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ORIGINAL ARTICLE

Serum HBV RNA predicts HBeAg clearance and seroconversion in patients with chronic hepatitis B treated with nucleos(t)ide analogues

Yang Wang[1,2] | Hao Liao[3,4] | Zhongping Deng[5,6] | Yanna Liu[3] | Dandan Bian[1,2] | Yan Ren[1,2] | Guangxin Yu[3] | Yingying Jiang[1,2] | Li Bai[1,2] | Shuang Liu[1,2] | Mei Liu[1,2] | Li Zhou[1,2] | Yu Chen[1,2] | Zhongping Duan[1,2] | Fengmin Lu[3] | Sujun Zheng[1,2]

[1]Liver Disease Center, Beijing YouAn Hospital, Capital Medical University, Beijing, China

[2]Beijing Municipal Key Laboratory of Liver Failure and Artificial Liver Treatment & Research, Beijing YouAn Hospital, Capital Medical University, Beijing, China

[3]Department of Microbiology and Infectious Disease Center, School of Basic Medical Sciences, Peking University Health Science Center, Beijing, China

[4]Intervention and Cell Therapy Center, Peking University Shenzhen Hospital, Shenzhen Peking University-The Hong Kong University of Science and Technology Medical Center, Shenzhen, China

[5]Academy for Advanced Interdisciplinary Studies, Peking University, Beijing, China

[6]Hunan Provincial Key Laboratory of Gene Diagnostic Technology, Changsha, China

Correspondence
Fengmin Lu, Department of Microbiology and Infectious Disease Center, School of Basic Medical Sciences, Peking University Health Science Center, 38 Xueyuan Road, Haidian 22 District, Beijing 100191, China.
Email: lu.fengmin@hsc.pku.edu.cn

Sujun Zheng, Liver Disease Center, Beijing YouAn Hospital, Capital Medical University, 8 Xitoutiao, Youwai Street, Beijing 100069, China.
Email: zhengsujun003@126.com.

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Abstract

This study evaluated the predictive value of serum HBV DNA, HBV RNA, HBcrAg, HBsAg, intrahepatic HBV DNA and cccDNA for HBeAg clearance and seroconversion during long-term treatment with nucleos(t)ide analogues (NAs) in patients with chronic hepatitis B (CHB). A single centre, prospective cohort of CHB patients was used for this study. Serum HBV RNA levels were retrospectively measured at baseline, 6, 12, 24, 36, 48, 60, 72 and 84 months post-NAs treatment. Serum HBsAg and HBcrAg levels were quantified at baseline, month 6, 60 and 72. Histological samples from liver biopsy at baseline and month 60 were analysed for intrahepatic HBV DNA and cccDNA. Eighty-three HBeAg-positive patients were enrolled with a median follow-up time of 108 months (range 18–138 months). Of them, 53 (63.86%) patients achieved HBeAg clearance, and 37 (44.58%) achieved HBeAg seroconversion. Cox multivariate analysis showed that only baseline HBV RNA was independently associated with HBeAg clearance and seroconversion (<5.45 $\log_{10}$copies/mL, HR = 5.06, 95% CI: 1.87–13.71, p = .001; HR = 3.38, 95% CI: 1.28–8.91, p = .01).

Abbreviations: ADV, Adefovir; ALT, alanine aminotransferase; AST, aspartate aminotransferase; AUC, area under the receiver operating characteristic curve; BMI, body mass index; cccDNA, covalently closed circular DNA; CHB, chronic hepatitis B; COI, cut-off index; HBcrAg, hepatitis B core-related antigen; HBeAg, hepatitis B e antigen; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; LLD, lower limit of detection; NAs, nucleos(t)ide analogues; pgRNA, pregenomic RNA; ROC, receiver operating characteristic curve.

Yang Wang, Hao Liao and Zhongping Deng equally contributed to this work.

The independent association with HBeAg clearance and seroconversion remained for HBV RNA levels at month 6 (<4.72 $\log_{10}$copies/mL, HR = 4.16, 95% CI: 1.61–10.72, $p = .003$; HR = 6.52, 95% CI: 1.85–22.94, $p = .003$) and month 12 (<4.08 $\log_{10}$ copies/mL, HR = 3.68, 95% CI: 1.96–6.90, $p < .001$; HR = 2.79, 95% CI: 1.31–5.94, $p = .008$). The AUCs of baseline HBV RNA for predicting the HBeAg clearance (0.83, 95% CI: 0.70–0.96, 0.83, 95% CI: 0.70–0.96 and 0.82, 95% CI: 0.69–0.95 respectively) and seroconversion (0.89, 95% CI: 0.77–1.00; 0.81, 95% CI: 0.66–0.95 and 0.84, 95% CI: 0.71–0.98 respectively) at month 36, 60 and 84 were higher than those of HBV DNA, HBsAg and HBcrAg. In conclusion, lower serum HBV RNA at baseline, month 6 and 12 post-NAs treatment could predict HBeAg clearance and seroconversion during long-term NAs treatment.

KEYWORDS
chronic hepatitis B, HBeAg clearance, HBeAg seroconversion, HBV RNA, nucleos(t)ide analogues

1 | INTRODUCTION

Hepatitis B virus (HBV) infection has been a global public health challenge.[1] Nucleos(t)ide analogues (NAs), which suppresses HBV DNA replication via inhibiting the reverse transcription of pregenomic RNA (pgRNA) into HBV DNA, are considered the first-line treatment option. However, NAs fails to clear the HBV covalently closed circular DNA (cccDNA) and RNA replication intermediates.[2] Thus, long-term and even indefinite NAs treatment is needed for sustained viral suppression in chronic hepatitis B (CHB) patients. The ideal anti-HBV treatment endpoint, namely functional cure is serum hepatitis B surface antigen (HBsAg) clearance.[2] For hepatitis B e antigen (HBeAg)-positive CHB patients, the realistic goal is to attain HBeAg clearance and further achieve seroconversion, which reflect partial immune control and a pre-requisite for the ideal treatment endpoint.[2] Therefore, biomarkers that potentially predict the possibility of HBeAg clearance and seroconversion during long-term NAs treatment may be useful in clinical practice.

Viral markers including serum HBV DNA, HBV RNA, hepatitis B core-related antigen (HBcrAg) and quantitative HBsAg are currently used to predict therapeutic response for CHB patients undergoing NAs therapy.[3,4] It has been reported that on-treatment decline of HBsAg level at 48 or 96 weeks may help predict HBeAg clearance or seroconversion in HBeAg-positive CHB patients treated with NAs.[5,6] Besides, HBV DNA levels at month 6 and 12 could predict serum HBeAg clearance in this population treated with Entecavir.[7,8] However, the predictability of HBsAg in Lee's study[9] and HBV DNA in Shin's study[10] for HBeAg seroconversion was not observed in Fung's study[11] in HBeAg-positive CHB patients treated with NAs.

