

# Serum HBV RNA Dynamic and Drug Withdrawal Predictor Value in Patients With Chronic HBV Infection on Long-term Nucleos(t)ide Analogue (NA) Therapy

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**Aims:** This study aimed to investigate the dynamic pattern of serum hepatitis B virus (HBV) RNA in chronic hepatitis B (CHB) patients on long-term nucleos(t)ide analogue (NA) therapy and evaluate predictor value of end-of-treatment (EOT) serum HBV RNA status on drug-withdrawal durability.

**Methods:** We carried out a real-life cohort study of 326 CHB patients on NA treatment between February 12, 2016 and February 21, 2018. Thirty of these patients discontinued NA treatment after enrollment, and were included in 2-year off-therapy follow-up. Serum HBV RNA levels were determined using the RNA simultaneous amplification testing method.

**Results:** Both serum HBV RNA and DNA levels declined significantly in long-term antiviral progress. When the treatment duration was longer than 3 years, the undetectable rates of HBV RNA and DNA were 55.10% and 97.0%, respectively. The serum HBV RNA-negative rate was 39.5%. The cumulative 2-year off-therapy viral and clinical relapse rate was 40.56%; 95% confidence interval (95% CI), 21.51-59.61 and 31.31%; 95% CI, 11.32-51.29 in all patients, respectively. Patients with EOT hepatitis B surface

antigen (HBsAg)  $\leq$  1000 IU/mL plus HBV RNA negativity had a relatively lower cumulative 2-year off-therapy viral relapse rate (23.01%; 95% CI, 0.17-45.99). EOT HBsAg  $\leq$  1000 IU/mL plus HBV RNA negativity showed obvious superiority for the EOT HBsAg  $\leq$  1000 IU/mL single in drug withdrawal durability prediction, with better specificity (18.18% vs. 72.73%,  $P=0.03$ ), and the positive predictive value and negative predictive value were 76.92% and 47.06%, respectively.

**Conclusions:** In the long-term antiviral process, both serum HBV RNA and DNA levels declined significantly. EOT serum HBV RNA negativity was not an independent drug withdrawal marker, but can complement the HBsAg titer to monitor drug withdrawal in CHB patients on long-term NA therapy.

**Key Words:** pregenome RNA, prediction, antiviral therapy, cessation  
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## BACKGROUND

As one of the first-line antiviral therapy strategies for chronic hepatitis B (CHB), nucleos(t)ide analogue (NA) can both efficiently inhibit hepatitis B virus (HBV) replication in almost all patients and ameliorate the development of liver fibrosis.<sup>1,2</sup> However, NA therapy rarely leads to a functional cure (ie, HBsAg loss) because of the limited effect of NA on covalently closed circular DNA (cccDNA), which is believed to be the main cause of viral persistence.<sup>3</sup> Accordingly, cessation of NA is often accompanied by various incidences of viral and clinical relapse.<sup>4-6</sup> Although the intrahepatic cccDNA level at the end of treatment (EOT) could be a strong predictor of sustained response to antiviral therapy in CHB patients, liver biopsy and lack of standard test progress have limited its use in clinical practice. Research to find surrogate markers to predict a sustained viral response is ongoing.

As an alternative index of HBV cccDNA transcriptional activity, serum hepatitis B surface antigen (HBsAg) has been advocated as a novel serum marker for monitoring treatment response in CHB patients, and clearance of HBsAg is deemed the ultimate goal of CHB antiviral therapy. All the main international guidelines for CHB infection recommend HBsAg loss, with or without antibodies to HBsAg (anti-HBs), as an optimal endpoint of NA treatment in patients with CHB, especially in patients who are hepatitis B e antigen (HBeAg) negativity.<sup>1,2,7</sup> In a recent meta-analysis,<sup>8</sup> an EOT HBsAg level  $<$  100 IU/mL was suggested as the optimal cut-off value for the cessation of NA treatment both HBeAg-positive and HBeAg-negative CHB patients.

Serum HBV RNA, which is transcribed from cccDNA, was also found to be correlated with the presence of cccDNA and associated with the response to NA.<sup>9-12</sup> Therefore, HBV RNA may be another clinical surrogate marker for intrahepatic cccDNA levels after long-term NA treatment and may be used

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Y.L.: recruited the patients and collected specimens, and carried out the data analysis. J.X.: enrolled the patients and followed the patients who discontinued antiviral therapy. W.L.: collected the specimens and edited the clinical data of the enrolled patients. H.Y.: participated in the design of the study and revision of the manuscript. X.L.: conceived the study and participated in the design of the study; carried out the data analysis; and drafted the manuscript.

The authors declare that they have nothing to disclose.

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to monitor NA therapy. However, a recent study<sup>13</sup> showed that although the serum HBV RNA level at baseline or its decline after 96 weeks of NA treatment correlated with the corresponding intrahepatic cccDNA level, the reduction was less than that observed with serum HBV DNA at baseline and HBsAg (or its decline) at 96 weeks after treatment. Furthermore, at 96 weeks after NA treatment, the correlation between the intrahepatic cccDNA and serum HBV RNA level disappeared. Therefore, further studies are needed to investigate the relationship between HBV RNA and intrahepatic cccDNA in patients with long-term NA treatment, and the value of serum HBV RNA levels in NA treatment management should also be further evaluated. Thus far, the status of serum HBV RNA during long-term NA therapy remains unclear, especially when the treatment duration is longer than 3 years. In the present study, serum HBV RNA levels at different time points in long-term NA-treated CHB patients were quantified. The correlation between serum HBV RNA levels in patients undergoing long-term NA treatment and the corresponding serum HBV DNA level, HBsAg status, and HBeAg status was analyzed. The predictive value of EOT serum HBV RNA level on viral or clinical relapse in CHB patients who discontinued NA therapy was evaluated and the supplementary value of HBV RNA to existing drug withdrawal predictor was also evaluated.

## METHODS

A real-life cohort study of 326 CHB patients on long-term NA treatment was carried out in Shanghai Changhai hospital between February 12, 2016 and February 21, 2018. Behavior of the serum HBV RNA during long-term NA treatment was determined in 326 CHB patients undergoing long-term NA therapy before enrollment and the predictive value of EOT HBV RNA on safe drug withdrawal was evaluated in 30 CHB patients who discontinued the NA therapy after enrollment.

