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Serum HBcrAg is better than HBV RNA and HBsAg in reflecting intrahepatic covalently closed circular DNA

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Summary

The correlation between serum HBcrAg and HBV RNA is unclear, and correlations of intrahepatic cccDNA with HBcrAg, HBV RNA and HBsAg are rarely reported in the same cohort. This study aimed to assess the correlation of HBcrAg with HBV RNA and HBsAg, and investigate whether serum HBcrAg is superior to serum HBV RNA and HBsAg in reflecting intrahepatic HBV cccDNA in HBeAg-positive and HBeAg-negative CHB patients. In this study, 85 HBeAg-positive and 25 HBeAg-negative patients who have never received antiviral therapy were included. Among HBeAg-positive patients, HBcrAg was correlated positively with HBsAg ($r = 0.564$, $P < 0.001$) and HBV RNA ($r = 0.445$, $P < 0.001$), and HBV RNA was also correlated positively with HBsAg ($r = 0.323$, $P = 0.003$). Among HBeAg-negative patients, no significant correlation was observed between HBcrAg, HBsAg and HBV RNA. By multivariable linear regression, HBcrAg ($\beta = -0.563$, $P < 0.001$), HBsAg ($\beta = -0.328$, $P < 0.001$) and HBV RNA ($\beta = 0.180$, $P = 0.003$) were all associated with cccDNA levels among HBeAg-positive patients, but only serum HBcrAg was associated with cccDNA level ($\beta = 0.774$, $P = 0.000$) among HBeAg-negative patients. HBcrAg was better correlated with cccDNA as compared to HBsAg and HBV RNA, irrespective of HBeAg status. Among HBeAg-positive patients, though HBcrAg level was influenced by hepatic inflammatory activity and HBV DNA levels, the good correlations of HBcrAg with cccDNA persisted after stratification by inflammatory activity and HBV DNA levels. In conclusion, correlations of serum HBcrAg, HBV RNA and HBsAg levels differ significantly between HBeAg-positive and HBeAg-negative patients, but serum HbcrAg correlates with cccDNA levels better than HBV RNA and HBsAg, irrespective of HBeAg status.

KEYWORDS

covalently closed circular DNA, HBV RNA, hepatitis B core-related antigen, hepatitis B surface antigen

Abbreviations: BCP, basal core promoter; cccDNA, covalently closed circular DNA; CHB, chronic hepatitis B; DR, direct repeat; HBcAg, hepatitis B core antigen; HBcrAg, hepatitis B core-related antigen; HBeAg, hepatitis B e antigen; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; NAs, nucleos(t)ide analogues; PC, precore; pgRNA, pregenomic RNA.

1 | INTRODUCTION

Hepatitis B virus (HBV) infection is a worldwide public health problem. It is estimated that 240 million people are chronically infected and at least 650 000 people die each year due to chronic hepatitis B (CHB) worldwide.[1] Though the currently approved nucleos(t)ide analogues can effectively reduce serum HBV DNA of CHB patients, HBV is difficult to eliminate due to the persistence of HBV covalently closed circular DNA (cccDNA) in the infected hepatocytes.[2] As a template for transcription of all viral RNAs, intrahepatic cccDNA can produce the offspring virion DNA and influence viral proteins synthesis.[2] Additionally, low levels of intrahepatic cccDNA also predict sustained virologic response after cessation of antiviral therapy.[2] Thus, dynamic monitoring of intrahepatic cccDNA level should be helpful to accurately assess the efficacy of antiviral therapy and disease progression risk.[3] However, because of the invasive nature of the procedure and potential for sampling error, dynamic liver biopsy is not well tolerated, which greatly limits the use of intrahepatic cccDNA in real-world clinical practice.[4] Therefore, many noninvasive convenient markers have been investigated to reflect intrahepatic cccDNA level.[5] As a classic indicator, serum hepatitis B surface antigen (HBsAg) levels have long been thought to be correlated with intrahepatic cccDNA, but this correlation is not strong.[6]

Hepatitis B core-related antigen (HBcrAg) is a new valuable serum marker of HBV, and it consists of three species of related proteins sharing an identical 149 amino acid sequence: hepatitis B core antigen (HBcAg), hepatitis B e antigen (HBeAg) and a truncated 22 kDa precore protein (p22Cr).[7] Like HBeAg, p22cr is also a processed product of the precore protein, but with protein processing at both the N- and C-terminals.[7] In our previous studies, early on-treatment serum HBcrAg level was found to be a good biomarker for predicting off-treatment HBeAg seroconversion in patients receiving peginterferon therapy,[8] and as compared to serum HBsAg level, serum HBcrAg has a better correlation with intrahepatic cccDNA.[6,9] In addition, patients with low HBcrAg and HBsAg levels are also reported to have a low relapse risk after cessation of antiviral therapy.[10,11]

Recently, serum HBV RNA is also attracting attention as a useful biomarker.[12,13] As early as 1996, serum HBV RNA was identified in HBV-infected patients but its nature and origin were unclear until recently when serum HBV RNA was confirmed to be pregenomic RNA (pgRNA). Undetectable serum HBV RNA level has been reported to be associated with sustained virological response to antiviral therapy, and it might serve as a new potential surrogate marker to reflect the status of intrahepatic cccDNA.[14,15] In addition, some scholars have called for a new definition of sustained virological response (using undetectable HBV DNA plus HBV RNA in place of undetectable HBV DNA).[16] It is worth mentioning that the majority of published studies are focussed on HBeAg-negative patients, and the correlation between serum HBV RNA and intrahepatic cccDNA is rarely reported in HBeAg-positive patients.

