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Serum HBV RNA and HBeAg are useful markers for the safe discontinuation of nucleotide analogue treatments in chronic hepatitis B patients

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Abstract

Background Treatment for chronic hepatitis B has improved drastically with the use of nucleot(s)ide analogues (NAs). However, NA therapy typically fails to eliminate Hepatitis B virus (HBV) completely, and it is difficult to discontinue these therapies. We previously demonstrated that NA therapy induced immature viral particles, including HBV RNA in sera of chronic hepatitis B patients. In the study reported here, we analyzed the association between HBV RNA titer and the recurrence rate of hepatitis after discontinuation of NA therapy.

Methods The study cohort comprised 36 patients who had discontinued NA therapy. Serum HBV DNA or DNA plus RNA levels were measured by real time PCR and statistical analyses were performed using clinical data and HBV markers.

Results At 24 weeks after discontinuation of NA therapy, HBV DNA rebound was observed in 19 of the 36 patients (52.8 %), and alanine aminotransferase (ALT) rebound was observed in 12 of 36 patients (33.3 %). Multivariate

statistical analysis was used to identify factors predictive of HBV DNA rebound. The HBV DNA + RNA titer following 3 months of treatment was significantly associated with HBV DNA rebound [$P = 0.043$, odds ratio (OR) 9.474, 95 % confidence interval (CI) 1.069–83.957]. Absence of hepatitis B e antigen (HBeAg) at the end of treatment was significantly associated with ALT rebound ($P = 0.003$, OR 13.500, 95 % CI 2.473–73.705). In HBeAg-positive patients, the HBV DNA + RNA titer after 3 months of treatment was marginally associated with ALT rebound ($P = 0.050$, OR 8.032, 95 % CI 0.997–64.683). **Conclusions** Monitoring of serum HBV DNA + RNA levels may be a useful method for predicting re-activation of chronic hepatitis B after discontinuation of NA therapy.

Keywords HBV · HBV RNA · Nucleotide analogue · HBV replication

Abbreviations

ADV Adefovir dipivoxil
ETV Entecavir

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HBeAg	Hepatitis B e antigen
HBsAg	Hepatitis B surface antigen
HBV	Hepatitis B virus
LMV	Lamivudine
NA	Nucleot(s)ide analogue
RT	Reverse transcriptase

Introduction

Hepatitis B virus (HBV) infection is a serious global health problem, with more than two billion people infected with HBV, of whom about 20 % remain chronically infected [1, 2]. Chronically infected individuals often develop chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma (HCC), and the incidence of HCC in chronically infected individuals is significantly higher than that in healthy individuals [3]. Once HBV infects human hepatocytes, HBV genomes are transported into the nucleus, and some viral genomes become integrated into human chromosomes [4–7]. Thus, complete elimination of the virus is difficult, and patients are generally treated with interferon and nucleot(s)ide analogues (NAs) that suppress viral replication and prevent the progression of liver disease by combating inflammation [8–10]. However, long-term treatment with NAs is known to lead to the development of drug-resistant viral mutants, with the possible occurrence of a serious hepatitis flare-up (breakthrough hepatitis) [11–21]. To avoid the development of drug-resistant HBV, Japanese guidelines currently recommend that patients with chronic hepatitis B be treated with the eventual goal of reaching a “drug-free state” involving discontinuation of NAs [9]. However, there are at the present time no criteria for safely discontinuing NA therapy.

It has previously been reported that HBV particles, including particles of HBV RNA, are released from hepatocytes during NA treatment and become detectable in sera [22–25]. Commonly, in the course of HBV replication, pregenome RNAs are encapsidated into HBV core particles in the cytoplasm, and all pregenome RNAs are reverse transcribed into plus-stranded genomic DNA in the core particle [26]. However, during NA therapy, it is thought that NA strongly interferes with reverse transcription, causing excessive accumulation of HBV RNA particles in hepatocytes and leading to release without reverse transcription. In our previous study, we found that the existence of HBV RNA particles was significantly associated with the development of drug-resistant viruses [22]. This finding led us to consider that the existence of HBV RNA particles might be associated with HBV replication activity and that viruses with high replication activity produce high

amounts of HBV RNA, leading to a greater opportunity for developing drug-resistance mutations. Therefore, we speculated that serum HBV RNA levels might be associated with HBV replication activity.

In the study reported here, several clinical parameters, including serum HBV DNA and HBV RNA titers, were analyzed with the aim of identifying factors predictive of the safe discontinuation of NA treatment. HBV replication activity and the deviation between serum HBV RNA and HBV DNA levels were found to be important predictors for the safe discontinuation of NA treatment.

Materials and methods

Patients

The study cohort comprised 36 Japanese chronic hepatitis B patients who had received NA therapy for more than 6 months at Hiroshima University Hospital or hospitals belonging to the Hiroshima Liver Study Group (http://home.hiroshima-u.ac.jp/naika1/research_profile/pdf/liver_study_group_e.pdf) and subsequently discontinued NA therapy. The discontinuation of NA therapy was decided at the discretion of the attending physicians, resulting in similar, but not uniform, criteria for discontinuation. In all analyses, the time of discontinuation was defined as the end of NA therapy. None of the patients were infected with other viruses, including human immunodeficiency virus or hepatitis C virus, and none had evidence of other liver diseases, such as auto-immune hepatitis or alcoholic liver disease. Patients with a total ethanol intake of >100 kg were excluded [27]. All patients gave written informed consent to participate in the study. The experimental protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the ethical committee of Hiroshima University Hospital.

Baseline characteristics of the 36 patients are shown in Table 1. Thirty-one patients were treated with 100 mg/day of lamivudine (LMV), three were treated with 0.5 mg/day of entecavir (ETV), and two were treated with 10 mg/day of adefovir (ADV) monotherapy or LMV + ADV combination therapy. Twenty-six patients underwent sequential therapy, which included 6 months of conventional interferon therapy from 1 month prior to discontinuation until 5 months after discontinuation of NA therapy. Twenty-three patients were male and 13 were female. Median age at the onset of treatment was 43 years. Sixteen patients were positive for hepatitis e antigen (HBeAg). Blood samples were obtained from the patients before the beginning of therapy and every 4 weeks during the follow-up period. Biochemical and hematological tests were performed by the Hiroshima University Hospital laboratory.

The remaining sera were stored at -80°C for further analysis.

Extraction and reverse transcription of HBV nucleic acid

Nucleic acid was extracted from 100 μL of serum by the SMITEST (Genome Science Laboratories, Tokyo, Japan)

Table 1 Clinical backgrounds of the study cohort

Characteristics ^a	Values
Gender (M:F)	23:13
HBV genotype (B:C:ND)	2:31:3
Age (years) ^b	43 (25–66)
Platelet ($\times 10^4/\mu\text{L}$) ^b	16.1 (9.6–28.0)
ALT (IU/L) ^b	139 (22–780)
HBV DNA (log copies/mL) ^b	6.9 (3.6–8.8)
HBsAg (IU/mL) ^b	3,088 (66–1,354,400)
HBeAg (+:–)	16:20
HBcrAg (log U/mL) ^b	6.2 (3.4–8.8)
Nucleot(s)ide analogues (LMV:LMV + ADV:ADV:ETV)	31:1:1:3
Sequential therapy (+:–)	26:10
Duration of NA therapy (weeks) ^b	36 (24–304)
Observation period (weeks) ^b	269 (73–508)
Re-elevation of HBV DNA within 24 weeks (+:–)	21:15
Re-elevation of ALT within 24 weeks (+:–)	13:23

M Male, F female, HBV hepatitis B virus, ND not determined ALT alanine aminotransferase, HBsAg hepatitis B surface antigen, HBeAg hepatitis B e antigen, HBcrAg HBV core-related antigen, LMV lamivudine, ADV adefovir, ETV entecavir, NA nucleot(s)ide analogues

^a Unless indicated otherwise, the values are given as the number (n) of patients

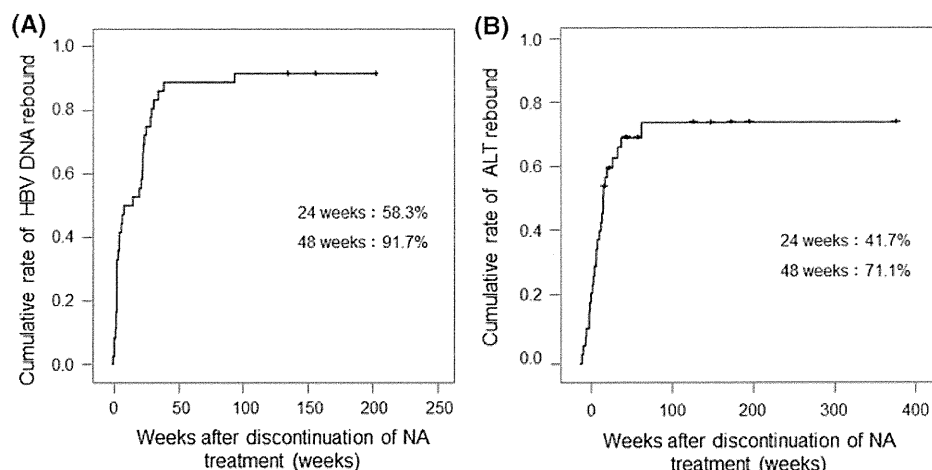
^b Mean (range)

and dissolved in 20 μL of H_2O . Each extracted solution was divided into two aliquots. An 8.8- μL aliquot of the nucleic acid solutions was used for measuring HBV RNA. The solutions were reverse-transcribed as previously described [22]. The nucleic acid solutions were then mixed with 25 pM of random primer (Takara Bio, Shiga, Japan) and incubated at 65°C for 5 min. The samples were set on ice for 5 min, then each sample was mixed with 4 μL of $5\times$ reverse transcription (RT) buffer, 2 μL of 10 mM dNTPs, 2 μL of 0.1 M dithiothreitol (DTT), 8 U of ribonuclease inhibitor, and 100 U of M-MLV reverse transcriptase (ReverTra Ace; TOYOBO Co., Osaka, Japan). The reaction mixture was incubated at 30°C for 10 min and 42°C for 60 min, followed by inactivation at 99°C for 5 min. The aliquots of the nucleic acid solutions were then used for the measurement of HBV DNA.