Serum HBV RNA is the encapsidated HBV pregenomic RNA (pgRNA) inside the maturing core particles.[12] It reflects the intrahepatic transcriptional activity of cccDNA and indicates changes of liver inflammation and fibrosis score in CHB patients receiving NAs therapy.[13] Studies have shown that HBV RNA quantification is correlated with HBeAg clearance and seroconversion after long-term NAs treatment in CHB patients.[14,15] Luo et al. reported that HBeAg seroconversion more likely occurred in CHB patients with HBV RNA levels below 4.12 $\log_{10}$ copies/mL before NAs treatment.[15] And patients who remained HBV RNA-positive after 48 weeks of NAs treatment had an increased risk of not achieving HBeAg clearance.[14]

Hepatitis B core-related antigen is also considered a novel serum marker for disease monitoring and prognosis of CHB.[16] Compared to serum HBsAg and HBV DNA level, serum HBcrAg had a better correlation with intrahepatic cccDNA.[17] Evidence is accumulating that HBcrAg could predict the response to NAs therapy, off-treatment viral relapse, HBeAg seroconversion and the risk of development to HCC.[18] Wang and colleagues revealed that the quantitative serum HBsAg and HBcrAg could predict the HBeAg seroconversion in HBeAg-positive patients treated with NAs.[19]

It has been reported that correlation between new markers (HBcrAg and HBV RNA) and HBeAg clearance or seroconversion is better than that of the traditional markers (HBsAg and HBV DNA).[19,20] The following issues still require further studies to be clarified: First, head-to-head to compare the predictive value of serum viral markers including HBV DNA, HBV RNA, HBcrAg and HBsAg for HBeAg clearance and seroconversion during long-term treatment with NAs. Second, the correlation between early on-treatment decline of new serum viral markers and HBeAg clearance or seroconversion after long-term NAs treatment need to be elucidated. In this single centre, longitudinal study, we evaluated the predictive value of serum viral markers including HBV DNA, HBV RNA, HBcrAg, HBsAg, intrahepatic HBV DNA and liver tissue cccDNA for HBeAg clearance and seroconversion during long-term treatment with NAs in HBeAg-positive CHB patients.

2 | MATERIAL AND METHODS

2.1 | Patients and study design

This study has been conducted using the database of patients receiving NAs monotherapy, prospectively recruited from Beijing YouAn Hospital, Capital Medical University (Beijing, China) between June 2007 and July 2008. Eligible patients were diagnosed with CHB according to the AASLD,[21] male or female patients aged ≥16 years. The exclusion criteria were as follows: (i) co-infection with other viruses, including hepatitis C or D, Epstein–Barr, cytomegalovirus and human immunodeficiency, the existence of autoimmune liver disease or alcoholic liver disease; (ii) with decompensated liver function (ascites, hepatic encephalopathy or upper gastrointestinal bleeding); (iii) with any diseases of other major organs, such as severe heart disease or kidney disease; (iv) poor compliance; (v) history of a malignancy, including hepatocellular carcinoma, carcinoma in situ and atypical hyperplastic nodules; (vi) with mental illness; (vii) had received corticosteroids, immunosuppressants or chemotherapeutic drugs ≤6 months prior to enrolment; and (viii) pregnant or breast-feeding women.

Patients were followed up every three months in the first year of therapy, and every 6 months thereafter. At each follow-up, serum specimens were collected for liver function tests and HBV DNA quantification. Serum HBsAg, anti-HBs, HBeAg, anti-HBe and anti-HBc were also determined. Remaining serum samples were stored at −80°C for subsequent research. At enrolment and 60 months on-treatment, percutaneous liver biopsy was performed to evaluate the histological changes, which were scored according to the modified Knodell and Ishak scoring system.[22]

Hepatitis B e antigen clearance was defined as the cut-off index (COI) of HBeAg lower than 1. HBeAg seroconversion was defined as the COI of HBeAg lower than 1 and the COI of anti-HBe was greater than 1.

With the foregoing collected blood, we retrospectively quantified HBsAg and HBcrAg levels at baseline, 6, 60 and 72 months, HBV RNA levels at baseline, 6, 12, 24, 36, 48, 60, 72 and 84 months, and intrahepatic HBV DNA and cccDNA levels at baseline and at 60 months by a method previously reported.[3,12,23]

The study was conducted in compliance with the Declaration of Helsinki. Use of the research samples was approved by the Medical Ethics Review Committee of Beijing YouAn Hospital. All patients provided written informed consent authorizing us to access their medical records and to store the serum specimens for research purposes.

2.2 | Assays for serological HBV markers, HBV DNA, HBV RNA and HBcrAg

Serum HBsAg, anti-HBs, HBeAg, anti-HBe and anti-HBc were determined on a Roche Cobas e601 analyser using an electrochemiluminescence immunoassay (Abbott Laboratories, Chicago, IL, USA). HBsAg was quantified using an Elecsys for HBsAg quantitation (Roche Diagnostics) with a lower limit of detection (LLD) of 0.05 IU/mL. The serum HBV DNA level was determined using the Cobas HBV Amplicor Monitor assay (Roche Diagnostics, Pleasanton, CA, USA), with a LLD of 50 IU/mL. Instead of using the dual targets described by Butler et al,[24] we chose a target region closer to the 5′ end of pgRNA to avoid the potential adverse effect brought by splicing variants and the easily lost X region.[12] Briefly, HBV RNA was isolated with the nucleic acid extraction or purification kit (Sansure Biotech, Changsha, China) and treated with DNase I (Thermo Fisher Scientific, Waltham, MA, USA). The specially modified super-cis nano-magnetic beads efficiently adsorbed and enriched nucleic acids from 200 μL serum. For DNase I treatment, every reaction mixture comprised 2 μL of DNase I Reaction Buffer (10×), 2 μL of DNase I (RNase-free) and 16 μL of total nucleic acids. The reaction was carried out at 37°C for 30 min. Next, each mixture was incubated at 75°C for 10 min to inactivate DNase I. Finally, DNase-I-treated HBV RNA was one-step of reverse-transcribed and real-time fluorescent quantitative PCR using the HBV pgRNA high-sensitivity quantitative kit (Sansure Biotech, Changsha, China). Serum HBV RNA level was determined as described previously.[3,12,23] The LLD of the assay was 200 copies/mL. Details for the HBV RNA assay can be found in Supplementary Materials. HBcrAg was determined using a chemiluminescent enzyme immunoassay in an automated analyser system (Lumipulse System, Fujirebio Inc., Tokyo, Japan). The LLD was 1000 U/mL with a linear range of 3–7 $\log_{10}$ U/mL.

2.3 | Quantitation of intrahepatic HBV DNA and cccDNA

About 30 μm formalin fixed paraffin-embedded liver biopsy tissue in sections of 6 μm each was used for DNA extraction. The DNA was extracted using the QIAamp FFPE DNA Mini Kit (QIAGEN, GmbH, Hilden, Germany) according to the instructions of the manufacturer. T5 Exonuclease (New England Biolabs, USA) was used to digest HBV rcDNA, replicative dsDNA and ssDNA. The reaction mixture contained 100 ng extracted DNA, 0.5 μL (10 units) T5 Exonuclease, 1 μL NEBuffer 4 (10×) with Nuclease-free H_2O to a final volume of 10 μL. The digestion was carried out at 37 °C for 1 h, and stopped with EDTA to at least 11 mmol/L. We combined 6.42 μL of digestion product, which was obtained in the previous step, with 7.50 μL QuantStudio™ 3D Digital PCR Master Mix, 0.06 μL of TaqMan Probe-RC-MGB (50 μmol/L), 0.06 μL TaqMan Probe-RNAseP-VIC (50 μmol/L), 0.24 μL primer of rc-F, 0.24 μL primer of rc-R, 0.24 μL primer of RNaseP-F and 0.24 μL primer of RNaseP-R. This sample mix of 15 μL was added on each chip and loaded on a ProFlex™ 2× Flat PCR System with the following program: Absolute quantification was determined using the QuantStudio™ 3D Digital PCR System (Thermo Fisher Scientific Inc., Waltham, Massachusetts, USA) and analysed with QuantStudio 3D AnalysisSuite Cloud Software. (https://china.apps.thermofisher.com/quantstudio3d/). All intrahepatic HBV cccDNA values were normalized to cell number assessed by RNase P copy number assay.

