

TABLE I. Clinical Characteristics of Hepatitis B Virus Patients and Healthy Controls

Characteristic	HBV patients (n = 198)		Healthy controls (n = 22)	
	N	Value	N	Value
Age (years) <sup>a</sup>	198	42 (13–71)	22	31.5 (25–39)
Sex (male/female)	198	140/58	22	10/12
Fibrosis (1/2/3/4)	198	58/75/43/22		
Activity (0/1/2/3)	198	2/53/109/34		
miR-122/cel-miR-238	198	0.144 (0.002–1.737)	22	0.02 (0.01–0.04)
miR-22/cel-miR-238	198	0.266 (0.019–1.652)	22	0.02 (0.11–0.49)
HBV DNA (LGE/ml) <sup>a</sup>	181	6.5 (2.6–8.8)		
AST (IU/l) <sup>a</sup>	197	51 (18–982)		
ALT (IU/l) <sup>a</sup>	197	73 (10–1,867)	20	16 (10–23)
γ-GT (IU/l) <sup>a</sup>	189	46 (9–536)		
ALB (g/dl) <sup>a</sup>	196	4.3 (2.6–5.2)		
PLT (×10 <sup>4</sup> /mm <sup>3</sup> ) <sup>a</sup>	197	17.1 (1.0–36.2)		
PT <sup>a</sup>	180	92 (19–146)		
AFP (ng/ml) <sup>a</sup>	186	6.5 (<5.0–8,928.0)		
HBsAg (IU/ml)	176	2,765 (<0.05–1,55,000)		
Anti-HBeAg (±/NA)	176	104/82/12		
HBeAb (±/NA)	176	85/96/17		

NA, not available.

<sup>a</sup>Median (range).

Austin, TX) according to the manufacturer's instructions. RNA was eluted in 80 μl of nuclease free water and reverse transcribed using TaqMan MicroRNA Reverse Transcription Kit (Life Technologies Japan Ltd, Tokyo, Japan). *Caenorhabditis elegans* miR-238 (cel-miR-238) was spiked to each sample as a control for extraction and amplification steps. The reaction mixture contained 5 μl of RNA solution, 2 μl of 10× reverse transcription buffer, 0.2 μl of 100 mM dNTP mixture, 4 μl of 5× RT primer, 0.25 μl of RNase inhibitor, and

7.22 μl of nuclease free water in a total volume of 20 μl. The reaction was performed at 16°C for 30 min followed by 42°C for 30 min. The reaction was terminated by heating the solution at 85°C for 5 min. miR-122 and miR-22 were amplified using primers and probes provided by Applied Biosystems (Foster City, CA) using TaqMan MicroRNA assays according to the manufacturer's instructions. The reaction mixture contained 12.5 μl of 2× Universal PCR Master Mix, 1.25 μl of 20× TaqMan Assay solution, 1 μl of reverse

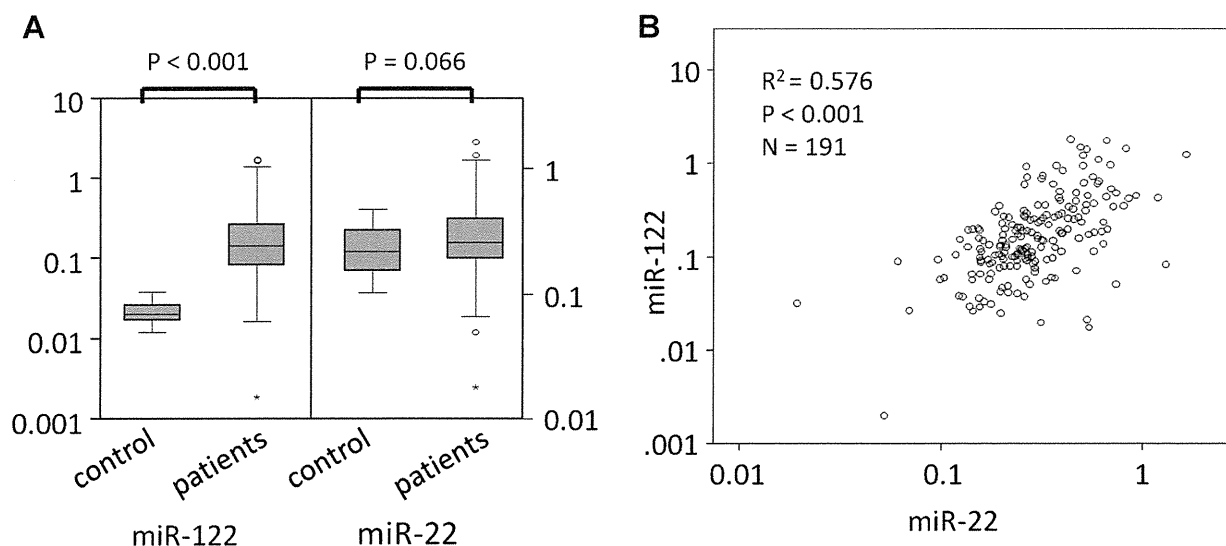


Fig. 1. Detection of miR-122 and miR-22 in patients infected with HBV and in healthy subjects and the relationship between miR-122 and miR-22. **A**: Serum levels of miR-122 and miR-22 in patients infected with HBV (171) and in healthy controls (22). Boxes represent 25–75 percentiles, and horizontal bars represent median values. Statistical analysis was performed using the Mann-Whitney U test. **B**: The relationship between miR-122 and miR-22 was analyzed using the Spearman rank correlation coefficient.