Measurement of serum HBV DNA and RNA by real-time PCR

The real-time PCR analyses were performed using the ABI Prism 7300 Sequence Detection System (Applied Biosystems, Foster City, CA) according to the instructions provided by the manufacturer. A 25- μL volume of reaction mixture containing SYBR Green PCR Master Mix (Applied Biosystems), 200 nM of forward primer (5'-TTT GGGGCATGGACATTGAC-3', nucleotides 1893–1912), 200 nM of reverse primer (5'-GGTGAACAATGGTCCG GAGAC-3', nucleotides 2029–2049), and 1 μL of DNA or cDNA solution was prepared. After incubation for 2 min at 50°C , the sample was heated for 10 min at 95°C for denaturing, followed by a PCR cycling program consisting of 40 two-step cycles of 15 s each at 95°C and 60 s at 60°C . The lower detection limit of this assay was 2.3 log copies/mL. In the statistical analyses, samples which included less than the quantitation limit of HBV

Fig. 1 Cumulative rate of hepatitis B virus (HBV) DNA (a) and alanine aminotransferase (ALT) rebound (b) in 36 chronic hepatitis B patients following discontinuation of nucleos(t)ide analogue (NA) therapy. Cumulative HBV DNA rebound rate and cumulative ALT rebound rate were analyzed using the Kaplan–Meier method



nucleotides were represented as 2.2 log copies/mL. By using these methods, we were able to measure the HBV DNA titers with DNA solutions and HBV DNA + RNA titers with cDNA solutions. In the present study, the ratios between HBV DNA + RNA to HBV DNA (DR ratio) was also assessed using the ratio of $\log_{10}(\text{HBV DNA} + \text{RNA})$ to $\log_{10}(\text{HBV DNA})$.

Measurement of HBV-related markers

Quantification of serum hepatitis B surface antigen (HBsAg) was performed with Elecsys HBsAg II Quant (Roche Diagnostics, Tokyo, Japan). High HBsAg titer was measured with 40,000-fold diluted serum. The quantitative range of HBsAg was 0.05–5,200,000 IU/mL. Serum HBcrAg levels were

Table 2 Multiple logistic regression for factors associated with HBV DNA rebound within 24 weeks after discontinuation of NA treatment

Factors ^a	DNA relapsed (n = 21)	DNA non-relapsed (n = 15)	Univariate <i>P</i> value ^b	Multiple logistic regression ^c	
				<i>P</i> value	OR (95 % CI)
Gender (M:F)	12:9	11:4	0.484 (chi-square test)		
HBV genotype (B:C:ND)	1:18:2	1:13:1	0.931 (chi-square test)		
Before treatment					
Age (years) ^d	41 (25–59)	47 (30–66)	0.252		
Platelet ($\times 10^4/\mu\text{L}$) ^d	17.6 (9.6–28.0)	14.8 (9.6–23.6)	0.104		
ALT (IU/L) ^d	161 (37–780)	114 (22–304)	0.324		
HBsAg (IU/mL) ^d	3,714 (462–1,354,400)	1,754 (66–10,109)	0.083	0.581	
HBeAg (+:–)	12:9	4:11	0.096 (chi-square test)	0.389	
HBcrAg (log U/mL) ^d	5.9 (4.8–8.8)	6.2 (3.4–7.9)	0.608		
HBV DNA (log copies/mL) ^d	9.1 (3.5–10.1)	7.4 (4.1–9.3)	0.547		
HBV DNA + RNA titers (log copies/mL)	7.9 (3.4–10.0)	7.0 (3.4–9.1)	0.704		
DR ratio	–0.2 (–1.4–0.5)	–0.4 (–1.5 to 0.0)	0.304		
After 3 months of treatment					
HBV DNA (log copies/mL) ^d	4.4 (2.2–7.3)	3.6 (2.2–5.4)	0.056	0.074	
HBV DNA + RNA titers (log copies/mL)	4.8 (2.2–8.2)	4.2 (2.2–5.8)	0.015	0.043	9.474 (1.069–83.957)
DR ratio	0.9 (–0.9–2.7)	0.4 (–0.7 to 1.4)	0.019	0.643	
End of treatment					
HBsAg (IU/mL) ^d	1,912 (481–16,301)	470 (<1.1–4,736)	0.036	0.070	
HBeAg (+:–)	11:10	3:12	0.083 (chi-square test)	0.637	
HBcrAg (log U/mL) ^d	4.9 (3.0–8.2)	4.2 (3.0–6.6)	0.516		
HBV DNA (log copies/mL) ^d	3.5 (2.2–9.2)	3.3 (2.2–7.1)	0.465		
HBV DNA + RNA titers (log copies/mL)	3.9 (2.2–8.7)	3.6 (2.2–6.5)	0.117		
DR ratio	0.7 (–1.0–2.7)	0.0 (–1.0 to 1.2)	0.102		
Sequential therapy (+:–)	13:8	13:2	0.142 (chi-square test)		
Duration of treatment (weeks) ^d	34 (24–221)	53 (24–304)	0.800		

DR ratio HBV DNA + RNA titers/HBV DNA, OR odds ratio, CI confidence interval

^a Unless indicated otherwise, the values are given as the number (*n*) of patients

^b Univariate analysis was performed with Mann-Whitney *U* test unless indicated otherwise

^c Multiple logistic regression analysis was performed using variables that were at least marginally significant ($P < 0.10$) in the univariate analysis

^d Median (range)

Table 3 Univariate analysis for factors associated with HBV DNA rebound within 48 weeks after discontinuation of NA treatment

Factors	DNA relapsed (n = 31)	DNA non-relapsed (n = 5)	Univariate P value
Gender (M:F)	21:10	2:3	0.328 ^b
HBV genotype (B:C:ND)	2:27:2	0:4:0	0.523 ^b
Before treatment			
Age (years) ^a	41 (25–66)	47 (30–62)	0.749
Platelet ($\times 10^4/\mu\text{L}$) ^a	15.6 (9.6–28.0)	17.3 (14.7–18.8)	0.679
ALT (IU/L) ^a	135 (22–780)	192 (94–296)	0.450
HBsAg (IU/mL) ^a	2,983 (66–1,354,400)	4,264 (1,172–10,109)	0.758
HBeAg (+:–)	14:17	2:3	1.000
HBcrAg (log U/mL) ^a	5.4 (3.4–8.8)	6.8 (5.4–7.9)	0.330
HBV DNA (log copies/mL) ^a	7.6 (3.5–10.1)	8.3 (6.7–9.1)	0.766
HBV DNA + RNA titers (log copies/mL)	7.4 (3.4–10.0)	8.0 (6.7–9.0)	0.522
DR ratio	–0.2 (–1.4–0.9)	–0.3 (–0.6 to –0.1)	0.596
After 3 months of treatment			
HBV DNA (log copies/mL) ^a	4.0 (2.2–7.3)	3.7 (3.2–4.2)	0.409
HBV DNA + RNA titers (log copies/mL)	4.8 (2.2–8.2)	4.3 (2.7–4.9)	0.507
DR ratio	0.7 (–0.9–2.7)	0.6 (–0.6–1.4)	0.464
End of treatment			
HBsAg (IU/mL) ^a	2,195 (48–16,301)	533 (<1.1–9,680)	0.105
HBeAg (+:–)	13:18	1:4	0.628 ^b
HBcrAg (log U/mL) ^a	4.7 (3.0–8.2)	4.6 (3.6–6.6)	0.657
HBV DNA (log copies/mL) ^a	3.5 (2.1–9.2)	3.0 (2.7–6.1)	0.818
HBV DNA + RNA titers (log copies/mL)	3.7 (2.2–8.7)	4.2 (2.2–5.7)	0.801
DR ratio	0.2 (–1.0–2.7)	0.4 (–0.8–1.2)	0.348
Sequential therapy (+:–)	23:8	3:2	0.603 ^b
Duration of treatment (weeks) ^a	36 (24–221)	86 (24–304)	0.278

ND not determined, DR ratio
HBV DNA + RNA titers/HBV
DNA

^a Median (range) univariate
analysis was performed with
Mann–Whitney *U* test

^b Chi-square test

measured using a CLEIA HBcrAg assay kit with a fully automated Lumipulse System analyzer (Fujirebio Inc, Tokyo, Japan), as described previously [28, 29].

Evaluation of rebound of HBV DNA and alanine aminotransferase after discontinuation of NA therapy

The rebound of HBV DNA after discontinuation of NA therapy was determined based on two criteria: (1) when the HBV DNA reached >4.0 log copies/mL after discontinuation of NA therapy in patients whose HBV DNA titers became negative (<2.6 log copies/mL) at the end of NA therapy; (2) when the HBV DNA increased to >1.0 log copies/mL after the discontinuation of NA therapy in patients whose HBV DNA titers were still positive (>2.7 log copies/mL) at the end of NA therapy.

