might be due to viral variation, host immune response, and the epigenetic modulation of cccDNA [28,43,44]. For host immune response, it has been reported that retinoic acid-inducible gene-I (RIG-I) can not only induce the expression of type III interferons, but also counteract the interaction of HBV polymerase with the 5′-ε region of pgRNA [45]. More than that, RIG-I can directly inhibit the formation of encapsidated pgRNA, and subsequently inhibit the production of Dane particles and HBV RNA virion-like particles, both of which may interfere with the correlation of intrahepatic cccDNA and serum HBV DNA or RNA.

Of note, though there were no significant differences in the transcription efficiency of cccDNA (HBV RNA/cccDNA) among four phases of chronic HBV infection, the HBV replication efficiency (HBV DNA/cccDNA) was lowest in HBeAg-negative chronic HBV infection phase. Moreover, the levels of serum HBV RNA was lower than that of serum HBV DNA in HBeAg-positive chronic HBV infection, HBeAg-positive chronic hepatitis B and HBeAg-negative chronic hepatitis B phases, but higher in HBeAg-negative chronic HBV infection phase, which indicated that the reverse transcription process might be affected in this phase. Therefore, the ratio of serum HBV RNA to DNA was analyzed. The result revealed that the ratio of serum HBV RNA to DNA in HBeAg-negative chronic HBV infection phase was higher than that of other three phases. Furthermore, high-frequency R193M and P196A mutations in RT region of HBV polymerase, which attenuated the reverse transcription of pgRNA, were frequently found in individuals in HBeAg-negative chronic HBV infection phase. The ratios of supernatant HBV RNA to HBV DNA of R193M or P196A mutated HBV were higher than that of wild type HBV, suggesting that both mutated HBV possessed lower reverse transcription efficiencies. Above results implicated that the mutation in RT region of HBV polymerase contributed to the lower HBV DNA levels and higher HBV RNA levels in HBeAg-negative chronic HBV infection phase.

Besides, the levels of serum HBV RNA, HBV DNA, HBsAg and intrahepatic cccDNA in 19 acute hepatitis B (AHB) patients were confirmed to be significantly lower than those of treatment-naïve chronic HBV-infected individuals. In line with the previous report, these results indicated that active anti-HBV immune response in AHB patients could

Table 4
Mutations of HBV polymerase RT Region in 5 chronic HBV-infected individuals of HBeAg negative chronic HBV infection phase.

Mutation sites	Mutations in RT Region of HBV polymerase				
	Individual 1	Individual 2	Individual 3	Individual 4	Individual 5
191	V191G		V191I		
192	R192L				
193	R193M	R193M R193L	R193K	R193M	R193M
	R193W	R193W			
194	A194G	A194G			
196	P196A		P196A	P196A	P196A
223				S223P	
224				I224V	
269			I269L		

effectively eliminate the infected hepatocytes and silence intrahepatic cccDNA as well [19].

Data in this study suggested that serum HBV DNA plus RNA showed superiority in reflecting intrahepatic cccDNA activity in treatment-naïve HBeAg-positive HBV-infected individuals. However, there were still several limitations in our study. For example, the study cohort was still needed to be enlarged and what's more, anti-HBc was also reported as a marker for HBV replication and cccDNA activity [46], the residual material was not enough for detecting this maker. Since the quantitative detection methods of serum HBV RNA have been well established, and the anchored sequence in cDNA reverse transcribed from the HBV RNA could significantly reduce the interference of HBV DNA [30,33], in the future, serum HBV RNA and HBV DNA plus RNA may be the potential serum viral markers used to guide the antiviral therapy and safe discontinuation of NAs therapy in clinical practice.

Ethical approval

This study was approved by the Ethics Committee of Peking University Health Science Center. Written informed consent was obtained from each patient.

Conflict of interest

The authors have declared that no competing interests exist.

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