At present, the correlation between HBcrAg and HBV RNA is unclear. The correlations of intrahepatic cccDNA with serum HBcrAg, HBV RNA and HBsAg are rarely reported in the same cohort. This study was designed to assess the correlation of serum HBcrAg with serum HBV RNA and HBsAg, and further investigate whether serum HBcrAg is superior to serum HBV RNA and HBsAg in reflecting intrahepatic HBV cccDNA in HBeAg-positive and HBeAg-negative CHB patients.

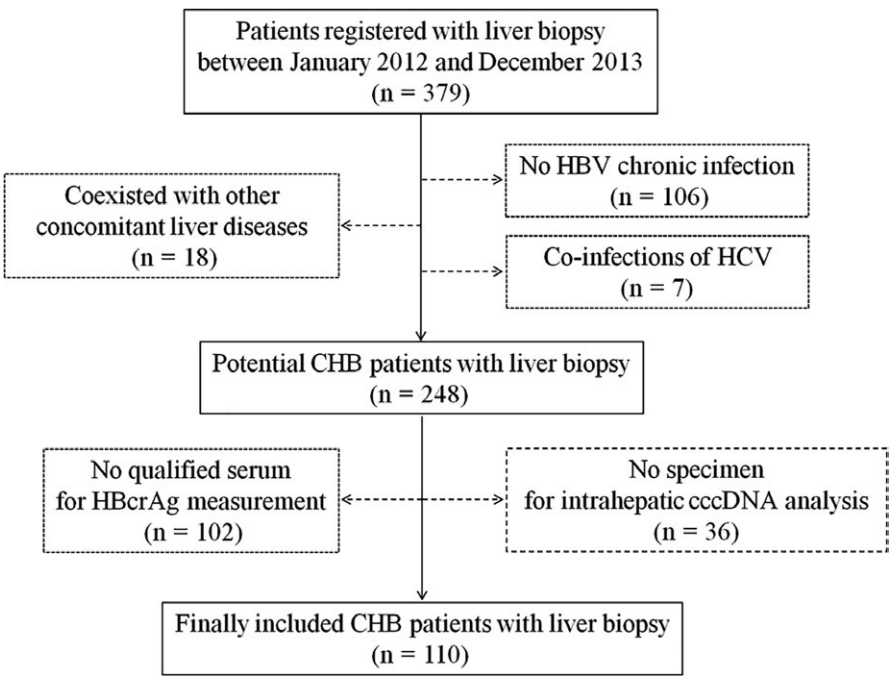

FIGURE 1 The acquisition process of registered patients with liver biopsy in present retrospective study

2 | PATIENTS AND METHODS

2.1 | Patients

This was a retrospective study of CHB patients who underwent percutaneous liver biopsy at the West China Hospital of Sichuan University between January 2012 and December 2013. All patients had not received antiviral therapy before the biopsy. Patients were excluded if they had any evidence of other concomitant liver diseases (including alcoholic liver disease, autoimmune liver disease and hepatocellular carcinoma) or markers of hepatitis C virus or human immunodeficiency virus co-infections. In addition, patients without serum for HBcrAg measurement and eligible liver tissue for intrahepatic HBV cccDNA measurement were also excluded. The detailed information of patient acquisition procedures is shown in Figure 1.

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

2.2 | General laboratory variables measurement

Serum biochemical indexes were measured according to standard procedures (Olympus AU5400, Olympus Corporation, Tokyo, Japan). Serum HBeAg status was assessed using electrochemiluminescence immunoassay (Roche Diagnostics, Indianapolis, IN, USA). Serum HBsAg level was quantitatively measured using Elecsys® HBsAg II Quant Assay (Roche Diagnostics, Penzberg, Germany). Serum HBV DNA concentration was quantitatively determined using Cobas TaqMan assay kit (Roche Diagnostics, Branchburg, NJ), with a lower limit of detection of 20 IU/mL. HBV genotypes were determined by direct S gene sequencing. The PC/BCP mutations of HBV (including A1762T and G1764A) were detected using commercially available Line Probe Assays (INNOGENETICS, Belgium).

2.3 | Serum HBcrAg measurement

The serum HBcrAg level was quantitatively measured using the fully automated CLEIA system (Fujirebio Inc., Tokyo, Japan), and the detailed process of serum HBcrAg measurement is as previously reported.[17] Since the assay's validated measurement range is from 1000 U/mL (3 log10 U/mL) to 10 000 000 U/mL (7 log10 U/mL), serial dilutions of the serum sample are required when serum HBcrAg level is above the detection limit.

2.4 | Serum HBV RNA measurement

The HBV RNA was detected by RNA simultaneous amplification testing method (HBV-SAT) based on real-time fluorescence detection of isothermal RNA amplification using HBV-SAT kit (Shanghai Rendu Biotechnology Co., Ltd. China) according to the manufacturer's recommendations. Briefly, RNA was extracted by magnetic microparticles with HBV-specific RNA oligonucleotides. The target RNA was reverse transcribed by MMLV enzyme, transcribed by T7 RNA polymerase and detected by RNA beacon probe labelled by fluorescence and quencher. The concentration of serum HBV RNA was calculated using normalization to the internal control (IC) nucleic acid, which was distinct from HBV genome and human genome. A fixed dose of IC was added to each same volume sample from nuclear acid extraction step. All the reagents of the whole assay procedure were sufficient to IC detection and the IC amplification results should be constant theoretically. The HBV RNA amplification results were calibrated by IC amplification result and avoid the effects of the variations in specimen processing, amplification and detection. The linear range was established by testing panels of armoured HBV RNA diluted in HBV-negative human serum. The linear concentration ranged from 2 log copies/mL to 8 log copies/mL. The R^2 value of linear equation is more than 0.95. The limit of detection is 50 copies/mL.