Alanine aminotransferase (ALT) rebound after discontinuation of NA therapy was defined using the following criteria: (1) when ALT reached >50 IU/L after

discontinuation of NA therapy in those patients whose ALT levels had normalized (≤ 35 IU/L) at the end of NA therapy; (2) when ALT increased by >80 IU/L (twofold of upper limit of normal) after discontinuation of NA therapy in those patients whose ALT levels were still high (>35 IU/L) at the end of NA therapy.

Statistical analysis

The baseline characteristics of the patients in the two groups were compared, and differences were assessed by the chi-square test with Yate's correction, Fisher's exact probability test, and the Mann–Whitney *U* test. All *P* values of <0.05 by the two-tailed test were considered to be significant. To identify predictors for HBV DNA or ALT rebound, univariate and multivariate logistic regression analyses were performed. Potential predictive factors included the following variables: age, gender, body mass index (BMI), platelet count, prothrombin time, total

Fig. 2 Change in HBV DNA and HBV DNA + RNA titers during NA therapy. **a, b** HBV DNA + RNA titers and HBV DNA titers were compared at each time point for the DNA non-relapse group (**a**) and DNA relapse group (**b**). **c** Changes in the HBV RNA + DNA/HBV DNA ratio were compared with each group. Statistical analyses were performed by the Mann–Whitney *U* test

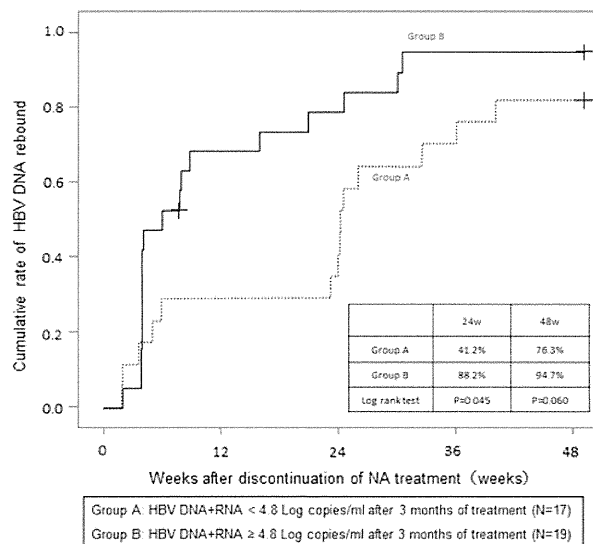
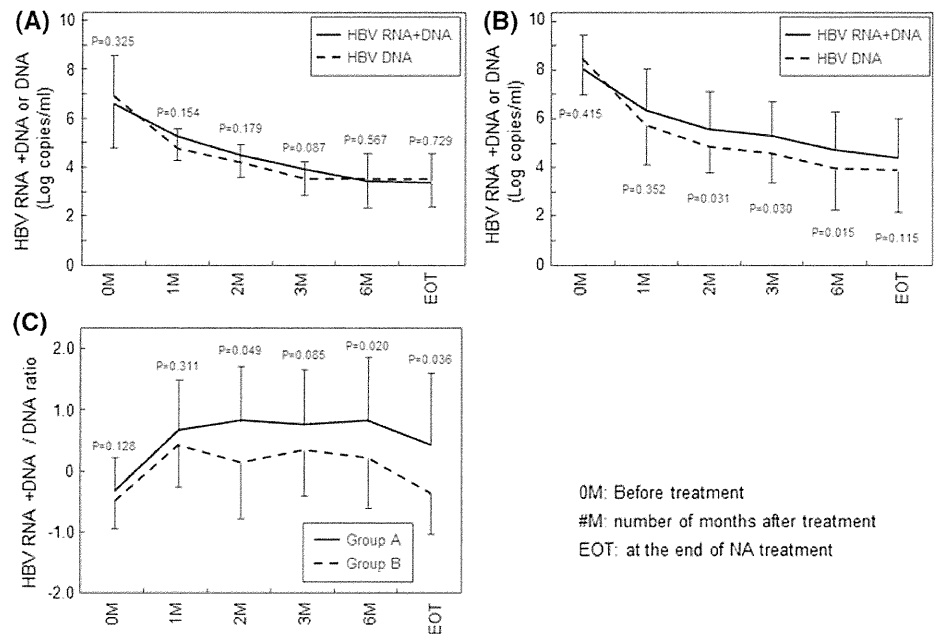


Fig. 3 Cumulative rate of HBV DNA rebound after discontinuation of NA treatment. Seventeen patients whose HBV DNA + RNA titers reached <4.8 log copies/mL after 3 months of treatment, were assigned to group A; the other 19 patients, whose HBV DNA + RNA titers were ≥4.8 log copies/mL after 3 months of treatment, were assigned to group B. The cumulative ALT rebound rate in HBeAg-positive chronic hepatitis B patients was analyzed using the Kaplan–Meier method

bilirubin, aspartate aminotransferase, ALT, lactate dehydrogenase, alkaline phosphatase, gamma-glutamyltranspeptidase, HBV DNA titer, HBV DNA + RNA titer, and

the DR ratio. As shown in a previous study, interferon treatment decreases the production of HBV RNA particles [23]. Thus, HBV RNA + DNA titer at 6 months of treatment was considered to be inappropriate for the statistical analyses in the present study, and these data were not included in these analyses. Odds ratios (OR) and 95 % confidence intervals (95 % CI) were also calculated. Variables with at least marginal significance ($P < 0.10$) in the univariate analysis were entered into the multiple logistic regression analysis to identify significant independent factors. Statistical analyses were performed using SPSS ver. 17.0 (SPSS, Chicago, IL).

Results

Analysis of HBV DNA and ALT rebound rates after discontinuation of NA therapy

Although NA therapy suppressed HBV replication and genomic HBV DNA synthesis, serum HBV DNA and ALT rebound occurred with a high frequency after therapy discontinuation. The cumulative HBV DNA and ALT rebound rates were analyzed to identify associated risk factors. As shown in Fig. 1a, the cumulative HBV DNA rebound rate increased in a time-dependent manner, reaching 58.3 and 91.7 % at 24 and 48 weeks after discontinuation of NA therapy, respectively. The cumulative

Table 4 Univariate analysis for factors associated with HBV DNA rebound within 24 weeks after discontinuation of NA treatment in those patients whose HBV DNA titer became negative at the end of NA treatment

Factors ^a	DNA relapsed (n = 5)	DNA non-relapsed (n = 6)	Univariate P value ^b
Gender (M:F)	3:2	4:1	0.545 (chi-square test)
HBV genotype (B:C:ND)	0:4:1	0:6:0	0.455 (chi-square test)
Before treatment			
Age (years) ^c	41 (3–52)	54 (32–66)	0.119
Platelet ($\times 10^4/\mu\text{L}$) ^c	18.8 (11.7–27.5)	14.8 (10.2–23.6)	0.221
ALT (IU/L) ^c	186 (79–303)	95 (48–270)	0.273
HBsAg (IU/mL) ^c	2,603 (2,064–9,400)	1,984 (406–7,016)	0.180
HBeAg (+:–)	2:3	1:5	0.545 (chi-square test)
HBcrAg (log U/mL) ^c	5.4 (5.0–7.8)	4.1 (3.4–7.9)	0.462
HBV DNA (log copies/mL) ^c	5.7 (3.8–9.2)	7.9 (5.7–9.7)	0.410
HBV DNA + RNA titers (log copies/mL)	5.6 (3.4–9.0)	7.5 (5.0–9.7)	0.583
DR ratio	–0.1 (–0.8–0.1)	–0.4 (–0.7–0.0)	0.527
After 3 months of treatment			
HBV DNA (log copies/mL) ^c	3.8 (2.2–4.8)	3.5 (2.2–4.4)	0.518
HBV DNA + RNA titers (log copies/mL)	4.0 (3.7–6.0)	3.6 (2.2–4.8)	0.313
DR ratio	1.2 (–0.1 to 1.4)	0.4 (–0.9 to 0.7)	0.272
End of treatment			
HBsAg (IU/mL) ^c	5,681 (684–16,301)	1,865 (85–5,711)	0.144
HBeAg (+:–)	1:4	1:5	1.000 (chi-square test)
HBcrAg (log U/mL) ^c	4.5 (3.6–4.9)	3.4 (3.0–5.6)	0.297
HBV DNA (log copies/mL) ^c	2.2 (2.2–2.2)	2.2 (2.2–2.7)	0.562
HBV DNA + RNA titers (log copies/mL)	3.4 (2.2–4.4)	2.6 (2.2–3.7)	0.463
DR ratio	1.3 (0.2–2.1)	0.5 (–0.1 to 1.6)	0.201
Sequential therapy (+:–)	3:2	6:0	0.182 (chi-square test)
Duration of treatment (weeks) ^c	31 (24–175)	24 (24–110)	0.291

^a Unless indicated otherwise, the values are given as the number (n) of patients

^b Univariate analysis was performed with Mann-Whitney U test unless indicated otherwise

^c Median (range)

ALT rebound rate was lower than that of HBV DNA rebound, but the rate also increased in a time-dependent manner. The cumulative ALT rebound rate reached 41.7 and 71.1 % at 24 and 48 weeks after discontinuation of NA therapy, respectively (Fig. 1b). Accordingly, it was difficult to discontinue NA therapy safely over a long period. Therefore, to identify factors associated with the safe discontinuation of NA therapy, we performed a number of analyses.