### Patients

The inclusion criteria were as follows: age 18 to 70 years, no gender restriction, serum HBsAg positivity for longer than 6 months, undergoing NA treatment for at least 6 months, ability to understand and sign informed consent, and good treatment compliance.

The exclusion criteria were as follows: coinfection with other hepatotropic viruses, such as hepatitis C virus, hepatitis D virus, hepatitis E, and hepatitis A, coinfection with HIV, positive for markers such as ceruloplasmin, antinuclear antibodies, and antimitochondrial antibodies for coexistent autoimmune and metabolic liver diseases, with hepatocellular carcinoma, with uncontrollable extrahepatic disease, had received glucocorticoid or other immune inhibitor therapy, and pregnancy.

The cessation criteria for NA treatment were as follows: NA treatment duration more than 3 years or less than 3 years, but with HBsAg titer <100 IU/mL for more than 12 months for HBeAg-negative CHB patients, undetectable serum HBV DNA level, and alanine aminotransferase (ALT) normalization on at least 3 occasions 6 months apart, HBeAg seroconversion and consolidated therapy for more than 12 months for HBeAg-positive CHB patients, no liver cirrhosis, and good treatment compliance.

### Follow-up of Patients Who Discontinued NA Therapy

After NA discontinuation, all patients were followed up with liver function tests and all viral indexes, including

HBsAg, HBeAg, anti-HBe, and serum HBV DNA. Follow-up was performed once a month in the first 3 months and subsequently every 3 months until 24 months after cessation of therapy or until clinical relapse.

### Relapse Definition

Viral relapse was defined as a serum HBV DNA level reappearance at >2000 IU/mL from undetectable levels at 2 consecutive visits during off-treatment follow-up. Viral relapse with an increase in ALT levels to >2× upper limit of normal was defined as a clinical relapse.

All patient demographics, liver biochemistry, quantitative HBsAg, and HBeAg status were collected and determined. Serum HBV DNA levels were obtained when serum samples were collected.

This study was carried out according to the guidelines of the Declaration of Helsinki and was approved by the Shanghai Changhai Hospital ethics research committee (CHEC2019-056). Written informed consent was obtained from each patient. The study was registered on the ClinicalTrials.gov (NCT03909191).

### HBV RNA Quantification

The HBV RNA was detected by the RNA simultaneous amplification testing method (HBV-SAT) using the HBV-SAT kit (Rendu Biotechnology, Shanghai, China) as previously described.<sup>14</sup> Primers and probes were designed to amplify an HBV pregenome RNA conserved region.<sup>15</sup> RNA extraction, amplification, and detection were processed on an automated AutoSAT system (Rendu Biotechnology). Calibration of the HBV RNA assay was performed using an armored HBV RNA standard traceable to HBV RNA standard in vitro transcripts. The minimum detection limit (MDL) was 100 copies/mL.

The serum HBV RNA test results were classified as (1) “target not detected”; (2) “<100 copies/mL”; and (3) “a numeric titer of >100 copies/mL.” The negative HBV RNA was strictly defined as HBV RNA “target not detected.” The positive HBV RNA was defined as HBV DNA <100 copies/mL or a numeric titer of >100 copies/mL.

### Serum HBsAg Quantification

Serum HBsAg levels were measured using commercially available kits (Abbott Laboratories, North Chicago, IL) in our clinical lab. The dynamic range was 0.05 to 250 IU/mL. The samples were diluted 1:500 or 1:1000 using the ARCHITECT HBsAg Manual Diluent (Abbott Diagnostics) if >250 IU/mL.

### HBV DNA Measurement

Serum HBV DNA was quantified using the Determination Kit for hepatitis B viral DNA (Sansure Biotech, China), with a detection range of  $1 \times 10^2$  to  $5 \times 10^8$  IU/mL.

### Statistical Analysis

All non-normal distribution data were presented as the median and interquartile range and normal distribution data were presented as mean  $\pm$  SD. The variables were compared between groups using the Mann-Whitney *U* and Fisher exact tests for univariate comparisons and the Kruskal-Wallis test for multivariate comparisons. The regression and Spearman correlation coefficients (*r*) were used to compare the correlation between 2 variables. Cumulative incidences of the outcomes were estimated and plotted by the Kaplan-Meier method with right censoring. Cox regression models

were used to identify predictors of off-therapy relapse. A *P*-value (2-tailed) of 0.05 was considered statistically significant. All statistical analyses were carried out using SPSS software (version 21.0.0; Chicago, IL).

## RESULTS

### Patient Characteristics

A total of 350 CHB patients on NA treatment were recruited for the present study from February to November 2016 in Shanghai Changhai Hospital. Finally, 326 CHB patients (234 males and 92 females) with a median of 36 months of NA treatment duration were enrolled, and of these, 48 were included in the cessation of NA treatment follow-up group. Ultimately, 30 patients completed at least on follow-up test and evaluation and were finally included in statistical analysis. One hundred twenty-four of the 326 cases enrolled were confirmed to have compensated liver cirrhosis by abdominal B ultrasound or computed tomography examination; of these, 100 cases were HBeAg negative. All of the patients at enrollment had received NA therapy for at least 6 months, and the majority of them (84.66%) had received first-line NA (ETV or TDF) treatment. In addition, 54 cases had received interferon treatment in the early stage of treatment and all of them had switched to NA for at least 6 months until enrollment.

At enrollment, serum HBV RNA and HBV DNA below the MDL rate in total patients was 43.86% (143/326) and 88.65% (289/326), respectively. Patients with HBeAg positivity had higher serum HBV DNA and RNA levels compared with patients with HBeAg negativity (*P*-values all <0.05). All patients undergoing antiviral therapy at the time of enrollment achieved good ALT reduction (Table 1).

### Changes in Viral and Biochemical Indicators During Long-term NA Treatment

As shown in Figure 1, in the NA treatment progress, both serum HBV RNA and DNA levels decreased significantly. When the treatment duration was longer than 3 years, the below MDL rates of serum HBV RNA and DNA were 55.10% and 97.00%, respectively (Figs. 1A, B).