2.5 | Intrahepatic HBV cccDNA measurement

Intrahepatic HBV cccDNA levels in paraffin-embedded liver tissue were measured with the real-time PCR method, as described previously.[6,18] For specifically amplification and quantification of cccDNA, we designed cccDNA-selective primers and a probe targeting the gap region between the viral genome direct repeat regions (DR1 and DR2). In the present study, the cellular DNA was quantified by determining the copy number of cellular house-keeping gene β-actin. To establish the standard curves for cccDNA quantitation, 10-fold serial dilutions (10^2-10^9 copies/mL) of a plasmid containing the entire wild-type HBV genotype C genome were used. Human liver tissue without HBV infection was used as negative controls. The amount of cccDNA was expressed as the number of copies per cell, with the estimation of 6.667 pg of DNA/cell. The detailed primers information for intrahepatic HBV cccDNA measurement is as previously reported.

2.6 | Statistical analyses

Continuous variables were expressed as median and range, and categorical variables were expressed as counts and percentages. Student's *t* test or Mann-Whitney test was used to analyse the differences between continuous variables, and paired samples *t* test was used to analyse the continuous variables before and after antiviral therapy. The correlation between two continuous variables was calculated using Spearman's bivariate correlation analysis, and the correlation is significant at the 0.01 level (2-tailed). Additionally, linear regression analysis was also performed to determine factors associated with intrahepatic HBV cccDNA levels. A *P*-value less than 0.05 was considered to indicate statistical significance. All statistical analyses were done with SPSS Version 18.0 (SPSS, Chicago, IL), and figures were drawn using GraphPad Prism 6 (GraphPad Software Inc., California, USA).

TABLE 1 Characteristics of patients included in this study

Variables	HBeAg positive (n = 85)	HBeAg negative (n = 25)	*P*-value
Age, median (range), years	31.00 (20.00-57.00)	38.00 (23.00-52.00)	0.000
Gender, male/female, n (%)	57 (67.05)/28(32.94)	17 (68.00)/8(32.00)	0.930
Inflammation grade[a], G < 2/G ≥ 2, n (%)	62 (72.94)/23(27.05)	9 (36.00)/16(64.00)	0.213
Fibrosis stage[a], S < 2/S ≥ 2, n (%)	69 (81.2)/16(18.8)	6 (24.00)/19(76.00)	0.240
HBV genotype, B/C, n (%)	55 (64.7)/30(35.3)	13 (52.00)/12(48.00)	0.250
HBV BCP mutations, yes/no, n (%)	21 (24.7)/64(75.3)	9 (36.00)/16(64.00)	0.265
ALT, median (range), IU/L	30.00 (6.00-97.00)	21.00 (8.00-56.00)	0.002
ALT, elevated/normal, n (%)	28 (32.9)/57(67.1)	5 (20.00)/20(80.00)	0.215
Serum HBV DNA, median (range), log10 IU/mL	7.91 (2.90-8.80)	3.68 (2.70-6.08)	0.000
Serum HBV RNA, median (range), log10 copies/mL	6.83 (4.20-8.15)	2.86 (1.70-6.60)	0.000
Serum HBsAg, median (range), log10 IU/mL	4.59 (0.82-5.10)	3.49 (0.99-4.01)	0.000
Serum HBeAg, median (range), log10 COI	2.81 (0.11-3.13)	0.41 (0.14-0.84)	0.000
Serum HBcrAg, median (range), log10 U/mL	10.30 (6.00-12.30)	5.40 (3.28-7.20)	0.000
HBV cccDNA, median (range), log10 copies/10^6 cell	7.46 (5.11-8.17)	6.03 (5.00-6.85)	0.000

[a]Pathological assessment of liver tissue using METAVIR score.

3 | RESULTS

3.1 | Patient's characteristics

A total of 110 eligible CHB patients were analysed, including 85 HBeAg-positive patients (median age 31 years [range 20-57]; 57 male [67.05%]) and 25 HBeAg-negative patients (median age 38 years [range 23-52]; 17 male [68.00%]). For HBeAg-positive patients, the median level was 7.91 log10 IU/mL for serum HBV DNA, 6.83 log10 copies/mL for serum HBV RNA, 4.59 log10 IU/mL for serum HBsAg, 2.81 log10 COI for serum HBeAg, 10.30 for serum HBcrAg log10 U/mL and 7.46 log10 copies/10^6 cell for intrahepatic HBV cccDNA. For HBeAg-negative patients, the median level was 3.68 log10 IU/mL for serum HBV DNA, 2.86 log10 copies/mL for serum HBV RNA, 3.49 log10 IU/mL for serum HBsAg, 5.40 for serum HBcrAg log10 U/mL and 6.03 log10 copies/10^6 cell for intrahepatic HBV cccDNA. The detailed characteristics of patients and comparison between HBeAg-positive and HBeAg-negative patients are shown in Table 1.