Predictive factors for HBV DNA rebound

To identify those factors associated with HBV DNA rebound, we divided the patients into two groups, namely,

a HBV DNA relapse and a non-relapse group, respectively, based on the timing of HBV DNA rebound. The 22 patients whose HBV DNA titers rebounded within 24 weeks after discontinuation of therapy were included in the relapse group, and the remaining 14 patients were included in the non-relapse group. As shown in Table 2, HBV DNA + RNA titers and the DR ratio after 3 months of treatment were both associated with HBV DNA rebound ($P = 0.015$ and $P = 0.019$, respectively). However, duration of treatment and HBsAg, HBcrAg, and HBV DNA levels at the end of treatment were not significant predictive factors. As shown in Fig. 1a, most HBV DNA rebound occurred within 48 weeks of treatment discontinuation. However, subsequent multivariate

Table 5 Multiple logistic regression for factors associated with HBV DNA rebound within 24 weeks after discontinuation of NA treatment in those patients whose HBV DNA did not become negative at the end of NA treatment

Factors ^a	DNA relapsed (<i>n</i> = 16)	DNA non-relapsed (<i>n</i> = 9)	Univariate <i>P</i> value ^b	Multiple logistic regression ^c	
				<i>P</i> value	OR (95 % CI)
Gender (M:F)	9:7	3:6	0.691 (chi-square test)		
HBV genotype (B:C:ND)	1:14:1	1:7:1	0.817 (chi-square test)		
Before treatment					
Age (years) ^d	41 (25–59)	39 (30–62)	0.777		
Platelet ($\times 10^4/\mu\text{L}$) ^d	17.4 (9.6–28.0)	14.7 (9.6–18.8)	0.183		
ALT (IU/L) ^d	148 (37–780)	118 (22–304)	0.610		
HBsAg (IU/mL) ^d	3,730 (462–1,354,400)	1,384 (66–10,109)	0.267		
HBeAg (+:–)	10:6	3:6	0.226 (chi-square test)		
HBcrAg (log U/mL) ^a	6.4 (4.8–8.8)	6.5 (3.7–7.4)	0.796		
HBV DNA (log copies/mL) ^d	8.4 (3.5–10.1)	7.7 (4.1–9.2)	0.294		
HBV DNA + RNA titers (log copies/mL)	7.9 (3.8–10.0)	7.1 (3.8–9.1)	0.497		
DR ratio	–0.2 (–1.4 to 0.9)	–0.3 (–1.3 to –0.1)	0.359		
After 3 months of treatment					
HBV DNA (log copies/mL) ^d	4.5 (2.4–7.3)	3.8 (3.1–4.6)	0.118		
HBV DNA + RNA titers (log copies/mL)	5.6 (3.7–8.2)	4.7 (2.4–6.2)	0.089	0.068	2.048 (0.949–4.419)
DR ratio	1.0 (–0.6 to 2.7)	0.0 (–0.7 to 1.4)	0.061	0.320	
End of treatment					
HBsAg (IU/mL) ^d	2,306 (481–11,607)	626 (<1.1–9,680)	0.064	0.839	
HBeAg (+:–)	10:6	2:7	0.097 (chi-square test)	0.490	
HBcrAg (log U/mL) ^d	5.1 (3.0–8.2)	5.1 (3.1–6.6)	1.000		
HBV DNA (log copies/mL) ^d	3.9 (2.8–9.2)	4.1 (2.8–7.1)	0.887		
HBV DNA + RNA titers (log copies/mL)	4.2 (3.1–8.7)	3.9 (2.2–6.5)	0.411		
DR ratio	0.3 (–1.0 to 2.8)	–0.4 (–0.8 to 1.2)	0.061	0.171	
Sequential therapy (+:–)	10:6	7:2	0.661 (chi-square test)		
Duration of treatment (weeks) ^d	35 (24–221)	86 (24–304)	0.164		

^a Unless indicated otherwise, the values are given as the number (*n*) of patients

^b Univariate analysis was performed with Mann-Whitney *U* test unless indicated otherwise

^c Multiple logistic regression analysis was performed using variables that were at least marginally significant ($P < 0.10$) in the univariate analysis

^d Median (range)

analysis aimed at identifying factors associated with HBV DNA rebound within 48 weeks after discontinuation of therapy did not identify any independent factors (Table 3).

Because HBV DNA rebound is assumed to be associated with HBV replication activity, HBV DNA and HBV DNA + RNA titers were compared at several points during treatment (Fig. 2). In the non-relapse group, HBV DNA and HBV DNA + RNA titers decreased rapidly, and

no divergence was observed during NA therapy (Fig. 2a). In comparison, while HBV DNA titer also declined rapidly in the relapse group, the reduction in HBV DNA + RNA titers occurred so gradually that the two titers had significantly diverged by 2 months after the start of treatment (Fig. 2b).

Multivariate analysis of HBV DNA rebound was performed using the following candidate factors: HBsAg and HBeAg before nucleotide treatment, HBV DNA, HBV

Table 6 Multiple logistic regression for factors associated with ALT rebound within 24 weeks after discontinuation of NA treatment

Factors ^a	ALT relapsed (<i>n</i> = 13)	ALT non-relapsed (<i>n</i> = 23)	Univariate <i>P</i> value ^b	Multiple logistic regression ^c	
				<i>P</i> value	OR (95 % CI)
Gender (M:F)	7:6	16:7	0.346 (chi-square test)		
HBV genotype (B:C:ND)	0:12:1	2:19:2	0.540 (chi-square test)		
Before treatment					
Age (years) ^d	40 (25–59)	47 (29–66)	0.149		
Platelet ($\times 10^4/\mu\text{L}$) ^d	19.1 (9.6–28.0)	14.8 (9.6–27.5)	0.205		
ALT (IU/L) ^d	35 (37–309)	143 (22–780)	0.795		
HBsAg (IU/mL) ^d	3,730 (462–1,354,400)	2,092 (66–10,109)	0.127		
HBeAg (+:–)	10:3	6:17	0.005 (chi-square test)	0.544	
HBcrAg (log U/mL) ^d	6.4 (5.5–8.8)	5.4 (3.4–7.9)	0.131		
HBV DNA (log copies/mL) ^d	7.7 (5.0–10.1)	7.7 (3.5–9.7)	0.434		
HBV DNA + RNA titers (log copies/mL)	7.8 (5.1–10.0)	7.5 (3.4–9.7)	0.397		
DR ratio	–0.2 (–1.4 to 0.9)	–0.4 (–1.4 to 0.5)	0.336		
After 3 months of treatment					
HBV DNA (log copies/mL) ^d	4.9 (2.4–7.3)	3.7 (2.2–4.8)	0.007	0.228	
HBV DNA + RNA titers (log copies/mL)	5.7 (3.8–8.2)	4.1 (2.2–6.3)	0.004	0.120	
DR ratio	0.9 (–0.2 to 2.7)	0.6 (–0.9 to 1.9)	0.115		
End of treatment					
HBsAg (IU/mL) ^d	2,306 (481–11,607)	824 (<1.1–11,600)	0.019	0.821	
HBeAg (+:–)	10:3	4:19	0.001 (chi-square test)	0.003	13.500 (2.473–73.705)
HBcrAg (log U/mL) ^d	5.4 (3.6–8.2)	4.3 (3.0–6.6)	0.085	0.264	
HBV DNA (log copies/mL) ^d	4.4 (2.2–9.2)	3.3 (2.2–7.1)	0.070	0.380	
HBV DNA + RNA titers (log copies/mL)	4.4 (3.1–8.7)	3.6 (2.2–6.5)	0.004	0.174	
DR ratio	0.4 (–1.0 to 2.8)	0.2 (–0.8 to 1.6)	0.434		
Sequential therapy (+:–)	9:4	17:6	0.527 (chi-square test)		
Duration of treatment (weeks) ^d	29 (24–221)	51 (24–304)	0.169		

^a Unless indicated otherwise, the values are given as the number (*n*) of patients

^b Univariate analysis was performed with Mann-Whitney *U* test unless indicated otherwise

^c Multiple logistic regression analysis was performed using variables that were at least marginally significant ($P < 0.10$) in the univariate analysis

^d Median (range)

DNA + RNA titers, and DR ratio after 3 months of treatment, and HBsAg and HBeAg at the end of treatment. As shown in Table 2, only HBV DNA + RNA titer after 3 months of treatment was identified as an independent predictive factor for the safe discontinuation of NA therapy without HBV DNA rebound ($P = 0.043$, OR 9.474, 95 % CI 1.069–83.957). HBsAg titer at the end of treatment and HBV DNA titer after 3 months of treatment were marginally associated ($P = 0.070$,

$P = 0.074$, respectively). These results suggest that HBV rebound is significantly associated with HBV replication activity during NA treatment.