transcription product, and 10.25  $\mu$ l of nuclease free water in a total volume of 25  $\mu$ l. Amplification conditions were 95°C for 10 min followed by 50 denaturing cycles for 15 sec at 95°C and annealing and extension for 60 sec at 60°C in an ABI7300 thermal cycler. For the cel-miR-238 assay, a dilution series using chemically synthesized microRNA was used to generate a standard curve that permitted absolute quantification of molecules. For miR-122 and miR-22, relative abundance was determined using standard curves generated with a dilution series of samples with high serum levels. miR-122 and miR-22 levels were calculated by normalizing based on cel-miR-238 measurement levels.

### Statistical Analysis

Data were analyzed using the Mann-Whitney U test for continuous variables and the chi-squared or Fisher exact test for categorical variables using the R statistics package (<http://www.r-project.org>). Factors associated with high miR-122 and miR-22 levels were analyzed by multiple regression analysis using the rms library. Forward/backward stepwise selection of factors with a  $P$ -value < 0.05 in univariate analysis was used for model selection. The Spearman rank correlation coefficient was used to evaluate the strength of the association between continuous variables.

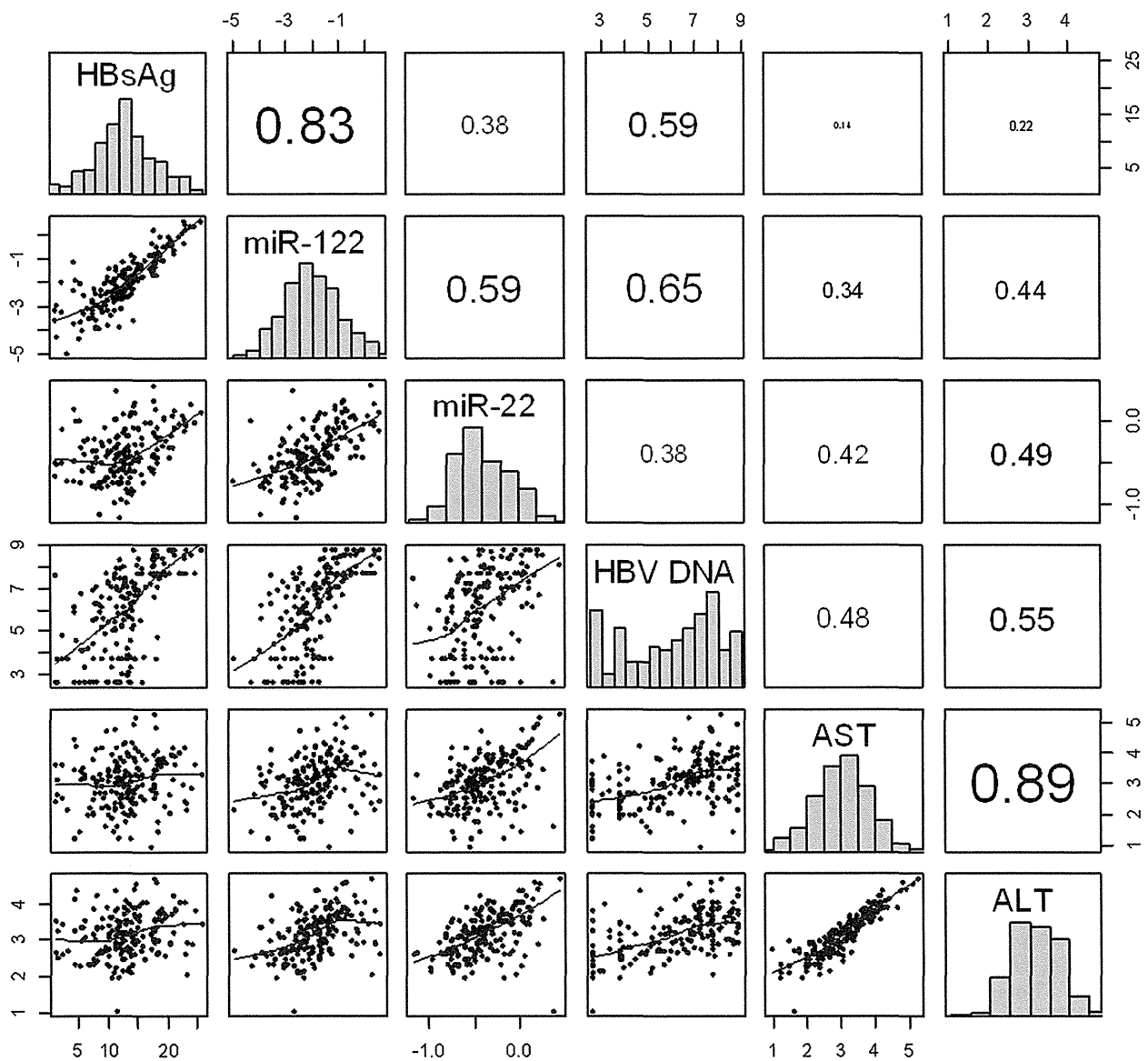


Fig. 2. Pairwise correlations of miR-122 and miR-22 with HBsAg, HBV DNA, ALT, and AST levels. Serum levels of miR-122 and miR-22 were compared with serum HBsAg and HBV DNA titers and with ALT and AST levels using the Spearman rank correlation coefficient.