2.4 | Statistical analysis

Hepatitis B surface antigen, HBV DNA, HBV RNA, HBcrAg, intrahepatic HBV DNA and cccDNA expression were logarithmically transformed for analysis. Continuous variables were expressed as mean ± SD or median (range). Categorical data were expressed as counting and/or proportion. Continuous quantitative data were compared between groups using *t* test or Mann–Whitney *U* test, the comparison between before and after treatment was performed using *Wilcox rank sum test*. The comparison between the counting data sets was performed using a *chi-square* test or *Fisher's exact probabilities*. Cox regression analysis was used to determine relevant factors associated with HBeAg clearance and seroconversion, using the moving forward LR method, with *p* values of entry and removal of .05 and .10 respectively. Patients were divided into two specified groups based on median value of continuous variables, in the aim to compare the accumulated HBeAg clearance and seroconversion rate of specified groups. Patients who were lost to follow-up before HBeAg clearance or seroconversion were considered as HBeAg non-clearance or non-seroconversion. The predictive capacity of these serum biomarkers were assessed by time-dependent area under the curve analysis using the R packages 'survival', 'survivalROC' and 'timeROC'. Comparisons between ROC curves were performed using DeLong's test. Based on cox multivariate regression analysis, combination of HBsAg, HBV DNA, HBV RNA and HBcrAg was evaluated to predict HBeAg clearance and seroconversion. Data were analysed using the IBM SPSS 22.0 (SPSS Inc., Chicago, IL, USA) or R software version 4.1.0 (R Foundation for Statistical Computing, Vienna, Austria). $p < .05$ was considered statistically significant in a two-tailed test.

3 | RESULTS

3.1 | Patients

A total of 83 eligible HBeAg-positive CHB patients were enrolled. Of them, 36 were NAs-naive patients, 47 were Lamivudine-treated patients. Forty-six (55.42%) patients were treated with Entecavir 0.5 mg once daily and the other 37 (44.58%) patients were treated with Adefovir dipivoxil 10 mg once daily. Sixty-eight (81.93%) patients were male. Average age was 36.42 ± 9.78 years. The median follow-up period was 108 months (range: 18–138 months). Thirty-nine patients had available genotype data, with 74.36% (29/39)

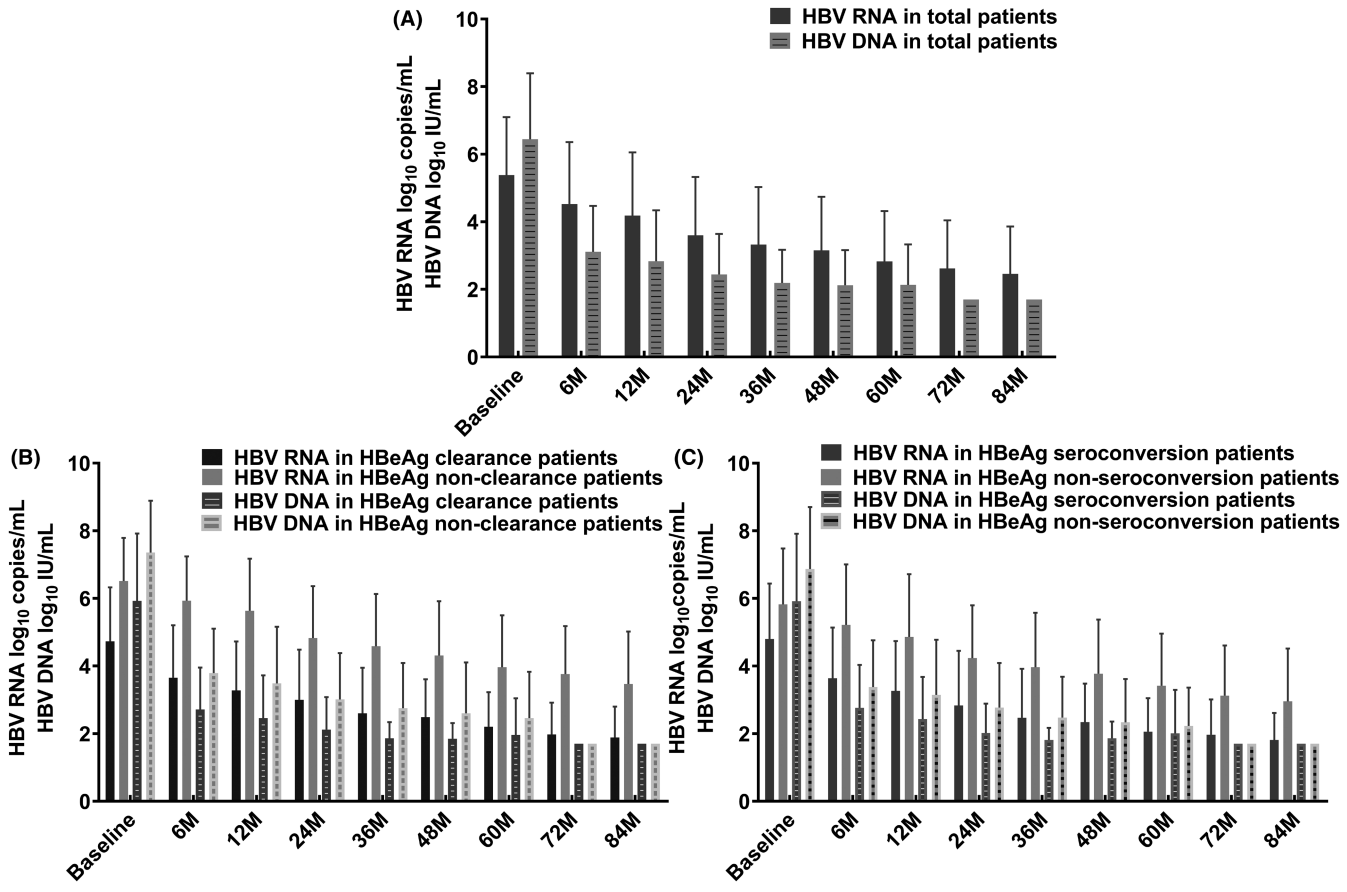

FIGURE 1 Dynamic changes of serum HBV RNA and HBV DNA levels in all patients (A), HBeAg clearance or non-clearance patients (B) and HBeAg seroconversion or non-seroconversion patients (C)

patients being genotype C. Fifty-nine patients had baseline liver biopsy; the median inflammation and fibrosis score were 7 (range: 2–15) and 3 (range: 1–5) respectively. Detailed demographic and clinical characteristics are shown in Table S1.

Percentages of patients followed up at each time point for HBeAg clearance and seroconversion are shown in Supplementary Materials and Figure S1.

3.2 | The changes of HBV viral markers during long-term NAs therapy

As shown in Figures 1 and 2, baseline serum HBV DNA, HBV RNA, HBcrAg and HBsAg levels were 6.44 ± 1.95 $\log_{10}$ IU/mL, 5.38±1.71 $\log_{10}$copies/mL, 7.15 ± 1.12 $\log_{10}$ U/mL and 3.60 ± 1.04 $\log_{10}$ IU/mL respectively. After 72 months of NAs treatment, these levels decreased to 1.70 ± 0.00 $\log_{10}$ IU/mL, 2.62 ± 1.42 $\log_{10}$copies/mL, 4.89 ± 1.02 $\log_{10}$ U/mL and 2.94 ± 1.18 $\log_{10}$ IU/mL respectively (all $p < .05$). Baseline serum intrahepatic HBV DNA and cccDNA levels were 6.37 ± 0.83 $\log_{10}$copies/10^5 cells and 4.74 ± 0.93 $\log_{10}$copies/10^5 cells respectively. At month 60, they were significantly decreased to 4.91 ± 0.50 $\log_{10}$copies/10^5 cells and 3.41 ± 0.60 $\log_{10}$copies/10^5 cells respectively (both $p < .05$). The levels of these markers were significantly decreased, regardless of the occurrence of HBeAg clearance or seroconversion (all $p < .05$).