The total concordance rate of the HBV RNA and DNA test results was 55.69% (93/167) in patients on NA treatment for more than 3 years. Furthermore, 39.5% (66/162) of patients on NA treatment for more than 3 years had a negative serum HBV RNA test when serum HBV DNA test results were lower than MDL irrespective of baseline HBV DNA levels, and 74.2% (49/66) of them were HBeAg negative and 62.12% (41/66) of them were HBsAg <100 IU/mL. During long-term NA therapy, almost all patients achieved on-treatment serum HBV DNA and RNA reductions at various treatment durations, but the corresponding HBsAg titer of these patients sustained high levels (Fig. 1C). When the treatment duration was longer than 3 years, the median HBsAg titer was 2.64 (1.89 to 3.18) log<sub>10</sub> IU/mL and only 28.14% (47/167) of patients achieved HBsAg titer <100 IU/mL. Patients undergoing NA treatment also achieved a good reduction in the biochemical marker levels, and the ALT normalization rate was more than 90% at all time points. For patients who had been treated for more than 3 years, even if they were based on AASLD's strict ALT normal criteria (30 for men and 19 for women), the ALT normalization rate was still 75% (Fig. 1D).

### Correlation Between Serum HBV RNA and HBV DNA or HBsAg in Patients on NA Treatment

Overall, during NA treatment serum HBV RNA weakly correlated with HBV DNA and HBsAg titer in total patients irrespective of treatment time and pretreatment HBeAg status (*r*=0.301 and 0.377, respectively, Figs. 2A, B). In addition, further stratifying analysis by HBeAg status, we found that during NA treatment, when HBeAg was negative and serum HBV RNA level had a very weak correlation with both serum HBV DNA level and HBsAg titer (*r*=0.157 and 0.123, respectively, Figs. 2C, D), but when HBeAg was positive, the serum HBV RNA level had a medium correlation with both serum HBV DNA and HBsAg levels (*r*=0.431 and 0.438, respectively, Figs. 2E, F).

Further stratification of analysis of the correlation between serum HBV RNA and HBsAg or HBV DNA level by NA treatment duration showed that the correlation between serum HBV RNA and serum HBV DNA and

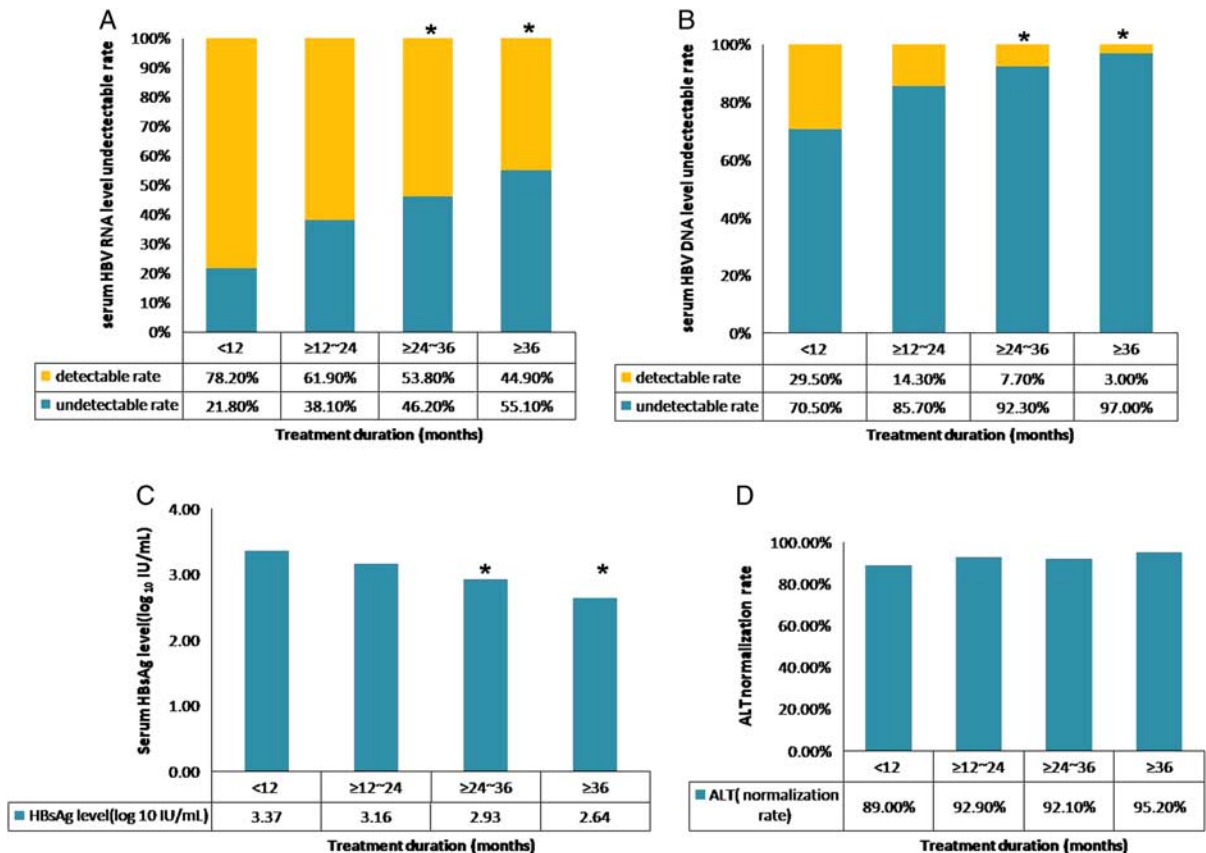
TABLE 1. Patients' Characteristics at Enrollment, by HBeAg Status

Characteristics	Total (n = 326)	HBeAg Positive (n = 125)	HBeAg Negative (n = 201)
Sex (M:F)	234:92	80:45	154:47
Age (y)	44 (35-53)	38 (31-44.5)	49 (40-58)
Cirrhosis, n (%)	124 (38.04)	24 (19.20)	100 (49.75)
HBsAg (IU/mL)	1032.35 (175-2564.44)	2561.95 (1077.10-4833.43)	456.01 (93.56-1325.21)
HBeAg (S/CO)	17.63 (3.06-102.94)	17.63 (3.06-102.94)	—
HBV RNA log <sub>10</sub> copies/mL	3.09 (2.30-4.14)	4.01 (3.07-4.88)	2.73 (2.04-3.33)
HBV RNA negative, n (%)	100 (30.67)	27 (21.6)	73 (36.32)
HBV DNA (log <sub>10</sub> IU/mL)	2.0 (2.0-2.0)	2.0 (2.0-2.0)	2.0 (2.0-2.0)
ALT (U/L)	25 (17-36.5)	28 (18-39.25)	24 (16-34)
AST (U/L)	23 (18-28)	23 (19-29)	22 (18-27)
Therapy strategy			
First-line NA, n (%)	237 (72.70)	109 (87.20)	128 (63.68)
Non-first-line NA, n (%)	89 (27.30)	16 (12.80)	73 (36.32)
IFN experience, n (%)*	54 (16.56)	35 (28.00)	19 (9.45)
NA treatment duration (mo)	36 (12-108)	20 (8.75-36)	56 (19.5-120)

Continuity data were shown as median (interquartile range).