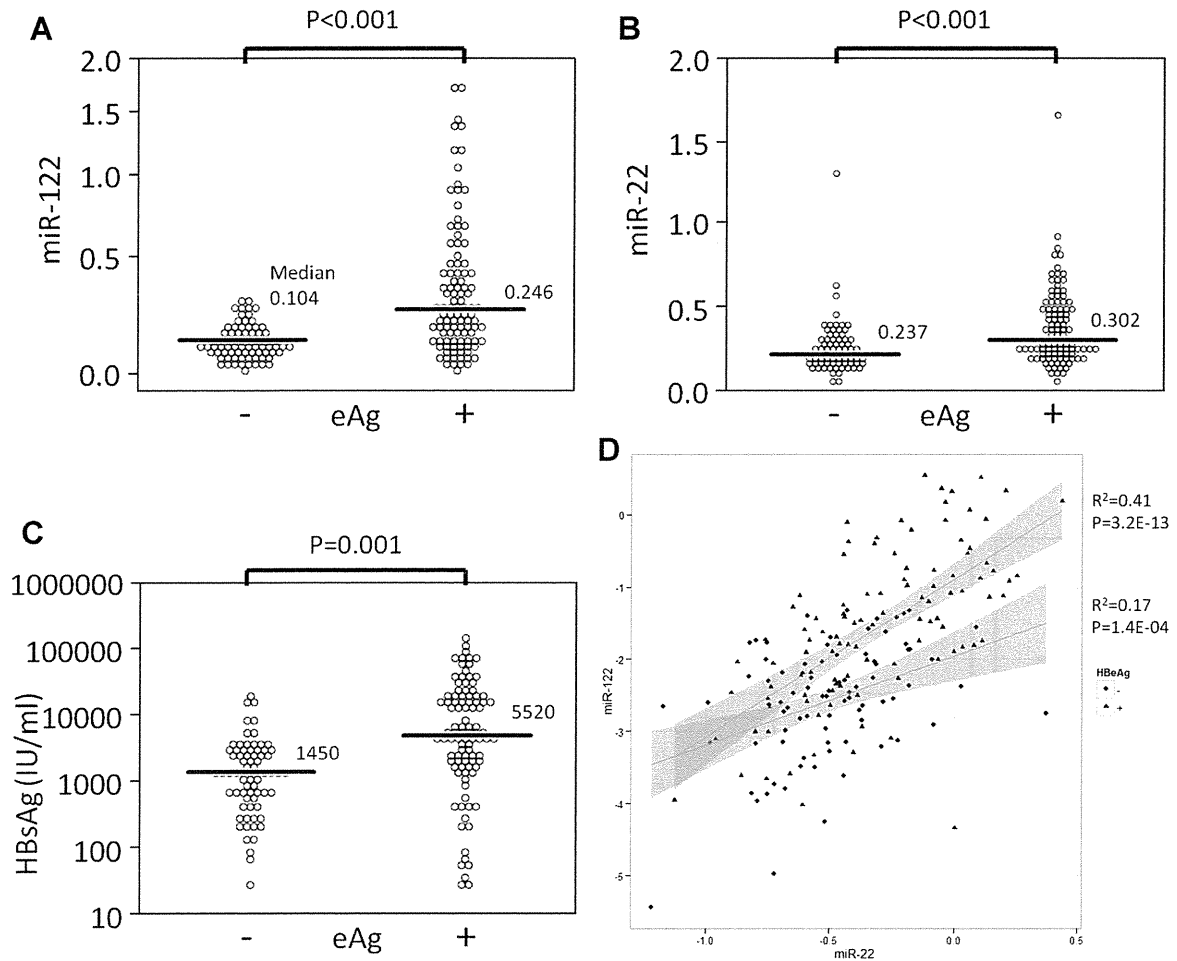


Fig. 3. Comparison of miR-122 and miR-22 with HBsAg levels between patients positive or negative for HBeAg. Serum levels of miR-122 (A), miR-22 (B), and HBsAg (C) were analyzed using the Mann-Whitney U test. Bars indicate median values.

**RESULTS**

**Detection of Circulating miR-122 and miR-22 and Their Correlation**

Both miR-122 and miR-22 were detectable in all HBV patients, and median values were higher than in normal controls (Table I; Fig. 1A,  $P < 0.001$  and  $P = 0.066$ , respectively). miR-122 and miR-22 expression levels were moderately correlated (Fig. 1B,  $R^2 = 0.576$ ,  $P < 0.001$ ).