3.3 | Relevance of viral markers at baseline and during early on-treatment for HBeAg clearance or seroconversion

During follow-up, 63.86% (53 of 83) of patients achieved HBeAg clearance, the median HBeAg clearance time was 66 months (95% CI: 37.84–94.16), and 44.58% (37/83) achieved HBeAg seroconversion, the median seroconversion time being 126 months (95% CI: 92.16–159.84). Clinical characteristics of HBeAg clearance and non-clearance patients are shown in Table S1. As shown in Figure 3, the cumulative HBeAg clearance rate increased gradually. Kaplan–Meier survival analysis showed that patients with lower baseline levels of HBV DNA (Figure 3A), HBsAg (Figure 3B), HBcrAg (Figure 3C) and HBV RNA (Figure 3D) had a higher accumulative HBeAg clearance rate than those with higher levels (all $p < .05$) Table 1. Patients with different levels of intrahepatic HBV DNA (Figure 3E) and cccDNA (Figure 3F) showed no statistical differences of accumulative HBeAg clearance rate (both $p > .05$). Cox multivariate analysis showed that an HBV RNA <5.45 $\log_{10}$ copies/mL was independently associated with HBeAg clearance (HR = 5.06, 95% CI: 1.87–13.71, $p = .001$), with median HBeAg clearance time of 36 months (95% CI: 22.71–49.29). Moreover, the serum HBV RNA <4.72 $\log_{10}$ copies/mL at month 6 was independently associated with HBeAg clearance (HR = 4.16, 95% CI: 1.61–10.72, $p = .003$), as well as the serum HBV RNA levels <4.08 $\log_{10}$copies/mL at month 12 (HR = 3.68, 95% CI 1.96–6.90, $p < .001$).

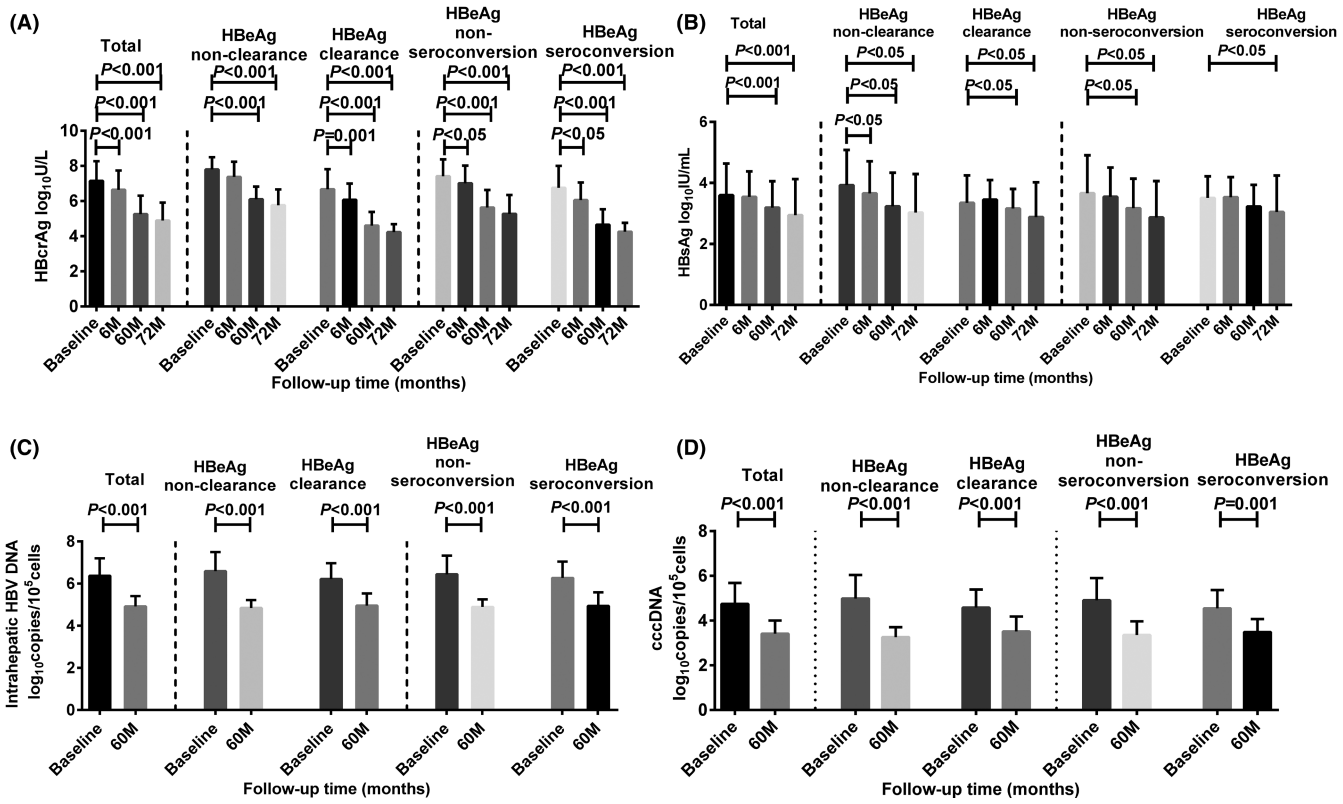

FIGURE 2 Changes of HBcrAg (A), HBsAg (B), intrahepatic HBV DNA (C) and cccDNA (D) in HBeAg clearance, HBeAg non-clearance, HBeAg seroconversion and HBeAg non-conversion patients during follow-up