To analyze the cumulative HBV DNA rebound rate, we divided the 36 subjects into two groups. Cut-off values for assigning patients to the groups were determined by inspection of the receiver operating characteristic (ROC) curve. According to this curve, the best cut-off value of HBV DNA + RNA after 3 months of treatment was

Table 7 Multiple logistic regression for factors associated with ALT rebound within 48 weeks after discontinuation of NA treatment

Factors ^a	ALT relapsed (<i>n</i> = 25)	ALT non-relapsed (<i>n</i> = 11)	Univariate <i>P</i> value ^b	Multiple logistic regression ^c	
				<i>P</i> value	OR (95 % CI)
Gender (M:F)	17:8	6:5	0.475 (chi-square test)		
HBV genotype (B:C:ND)	2:21:2	0:10:1	0.627 (chi-square test)		
Before treatment					
Age (years) ^d	41 (25–64)	45 (29–66)	0.877		
Platelet ($\times 10^4/\mu\text{L}$) ^d	15.6 (9.6–28.0)	16.5 (9.6–27.5)	0.768		
ALT (IU/L) ^d	143 (22–402)	118 (48–780)	0.945		
HBsAg (IU/mL) ^d	2,878 (66–1,354,400)	4,908 (1,172–10,109)	0.490		
HBeAg (+:–)	12:13	4:7	0.718 (chi-square test)		
HBcrAg (log U/mL) ^d	6.3 (4.0–8.8)	5.8 (3.4–7.9)	0.518		
HBV DNA (log copies/mL) ^d	7.7 (3.5–10.1)	7.7 (3.8–9.6)	0.353		
HBV DNA + RNA titers (log copies/mL)	7.8 (3.8–10.0)	7.4 (3.4–9.0)	0.429		
DR ratio	–0.2 (–1.4 to 0.9)	–0.4 (–1.3 to 0.5)	0.201		
After 3 months of treatment					
HBV DNA (log copies/mL) ^d	4.2 (2.2–7.3)	3.6 (2.2–4.6)	0.082	0.106	
HBV DNA + RNA titers (log copies/mL)	4.8 (2.2–8.2)	4.2 (2.2–6.3)	0.271		
DR ratio	0.7 (–0.9 to 2.7)	0.6 (–0.7 to 1.9)	0.757		
End of treatment					
HBsAg (IU/mL) ^d	2,387 (48–16,301)	812 (<1.1–11,600)	0.183		
HBeAg (+:–)	13:12	2:9	0.142 (chi-square test)		
HBcrAg (log U/mL) ^d	5.1 (3.0–8.2)	3.9 (3.0–6.6)	0.291		
HBV DNA (log copies/mL) ^d	3.6 (2.1–9.2)	3.3 (2.2–7.1)	0.782		
HBV DNA + RNA titers (log copies/mL)	3.7 (2.2–8.7)	3.6 (2.2–6.5)	0.655		
DR ratio	0.3 (–1.0 to 2.8)	–0.1 (–0.8 to 1.3)	0.135		
Sequential therapy (+:–)	20:5	6:5	0.224 (chi-square test)		
Duration of treatment (weeks) ^d	31 (24–221)	91 (24–304)	0.028	0.034	1.014 (1.001–1.027)

^a Unless indicated otherwise, the values are given as the number (*n*) of patients

^b Univariate analysis was performed with Mann-Whitney *U* test unless indicated otherwise

^c Multiple logistic regression analysis was performed using variables that were at least marginally significant ($P < 0.10$) in the univariate analysis

^d Median (range)

4.8 log copies/mL (sensitivity 0.733, specificity 0.619, positive predictive value 0.578, negative predictive value 0.765). Seventeen subjects who achieved a titer of <4.8 log copies/mL of HBV DNA + RNA after 3 months of treatment were assigned to group A; the remaining 19 subjects were assigned to group B. The cumulative HBV DNA rebound rate of group A was significantly lower than that of group B at 24 weeks after discontinuation ($P = 0.045$, Fig. 3).

To address potential bias in the study criteria, we analyzed subjects separately depending on whether HBV DNA titer became negative or not at the end of treatment to identify factors associated with HBV DNA rebound. No significant factors for HBV DNA rebound were identified in patients whose HBV DNA titer became negative at the end of NA treatment ($n = 11$) (Table 4). In patients whose HBV DNA did not become negative at the end of NA treatment ($n = 25$), HBV DNA + RNA titer after

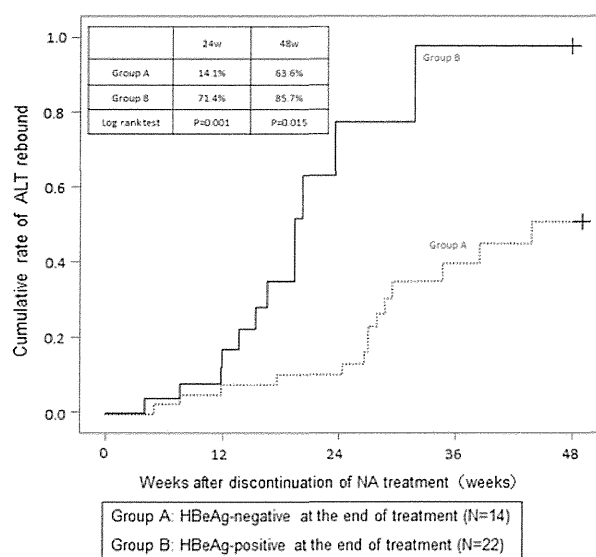


Fig. 4 Cumulative rate of ALT rebound after discontinuation of NA treatment. Fourteen patients who were hepatitis B virus e antigen (HBeAg) negative at the end of treatment were assigned to group A; the other 22 patients, who were positive to HBeAg at the end of treatment, were assigned to group B. The cumulative ALT rebound rate in HBeAg-positive chronic hepatitis B patients was analyzed using the Kaplan–Meier method

3 months of treatment was identified as a marginally significant predictive factor for safe discontinuation of NA therapy without HBV DNA rebound ($P = 0.068$, OR 2.048, 95 % CI 0.949–4.419) (Table 5).

Predictive factors for ALT rebound

To identify predictive factors for ALT rebound, patients were divided into two groups based on the timing of ALT elevation. The 13 patients whose ALT levels rebounded within 24 weeks after discontinuation of therapy were assigned to the ALT relapse group, and the remaining 23 patients were assigned to the ALT non-relapse group. As shown in Table 6, HBeAg presence before treatment, HBV DNA and HBV DNA + RNA titers after 3 months of treatment, and HBeAg presence, HBV DNA + RNA levels, and HBsAg titer at the end of treatment were significantly associated with ALT relapse in the univariate analysis. However, ALT, duration of treatment, and DR ratio at the end of treatment were not significant.

As shown in Table 6, multivariate analysis of ALT rebound was performed using the following candidate factors: HBeAg presence before treatment, HBV DNA and HBV DNA + RNA levels after 3 months of treatment, and HBeAg presence, HBV DNA and DNA + RNA levels, HBcrAg titer, and HBsAg titer at the end of treatment. Only the presence of HBeAg at the end of treatment was identified

as an independent predictive factor for safe discontinuation of NA therapy without ALT rebound ($P = 0.003$, OR 13.500, 95 % CI 2.473–73.705). These results suggest that ALT rebound is also significantly associated with HBV replication activity during NA therapy.

As shown in Fig. 1b, most ALT rebound also occurred within 48 weeks. We performed further analysis to identify factors associated with ALT rebound within 48 weeks after discontinuation of NA therapy. In the univariate analysis, duration of NA treatment was significantly associated with ALT relapse, and HBV DNA level after 3 months of treatment was marginally associated with ALT relapse. Only duration of NA treatment was identified as an independent predictive factor for safe discontinuation of NA therapy without ALT rebound by multivariate analysis ($P = 0.034$, OR 1.014, 95 % CI 1.001–1.027) (Table 7).

To analyze the cumulative ALT rebound rate, the 36 subjects were divided into two groups based on HBeAg presence. Twenty-two subjects who were HBeAg-negative at the end of treatment were assigned to group A, and the remaining 14 subjects were assigned to group B. The cumulative ALT rebound rate of group A was significantly lower than that of group B at 24 and 48 weeks after discontinuation of therapy ($P = 0.001$, $P = 0.015$, respectively; Fig. 4).

To account for potential bias in the study criteria, we analyzed subjects separately based on whether ALT was normalized or not at the end of treatment, with the aim of identifying factors for ALT rebound. In patients whose ALT was normalized at the end of NA treatment ($n = 25$), HBeAg presence before treatment, HBV DNA and HBV DNA + RNA titers after 3 months of treatment, and HBeAg presence at the end of treatment were significantly associated with ALT relapse in the univariate analysis. HBeAg presence at the end of treatment was identified as an independent predictive factor for safe discontinuation of NA therapy without ALT relapse (Table 8). In patients whose ALT was not normalized at the end of NA treatment ($n = 11$), only HBV DNA titer after 3 months of treatment was marginally associated with ALT relapse in the univariate analysis ($P = 0.052$; Table 9).

Predictive factors for ALT rebound in HBeAg-positive patients

Because the cumulative rate of ALT rebound in HBeAg-positive CHB patients was significantly higher than that in HBeAg-negative patients, we focused on the 16 HBeAg-positive patients to identify factors associated with ALT rebound in these patients. As shown in Table 10, only the HBV DNA + RNA titer after 3 months of treatment was significant in the univariate analysis. However, in multivariate analysis, the HBV DNA + RNA titer after

Table 8 Multiple logistic regression for factors associated with HBV DNA rebound within 24 weeks after discontinuation of NA treatment in those patients whose ALT levels had normalized at the end of NA treatment