**miR-122 and miR-22 Levels and Viral Markers**

Relationships between miR-122 and miR-22 levels and HBsAg, HBeAg, and ALT levels were examined (Fig. 2A). There was a strong linear correlation between HBsAg and miR-122 levels ( $R^2 = 0.824$ ,  $P < 0.001$ ). There was also a correlation between HBsAg and miR-22 levels ( $R^2 = 0.394$ ,  $P < 0.001$ ), although the correlation was not as strong as with miR-122. Both miR-122 and miR-22 were also correlated with HBV DNA titers ( $R^2 = 0.694$ ,  $P < 0.001$ ).

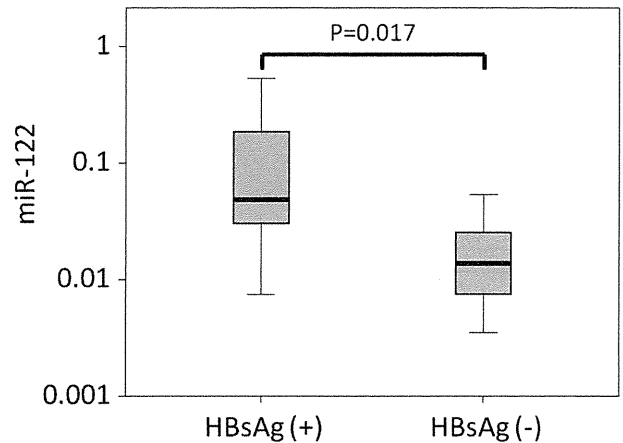


Fig. 4. miR-122 and HBsAg elimination. miR-122 levels before and after HBsAg elimination are shown for patients who became negative for HBsAg ( $n = 13$ ). Bars represent median, minimum, and maximum levels, and boxes represent the 25th and 75th percentiles. Data were analyzed using the Mann-Whitney U test.

and  $R^2 = 0.421$ ,  $P < 0.001$ , respectively) and ALT levels ( $R^2 = 0.498$ ,  $P < 0.001$  and  $R^2 = 0.528$ ,  $P < 0.001$ , respectively). The correlation with ALT was slightly stronger with miR-22 ( $R^2 = 0.528$ ) than with miR-122 ( $R^2 = 0.498$ ). Patients who were positive for HBeAg had elevated levels of both miR-122 and miR-22

(Fig. 3A and B;  $P < 0.001$  and  $P < 0.001$ ) and had higher HBsAg titers (Fig. 3C;  $P = 0.001$ ). The correlation between miR-122 and miR-22 expression was also stronger in HBeAg positive patients (Fig. 3D,  $R^2 = 0.41$ ,  $P = 3.2E-13$ ) compared to HBeAg negative patients ( $R^2 = 0.17$ ,  $P = 1.4E-04$ ).