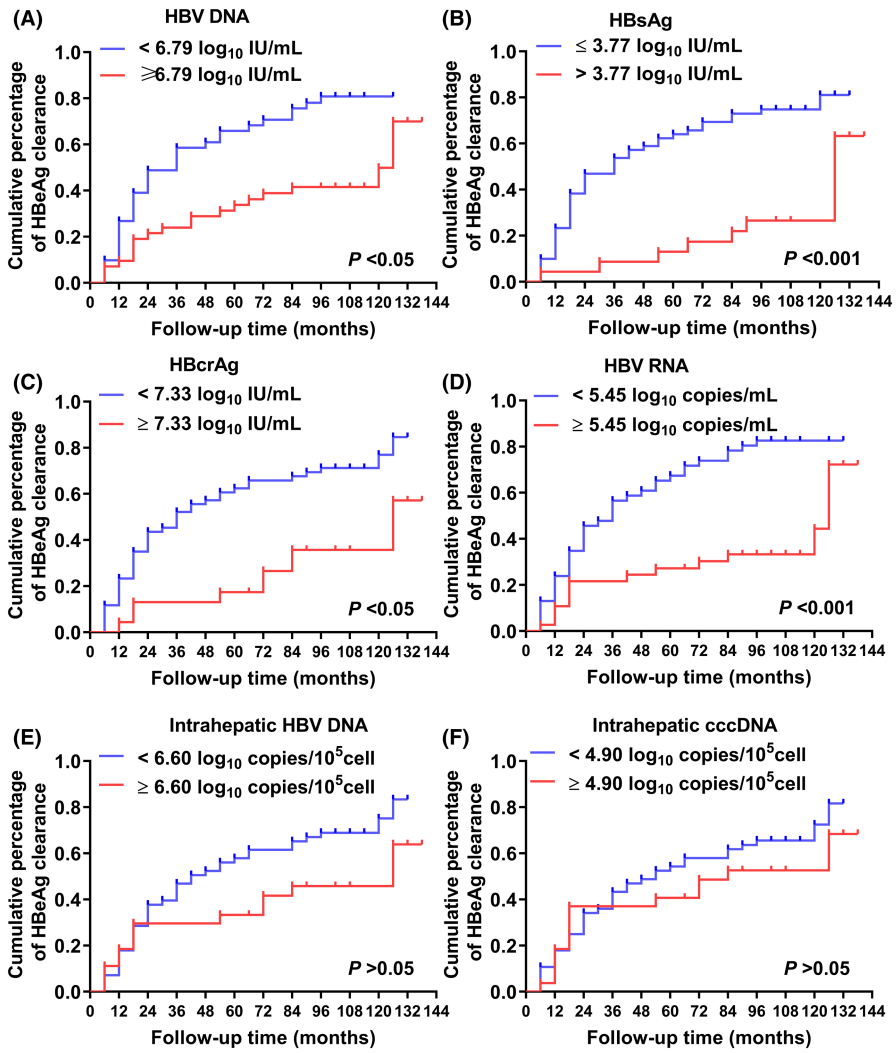

FIGURE 3 Kaplan–Meier survival analysis of the cumulative HBeAg clearance rate in different groups determined by HBV DNA (A), HBsAg (B), HBcrAg (C), HBV RNA (D), intrahepatic HBV DNA (E) and cccDNA (F) levels

Regarding HBeAg seroconversion, clinical characteristics of HBeAg seroconversion and non-seroconversion patients are shown in Table S2. The cumulative HBeAg seroconversion rate was gradually increased (Figure 4). Kaplan–Meier survival analysis showed that patients with lower baseline level of serum HBV DNA (Figure 4A), higher baseline level of HBsAg (Figure 4B), and lower baseline level of HBV RNA (Figure 4C) had a higher accumulative HBeAg seroconversion rate (all $p < .05$) Table 2, while patients with different levels of HBcrAg (Figure 4D), intrahepatic HBV DNA (Figure 4E) and cccDNA (Figure 4F) had no statistical differences in cumulative HBeAg seroconversion rate (all $p > .05$). Cox multivariate analysis showed that baseline HBV RNA <5.45 $\log_{10}$copies/mL was independently associated with HBeAg seroconversion (HR = 3.38, 95% CI: 1.28–8.91, $p = .01$), with median HBeAg seroconversion time of 66 months (95% CI: 28.78–103.21). Interestingly, the results revealed that in addition to the baseline, serum HBV RNA <4.72 $\log_{10}$ copies/mL at month 6 (HR = 6.52, 95% CI: 1.85–22.94, $p = .003$) and <4.08 $\log_{10}$ copies/mL at month 12 (HR = 2.79, 95% CI 1.31–5.94, $p = .008$) remained to be independently associated with HBeAg seroconversion.

The decline of HBV RNA during the first 6 months of treatment was significantly higher in HBeAg clearance patients than that in non-clearance patients; however, this value was comparable between HBeAg seroconversion and non-seroconversion (all $p > .05$). The declines of HBV RNA after 12 months treatment were not significantly different between HBeAg clearance/seroconversion and non-clearance/non-seroconversion (all $p > .05$) (Supplementary Materials and Table S3).

3.4 | Predicting potentials of baseline and early on-treatment viral markers quantification for HBeAg clearance and seroconversion

The timeAUC of baseline HBV RNA for predicting HBeAg clearance at month 36, 60 and 84 were 0.83 (95% CI: 0.70–0.96), 0.83 (95% CI: 0.70–0.96) and 0.82 (95% CI: 0.69–0.95), respectively, higher than those of HBsAg, HBV DNA and HBcrAg at the corresponding time points. The difference between timeAUCs of these indicators and HBV RNA was not significant (all $p > .05$). Similarly, timeAUCs of

TABLE 1 Cox regression analysis of clinical variables related to HBeAg clearance

Variables	Univariate analysis	
	HR (95% CI)	*p* Value[†]
Baseline characteristics		
Age, years <35	0.62 (0.36–1.08)	.088
Sex, Male	0.71 (0.37–1.40)	.323
NAs, ETV	0.69 (0.40–1.18)	.174
NAs naive	1.07 (0.62–1.84)	.816
BMI Kg/m^2 <24	1.02 (0.60–1.76)	.937
HBV genotype[†] genotype C	1.93 (0.45–8.38)	.379
ALT, U/L, <80	0.75 (0.43–1.30)	.304
AST, U/L, <80	0.60 (0.32–1.13)	.111
AST/ALT ratio, <1	0.77 (0.41–1.41)	.391
TBIL, μmol/L, <20	0.63 (0.34–1.16)	.141
ALP, U/L, <88	0.77 (0.45–1.34)	.357
HBsAg (log_{10} IU/mL) ≤3.77	5.24 (2.06–13.30)	**.001**
HBV DNA (log_{10} IU/mL) <6.79	2.51 (1.43–4.43)	**.001**
HBV RNA (log_{10} copies/mL) <5.45	3.42 (1.83–6.42)	**<.001**
HBcrAg (log_{10} U/mL) <7.33	3.49 (1.49–8.16)	**.004**
Intrahepatic HBV DNA (log_{10}copies/10^5 cell), <6.60	1.70 (0.83–3.46)	.144
cccDNA (log_{10}copies/10^5 cell), <4.9	1.22 (0.61–2.46)	.574
Inflammation scores[‡] ≤7	0.33 (0.16–0.68)	**.003**
Fibrosis scores[‡] ≤3	0.36 (0.18–0.72)	**.004**
At 6-month time point		
HBsAg (log_{10} IU/mL) ≤3.65	2.82 (1.21–6.57)	**.017**
HBV DNA (log_{10} IU/mL) <2.90	2.67 (1.51–4.74)	**.001**
HBV RNA (log_{10} copies/mL) <4.72	4.05 (2.13–7.70)	**<.001**
HBcrAg (log_{10} IU/mL) <6.64	5.43 (2.03–14.56)	**.001**
Decrease after 6 months treatment		
HBsAg (log_{10} IU/mL) ≤0.02	1.81 (0.83–3.93)	.133
HBV DNA (log_{10} IU/mL) <3.50	1.24 (0.72–2.15)	.434
HBV RNA (log_{10} copies/mL) ≤0.56	0.46 (0.25–0.85)	**.013**
HBcrAg (log_{10} IU/mL) ≤0.23	0.91 (0.42–1.97)	.812
At 12-month time point		
HBV DNA (log_{10} IU/mL) <2.07	2.64 (1.43–4.87)	**.002**
HBV RNA (log_{10} copies/mL) <4.08	3.17 (1.65–6.09)	**.001**
Decrease after 12 months treatment		
HBV DNA (log_{10} IU/mL) <3.92	1.17 (0.68–2.03)	.564
HBV RNA (log_{10} copies/mL) <0.63	0.66 (0.36–1.20)	.172

Abbreviations: ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; cccDNA, covalently closed circular DNA; ETV, Entecavir; HBcrAg, hepatitis B core-related antigen; HBeAg, hepatitis B e antigen; HBsAg, hepatitis B surface antigen; HBV RNA, hepatitis B virus ribonucleic acid; HBV, hepatitis B virus; TBIL, total bilirubin.

† Thirty-nine patients with available genotype data were analysed.

‡ Fifty-nine patients with available liver biopsy data were analysed. Hepatic inflammation grade and fibrosis stage were diagnosed according to the modified Knodell and Ishak scoring system respectively.

$p < .05$ were marked as bold values.

baseline HBV RNA for predicting HBeAg seroconversion at month 36, 60 and 84 were 0.89 (95% CI: 0.77–1.00), 0.81 (95% CI: 0.66–0.95) and 0.84 (95% CI: 0.71–0.98), respectively, higher than those of HBsAg, HBV DNA and HBcrAg. Again, the difference between timeAUCs of these indicators and HBV RNA was not significant (all $p > .05$) (Table 3).