3.2 | Regression analysis of factors associated with intrahepatic cccDNA level

Among HBeAg-positive patients, by univariable linear regression, factors associated with intrahepatic cccDNA were age, inflammatory grade, serum HBeAg, HBcrAg, HBsAg, HBV RNA and HBV DNA, while gender, fibrosis stage, ALT levels and HBV genotype were not associated with intrahepatic cccDNA level (Table 2). By multivariable linear regression, factors of serum HBcrAg, HBsAg and HBV RNA were all associated with intrahepatic cccDNA, and the performance of serum HBcrAg (β = 0.563, *P* < 0.001) was superior to that of serum HBsAg (β = 0.328, *P* < 0.001)and HBV RNA (β = 0.180, *P* = 0.003; Table 2).

Among HBeAg-negative patients, by univariable linear regression, factors associated with intrahepatic cccDNA were serum HBcrAg and HBsAg, while age, gender, inflammatory grade, fibrosis stage, ALT levels, HBV genotype, HBV RNA and HBV DNA were all not associated with intrahepatic cccDNA level. By multivariable linear regression, only serum HBcrAg was associated with intrahepatic cccDNA level (β = 0.774, *P* = 0.000) (Table 2).

3.3 | Correlation analysis among serum HBcrAg, HBV RNA and HBsAg levels

The correlation of different HBV serum markers among HBeAg-positive and HBeAg-negative patients is shown in Figure 2. Among HBeAg-positive patients, serum HBcrAg correlated strongly with serum HBsAg (*r* = 0.564, *P* < 0.001). Both serum HBcrAg (*r* = 0.445, *P* < 0.001) and HBsAg (*r* = 0.323, *P* = 0.003) correlated moderately with serum HBV RNA. It was worth to mention that the correlation was moderate for serum HBcrAg and HBV DNA(*r* = 0.445, *P* < 0.001), strong for serum HBsAg and HBV DNA(*r* = 0.654, *P* < 0.001), but not significant correlation for serum HBV RNA and HBV DNA (*r* = 0.271, *P* = 0.012). Additionally, we also analysed the correlation of serum HBeAg level with serum HBcrAg, HBsAg, HBV RNA and HBV DNA. And we found that serum HBeAg strongly correlated with HBV DNA (*r* = 0.540, *P* < 0.001) and HBsAg ((*r* = 0.520, *P* < 0.001), moderately with serum HBcrAg (*r* = 0.491, *P* < 0.001) and weakly correlated with serum HBV RNA (*r* = 0.299, *P* = 0.005).

Among HBeAg-negative patients, serum HBcrAg correlated strongly with serum HBsAg (*r* = 0.552, *P* = 0.007), but not significantly correlated with serum HBV RNA (*r* = −0.017, *P* = 0.937) and HBV DNA (*r* = −0.187, *P* = 0.370). Additionally, no significant correlation was observed between serum HBsAg and HBV RNA(*r* = 0.156, *P* = 0.457), serum HBsAg and HBV DNA(*r* = −0.038, *P* = 0.856), and serum HBV RNA and HBV DNA(*r* = 0.398, *P* = 0.049).

TABLE 2 Univariable and multivariable linear regression analysis of factors associated with intrahepatic cccDNA

	Univariable			Multivariable			
Parameter	**B**	**95%CI**	***P*-value**	**B**	**95%CI**	**B**	***P*-value**
HBeAg positive							
Age, year	−0.023	−0.041 to −0.005	0.019	0.001	−0.008 to 0.011	0.016	0.779
Gender	−0.009	−0.272 to 0.274	0.943				
Inflammation grade	−0.486	−0.779 to −0.213	0.004	−0.031	−0.130 to 0.068	−0.041	0.530
Fibrosis stage	−0.326	−0.654 to −0.016	0.053	0.001	−0.081 to 0.084	0.003	0.971
ALT, IU/mL	−0.004	−0.011 to 0.002	0.240				
HBV genotype	−0.250	−0.558 to 0.034	0.098	−0.017	−0.154 to 0.120	−0.014	0.803
Serum HBV DNA, log10 IU/mL	0.272	0.158 to 0.461	0.002	−0.007	−0.086 to 0.071	−0.014	0.924
Serum HBV RNA, log10 copies/mL	0.416	0.275 to 0.600	0.001	0.139	0.050 to 0.227	0.180	0.003
Serum HBeAg, log10 COI	0.460	0.290–0.629	0.000	0.016	0.156 to 0.269	0.017	0.794
Serum HBsAg, log10 IU/mL	0.578	0.358 to 1.084	0.022	0.267	0.136 to 0.399	0.328	0.000
Serum HBcrAg, log10 U/mL	0.318	0.261 to 0.380	0.001	0.212	0.156 to 0.269	0.563	0.000
HBeAg negative							
Age, year	−0.015	−0.050 to 0.020	0.397				
Gender	0.050	−0.451 to 0.550	0.839				
Inflammation grade	−0.146	−0.386 to 0.094	0.221				
Fibrosis stage	−0.070	−0.260 to 0.119	0.450				
ALT, IU/mL	−0.005	−0.023 to 0.013	0.585				
HBV genotype	0.104	−0.361 to 0.570	0.648				
Serum HBV DNA, log10 IU/mL	−0.054	−0.324 to 0.216	0.681				
Serum HBV RNA, log10 copies/mL	−0.014	−0.190 to 0.161	0.869				
Serum HBsAg, log10 IU/mL	0.493	0.194 to 0.793	0.002	0.149	−0.062 to 0.361	0.175	0.157
Serum HBcrAg, log10 U/mL	0.476	0.357 to 0.595	0.000	0.425	0.289 to 0.562	0.774	0.000

3.4 | Serum HBcrAg, HBV RNA and HBsAg distribution stratified by demographic and other clinical characteristics