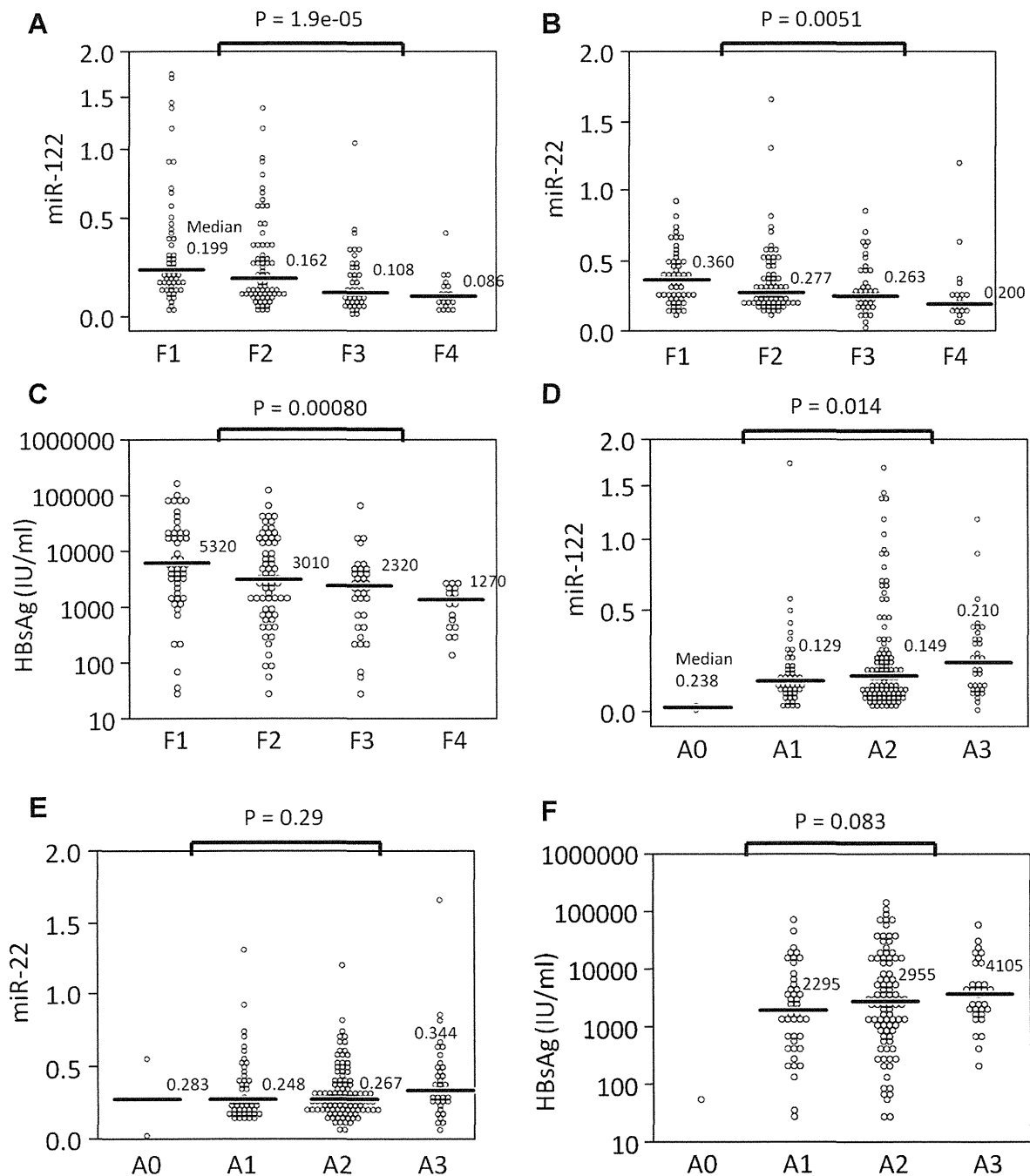


Fig. 5. Stage of fibrosis and histological inflammation activity by liver biopsy and miR-122, miR-22, and HBsAg levels. Serum levels of miR-122, miR-22, and HBsAg were plotted according to the stage of fibrosis (A, B, and C, respectively) and inflammation activity (D, E, and F). Median values are indicated as horizontal bars. Statistical analysis was performed using the Kruskal–Wallis non-parametric analysis of variance test.

### miR-122 Levels in Patients Who Became Negative for HBsAg

To examine if the high miR-122 levels seen in chronic hepatitis B patients with high HBsAg levels result from active HBsAg production or represent individual characteristics that allow high-level HBsAg production, miR-122 levels were measured before and after elimination of HBsAg (observation period 4.5–16.5 years [median 9.0 years]). As shown in Figure 4, miR-122 levels in these patients declined significantly when they became negative for HBsAg ( $P = 0.017$ ).

### miR-122 and miR-22 Levels and Histological Findings

As shown in Figure 5A and B, both miR-122 and miR-22 were observed at progressively lower serum levels at more advanced stages of fibrosis ( $P < 0.001$  and  $P = 0.001$ , respectively). HBsAg levels were also lower in patients with advanced fibrosis (Fig. 5C;  $P = 0.001$ ). In contrast, serum levels of miR-122 and miR-22 were higher in patients with higher inflammatory activity (Fig. 5D and E;  $P = 0.025$  and  $P = 0.170$ ,

respectively), although for miR-22 the difference was not significant. HBsAg levels were also marginally higher in patients with higher inflammatory activity (Fig. 5F;  $P = 0.079$ ).

### Factors Associated with Higher Serum miR-122 and miR-22 Levels

Clinical factors associated with elevated miR-122 and miR-22 levels were examined using multiple linear regression. As shown in Table II, HBsAg was most strongly associated with miR-122 ( $P = 1.1\text{E}-67$ ), whereas serum AST levels were most strongly associated with miR-22 ( $P = 4.7\text{E}-19$ ).

### miR-122 and miR-22 Levels in Patients with Acute HBV Infection, Cirrhosis, and HCC

To examine miR-122 and miR-22 levels in patients with and without HBV infection, miR-122 and miR-22 levels were also measured in the following groups of patients: healthy controls (5), patients with acute (9) or chronic (9) HBV infection, liver cirrhosis (24),

TABLE II. Univariate and Multivariate Regression Analysis of Predictive Factors for MicroRNA-122 and MicroRNA-22 Expression Levels Relative to cel-miR-238

MicroRNA	Variable	Univariate			Multivariate	
		N	Coef.	P	Coef.	P
miR-122	Female	198	0.076	6.6E-01		
	Age	198	-0.030	2.9E-07***	0.007	1.7E-02*
	Fibrosis	198	-0.391	8.9E-07***	-0.143	3.8E-04***
	Activity	198	0.331	4.0E-03**		
	HBsAg	176	0.177	6.7E-46***	0.137	3.3E-32***
	HBeAg (±)	186	1.010	3.5E-11***		
	Anti-HBeAb (±)	181	-0.801	2.5E-07***		
	HBV DNA	181	0.357	2.1E-21***	0.064	1.4E-02*
	AST	197	0.472	6.1E-07***		
	ALT	197	0.816	2.0E-11***	0.281	4.1E-04***
	γ-GT	189	0.187	3.8E-01		
	Total bilirubin	196	-1.020	3.5E-02*	-0.596	9.8E-03**
	ALB	196	0.137	3.2E-02**		
	PT	180	0.020	1.3E-05***		
	AFP	186	0.000	1.5E-01		
	miR-22	198	2.010	1.9E-19***	0.739	4.2E-07***
	miR-22	Female	198	-0.080	1.1E-01	
Age		198	-0.009	3.8E-07***	-0.005	7.4E-04***
Fibrosis		198	-0.085	2.9E-04***		
Activity		198	0.053	1.1E-01		
HBsAg		176	0.023	1.6E-07***	-0.016	2.1E-02*
HBeAg (±)		186	0.192	1.8E-05***		
Anti-HBeAb (±)		181	-0.143	1.5E-03**	-0.044	2.8E-01
HBV DNA		181	0.058	4.6E-07***	-0.025	7.9E-02
AST		197	0.161	1.5E-09***	0.116	2.6E-05***
ALT		197	0.255	9.1E-14***		
γ-GT		189	0.129	3.0E-02*		
Total bilirubin		196	-0.170	2.2E-01		
ALB		196	0.058	1.3E-03**		
PT		180	0.006	1.6E-06***	0.004	2.3E-04***
AFP		186	0.000	2.5E-01		
miR-122		198	0.170	1.9E-19***	0.162	5.5E-06***