\*IFN treatment experience means that during antiviral treatment, patients had received interferon treatment for 3 to 6 months, but had been switched to NA treatment for at least 6 months at the time of enrollment.

ALT indicates alanine aminotransferase; AST, aspartate aminotransferase; HBeAg, hepatitis B e antigen; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; NA, nucleos(t)ide analogue.



**FIGURE 1.** Clinical characteristics of chronic hepatitis B patients on nucleos(t)ide analogue (NA) treatment. A, The rate of serum hepatitis B virus (HBV) RNA below detection limit at different treatment time points. B, The rate of serum HBV DNA below detection limit at different treatment time points. C, Serum hepatitis B surface antigen (HBsAg) level at different treatment time points. D, Alanine aminotransferase (ALT) normalization rate at different treatment time points. \*Compared with the rate of patients on NA treatment shorter than 12 months;  $P < 0.015$  was considered statistically significant.

HBsAg remained in the early period of treatment. However, when the treatment duration exceeded 36 months, the relationship between serum HBV RNA and HBV DNA and HBsAg titer disappeared in all CHB patients and patients with HBeAg negativity (Spearman  $r = 0.101$  and  $0.032$  vs.  $r = 0.126$  and  $-0.065$ , respectively,  $P$ -value all  $> 0.05$ ) (Table S1, Supplemental Digital Content 1, <http://links.lww.com/JCG/A572>).

### Characteristics of Patients Who Discontinued Long-term Antiviral Treatment

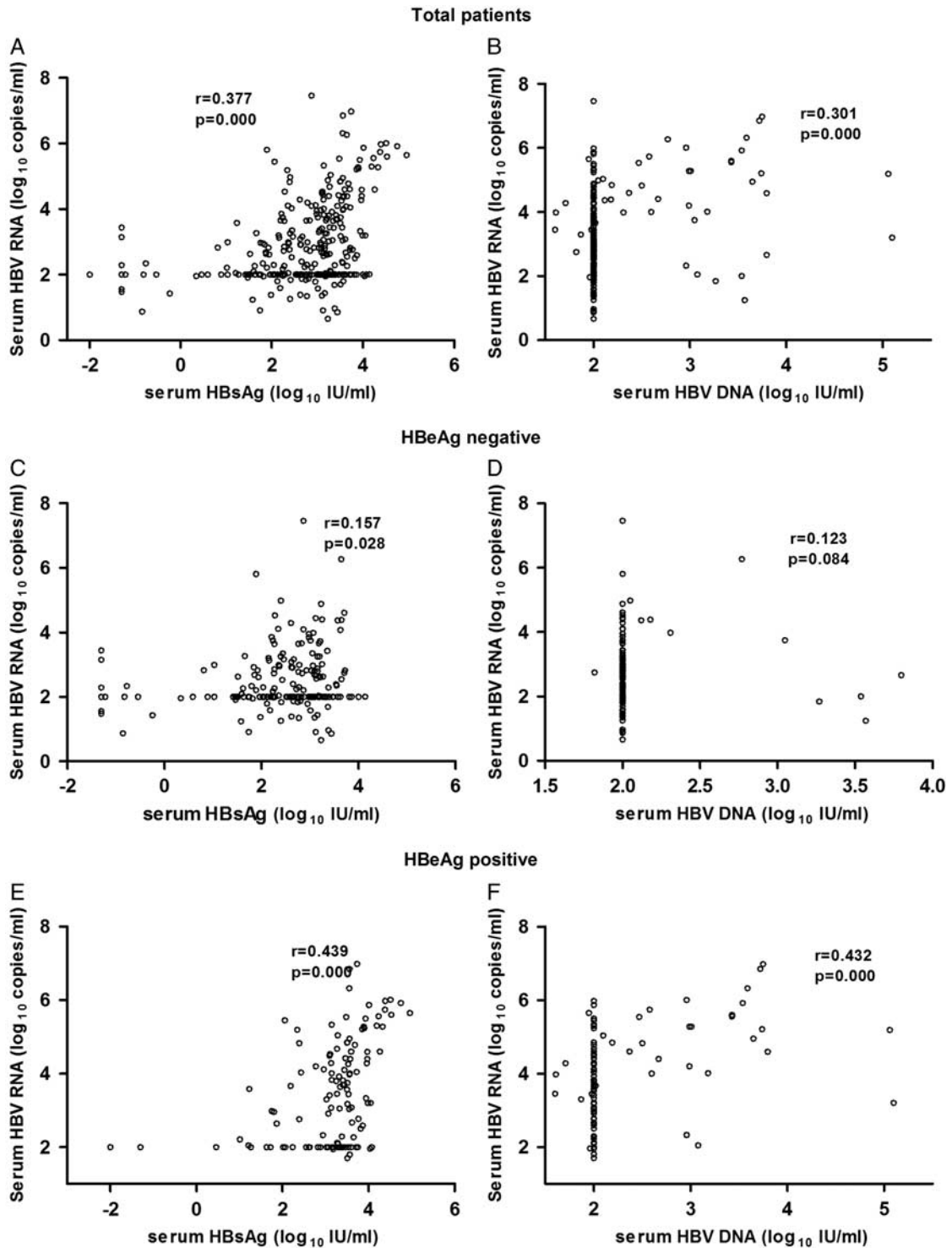
After consultations with liver disease experts, a total of 30 CHB patients who had no liver cirrhosis and showed good antiviral response and compliance discontinued NA treatment. The median age of these patients was 46 (35.75 to 59) years, most were male (70%), and the median treatment duration was 57.7 (30.25 to 77.5) months. Forty percent (12/30) of the patients had experience with interferon in the initial stage of antiviral treatment process and 20% (6/30) of the patients experienced drug-related resistance gene mutation and viral breakthrough during long-term NA treatment progress. As shown in Table 2, irrespective of the virological status at the initiation of antiviral therapy, all 30 CHB patients achieved good viral response and ALT normalization. At EOT, the median serum HBsAg titer was 1.91 ( $-0.62$  to  $2.89$ )  $\log_{10}$  IU/mL, and 17 (56.7%) patients