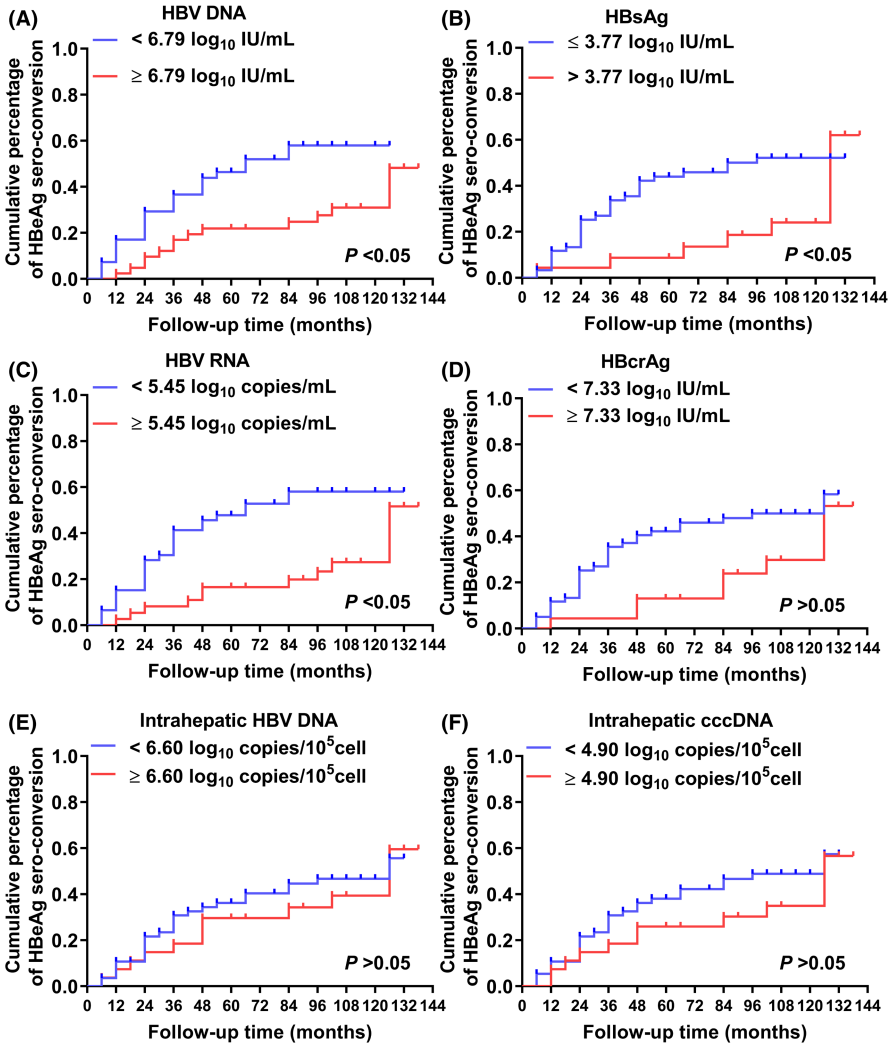

FIGURE 4 Kaplan–Meier survival analysis of the cumulative HBeAg seroconversion rate in different groups determined by HBV DNA (A), HBsAg (B), HBV RNA (C), HBcrAg (D), intrahepatic HBV DNA (E) and cccDNA (F) levels

The timeAUC of HBV RNA at month 6 and 12 for predicting HBeAg clearance and seroconversion at month 36, 60 and 84 are shown in Tables S4 and S5.

4 | DISCUSSION

To our knowledge, the present study was the first head-to-head comparison of these serum markers quantitation in predicting HBeAg clearance and seroconversion. The results showed that only serum HBV RNA levels were independently and negatively associated with HBeAg clearance and seroconversion at baseline, at month 6 and 12 respectively. Besides, the AUC of HBV RNA levels for HBeAg clearance and seroconversion was the highest at baseline among these viral markers.

According to our results, HBeAg clearance patients had significantly lower baseline HBsAg, HBV DNA, HBV RNA and HBcrAg than those in non-clearance patients. However, HBeAg seroconversion patients did not show significant lower baseline HBsAg and HBcrAg than those with non-seroconversion. The results of HBsAg in patients with HBeAg seroconversion seem consistent with previous studies that patients with HBeAg seroconversion have lower baseline HBsAg than patients without seroconversion.[9,11,20] We also noticed that patients with higher levels of baseline HBsAg had a higher cumulative HBeAg seroconversion rate. This discrepancy maybe due to the relative small sample size of patients. Our results of HBcrAg was in line with Wang's study that HBcrAg baseline level of patients who achieved HBeAg seroconversion was not significantly lower than patients with non-seroconversion.[19]

The predictive performance of HBV RNA for HBeAg clearance and seroconversion was better than that of HBsAg, HBV DNA or HBcrAg in our study. The underlying mechanism may be as follows; firstly, HBsAg is produced both from cccDNA and HBV DNA integrated into the host genome,[2] negatively affecting the prediction for HBeAg clearance and seroconversion. Secondly, circulating HBV DNA declined rapidly after NAs treatment. This characteristic restricted its use for the prediction of HBeAg clearance and seroconversion after long-term NAs treatment. Thirdly, due to LAM treatment, the proportion of drug-resistant mutants and viral quasispecies were not detected, especially the precore/core sequences, which may influence HBeAg seroconversion as reported previously[25] and may affect the predictive performance of HBcrAg for HBeAg

TABLE 2 Cox regression analysis of clinical variables related to HBeAg seroconversion

Variables	Univariate analysis	
	HR (95% CI)	*p* Value[†]
Baseline characteristics		
Age, years <35	0.73 (0.38–1.40)	.340
Sex, Male	0.56 (0.26–1.20)	.136
NAs, ETV	0.70 (0.36–1.33)	.272
NAs naive	0.89 (0.47–1.71)	.733
BMI Kg/m^2 <24	0.68 (0.36–1.31)	.254
HBV genotype[†] genotype C	0.98 (0.22–4.38)	.974
ALT, U/L, <80	0.85 (0.44–1.65)	.633
AST, U/L, <80	0.65 (0.31–1.38)	.261
AST/ALT ratio, <1	0.78 (0.38–1.61)	.497
TBIL, μmol/L, <20	0.65 (0.32–1.35)	.251
ALP, U/L, <88	1.12 (0.59–2.16)	.726
HBsAg (log_{10} IU/mL) ≤3.77	2.68 (1.05–6.85)	**.039**
HBV DNA (log_{10} IU/mL) <6.79	2.31 (1.17–4.55)	**.016**
HBV RNA (log_{10} copies/mL) <5.45	2.62 (1.25–5.47)	**.010**
HBcrAg (log_{10} U/mL) <7.33	2.18 (0.84–5.65)	.110
Intrahepatic HBV DNA (log_{10}copies/10^5 cell), <6.60	1.31 (0.59–2.92)	.514
cccDNA (log_{10}copies/10^5 cell), <4.9	1.67 (0.74–3.76)	.220
Inflammation scores[‡] ≤7	0.40 (0.18–0.90)	**.026**
Fibrosis scores[‡] ≤3	0.32 (0.14–0.73)	**.007**
At 6-month time point		
HBsAg(log_{10} IU/mL) ≤3.65	1.63 (0.66–4.01)	.287
HBV DNA (log_{10} IU/mL) <2.90	2.13 (1.08–4.22)	**.029**
HBV RNA (log_{10} copies/mL) <4.72	3.69 (1.70–8.02)	**.001**
HBcrAg (log_{10} IU/mL) <6.64	4.81 (1.39–16.68)	**.013**
Decrease after 6 months treatment		
HBsAg (log_{10} IU/mL) ≤0.02	1.43 (0.59–3.47)	.426
HBV DNA (log_{10} IU/mL) <3.50	1.24 (0.64–2.39)	.519
HBV RNA (log_{10} copies/mL) ≤0.56	0.54 (0.26–1.12)	.097
HBcrAg (log_{10} IU/mL) ≤0.23	0.56 (0.52–3.38)	.556
At 12-month time point		
HBV DNA (log_{10} IU/mL) <2.07	2.06 (1.05–4.08)	**.037**
HBV RNA (log_{10} copies/mL) <4.08	2.79 (1.31–5.94)	**.008**
Decrease after 12 months treatment		
HBV DNA (log_{10} IU/mL) <3.92	1.19 (0.62–2.29)	.609
HBV RNA (log_{10} copies/mL) <0.63	0.70 (0.34–1.45)	.332