The distribution of serum HBcrAg, HBV RNA and HBsAg levels among HBeAg-positive patients is shown in Table. 3. In the present study, there was no statistically significant difference in serum level of HBV RNA stratified by age ($P = 0.707$), gender ($P = 0.631$), inflammatory grade ($P = 0.143$), fibrosis stage ($P = 0.183$), ALT ($P = 0.419$), viral genotype ($P = 0.363$), BCP mutation ($P = 0.805$) and HBV DNA levels ($P = 0.110$). The level of serum HBsAg was significantly higher in patients with young age ($P = 0.005$), low inflammatory grade ($P = 0.013$) and high serum HBV DNA levels ($P < 0.001$), but similar between patients with different gender ($P = 0.545$), fibrosis stage ($P = 0.060$), ALT ($P = 0.186$), viral genotype ($P = 0.057$) and BCP mutation ($P = 0.063$). Interestingly, the distribution of serum HBcrAg was significantly different among patients with different inflammatory grade ($P < 0.001$) and serum HBV DNA ($P = 0.004$), but not influenced by age ($P = 0.073$), gender ($P = 0.433$), fibrosis stage ($P = 0.154$), ALT level ($P = 0.829$), viral genotype ($P = 0.066$) and BCP mutations ($P = 0.071$).

3.5 | Intrahepatic cccDNA in relation to serum HBcrAg, HBsAg, HBV RNA and HBV DNA

Among HBeAg-positive patients, the level of intrahepatic cccDNA strongly correlated with serum HBcrAg ($r = 0.843$, $P < 0.001$) and serum HBsAg ($r = 0.710$, $P < 0.001$), and moderately correlated with serum HBV RNA ($r = 0.541$, $P < 0.001$), serum HBV DNA ($r = 0.507$, $P < 0.001$) and serum HBeAg ($r = 0.510$, $P < 0.001$) (Figure 3). The correlations of intrahepatic cccDNA with serum HBcrAg, HBsAg and HBV RNA, stratified by inflammatory grade and serum HBV DNA levels, are shown in Table 4. The correlation of serum HBcrAg with intrahepatic cccDNA was comparable for patients with different inflammatory grade (G < 2 vs G ≥ 2) or serum HBV DNA levels (<8 vs ≥8 log10 IU/mL). The correlation of serum HBV RNA with intrahepatic cccDNA was also not influenced by inflammatory grade and serum HBV DNA levels. However, the correlation of serum HBsAg with intrahepatic cccDNA was HBV DNA-dependent, indicated by a moderate correlation within HBV DNA < 8 log10 IU/mL ($r = 0.575$, $P < 0.001$), but strong correlation within HBV DNA ≥ 8 log10 IU/mL ($r = 0.926$, $P < 0.001$).Among HBeAg-negative patients, the level of intrahepatic cccDNA strongly correlated with serum HBcrAg ($r = 0.865$, $P < 0.001$)