Factors ^a	ALT relapsed (<i>n</i> = 6)	ALT non-relapsed (<i>n</i> = 19)	Univariate <i>P</i> value ^b	Multiple logistic regression ^c	
				<i>P</i> value	OR (95 % CI)
Gender (M:F)	5:1	12:7	0.073 (chi-square test)	0.073	
HBV genotype (B:C:ND)	0:6:0	2:16:1	0.584 (chi-square test)		
Before treatment					
Age (years) ^d	41 (31–59)	46 (29–66)	0.545		
Platelet ($\times 10^4/\mu\text{L}$) ^d	20.3 (9.6–28.0)	14.7 (9.6–27.5)	0.484		
ALT (IU/L) ^d	161 (62–309)	118 (22–780)	0.750		
HBsAg (IU/mL) ^d	3,573 (462–1,354,400)	2,485 (66–0.109)	0.201		
HBeAg (+:–)	5:1	5:14	0.023 (chi-square test)	0.707	
HBcrAg (log U/mL) ^d	7.1 (6.5–7.8)	5.3 (3.4–7.9)	0.264		
HBV DNA (log copies/mL) ^d	9.1 (6.8–10.0)	8.1 (3.5–9.6)	0.252		
HBV DNA + RNA titers (log copies/mL)	8.3 (6.1–9.7)	7.5 (3.4–9.2)	0.477		
DR ratio	–0.5 (–1.4 to 0.0)	–0.4 (–1.4 to 0.5)	0.503		
After 3 months of treatment					
HBV DNA (log copies/mL) ^d	3.7 (2.4–6.9)	3.7 (2.2–4.8)	0.503		
HBV DNA + RNA titers (log copies/mL)	3.7 (2.4–6.9)	4.2 (2.2–6.3)	0.041	0.413	
DR ratio	1.4 (–0.2 to 1.9)	0.7 (–0.9 to 1.9)	0.111		
End of treatment					
HBsAg (IU/mL) ^d	2,978 (481–16,301)	812 (<1.1–11,600)	0.127		
HBeAg (+:–)	5:1	3:16	0.006 (chi-square test)	0.009	26.667 (2.242–317.147)
HBcrAg (log U/mL) ^d	4.1 (3.6–5.8)	3.7 (3.0–6.6)	0.406		
HBV DNA (log copies/mL) ^d	3.3 (2.2–6.3)	3.4 (2.2–6.1)	0.632		
HBV DNA + RNA titers (log copies/mL)	4.1 (3.2–7.1)	3.6 (2.2–5.7)	0.064	0.444	
DR ratio	0.6 (–1.0 to 2.8)	0.2 (–0.8 to 1.5)	0.340		
Sequential therapy (+:–)	3:3	13:6	0.630 (chi-square test)		
Duration of treatment (weeks) ^d	59 (25–221)	51 (24–304)	0.702		

^a Unless indicated otherwise, the values are given as the number (*n*) of patients

^b Univariate analysis was performed with Mann-Whitney *U* test unless indicated otherwise

^c Multiple logistic regression analysis was performed using variables that were at least marginally significant ($P < 0.10$) in the univariate analysis

^d Median (range)

3 months of treatment was only marginally associated with the safe discontinuation of NA therapy without ALT rebound ($P = 0.050$, OR 8.032, 95 % CI 0.997–64.683). These results suggest that ALT rebound in HBeAg-positive patients might be associated with HBV replication activity during the NA treatment.

To analyze the cumulative ALT rebound rate in HBeAg-positive chronic hepatitis B patients, the 16 subjects were

divided into two groups based on HBV DNA + RNA levels. The cut-off value of HBV DNA + RNA after 3 months of treatment (4.8 log copies/mL) was determined by inspection of the ROC curve (sensitivity 0.833, specificity: 0.889, positive predictive value 0.833, negative predictive value 0.889). Six subjects who achieved <5.0 log copies/mL of HBV DNA + RNA levels after 3 months of treatment were assigned to group A and the remaining

Table 9 Univariate analysis for factors associated with HBV DNA rebound within 24 weeks after discontinuation of NA treatment in the patients in whom ALT levels did not normalize at the end of NA treatment

Factors	ALT relapsed (<i>n</i> = 7)	ALT non-relapsed (<i>n</i> = 4)	Univariate <i>P</i> value
Gender (M:F)	6:1	4:0	1.000 ^b
HBV genotype (B:C:ND)	0:6:1	0:3:1	1.000 ^b
Before treatment			
Age (years) ^a	36 (25–56)	50 (30–64)	0.218
Platelet ($\times 10^4/\mu\text{L}$) ^a	17.0 (13.1–27.5)	16.1 (15.6–16.5)	0.770
ALT (IU/L) ^a	101 (37–303)	148 (114–270)	0.571
HBsAg (IU/mL) ^a	11,113 (1,180–40,967)	1,384 (406–7,016)	0.197
HBeAg (+: –)	5:2	1:3	0.242 ^b
HBcrAg (log U/mL) ^a	5.9 (5.5–8.8)	6.7 (5.0–7.7)	1.000
HBV DNA (log copies/mL) ^a	7.1 (5.0–10.1)	6.7 (5.7–9.7)	0.635
HBV DNA + RNA titers (log copies/mL)	6.9 (5.1–10.0)	6.3 (5.0–9.7)	0.571
DR ratio	–0.1 (–0.2–0.9)	–0.4 (–0.7–0.0)	0.279
After 3 months of treatment			
HBV DNA (log copies/mL) ^a	5.1 (3.8–7.3)	4.2 (2.2–4.4)	0.052
HBV DNA + RNA titers (log copies/mL)	5.7 (3.9–8.2)	4.4 (2.9–6.2)	0.185
DR ratio	0.6 (–0.2–2.7)	0.1 (–0.1–0.6)	0.255
End of treatment			
HBsAg (IU/mL) ^a	4,317 (2,306–11,607)	5,209 (85–5,711)	0.915
HBeAg (+: –)	5:2	1:3	0.242 ^b
HBcrAg (log U/mL) ^a	5.4 (3.6–8.2)	5.6 (4.9–5.9)	1.000
HBV DNA (log copies/mL) ^a	4.4 (2.2–9.2)	2.2 (2.2–7.1)	0.178
HBV DNA + RNA titers (log copies/mL)	4.9 (3.1–8.7)	3.0 (2.2–6.5)	0.131
DR ratio	–0.1 (–0.5–2.7)	0.1 (–0.6–1.6)	0.850
Sequential therapy (+: –)	6:1	4:0	1.000 ^b
Duration of treatment (weeks) ^a	24 (24–36)	44 (24–110)	0.091

ND not determined, DR ratio HBV DNA + RNA titers/HBV DNA

^a Median (range) univariate analysis was performed with Mann-Whitney *U* test

^b Chi-square test

ten subjects were assigned to group B. The cumulative ALT rebound rate of group A was significantly lower than that of group B at 24 and 48 weeks after the discontinuation of therapy ($P = 0.008$, $P = 0.024$, respectively, Fig. 5).

Prediction of ALT rebound after discontinuation of therapy using two extracted factors

To predict successful discontinuation of therapy, we analyzed cumulative ALT rebound by using HBV DNA plus RNA levels at 3 months of NA treatment and existence of HBeAg at the end of treatment. Fourteen subjects who achieved both <4.8 log copies/mL of HBV DNA + RNA levels after 3 months of treatment and negative HBeAg at

the end of treatment were assigned to group A and the remaining 22 subjects were assigned to group B. The cumulative ALT rebound rate of group A was significantly lower than that of group B among all observation periods ($P = 0.046$, Fig. 6).

Discussion

Since the introduction of NAs, chronic hepatitis B progression has been drastically suppressed. NAs strongly suppress HBV replication in human hepatocytes and rapidly decrease serum HBV DNA titers to undetectable levels [30–33]. However, even if HBV DNA is continuously maintained at undetectable levels, it is difficult to

Table 10 Multiple logistic regression for factors associated with ALT rebound within 24 weeks after discontinuation of NA therapy in HBsAg-positive patients ($n = 16$)

Factors ^a	ALT relapsed ($N = 10$)	ALT non-relapsed ($N = 6$)	Univariate P value ^b	Multiple logistic regression ^c	
				P value	OR (95 % CI)
Gender (M:F)	5:5	3:3	0.696 (chi-square test)		
HBV genotype (B:C)	0:10	0:6	1.000 (chi-square test)		
Before treatment					
Age (years) ^d	35 (25–56)	38 (29–47)	0.957		
Platelets ($\times 10^4/\mu\text{L}$) ^d	20.3 (9.6–28.0)	17.3 (14.5–27.5)	0.768		
ALT (IU/L) ^d	148 (37–309)	155 (46–270)	0.958		
HBsAg (IU/mL) ^d	11,113 (462–1,354,400)	6,283 (66–10,109)	0.662		
HBcrAg (log U/mL) ^d	7.1 (5.5–8.8)	7.4 (5.2–7.7)	0.714		
HBV DNA (log copies/mL) ^d	9.1 (6.5–10.1)	8.8 (3.8–9.7)	0.792		
HBV DNA + RNA titers (log copies/mL)	8.3 (6.1–10.0)	8.6 (3.4–9.7)	0.958		
DR ratio	–0.2 (–1.4 to 0.9)	–0.3 (–0.7 to 0.0)	0.776		
After 3 months of treatment					
HBV DNA (log copies/mL) ^d	5.0 (3.5–7.3)	4.1 (2.2–4.4)	0.056	0.897	
HBV DNA + RNA titers (log copies/mL)	5.8 (4.8–8.2)	4.7 (3.7–6.3)	0.011	0.050	8.032 (0.997–64.683)
DR ratio	1.1 (–0.2 to 2.7)	1.1 (–0.6 to 1.9)	0.792		
End of treatment					
HBsAg (IU/mL) ^d	4,736 (823–16,301)	3,523 (48–11,600)	0.529		
HBsAg (+:–)	10:0	4:2	0.125 (chi-square test)		
HBcrAg (log U/mL) ^d	5.6 (4.1–8.2)	5.3 (4.0–6.6)	0.310		
HBV DNA (log copies/mL) ^d	4.4 (2.2–9.2)	3.7 (2.1–6.1)	0.220		
HBV DNA + RNA titers (log copies/mL)	4.9 (3.7–8.7)	3.9 (3.4–5.7)	0.093	0.543	
DR ratio	0.5 (–1.0 to 2.8)	0.2 (–0.8 to 1.6)	0.635		
Sequential therapy (+:–)	7:3	4:2	0.654 (chi-square test)		
Duration of treatment (weeks) ^d	29 (24–221)	119 (24–175)	0.169		

^a Unless indicated otherwise, the values are given as the number (n) of patients

^b Univariate analysis was performed with Mann-Whitney U test unless indicated otherwise

^c Multiple logistic regression analysis was performed using variables that were at least marginally significant ($P < 0.10$) in the univariate analysis

^d Median (range)

completely eliminate HBV from the liver. The goal of NA therapy is therefore to reduce the HBV DNA titer and to induce an inactive state of hepatitis, but, as a result, it is necessary that NA therapy should be continued for a long period of time. As it is well known that long-term treatment with NAs increases the incidence of HBV drug resistance [14], we propose that patients who maintain an inactive state of hepatitis with NA therapy may be able to discontinue the NA therapy to prevent the appearance of drug-

resistant strains. However, as shown in Fig. 1, in our patient cohort, hepatitis was re-activated after discontinuation of the therapy in more than 70 % of the patients who discontinued the NA therapy. Therefore, in this study, we analyzed predictive factors for the safe discontinuation of NA therapy.