Forward/backward stepwise selection was used for model selection.

\* $P < 0.05$ .

\*\* $P < 0.01$ .

\*\*\* $P < 0.001$ .

HCV-related HCC (12), and HBV-related HCC (12). Both miR-122 and miR-22 were significantly elevated in patients with acute or chronic HBV infection compared to other case types (Fig. 6A and B) and were more strongly correlated (Fig. 6C).

## DISCUSSION

In this study, expression levels of miR-122 and miR-22 were correlated with each other, as well as

with markers of HBV infection, including HBsAg and HBV DNA titers (Fig. 2). Circulating levels of both microRNAs were also higher in patients who were positive for HBeAg. Although this suggests that these microRNAs may be up-regulated in cells infected with HBV, it will be necessary to compare serum and liver microRNA level to confirm this, as many other factors may influence circulating microRNA levels.

A notable result of this study is the strong linear association between miR-122 and serum HBsAg levels

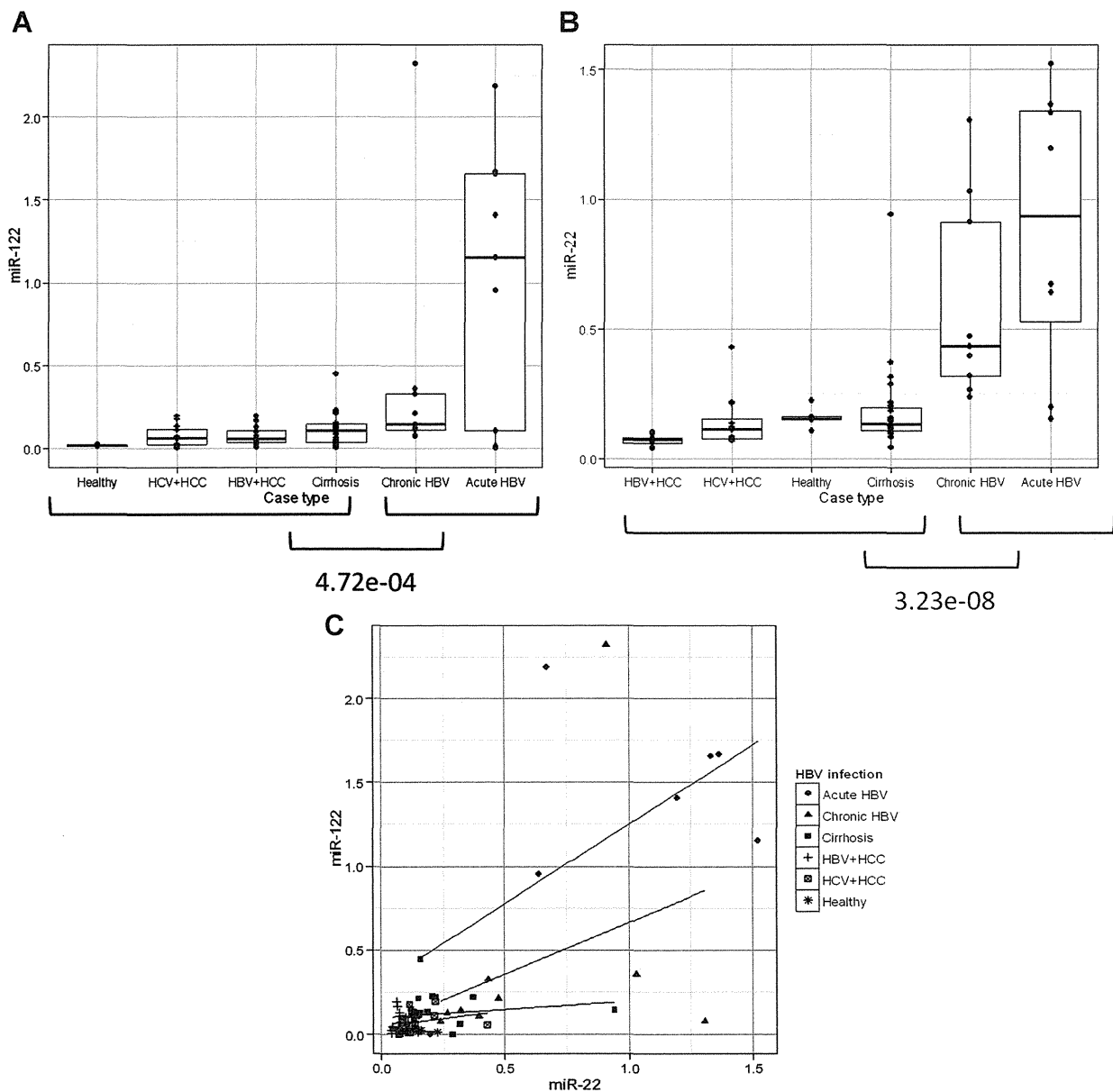


Fig. 6. miR-122 and miR-22 levels by case type. To examine the association of serum miR-122 and miR-22 with HBV infection, expression levels of miR-122 (A) and miR-22 (B) were compared among healthy controls, patients with acute or chronic HBV infection, liver cirrhosis, and HCC associated with either HBV or HCV. Both miR-122 and miR-22 were significantly higher in patients with acute or chronic HBV infection compared to patients with other case types, including patients with HBV-associated HCC. C: miR-122 and miR-22 also appear to be more strongly correlated in patients with acute or chronic HBV infection than in healthy controls or patients with cirrhosis or HCC.

(Table II; Fig. 2A). miR-122 has recently been shown to bind to a highly conserved HBV RNA sequence and negatively regulates viral gene expression and replication [Qiu et al., 2010; Chen et al., 2011]. Loss of miR-122 expression has also been shown to enhance HBV replication indirectly through cyclin G1-modulated p53 activity [Wang et al., 2011]. If miR-122 suppresses HBV replication, an inverse relationship between HBsAg titer and miR-122 levels might be expected, but instead a strong positive correlation was observed in this study. Although the reason for higher levels of miR-122 in patients with high HBsAg production is unclear, the innate immune response in liver cells against HBV replication may potentially induce higher expression of miR-122, which might be reflected in serum levels. Another possibility is that HBV might evade miR-122 suppression by sequestering and excreting miR-122 within the massively over-produced HBsAg particles in serum, in which case serum levels might be proportional to HBsAg levels but may not reflect miR-122 levels in the liver. It will be necessary to compare matched serum and liver miR-122 levels to address this issue.