Abbreviations: ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; cccDNA, covalently closed circular DNA; ETV, Entecavir; HBcrAg, hepatitis B core-related antigen; HBeAg, hepatitis B e antigen; HBsAg, hepatitis B surface antigen; HBV RNA, hepatitis B virus ribonucleic acid; HBV, hepatitis B virus; TBIL, total bilirubin.

† Thirty-nine patients with available genotype data were analysed.

‡ Fifty-nine patients with available liver biopsy data were analysed. Hepatic inflammation grade and fibrosis stage were diagnosed according to the modified Knodell and Ishak scoring system respectively.

p< .05 were marked as bold values.

clearance and seroconversion. Last but not least, anti-HBe antibodies are cross-reactive with HBcAg due to the amino acid sequence homology,[26] and p22cr (also detected as a part of HBcrAg) was found in 'empty' HBV DNA-negative Dane particles.[27] These factors may result in high interference against accurate measurement of the level of HBcrAg. All these factors may hinder the use of these markers for predicting HBeAg clearance and seroconversion. However, further research is needed to clarify the underlying mechanism.

TABLE 3 Time-dependent area under the curve analysis of baseline indicators for HBeAg clearance and seroconversion at month 36, 60 and 84

Indicators	Predict time point	AUC (95% CI)	Cut-off value	Se	Sp	PPV	NPV
HBeAg clearance							
HBsAg	36 m	0.77 (0.62–0.92)	3.99	1.00	0.54	0.56	1.00
	60 m	0.74 (0.58–0.91)	3.99	0.94	0.55	0.60	0.92
	84 m	0.81 (0.65–0.98)	3.99	0.95	0.63	0.75	0.91
HBV DNA	36 m	0.71 (0.54–0.79)	7.50	0.93	0.58	0.57	0.93
	60 m	0.66 (0.60–0.83)	7.50	0.88	0.55	0.58	0.86
	84 m	0.67 (0.60–0.84)	7.50	0.80	0.56	0.69	0.70
HBV RNA	36 m	0.83 (0.70–0.96)	5.43	0.86	0.63	0.57	0.88
	60 m	0.83 (0.70–0.96)	3.59	0.50	0.95	0.89	0.72
	84 m	0.82 (0.69–0.95)	5.47	0.80	0.63	0.72	0.72
HBcrAg	36 m	0.82 (0.67–0.96)	7.09	0.80	0.72	0.60	0.88
	60 m	0.78 (0.62–0.95)	6.39	0.53	0.93	0.82	0.76
	84 m	0.78 (0.63–0.93)	6.39	0.52	1.00	1.00	0.67
Combination	36 m	0.85 (0.73–0.97)	1.69	0.64	0.88	0.75	0.81
	60 m	0.83 (0.69–0.97)	1.69	0.63	0.91	0.83	0.77
	84 m	0.82 (0.68–0.96)	1.69	0.60	0.94	0.92	0.66
HBeAg seroconversion							
HBsAg	36 m	0.77 (0.61–0.93)	3.99	1.00	0.43	0.29	1.00
	60 m	0.78 (0.63–0.93)	3.59	0.82	0.71	0.52	0.91
	84 m	0.75 (0.57–0.92)	3.99	1.00	0.53	0.55	1.00
HBV DNA	36 m	0.72 (0.54–0.90)	7.50	1.00	0.50	0.31	1.00
	60 m	0.70 (0.53–0.88)	7.50	0.91	0.50	0.42	0.93
	84 m	0.75 (0.59–0.92)	7.50	0.93	0.53	0.53	0.92
HBV RNA	36 m	0.89 (0.77–1.00)	4.70	0.86	0.73	0.43	0.96
	60 m	0.81 (0.66–0.95)	5.43	0.91	0.58	0.46	0.94
	84 m	0.84 (0.71–0.98)	5.47	0.93	0.58	0.56	0.93
HBcrAg	36 m	0.80 (0.57–1.02)	7.09	0.86	0.63	0.35	0.95
	60 m	0.74 (0.55–0.92)	5.69	0.45	0.92	0.68	0.81
	84 m	0.78 (0.61–0.94)	6.00	0.55	0.89	0.75	0.78
Combination	36 m	0.86 (0.74–0.99)	1.17	0.86	0.70	0.40	0.95
	60 m	0.79 (0.64–0.94)	1.09	0.82	0.71	0.52	0.91
	84 m	0.84 (0.70–0.98)	0.82	0.93	0.63	0.59	0.94

Abbreviations: AUC, area under curve; CI, confidence interval; HBcrAg, hepatitis B core-related antigen; HBeAg, hepatitis B e antigen; HBsAg, hepatitis B surface antigen; HBV RNA, hepatitis B virus ribonucleic acid; HBV, hepatitis B virus; m, months; NPV, negative predictive value; PPV, positive predictive value; ROC, receiver operating characteristic curve; Se, sensitivity; Sp, specificity.

Rapid decrease of HBV RNA in the early stages of NAs treatment are likely associated with HBeAg clearance and seroconversion.[20] Whether or not the differences were significant, our results showed that patients who achieved HBeAg clearance or seroconversion have higher HBV RNA decline after 6 or 12 months treatment than those with non-clearance or non-seroconversion, which seems consistent with Wang's study.[20] Moreover, we also found that the timeAUCs of combination models based on HBV RNA, HBsAg, HBV DNA or HBcrAg did not increase significance compared with HBV RNA alone. However, in a previous study,[19] a combination of HBsAg and HBcrAg had the greatest predictive value for HBeAg seroconversion, with an AUC of 0.769 at month 6, 0.807 at month 12. The discrepancy maybe due to differences in the combination of variables, the genotype make-up of patients, and HBV RNA which were not included in that study. Thirdly, from the viewpoint of HBV RNA, although the differences were not significant, the timeAUCs tended to numerically increase from baseline to month 6 for the prediction of HBeAg clearance and seroconversion, which was still consistent with Wang's study.[20] Due to the deficiency of HBsAg and HBcrAg quantification at month 12, whether the predictive value of

serum HBV RNA is still numerically higher than HBsAg and HBcrAg at month 12 for the prediction of HBeAg clearance and seroconversion warrants additional study.

Our study has limitations. Firstly, the research is a retrospective, single centre study with relatively small sample size; and the genotype of most patients was B or C. Thus, the results of this study should be carefully extrapolated to patients with genotype A and D and with non-Chinese ethnicity. Secondly, the increased number of lost to follow-up patients after 96 months might underestimate the cumulative HBeAg clearance or seroconversion rate. Future studies with larger sample size and a prospective design are needed to confirm our conclusion.

In conclusion, lower serum HBV RNA at baseline and early on-treatment predict HBeAg clearance and seroconversion during long-term NAs treatment. This result deepens our knowledge and understanding of clinical significance of HBV RNA in HBeAg-positive patients receiving long-term NAs treatment.