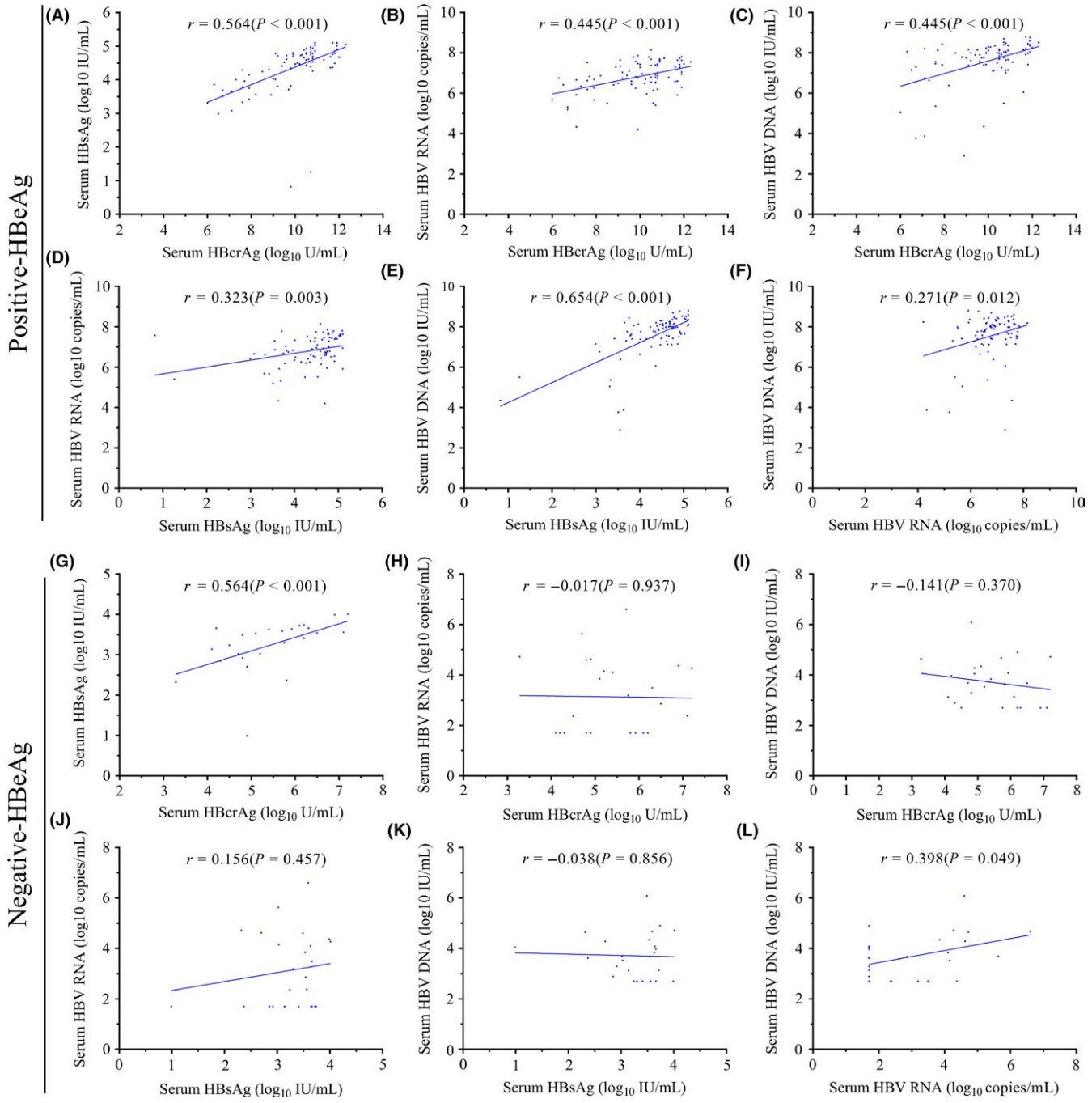

FIGURE 2 Correlations among different serum markers of HBV in HBeAg-positive (A-F) and HBeAg-negative patients (G-L). A, G, HBcrAg and HBsAg; B, H, HBcrAg and HBV RNA; C, I, HBcrAg and HBV DNA; D, J, HBsAg and HBV RNA; E, K, HBsAg and HBV DNA; F, L, HBV RNA and HBV DNA

and moderately correlated with serum HBsAg ($r = 0.579$, $P = 0.002$), but not significantly correlated with serum HBV RNA ($r = -0.028$, $P = 0.892$) and HBV DNA ($r = -0.086$, $P = 0.681$) (Figure 3).

4 | DISCUSSION

In the past decades, the fundamental role of intrahepatic cccDNA, as a template for transcription of all viral RNAs and further synthesis of viral proteins, has been recognized.[17] Thus, monitoring intrahepatic cccDNA levels could reflect the real activity of HBV replication in patients, and low levels of intrahepatic cccDNA could also predict sustained virologic response after cessation of antiviral therapy.[17] In recent years, several HBV-associated serum markers (including serum HBcrAg, HBsAg and HBV RNA) were all reported to be a potential indicator of intrahepatic cccDNA activity in different studies,[5,16] and low level of serum HBcrAg or loss of serum HBV RNA also might indicate exhausting or transcription silencing of cccDNA

TABLE 3 Serum HBcrAg, HBV RNA and HBsAg distribution stratified by demographic and other clinical characteristics among HBeAg-positive patients

Variable	HBcrAg (Log10 U/mL)	*P*-value	HBV RNA (Log10 copies/mL)	*P*-value	HBsAg (Log10 IU/mL)	*P*-value
Age, years						
<30 (n = 35)	6.30 (6.00-12.30)	0.073	6.90 (4.20-7.80)	0.707	4.68 (2.99-5.10)	0.005
≥30 (n = 50)	9.95 (6.70-11.90)		6.79 (4.33-8.15)		4.45 (0.82-5.10)	
Gender, n (%)						
Male (n = 57)	10.10 (6.00-12.30)	0.433	6.78 (4.20-8.15)	0.631	4.59 (0.82-5.10)	0.545
Female (n = 28)	10.65 (6.70-12.00)		6.89 (5.32-7.80)		4.58 (3.08-5.10)	
Inflammatory grade						
<2 (n = 62)	10.55 (6.00-12.30)	0.000	6.88 (4.20-7.80)	0.143	4.67 (0.82-5.10)	0.013
≥2 (n = 23)	9.20 (6.30-11.80)		6.74 (4.33-8.15)		4.29 (1.26-4.80)	
Fibrosis stage						
<2 (n = 69)	10.50 (6.00-12.30)	0.154	6.86 (4.33-8.15)	0.183	4.61 (0.82-5.10)	0.060
≥2 (n = 16)	9.85 (6.30-11.80)		6.80 (4.20-7.52)		4.34 (1.26-4.80)	
ALT						
Elevated (n = 57)	10.40 (6.00-12.00)	0.829	6.78 (4.20-8.15)	0.419	4.61 (3.31-5.10)	0.186
Normal (n = 28)	10.20 (6.30-12.30)		6.95 (5.40-7.74)		4.46 (0.82-5.10)	
HBV genotype						
B (n = 55)	10.40 (6.70-12.30)	0.066	6.81 (4.20-7.86)	0.363	4.66 (1.26-5.10)	0.057
C (n = 30)	9.85 (6.00-11.90)		6.87 (5.32-8.15)		4.31 (0.82-5.10)	
HBV BCP mutations						
No (n = 64)	10.50 (6.70-12.00)	0.071	6.81 (4.20-8.15)	0.805	4.61 (1.26-5.10)	0.063
Yes (n = 21)	9.80 (6.00-12.30)		6.94 (4.33-7.86)		4.00 (0.82-5.10)	
Serum HBV DNA, log10 IU/mL						
<8 (n = 49)	9.80 (6.00-11.80)	0.004	6.69 (4.33-7.79)	0.110	4.35 (0.82-5.02)	0.000
≥8 (n = 36)	10.70 (6.30-12.30)		6.95 (4.20-8.15)		4.73 (3.65-5.10)	