In contrast to miR-122 levels, miR-22 expression was most strongly correlated with ALT and AST levels (Fig. 2; Table II). As it is known that miR-122 is expressed primarily or exclusively in hepatocytes [Mariana et al., 2002], the higher levels of miR-122 might reflect liver cell damage caused in the course of chronic hepatitis, and the same may be true for miR-22. Tissue-specificity of miR-22 is less clear, although it appears to be strongly expressed in hepatocellular carcinoma cell lines [Landgraf et al., 2007]. However, the fact that the levels of miR-22 are more strongly associated with ALT levels than miR-122 suggests that miR-122 is more likely to be over-expressed in liver cells infected with HBV. In this sense, miR-22 might be a better marker of liver injury than miR-122, although the lack of correlation of miR-22 with inflammatory activity complicates this association. Therefore, miR-122 and miR-22 may reflect different aspects of HBV infection and disease progression. miR-122 and miR-22 were expressed more strongly in acute and chronic HBV infection than in healthy controls or in patients with cirrhosis or HCC, suggesting an association with HBV infection, but notably miR-22 expression was comparatively higher in chronic HBV infection than miR-122 (Fig. 6). Measuring expression levels of one or both of these microRNAs may aid in assessment of disease severity [Waidmann et al., 2012].

In this study, miR-122 and miR-22 levels were both associated with HBV replication and liver injury. This suggests the need for a more systematic approach to examining multiple microRNAs under various chronic hepatitis B conditions and possibly in HBV-associated hepatocellular carcinoma. Further study is needed to establish a system to evaluate various disease conditions or prognoses in chronic HBV infection using microRNA biomarkers. It may also be

of interest to determine the mechanism underlying the strong linear correlation between HBsAg and miR-122 levels to improve understanding of HBV virology.

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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/naikal/research\\_profile/pdf/liver\\_study\\_group\\_e.pdf](http://home.hiroshima-u.ac.jp/naikal/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 g 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  $-80^{\circ}\text{C}$  for further analysis.

Extraction and reverse transcription of HBV nucleic acid

Nucleic acid was extracted from 100  $\mu\text{L}$  of serum by the SMITEST (Genome Science Laboratories, Tokyo, Japan)

**Table 1** Clinical backgrounds of the study cohort

Characteristics <sup>a</sup>	Values
Gender (M:F)	23:13
HBV genotype (B:C:ND)	2:31:3
Age (years) <sup>b</sup>	43 (25–66)
Platelet ( $\times 10^4/\mu\text{L}$ ) <sup>b</sup>	16.1 (9.6–28.0)
ALT (IU/L) <sup>b</sup>	139 (22–780)
HBV DNA (log copies/mL) <sup>b</sup>	6.9 (3.6–8.8)
HBsAg (IU/mL) <sup>b</sup>	3,088 (66–1,354,400)
HBeAg (+:–)	16:20
HBcrAg (log U/mL) <sup>b</sup>	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) <sup>b</sup>	36 (24–304)
Observation period (weeks) <sup>b</sup>	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

<sup>a</sup> Unless indicated otherwise, the values are given as the number (n) of patients