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CONFLICT OF INTEREST

The authors declare no conflicts of interests related to this article.

AUTHOR CONTRIBUTIONS

Yang Wang, Hao Liao and Zhongping Deng equally contributed to the visualization, methodology, formal analysis, drafting and editing of the manuscript. Yanna Liu, Dandan Bian, Yan Ren, Guangxin Yu, Yingying Jiang and Li Bai contributed to data curation and acquisition, review and editing of the manuscript. Shuang Liu, Mei Liu, Li Zhou, Yu Chen and Zhongping Duan contributed to project administration, resources, review and editing of the manuscript. Sujun Zheng and Fengmin Lu contributed to conceptualization, funding acquisition, study design, experiment supervision and critical revision of the manuscript. All authors reviewed and approved the final version of manuscript to be published.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ORCID

Yingying Jiang https://orcid.org/0000-0002-3688-1052
Fengmin Lu https://orcid.org/0000-0002-1832-3209
Sujun Zheng https://orcid.org/0000-0002-6367-5764

REFERENCES

1. Polaris Observatory C. Global prevalence, treatment, and prevention of hepatitis B virus infection in 2016: a modelling study. *Lancet Gastroenterol Hepatol*. 2018;3:383-403.
2. Lu F, Wang J, Chen X, Xu D, Xia N. Potential use of serum HBV RNA in antiviral therapy for chronic hepatitis B in the era of nucleos(t)ide analogs. *Front Med*. 2017;11:502-508.
3. Liao H, Liu Y, Li X, et al. Monitoring of serum HBV RNA, HBcrAg, HBsAg and anti-HBc levels in patients during long-term nucleoside/nucleotide analogue therapy. *Antivir Ther*. 2019;24:105-115.
4. Wang J, Du M, Huang H, et al. Reply to: "Serum HBV pgRNA as a clinical marker for cccDNA activity": Consistent loss of serum HBV RNA might predict the "para-functional cure" of chronic hepatitis B. *J Hepatol*. 2017;66:462-463.
5. Gish RG, Chang TT, Lai CL, et al. Quantitative hepatitis B surface antigen analysis in hepatitis B e antigen-positive nucleoside-naive patients treated with entecavir. *Antivir Ther*. 2013;18:691-698.
6. Zoulim F, Carosi G, Greenbloom S, et al. Quantification of HBsAg in nucleos(t)ide-naive patients treated for chronic hepatitis B with entecavir with or without tenofovir in the BE-LOW study. *J Hepatol*. 2015;62:56-63.
7. Huang YJ, Chang CS, Peng YC, Yeh HZ, Yang SS. On-treatment HBV DNA level could predict HBeAg seroclearance in patients with HBeAg-positive chronic hepatitis B with entecavir therapy. *J Chin Med Assoc*. 2017;80:341-346.
8. Peng CY, Hsieh TC, Hsieh TY, et al. HBV-DNA level at 6 months of entecavir treatment predicts HBeAg loss in HBeAg-positive chronic hepatitis B patients. *J Formosan Med Assoc*. 2015;114:308-313.
9. Lee MH, Lee DM, Kim SS, Cheong JY, Cho SW. Correlation of serum hepatitis B surface antigen level with response to entecavir in naive patients with chronic hepatitis B. *J Med Virol*. 2011;83:1178-1186.
10. Fung J, Lai CL, Young J, et al. Quantitative hepatitis B surface antigen levels in patients with chronic hepatitis B after 2 years of entecavir treatment. *Am J Gastroenterol*. 2011;106:1766-1773.
11. Shin JW, Jung SW, Park BR, et al. Prediction of response to entecavir therapy in patients with HBeAg-positive chronic hepatitis B based on on-treatment HBsAg, HBeAg and HBV DNA levels. *J Viral Hepatitis*. 2012;19:724-731.
12. Wang J, Shen T, Huang X, et al. Serum hepatitis B virus RNA is encapsidated pregenome RNA that may be associated with persistence of viral infection and rebound. *J Hepatol*. 2016;65:700-710.
13. Wang J, Yu Y, Li G, et al. Relationship between serum HBV-RNA levels and intrahepatic viral as well as histologic activity markers in entecavir-treated patients. *J Hepatol*. 2017;S0168–8278(17):32261-32264.
14. Luo H, Tan N, Kang Q, et al. Hepatitis B virus pregenomic RNA status can reveal the long-term prognoses of chronic hepatitis B patients treated with nucleos(t)ide analogues. *J Viral Hepatitis*. 2020;27:323-328.
15. Luo H, Zhang XX, Cao LH, et al. Serum hepatitis B virus RNA is a predictor of HBeAg seroconversion and virological response with entecavir treatment in chronic hepatitis B patients. *World J Gastroenterol*. 2019;25:719-728.
16. Mak LY, Wong DK, Cheung KS, Seto WK, Lai CL, Yuen MF. Review article: hepatitis B core-related antigen (HBcrAg): an emerging marker for chronic hepatitis B virus infection. *Aliment Pharmacol Ther*. 2018;47:43-54.
17. Chen EQ, Wang ML, Tao YC, et al. Serum HBcrAg is better than HBV RNA and HBsAg in reflecting intrahepatic covalently closed circular DNA. *J Viral Hepatitis*. 2019;26:586-595.
18. Wang ML, Chen EQ, Tao CM, Tang H. Letter: serum HBcrAg is a useful marker for disease monitoring, predicting treatment response and disease outcome of chronic hepatitis B virus infection. *Aliment Pharmacol Ther*. 2018;47:1719-1720.
19. Wang B, Carey I, Bruce M, Montague S, Dusheiko G, Agarwal K. HBsAg and HBcrAg as predictors of HBeAg seroconversion in HBeAg-positive patients treated with nucleos(t)ide analogues. *J Viral Hepatitis*. 2018;25:886-893.
20. Wang X, Wang Z, Chi X, et al. Efficacy of a combination of HBV RNA and HBeAg in predicting HBeAg seroconversion in patients treated with entecavir for 144 weeks. *Int J Infect Dis*. 2020;99:171-178.

21. Lok AS, McMahon BJ. Chronic hepatitis B. *Hepatology*. 2007;45:507-539.
22. Goodman ZD. Grading and staging systems for inflammation and fibrosis in chronic liver diseases. *J Hepatol*. 2007;47:598-607.
23. Huang H, Wang J, Li W, et al. Serum HBV DNA plus RNA shows superiority in reflecting the activity of intrahepatic cccDNA in treatment-naive HBV-infected individuals. *J Clin Virol*. 2018;99–100:71-78.
24. Butler EK, Gersch J, McNamara A, et al. Hepatitis B virus serum DNA andRNA levels in nucleos(t)ide analog-treated or untreated patients during chronic and acute infection. *Hepatology*. 2018;68:2106-2117.
25. Sonneveld MJ, Rijckborst V, Zeuzem S, et al. Presence of precore and core promoter mutants limits the probability of response to peginterferon in hepatitis B e antigen-positive chronic hepatitis B. *Hepatology*. 2012;56:67-75.
26. Wang SJ, Chen ZM, Wei M, et al. Specific determination of hepatitis B e antigen by antibodies targeting precore unique epitope facilitates clinical diagnosis and drug evaluation against hepatitis B virus infection. *Emerg Microbes Infect*. 2021;10:37-50.
27. Kimura T, Ohno N, Terada N, et al. Hepatitis B virus DNA-negative dane particles lack core protein but contain a 22-kDa precore protein without C-terminal arginine-rich domain. *J Biol Chem*. 2005;280:21713-21719.

SUPPORTING INFORMATION

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