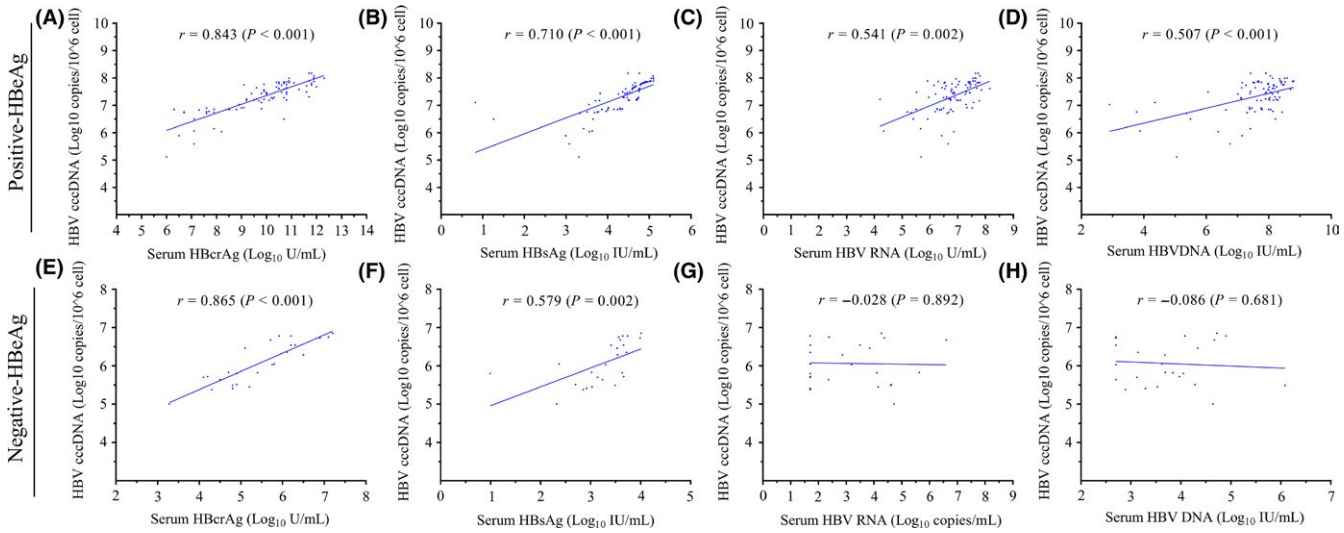

FIGURE 3 Correlations of serum viral proteins with intrahepatic cccDNA in HBeAg-positive (A-D) and HBeAg-negative patients (E-H). A, E, Serum HBcrAg; B, F, Serum HBsAg; C, G, Serum HBV RNA; D, H, Serum HBV DNA

reservoirs.[7,16] To our knowledge, the present study represents a truly "real-life" correlation analysis of serum HBcrAg and HBV RNA levels, and a first head-to-head comparison of serum HBcrAg, HBsAg and HBV RNA levels in reflecting intrahepatic cccDNA levels. The main findings from the present study are as follows: serum HBcrAg level is positively correlated with serum HBsAg or HBV RNA among

TABLE 4 Correlation of intrahepatic cccDNA with HBcrAg, HBsAg and HBV RNA stratified by inflammatory grade and HBV DNA level among HBeAg-positive patients

	cccDNA				cccDNA			
	G < 2		G ≥ 2		HBV DNA <8 log10 IU/mL		HBV DNA ≥8 log10 IU/mL	
Parameter	*r*	*P*-value	*r*	*P*-value	*r*	*P*-value	*r*	*P*-value
Serum HBcrAg	0.852	0.000	0.743	0.000	0.848	0.000	0.828	0.000
Serum HBsAg	0.650	0.000	0.750	0.000	0.632	0.000	0.926	0.000
Serum HBV RNA	0.513	0.000	0.551	0.006	0.575	0.000	0.417	0.011

HBeAg-positive patients, but not among HBeAg-negative patients; serum HBcrAg and HBsAg levels are both significantly influenced by inflammatory grade and serum HBV DNA levels among HBeAg-positive patients, but serum HBV RNA level is not; serum HBcrAg, HBsAg and HBV RNA levels are all associated with intrahepatic cccDNA levels among HBeAg-positive patients, but only serum HBcrAg was associated with cccDNA level among HBeAg-negative patients.

For a long time, the quantitative measurement of serum HBV DNA was widely used to estimate the activity of viral replication and antiviral efficacy of nucleos(t)ide analogues (NAs) treatment. However, NAs only act on limited steps of the viral replication cycle, and production of viral intermediate proteins may not be affected significantly.[19] Therefore, measurement of viral proteins can be useful in monitoring HBV activities, especially in patients receiving NAs when HBV DNA levels are undetectable. Currently, the most attractive viral proteins should be serum HBcrAg and HBsAg, and both of them can be found in mature virions as well as HBV DNA-negative empty particles. The effectiveness of either serum HBcrAg or HBsAg in reflecting intrahepatic cccDNA level had been identified in cohorts of patients with different races and viral genotypes.[7,20-22] Though a positive correlation was reported between HBcrAg and HBsAg in HBeAg-positive patients without antiviral therapy, the intensity of correlation was moderate ($r = 0.564$), which indicated that serum HBcrAg and HBsAg could not replace each other.[6] Additionally, serum HBcrAg and HBsAg had unique patterns of distribution throughout the five disease phases of CHB, including high detectability rates of serum HBcrAg after HBsAg seroclearance, which decided the different possibilities for their applicability in clinical practice.[7] Thus, a difference may exist between serum HBcrAg and HBsAg in reflecting intrahepatic cccDNA level. In fact, we previously investigated the correlations of serum HBcrAg and HBsAg level with intrahepatic cccDNA among 139 patients with liver biopsy and found that serum HBcrAg had a much stronger correlation with intrahepatic cccDNA than serum HBsAg, either before or during NAs treatment.[6] Importantly, the better performance of serum HBcrAg than HBsAg in reflecting intrahepatic cccDNA level was also observed in this cohort. As we know, serum HBsAg also could originate from the expression of the integrated HBV S gene in patients with S gene integration,[23,24] and this may partly cause the performance of HBsAg to be inferior to that of HBcrAg in reflecting intrahepatic cccDNA level and activity.