had HBsAg titer  $\leq 100$  IU/mL. Serum HBV RNA tests were performed on 29 patients who had available samples at EOT, of which 55.2% (16/29) were negative. The percent of patients with both serum HBV RNA test negative and HBsAg  $\leq 100$  IU/mL was 34.48% (10/29) at EOT (Table 2). At baseline (time of initiation of antiviral therapy), 13 (43.33%) patients were HBeAg positive and 17 (56.67%) patients were HBeAg negative. The characteristics of HBeAg-positive and HBeAg-negative patients were largely comparable at the EOT.

### Off-therapy Relapse

During the 24-month off-therapy follow-up, no patients experienced liver decomposition or died and 11 and 7 patients developed viral and clinical relapses, respectively. On the basis of HBV DNA reappearance from undetectable to  $> 2000$  IU/mL, the cumulative viral relapse rate was 40.56% [95% confidence interval (95% CI), 21.51%-59.61%] and the cumulative clinical relapse rate was 31.31% (95% CI, 11.32%-51.29%) at the second year off-therapy, respectively (Table S2, Supplemental Digital Content 1, <http://links.lww.com/JCG/A572>).

The risk of relapse between patients with different EOT HBV RNA status only differed in clinical relapse [17.50% (95% CI, 0.089%-64.87%) vs. 38.27% (95% CI, 6.83%-70.90%);  $P = 0.042$ ] (Table S2, Supplemental Digital Content 1, <http://links.lww.com/JCG/A572> and Fig. 3B). In addition, compared



**FIGURE 2.** Correlation between serum hepatitis B virus (HBV) RNA level and HBV DNA or hepatitis B surface antigen (HBsAg) level in chronic hepatitis B patients on nucleos(t)ide analogues treatment. A and B, Total chronic hepatitis B patients. C and D, Chronic hepatitis B patients with hepatitis B e antigen (HBeAg) negative at enrollment. E and F, Chronic hepatitis B patients with HBeAg positive at enrollment.

with EOT HBsAg titer alone, the EOT HBV RNA status showed no advantage in predicting off-therapy viral response durability (Table S3, Supplemental Digital Content 1, <http://links.lww.com/JCG/A572>). However, the risk of relapse significantly

differed in patients with different EOT HBsAg titers. Patients with EOT HBsAg titer  $\leq 100$  IU/mL had a significantly lower viral rebound rate through the 24-month off-therapy follow-up than patients with EOT HBsAg  $> 100$  IU/mL (Fig. 3C). The

**TABLE 2.** Clinical Characteristics of Patients Who Discontinued NA Treatment

Characteristics	Total	HBeAg Negative	HBeAg Positive	P
Patients, n	30	13	17	
Age, mean $\pm$ SD (y)	46 (35.7-59)	50 (39.5-59.5)	46 (32.5-57.5)	0.31
Sex (M:F)	21:9	9:4	12:5	1.00
Current antiviral therapy strategy, n (%)				
ETV	17 (56.67)	5	12	—
LAM	8 (26.67)	6	2	—
ADV	2 (6.67)	1	1	—
ADV+LAM	3 (10.00)	1	2	—
IFN experience	12 (40)	4 (30.8)	8 (47.1)	0.46
Drug-related mutation	6 (20)	4 (30.8)	2 (11.8)	0.36
Treatment duration, median (IQR) (mo)	57.5 (30.2-77.5)	57 (36.5-84)	60 (29.5-83)	0.81
EOT HBsAg (IQR) (log IU/mL)	1.91 (-0.6 to 2.8)	1.98 (-0.8 to 2.8)	1.84 (-0.2 to 2.8)	0.98
HBsAg $\leq$ 100 IU/mL, n (%)	17 (56.7)	7 (53.8)	10 (58.8)	1.00
HBV RNA negative, n (%)	16 (55.2)	6 (46.2)	10 (58.8)	0.46
HBV RNA negative and DNA below MDL, n (%)	16 (55.2)	6 (46.2)	10 (58.8)	0.46
HBV RNA negative and HBsAg $\leq$ 100 IU/mL, n (%)	10 (33.3)	4 (30.8)	6 (35.3)	1.00
Virological relapse, n (%)	11 (36.67)	7 (53.8)	4 (23.5)	0.132
Clinical relapse, n (%)	7 (23.33)	4 (30.8)	3 (17.6)	0.666
ALT level (U/L)	26 (19-38)	28 (24-38)	23.5 (15.5-37.5)	0.42
AST level (U/L)	21 (18-27)	22 (20-26)	19 (16.25-27.75)	0.40

ADV indicates adefovir dipivoxil; ALT, alanine aminotransferase; AST, aspartate aminotransferase; EOT, end of treatment; ETV, entecavir; HBeAg, hepatitis B e antigen; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; IFN, interferon; IQR, interquartile range; LAM, lamivudine; MDL, minimum detection limit; NA, nucleos(t)ide analogue.

P-value: compared between patients with pretreatment HBeAg positivity or negativity,  $P < 0.05$  was considered statistically significant.

cumulative viral relapse rates also significantly differed between pretreatment HBeAg-positive and HBeAg-negative patients [29.29% (95% CI, 4.54%-54.04%) vs. 55.13% (95% CI, 27.42%-82.84%), respectively (Fig. 3E, Table S2, Supplemental Digital Content 1, <http://links.lww.com/JCG/A572>).