After discontinuation of NA therapy, serum HBV DNA titers increased in 91.7 % of our patients within 48 weeks (Fig. 1a). In the multivariate logistic regression, the HBV

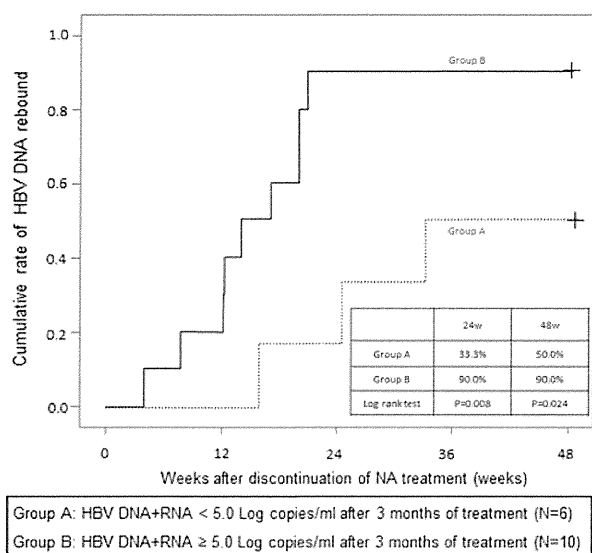


Fig. 5 Cumulative rate of ALT rebound after discontinuation of NA treatment in HBeAg-positive chronic hepatitis B patients. Six patients whose HBV DNA + RNA titers reached <5.0 log copies/mL after 3 months of treatment were assigned to group A; the other ten patients, whose HBV DNA + RNA titers were ≥5.0 log copies/mL after 3 months of treatment, were assigned to group B. The cumulative ALT rebound rate in HBeAg-positive chronic hepatitis B patients was analyzed using the Kaplan–Meier method

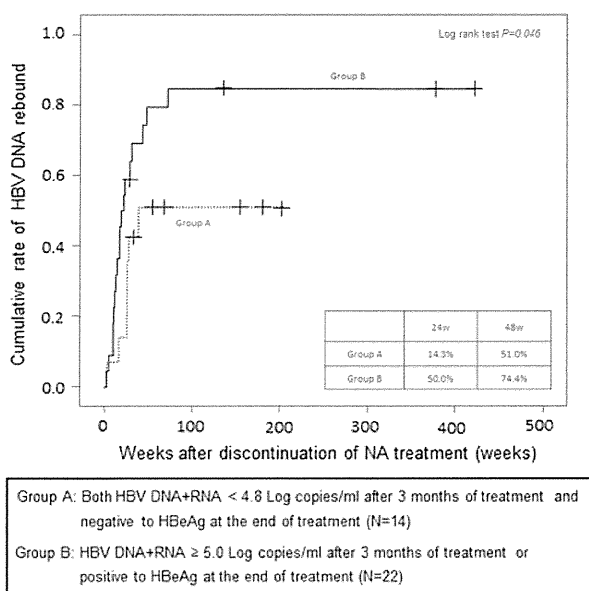


Fig. 6 Cumulative rate of ALT rebound after discontinuation of NA treatment by using combined criteria. The subjects were divided using combined criteria. Fourteen patients whose HBV DNA + RNA titers reached <5.0 log copies/mL after 3 months of treatment and who were HBeAg negative at the end of NA treatment were assigned to group A; the other 22 patients were assigned to group B. The cumulative ALT rebound rate in HBeAg-positive chronic hepatitis B patients was analyzed using the Kaplan–Meier method

DNA + RNA titer after 3 months of treatment was found to be significantly associated with HBV DNA rebound ($P = 0.043$, $OR = 9.474$; Table 2). Two other factors, HBV DNA titer after 3 months of treatment and HBeAg titer at the end of treatment, were marginally associated with HBV DNA rebound ($P = 0.074$, $P = 0.070$, respectively). After 3 months of NA treatment, HBV DNA titers decreased in both the HBV DNA relapse and non-relapse groups, but HBV DNA + RNA levels in the relapse group remained high. NA therapy suppressed the production of mature HBV particles in both groups, but in the HBV DNA relapse group, high HBV replication activity was likely maintained during the treatment, and immature HBV particles associated with HBV RNA genomes were continuously produced and accumulated in hepatocytes. After discontinuation of the treatment, these accumulated immature HBV particles may have been matured and been released from the hepatocytes. Thus, rebound of HBV DNA titers occurred rapidly after the discontinuation of NA therapy.

Although the presence of HBeAg before treatment, HBV DNA and DNA + RNA titers after 3 months of treatment, and the presence of HBeAg, HBeAg titer, and HBV DNA + RNA titer at the end of treatment were all significantly associated with ALT rebound in the univariate analysis, only the presence of HBeAg at the end of

treatment was identified as an independent predictive factor for ALT rebound following multivariate analysis (Table 4). HBeAg is commonly strongly associated with the activity of HBV replication, and HBV DNA levels are high in HBeAg-positive HBV carriers. Thus, HBe seroconversion usually indicates suppression of HBV activity, and the absence of HBeAg is thought to indicate the inactivation of HBV replication.

ALT rebound following the discontinuation of NA therapy was not observed in six of the 16 patients (37.5 %) who were HBeAg-positive at the end of treatment. After examining predictive factors for ALT rebound in these HBeAg-positive patients, only the HBV DNA + RNA titer after 3 months of treatment was identified as an independent predictive factor for ALT rebound in HBeAg-positive patients (Table 6). Although the presence of HBeAg indicates high activities of HBV replication and hepatitis, it is expected to be difficult to discontinue NA therapy without ALT rebound in these patients. However, these results indicate that HBV replication activities vary greatly among individuals and suggest that it might be possible to predict future replication activity based on HBV DNA + RNA titers after 3 months of treatment.

A limitation of this study is the small sample size; as such, selection bias might have affected the internal validity of the study. As it is not common to discontinue

NA therapy in Japan, we were only able to examine 36 subjects in our study. Because HBV-related markers such as HBsAg, HBcrAg, and HBV DNA + RNA titers varied widely among individuals, HBeAg and HBV DNA + RNA titers were only marginally associated with HBV DNA or ALT rebound after the discontinuation of NA therapy. In a previous study, Matsumoto et al. [34] analyzed predictive factors for the safe discontinuation of NA therapy in 126 clinical HBeAg-negative subjects from 12 clinical centers. These authors reported that HBsAg and HBcrAg titers at the end of treatment were predictive factors for the safe discontinuation of therapy. In our study, we also found that the absence of HBeAg at the end of treatment was important for the safe discontinuation of NA therapy, but we found no association between safety and HBsAg or HBcrAg titers. However, while HBsAg and HBcrAg are known to be associated with HBV replication activity, our results involving HBeAg and HBV DNA + RNA titers as important factors for safe discontinuation appear to be consistent.

In our study, the duration of NA therapy was quite short (mean duration was 36 weeks). Similar results might be observed if the NA therapy was extended, but it might be difficult to depress the potential of infected HBV replication with long-term NA therapy. HBsAg titers represent HBV replication in human hepatocytes, and it is difficult to decrease HBsAg levels by NA therapy. Thus, HBV DNA + RNA levels might be an important factor for predicting the HBV DNA or ALT rebounds.

As it may be difficult to discontinue therapy in patients with advanced liver fibrosis, our study subjects were selected based on liver spare capacities. As shown in Fig. 1, ALT rebound is likely to occur in most patients following the discontinuation of NA therapy, and severe hepatitis could occur in some patients. Thus, if the liver spare capacity were low, NA therapy would not be discontinued; the patients in this study were selected solely based on clinical aspects, which may have influenced our interpretation of the results.

In conclusion, HBV replication activity was found to be an important predictor of safe discontinuation of NA therapy. These findings suggest that monitoring of serum HBV DNA + RNA levels would be a useful method for predicting the re-activation of chronic hepatitis B following discontinuation of NA therapy.

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Conflict of interest None to declare.

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Genetic Association of Human Leukocyte Antigens with Chronicity or Resolution of Hepatitis B Infection in Thai Population

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Abstract

Background: Previous studies showed that single nucleotide polymorphisms (SNPs) in the *HLA-DP*, *TCF19* and *EHMT2* genes may affect the chronic hepatitis B (CHB). To predict the degree of risk for chronicity of HBV, this study determined associations with these SNPs.