In theory, intrahepatic HBV RNA could well correlate with intrahepatic cccDNA, but the biggest problems facing intrahepatic HBV RNA application are the difficulty in measurement, as the latter relies on liver biopsy. As we know, serum HBV RNA is also transcribed from intrahepatic cccDNA, and thus, it may be a potential alternative marker for intrahepatic cccDNA. Though a recent study showed that serum HBV RNA level could reflect intrahepatic cccDNA transcriptional activity, it was nevertheless inferior to serum HBV DNA in reflecting the level of intrahepatic cccDNA before treatment.[14] In the present study, as compared to the strong correlation of serum HBcrAg ($r = 0.843$) and HBsAg ($r = 0.710$) with intrahepatic cccDNA, the similar moderate correlation intensity for serum HBV RNA ($r = 0.541$) and HBV DNA ($r = 0.507$) with intrahepatic cccDNA suggested that serum HBV RNA was not a good indicator of intrahepatic cccDNA. Though the weak correlation of serum HBV RNA with both serum HBcrAg and HBsAg indicated a different possibility for its applicability in future, serum HBV RNA should be at least not an ideal indicator for the activity of HBV replication in liver tissue. In fact, the poor efficacy of serum HBV RNA in reflecting intrahepatic cccDNA activity was also reported in another recently published study.[15]

A large number of studies have showed that the hepatic inflammation and fibrosis are prominent features in chronic viral hepatitis, and the severity of inflammation and fibrosis plays important roles in the decision making of antiviral treatment and risk assessment of disease progression. In the present study, the levels of serum HBV RNA were similar between patients with different inflammation grade and fibrosis stage. However, Prof. Zhang WH and his team reported an association between serum HBV RNA levels and liver histological changes ($r = 0.665$ for grading and $r = 0.722$ for staging) in patients receiving NAs therapy.[13] Due to the fact that there were limited studies and small samples were provided, more data are still required to clarify whether the level of serum HBV RNA could effectively predict severe inflammation and advanced fibrosis. In the present study, we found that serum HBcrAg and HBsAg levels were influenced by liver histological changes, and both serum HBcrAg and HBsAg correlated strongly with intrahepatic cccDNA, regardless of inflammation grade. Among patients with severe inflammation, though serum HBcrAg showed a relative good correlation with intrahepatic cccDNA ($r = 0.743$), its accuracy for predicting intrahepatic cccDNA levels

may be further improved if combined with serum HBsAg. It is worth mentioning that the correlation between serum HBsAg and intrahepatic cccDNA would be affected by serum HBV DNA levels, and the correlation in patients with low serum HBV DNA levels was notably less than that in patients with high serum HBV DNA levels. On the contrary, the correlations between serum HBcrAg and intrahepatic cccDNA levels were not only excellent but also not influenced by serum HBV DNA levels. Thus, to a certain extent, serum HBcrAg may be more stable than serum HBsAg in reflecting intrahepatic cccDNA, especially among patients with high serum HBV DNA levels.

In the present study, multivariable linear regression analysis also showed that serum HBcrAg had the highest performance of association with intrahepatic cccDNA levels, which were followed by serum HBsAg and HBV RNA. Indeed, this finding was also highly consistent with the result of the correlation analysis. In previous studies, HBV genotypes, serum HBV DNA levels and liver histological changes were reported to be associated with the levels of serum HBcrAg, HBsAg or HBV RNA.[7,21,25] However, they had no significant association with intrahepatic cccDNA levels among HBeAg-positive patients in this study. Theoretically, the prediction efficiency should be better for the combination of several correlated variables than single variable. Thus, if serum HBcrAg, HBsAg and HBV RNA were combined together, the intrahepatic cccDNA levels should be more reliably and more accurately reflected.

Several limitations exist in this study. First, the sample size is relatively small, and there was a heterogeneous sample size between HBeAg-positive and HBeAg-negative patients, so further large sample size cohort studies are required to confirm the present findings. Second, the methods for serum HBV RNA and intrahepatic cccDNA quantification are not standardized, which may lead to inconsistent results with other studies; thus, standardized testing methods and agents are urgently needed. Third, as not all intrahepatic cccDNA molecules are equally transcriptional active, present findings just suggest a correlation of serum HBV serum markers with total amount of intrahepatic cccDNA but not the transcriptional activity of intrahepatic cccDNA.

In summary, the present study is the first head-to-head comparison of serum HBcrAg, HBsAg and HBV RNA levels in reflecting intrahepatic cccDNA levels. We find that serum HBcrAg, HBsAg and HBV RNA levels are significantly correlated with each other among HBeAg-positive patients but not among HBeAg-negative patients and serum HBcrAg is better than HBV RNA and HBsAg in correlation with cccDNA level, irrespective of HBeAg status. Thus, based on the findings of the present study and previous reports, serum HBcrAg is likely to be the most useful marker for disease monitoring, predicting treatment response and disease outcome of CHB.

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CONFLICT OF INTEREST

The authors do not have any conflict of interest.

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