Compared with EOT HBsAg titer alone, combining EOT HBsAg titer and HBV RNA status could better predict drug withdrawal durability. Patients with EOT HBsAg  $\leq$  100 IU/mL plus HBV RNA negativity had the lowest cumulative 24-month off-therapy viral relapse, 10%; 95% CI, 0%-28.598%. Even patients with EOT HBsAg  $\leq$  1000 IU/mL plus HBV RNA negativity also had relatively lower cumulative 24-month off-therapy viral relapse; 23.077%; 95% CI, 0.168%-45.986% (Fig. 3G, Table S2, Supplemental Digital Content 1, <http://links.lww.com/JCG/A572>). EOT HBsAg  $\leq$  1000 IU/mL plus HBV RNA negativity only showed obvious superiority for EOT HBsAg  $\leq$  1000 IU/mL alone, but not for HBsAg  $\leq$  100 IU/mL alone in drug-withdrawal durability prediction in patients on long-term NA therapy, with better specificity (18.18% vs. 72.73%,  $P = 0.030$ ) and the positive predictive value and negative predictive value were 76.92% and 47.06%, respectively (Table S3, Supplemental Digital Content 1, <http://links.lww.com/JCG/A572>).

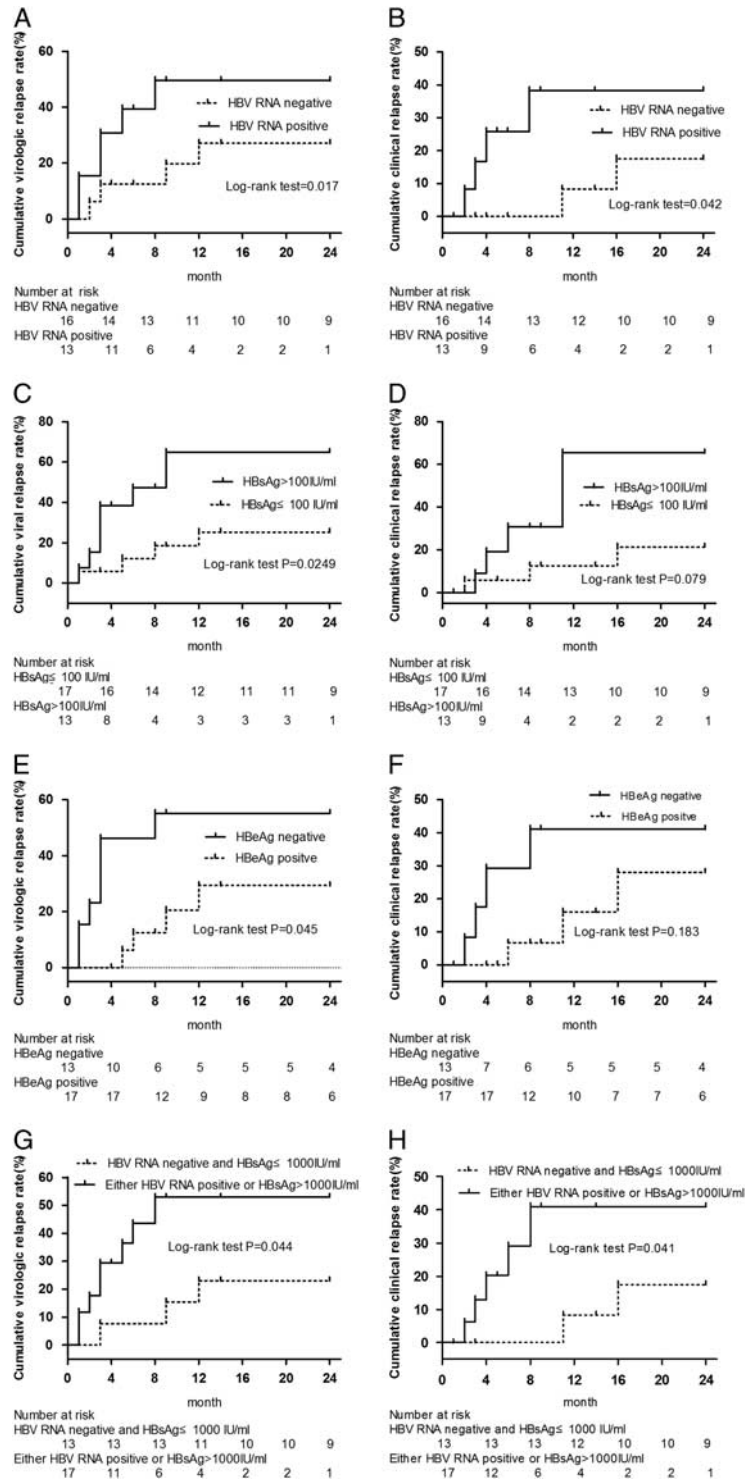
### Association of Serum HBV RNA Level and HBsAg Titer With Off-therapy Relapse

To compare the predictive value of EOT HBV RNA and HBsAg titer with other reported predictors, a Cox regression analysis was carried out. The univariate analyses showed that EOT HBsAg titer was associated with both viral and clinical relapse. EOT serum HBV RNA status was not associated with either viral or clinical relapse in all CHB patients. Further analysis of the predictive value of EOT HBV RNA in viral and clinical relapses on the basis of baseline HBeAg status showed that for both HBeAg-positive and HBeAg-negative patients, there was no clear correlation between EOT HBV RNA negativity and viral or

clinical relapse after drug withdrawal (Table S4, Supplemental Digital Content 1, <http://links.lww.com/JCG/A572>). The multivariate analysis showed that after adjustment for age, treatment duration, and pretreatment HBeAg status, the EOT HBsAg titer and HBV RNA status (HBsAg  $\leq$  1000 IU/mL and RNA negative vs. either HBsAg  $>$  1000 IU/mL or RNA positive) were still significantly associated with viral relapse ( $P = 0.038$ ). Besides EOT HBsAg titer and HBV RNA status, baseline HBeAg status was also strongly associated with virological relapse (HR, 4.633; 95% CI, 1.133-18.938) (Table 3).