Methods: The participants for this study were defined into 4 groups; HCC (n = 230), CHB (n = 219), resolved HBV infection (n = 113) and HBV uninfected subjects (n = 123). The *HLA-DP* SNPs (rs3077, rs9277378 and rs3128917), *TCF19* SNP (rs1419881) and *EHMT2* SNP (rs652888) were genotyped.

Results: Due to similar distribution of genotype frequencies in HCC and CHB, we combined these two groups (HBV carriers). The genotype distribution in HBV carriers relative to those who resolved HBV showed that rs3077 and rs9277378 were significantly associated with protective effects against CHB in minor dominant model (OR = 0.45, $p < 0.001$ and OR = 0.47, $p < 0.001$). The other SNPs rs3128917, rs1419881 and rs652888 were not associated with HBV carriers.

Conclusions: Genetic variations of rs3077 and rs9277378, but not rs3128917, rs1419881 and rs652888, were significantly associated with HBV carriers relative to resolved HBV in Thai population.

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Introduction

The hepatitis B virus (HBV) is one of the most common causes of chronic hepatitis B (CHB), liver cirrhosis and hepatocellular carcinoma (HCC). Globally more than 2 billion people have been infected with HBV and 378 million are suffering from chronic hepatitis. Over 600,000 people die each year because of HBV infection. In high prevalence areas such as the central Asian republics, Southeast Asia, Sub-Saharan Africa and the Amazon basin over 8% of the population may be HBV carriers [1]. The main route of HBV infection is vertical transmission from mother to infant and horizontal transmission between children, whereby 90% will develop chronic hepatitis as infants or in early childhood and never clear the virus [1–3]. In contrast, 15% of HBV

infections in adulthood develop into chronic hepatitis with viral persistence.

The frequency of HBV infection which develops into chronic hepatitis depends on the age at which the person is infected [1,2]. However, the factors determining HBV persistence or clearance are not clearly understood [4–6]. Risk factors for viral persistence include the following: virological factors (viral load, genotype, viral gene mutations and co-infection with another virus), host factors (age at infection, gender, immune status and genetic variability) and extrinsic factors (e.g. alcohol consumption and chemotherapy) [7]. Whether viral infection results in acute or chronic infection also depends on cellular immune responses influenced by human leukocyte antigen (*HLA*) class I and II molecules which must present the viral antigens to CD8+ T cells and CD4+ T cells, respectively [8]. The genes encoding *HLA* are the most

polymorphic in the human genome, presumably in order to be able to respond to all potential foreign antigens [9].

Recently, many genome-wide association studies (GWAS) have been performed to seek associations between human genetic variation and the outcome of HBV infection [10–15]. Studies in the Japanese population showed that 11 single nucleotide polymorphisms (SNPs) located within or around the *HLA-DPA1* and *HLA-DPB1* loci are significantly associated with the occurrence of CHB. Of these 11 SNPs, the most strongly associated with the outcome of HBV infection were rs9277535 and rs3128917 in *HLA-DPB1* and rs3077 in *HLA-DPA1* [10].

Thereafter, GWAS studies in the Korean population confirmed the presence of these host factors related to HBV outcome and reported two new SNPs significantly associated with CHB within the *HLA* region, namely rs1419881 and rs652888 in transcription factor 19 (*TCF19*) and euchromatic histone-lysine methyltransferase 2 (*EHMT2*), respectively [16]. *TCF19* (or transcription factor SC1) is a *trans*-activating factor that mainly influences the transcription of genes required for late growth regulation at the G1-S checkpoint and during S phase [17]. *EHMT2* is a histone methyltransferase responsible for mono- and di-methylation of H3K9 (lysine at 9th residue of histone subunit 3) in euchromatin [18], which modifies the conformation of chromatin from its tightly packed form, heterochromatin, and thus influences gene repression or transcriptional silencing [19].

In the present study, we determined associations between the SNPs of *HLA-DPA1* (rs3077), *HLA-DPB1* (rs9277378 and rs3128917), *TCF19* (rs1419881) and *EHMT2* (rs652888) in HBV infected patients compared to those with resolved infections and those who had never been infected.

Materials and Methods

Ethics Statement

This study was approved by the Institutional Review Board of the Faculty of Medicine, University (Bangkok, Thailand) code IRB.455/54. Written informed consent was obtained from each patient and all samples were anonymized.

Sample Collection

All blood samples were negative for hepatitis C virus and human immunodeficiency virus. Subjects were defined into 4 groups: 230 hepatitis B surface antigen (HBsAg)-positive HCC, and 219 CHB who had been HBsAg-positive for at least 6 months were recruited at the King Chulalongkorn Memorial Hospital, whereas patients with resolved HBV and uninfected subjects were from the Thai Red Cross Society and from the north-eastern part of Thailand (age >40 years) which had been screened by Immunoassay (Architect i2000SR, Abbott, USA.) for HBsAg, antibody to hepatitis B surface antigen (anti-HBs) and antibody to hepatitis B core protein (anti-HBc). Of these subjects, 113 were negative for HBsAg but positive for anti-HBc and/or positive for anti-HBs after resolution of infection, while 123 uninfected subjects were all negative for HBsAg, anti-HBc and anti-HBs. All samples in this study were collected from subjects who have lived at the same area in Thailand, suggesting that the genetic background would be balanced between a case and control.

Genotyping assays

DNA was extracted from peripheral blood mononuclear cell using phenol-chloroform DNA extraction. The concentration of DNA was determined by NanoDrop 2000c spectrophotometer (Thermo Scientific, Wilmington, DE). We determined SNPs of *HLA-DPA1* (rs3077), *HLA-DPB1* (rs9277378 and rs3128917), and

the genes *TCF19* (rs1419881) and *EHMT2* (rs652888) by commercial TaqMan PCR assays (Applied Biosystems, USA). In this study we investigated *HLA-DPB1* (rs9277378) because this SNP had a high level of linkage disequilibrium with rs9277535 ($D' = 1.00$, $R^2 = 0.954$) [20] and was clearly detectable by the TaqMan assay rather than rs9277535.

Statistical analyses

In this study, Hardy-Weinberg equilibrium was performed on each SNP. The Chi-square test of independence and Odds Ratio (OR) from two-by-two tables for comparisons between case and control groups was performed using Microsoft Excel. Statistical significance was defined by $P < 0.05$. The calculated of possibility level was established using Chi-square contingency table analysis.

Results

Subjects were defined into 4 groups: group 1) HCC (age = 58.2 ± 12 years, 190/230 (82.6%) male); group 2) CHB (age = 46.6 ± 10 years, 144/219 (65.7%) male); group 3) those with resolved HBV (age = 48.2 ± 6 years, 83/113 (73.5%) male); and group 4) HBV uninfected subjects (age = 46.7 ± 6 years, 73/123 (59.3%) male). The details are given in Table 1. To find the genetic factor associated with chronicity of HBV infection, however, the two groups (group 1 and 2) were combined (designated “HBV carriers”). Indeed, according to the frequencies of minor alleles of the SNPs in the *HLA-DP*, *TCF19* and *EHMT2* genes listed in Table 2, the frequencies of minor alleles of these 5 SNPs in HCC and CHB were similar (data shown in Table S1). The composite HBV carriers group had a minor allele frequency for rs3077 and rs9277378 lower than in groups 3 and 4 (OR = 0.57, 95% CI = 0.42–0.78, $p < 0.001$ and OR = 0.63, 95% CI = 0.47–0.85, $p = 0.008$ for rs3077, OR = 0.59, 95% CI = 0.44–0.81, $p = 0.001$ and OR = 0.56, 95% CI = 0.42–0.75, $p < 0.001$ for rs9277378, respectively). In contrast, the minor allele frequency for rs1419881 in HBV carriers was similar to group 3 (OR = 0.80, 95% CI = 0.60–1.08, $p = 0.142$) but lower than in group 4 (OR = 0.64, 95% CI = 0.48–0.85, $p = 0.002$). Moreover, minor allele frequency for rs3128917 and rs652888 in HBV carriers was comparable to groups 3 and 4 (OR = 1.14, 95% CI = 0.85–1.53, $p = 0.371$ and OR = 1.06, 95% CI = 0.80–1.41, $p = 0.673$ for rs3128917; OR = 1.14, 95% CI = 0.84–1.55, $p = 0.400$ and OR = 1.12, 95% CI = 0.83–1.50, $p = 0.471$ for rs652888, respectively).

The results of Hardy-Weinberg equilibrium analysis of each SNPs were shown in Table 3. All data were over 0.01 ($p > 0.01$), indicating that the frequencies did not deviate from Hardy-Weinberg equilibrium. The genotype distribution in HBV carriers compared to subjects with HBV resolution showed that both rs3077 and rs9277378 were significantly associated with protective effects against CHB in minor dominant model (OR = 0.45, 95% CI = 0.30–0.69, $p < 0.001$ for rs3077 and OR = 0.47, 95% CI = 0.31–0.72, $p < 0.001$ for rs9277378, are described in Table 3), suggesting that major homozygous genotypes were risk factors with the chronicity of HBV. The other SNPs rs3128917, rs1419881 and rs652888 were not associated against HBV carrier status (OR = 1.22, 95% CI = 0.76–1.97, $p = 0.413$ for rs3128917, OR = 0.67, 95% CI = 0.42–1.06, $p = 0.084$ for rs1419881 and OR = 1.31, 95% CI = 0.87–2.00, $p = 0.198$ for rs652888, respectively).

The genotype frequencies for 5 SNPs are shown in Table 3. Comparing HBV carriers with uninfected subjects showed that rs3077, rs9277378 and rs1419881 were all protectively associated with chronic HBV infection (OR = 0.63, 95% CI = 0.42–0.95,