## DISCUSSION

Although there is much heterogeneity in the methods and findings, it has been acknowledged that HBV RNA may be a new viral marker for monitoring of CHB patients, including antiviral therapy. In the present study, we cross-sectionally determined the serum HBV RNA levels in 326 patients with CHB on NA treatment for a median of 36 (interquartile range: 12 to 108) months using HBV-SAT and determined the behavior of the serum HBV RNA during NA treatment. Data from the present study showed that both serum HBV RNA and HBV DNA levels significantly decreased in the NA therapy progress, and in the long-term NA therapy progress, serum HBV RNA weakly correlated with serum HBV DNA and HBsAg titer in all CHB patients irrespective of the HBeAg status and treatment duration. The correlation between serum HBV RNA and HBV DNA and HBsAg remained until the treatment time exceeded 36 months. Our results were consistent with previous research results of Rokuhara et al<sup>16</sup> and Chayama and colleagues.<sup>17,18</sup> All these results indicated that during NA therapy, the serum HBV RNA and DNA reduced through different mechanisms. Furthermore, in the present study, we found that nearly half the patients (55.6%) achieved both serum HBV RNA and DNA below MDL when the NA treatment duration was more than 36 months, but only 39.5% of patients achieved a serum HBV RNA test result negative when serum



**FIGURE 3.** Virological and clinical relapse in patients who discontinued nucleos(t)ide analogues treatment by hepatitis B virus (HBV) RNA status and hepatitis B surface antigen (HBsAg) titer. A, Two-year off-therapy virological relapse in patients by end-of-treatment serum HBV RNA status. B, Two-year off-therapy clinical relapse in patients by end-of-treatment serum HBV RNA status. C, Two-year off-therapy virological relapse in patients by end-of-treatment HBsAg titer. D, Two-year off-therapy clinical relapse in patients by end-of-treatment HBsAg titer. E, Two-year off-therapy virological relapse in patients by baseline hepatitis B e antigen (HBeAg) status. F, Two-year off-therapy clinical relapse in patients by baseline HBeAg status. G, Two-year off-therapy virological relapse in patients by end-of-treatment HBV RNA status and HBsAg titer. H, Two-year off-therapy clinical relapses in patients by end-of-treatment HBV RNA status and HBsAg titer.

**TABLE 3.** Cox Regression Analysis for 24-Month Off-therapy Virological and Clinical Relapse Risk Factors in Patients Who Discontinued NAs Therapy

Characteristics	Univariate Analysis			Multivariate Modeling		
	HR	95% CI	P	HR	95% CI	P
<b>Virological relapse</b>						
Sex	1.525	0.445-5.220	0.502	2.652	0.645-10.897	0.176
Age	1.748	0.463-6.596	0.410	0.973	0.911-1.038	0.406
Treatment duration	1.005	0.987-1.023	0.609	1.002	0.984-1.021	0.840
Drug-related mutation experience	0.081	0.022-0.295	0.000			
IFN experience	2.195	0.578-8.336	0.248			
Baseline HBeAg status	3.257	0.946-11.212	0.061	4.633	1.133-18.938	0.033
EOT HBsAg titer (log IU/mL)	2.438	1.195-4.977	0.014			
EOT HBsAg ≤ 100 IU/mL	0.254	0.070-0.928	0.038			
EOT HBV RNA negative	0.373	0.102-1.368	0.137			
HBV RNA negative and HBsAg ≤ 1000 IU/mL	0.265	0.066-1.06	0.060	0.202	0.045-0.909	0.037
ALT (U/L)	0.994	0.959-1.031	0.759			
AST (U/L)	1.001	0.944-1.062	0.968			
<b>Clinical relapse</b>						
Sex	1.042	0.201-5.408	0.961	2.549	0.364-17.879	0.346
Age	4.078	0.490-33.946	0.194	0.975	0.897-1.059	0.549
Treatment duration (> 3 y)	0.399	0.085-1.878	0.245	0.999	0.974-1.024	0.930
Drug-related mutation experience	0.054	0.009-0.301	0.001			
IFN experience	1.302	0.286-5.918	0.733			
Baseline HBeAg status	2.675	0.594-12.046	0.200	4.664	0.817-26.606	0.083
EOT HBsAg titer (log IU/mL)	2.836	1.090-7.375	0.033			
EOT HBsAg ≤ 100 IU/mL	0.261	0.053-1.278	0.097			
EOT HBV RNA negative	0.174	0.028-1.085	0.061			
HBV RNA negative and HBsAg ≤ 1000 IU/mL	0.173	0.029-1.055	0.057	0.101	0.012-0.884	0.038
ALT (U/L)	1.000	0.959-1.043	0.983			
AST (U/L)	1.017	0.952-1.085	0.623			

ALT indicates alanine aminotransferase; AST, aspartate aminotransferase; CI, confidence interval; EOT, end of treatment; HBeAg, hepatitis B e antigen; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; HR, hazard ratio; IFN, interferon; NA, nucleos(t)ide analogue.  
*P* < 0.05 was considered statistically significant.

HBV DNA was below MDL by clinically available polymerase chain reaction (MDL, 100 IU/mL). However, it was worth noting that lack of high-sensitive detection methods for serum HBV RNA and DNA in practice resulted in a high proportion of patients with serum HBV RNA and DNA levels below MDL which may lead to deviations in the correlation between serum HBV RNA and DNA. Therefore, in the future, high-sensitivity detection methods need to be used to further determine the correlation between serum HBV RNA and other viral markers in patients with CHB and on NA therapy longer than 36 months.

In view of the correlation between serum HBV RNA and intrahepatic cccDNA,<sup>9-12</sup> consistent loss of serum HBV RNA was recommended as a surrogate marker for a clinical status of potential elimination or transcriptional silencing of cccDNA, and the loss of serum HBV RNA was suggested by some researchers to be a potential marker for the safe discontinuation of NA treatments.<sup>11,19,20</sup> For example, Wang et al<sup>11</sup> determined the EOT serum HBV RNA levels in 33 CHB patients with standard NA treatment for more than 3 years and found that 63.64% (21/33) of patients were serum HBV RNA positive at the end of the treatment, and all of the patients developed viral rebound during the 24 weeks off-therapy. In addition, the research team reported in another study<sup>19</sup> that serum HBV RNA was positive in 7 patients who achieved both HBsAg and HBV DNA clearance at the end of antiviral therapy, and all 7 patients developed drug withdrawal HBsAg composite reversion. However, Tsuge et al<sup>21</sup> found that only serum HBV RNA titer after 3 months of NA treatment was an independent predictor for 24 weeks of off-therapy virus rebound, but there was no difference in the EOT serum HBV RNA titer between

patients with and without off-therapy viral rebound. In the present study, on the basis of the overall status of serum HBV RNA status in patients with standard NA therapy for more than 3 years, we further determined the EOT serum HBV RNA titer in 29 patients who discontinued NA after a median of 57.5 months of NA treatment and found that 55.2% (16/29) of these patients achieved a negative EOT serum HBV RNA test. However, there was no difference in the EOT serum HBV RNA-negative rate between patients with and without 24-month off-therapy viral rebound. The heterogeneity of these studies suggests that the predictive value of EOT serum HBV RNA levels remains to be confirmed. Recently, Gao et al<sup>13</sup> investigated the correlation between serum HBV RNA levels and intrahepatic cccDNA before and after NA treatment in 62 patients and found that at baseline, serum HBV RNA was correlated with intrahepatic cccDNA levels in HBeAg-positive patients, but at 96 weeks after NA treatment, intrahepatic cccDNA only correlated with serum HBsAg titer, and the correlation between intrahepatic cccDNA and serum HBV RNA levels disappeared. These findings suggest that EOT serum HBV RNA levels may not be a good surrogate for intrahepatic cccDNA after NA treatment and indirectly suggested that the predictive value of EOT serum HBV RNA levels for off-therapy viral rebound requires further large-scale studies for confirmation.

To evaluate the value of EOT serum HBV RNA for the safe discontinuation of NA treatment, the EOT serum HBV RNA levels in 29 patients who discontinued long-term NA treatment were determined. At the EOT, all 30 patients had undetectable levels of HBV DNA, and 55.2% (16/29)



achieved a negative serum HBV RNA test. Univariate and multivariate analyses suggested that EOT serum HBV RNA status was not an independent risk factor for off-therapy viral or clinical relapse. In addition, compared with EOT HBsAg titer alone, EOT serum HBV RNA negativity had no advantage in predicting the viral and clinical off-therapy relapse in CHB patients on long-term NA therapy. Our results in agreement with the results of Tsuge et al,<sup>21</sup> but are inconsistent with the results of Wang et al.<sup>11</sup> In Wang study, viral rebound occurred in all (21/21) patients who were EOT serum HBV RNA positive, whereas only 25% (3/12) of patients who were EOT serum HBV RNA negative developed viral relapse ( $P < 0.01$ ). The reason for the difference in these findings may be the heterogeneity of the withdrawal baseline data, such as the HBeAg status and the EOT HBsAg titer, and the heterogeneity of HBV RNA test sensitivity.

Although our results did not support EOT HBV RNA as an independent predictor of safe drug withdrawal, it is interesting that we found that EOT serum HBV RNA negativity plus HBsAg  $< 1000$  IU/mL or HBsAg  $\leq 100$  IU/mL could independently predict safe drug withdrawal. Recently, Fan et al<sup>22</sup> carried out a post hoc analysis of data from a multicenter randomized-controlled trial of 130 patients with HBeAg positivity and evaluated the utility of the EOT HBV DNA and RNA levels in predicting the durability of response after discontinuation of NA in CHB patients. They found that EOT HBV DNA plus RNA level, namely, overall serum HBV nucleic acid level, was the strongest independent predictor for off-treatment durability. All these results suggested that EOT serum HBV RNA status may be one supplementary indicator of existing forecast indicators.

As a widely accepted surrogate marker for intrahepatic cccDNA, serum HBsAg quantification is pivotal in CHB management, especially for the safe cessation of treatment.<sup>23–27</sup> HBsAg loss is deemed the ultimate goal of CHB antiviral therapy,<sup>1,2</sup> and an EOT HBsAg level  $< 100$  IU/mL is suggested to be the optimal cut-off value for the cessation of NA treatment in both HBeAg-positive and HBeAg-negative CHB patients.<sup>8</sup> Here, we confirmed that the EOT HBsAg titer is an independent marker to monitor the safe cessation of NA in CHB patients undergoing long-term NA treatment. A lower HBsAg titer at the EOT indicates lasting off-therapy remission.

Because of the lack of a direct effect on cccDNA, and despite guidelines on when and in whom to stop NA therapy, a significant proportion of patients will experience viral and/or biochemical relapse.<sup>1,7</sup> In the present study, 30 patients discontinued NA treatment, the 24-month off-therapy cumulative virological relapse rate was 40.56%; 95% CI, 21.51%–59.609%, and the 24-month off-therapy cumulative clinical relapse rate was 31.305%; 95% CI, 11.32%–51.29%. The off-therapy durability in this study was comparable to the previous reports.<sup>4–6,28</sup>

Previous studies reported that some patients may show clearance of HBsAg after stopping treatment due to the activation of host immune response along with the off-treatment relapse.<sup>6,29</sup> In this study, only 1 patient with baseline HBeAg negativity achieved HBsAg clearance during the 24-month off-therapy follow-up. Patients in this study were retreated once clinical relapse occurred, which may explain the low off-therapy HBsAg clearance. In addition, previous studies have indicated that stopping NA treatment in white CHB patients after HBeAg seroconversion is associated with high relapse rates and fatal outcomes.<sup>30</sup> In this study, we found that patients with

HBeAg seroconversion had lower cumulative 24-month off-therapy viral relapse compared with patients with HBeAg negativity and multivariate analyses showed that baseline HBeAg negativity was an independent risk factor for withdrawal virological rebound.

Our research as a real-life data had certain advantages, such as inclusion of patients with HBeAg-negative CHB in the NA withdrawal group, and evaluation of clinical popular indicators including HBsAg level and HBV DNA. Nonetheless, it should be noted that in the present study, it was not possible to evaluate the dynamic change in the pattern of serum HBV RNA and its predictive value on durable off-therapy remission because the serum HBV RNA results at different time points during NA therapy were derived from a cross-sectional study. In addition, we did not assess the predictive effect of the hepatitis B core-related antigen, which is considered to be promising.<sup>31</sup> In addition, the sample size of the drug withdrawal follow-up cases in this study is small, and the significance of serum HBV RNA prediction needs to be further verified in larger sample sizes.

In conclusion, this study preliminarily revealed that both serum HBV RNA and DNA levels decreased significantly throughout the long-term antiviral therapy. However, only a small percentage of patients achieved a negative HBV RNA test result. Existing drug withdrawal prediction indicators had limitations. At the EOT, serum HBV RNA status may be one supplementary indicator of existing prediction indicators.

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