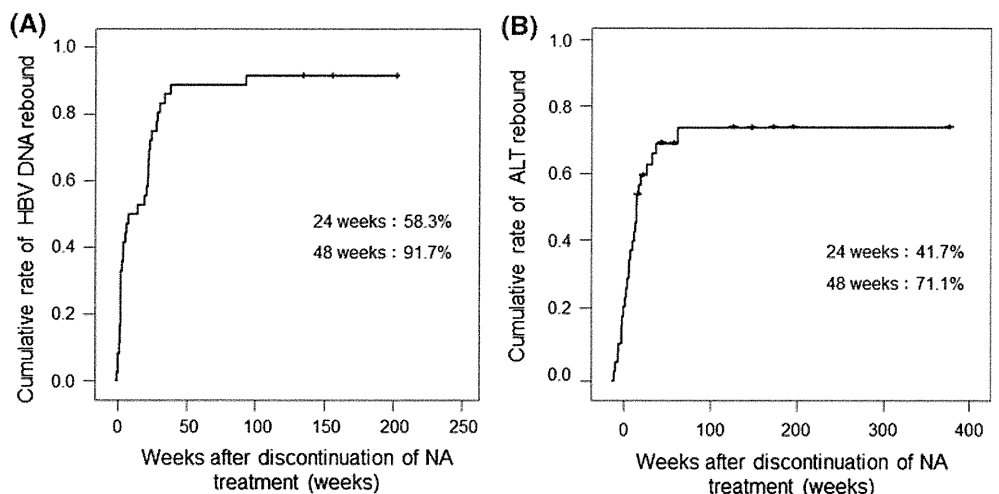
<sup>b</sup> Mean (range)

and dissolved in 20  $\mu\text{L}$  of  $\text{H}_2\text{O}$ . Each extracted solution was divided into two aliquots. An 8.8- $\mu\text{L}$  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  $65^{\circ}\text{C}$  for 5 min. The samples were set on ice for 5 min, then each sample was mixed with 4  $\mu\text{L}$  of  $5\times$  reverse transcription (RT) buffer, 2  $\mu\text{L}$  of 10 mM dNTPs, 2  $\mu\text{L}$  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  $30^{\circ}\text{C}$  for 10 min and  $42^{\circ}\text{C}$  for 60 min, followed by inactivation at  $99^{\circ}\text{C}$  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- $\mu\text{L}$  volume of reaction mixture containing SYBR Green PCR Master Mix (Applied Biosystems), 200 nM of forward primer (5'-TTTGGGGCATGGACATTGAC-3', nucleotides 1893–1912), 200 nM of reverse primer (5'-GGTGAACAATGGTCCG GAGAC-3', nucleotides 2029–2049), and 1  $\mu\text{L}$  of DNA or cDNA solution was prepared. After incubation for 2 min at  $50^{\circ}\text{C}$ , the sample was heated for 10 min at  $95^{\circ}\text{C}$  for denaturing, followed by a PCR cycling program consisting of 40 two-step cycles of 15 s each at  $95^{\circ}\text{C}$  and 60 s at  $60^{\circ}\text{C}$ . 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 <sup>a</sup>	DNA relapsed (n = 21)	DNA non-relapsed (n = 15)	Univariate <i>P</i> value <sup>b</sup>	Multiple logistic regression <sup>c</sup>	
				<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) <sup>d</sup>	41 (25–59)	47 (30–66)	0.252		
Platelet ( $\times 10^4/\mu\text{L}$ ) <sup>d</sup>	17.6 (9.6–28.0)	14.8 (9.6–23.6)	0.104		
ALT (IU/L) <sup>d</sup>	161 (37–780)	114 (22–304)	0.324		
HBsAg (IU/mL) <sup>d</sup>	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) <sup>d</sup>	5.9 (4.8–8.8)	6.2 (3.4–7.9)	0.608		
HBV DNA (log copies/mL) <sup>d</sup>	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) <sup>d</sup>	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) <sup>d</sup>	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) <sup>d</sup>	4.9 (3.0–8.2)	4.2 (3.0–6.6)	0.516		
HBV DNA (log copies/mL) <sup>d</sup>	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) <sup>d</sup>	34 (24–221)	53 (24–304)	0.800		

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

<sup>a</sup> Unless indicated otherwise, the values are given as the number (*n*) of patients

<sup>b</sup> Univariate analysis was performed with Mann-Whitney *U* test unless indicated otherwise

<sup>c</sup> Multiple logistic regression analysis was performed using variables that were at least marginally significant (*P* < 0.10) in the univariate analysis

<sup>d</sup> Median (range)

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

Factors	DNA relapsed ( <i>n</i> = 31)	DNA non-relapsed ( <i>n</i> = 5)	Univariate <i>P</i> value
Gender (M:F)	21:10	2:3	0.328 <sup>b</sup>
HBV genotype (B:C:ND)	2:27:2	0:4:0	0.523 <sup>b</sup>
Before treatment			
Age (years) <sup>a</sup>	41 (25–66)	47 (30–62)	0.749
Platelet ( $\times 10^4/\mu\text{L}$ ) <sup>a</sup>	15.6 (9.6–28.0)	17.3 (14.7–18.8)	0.679
ALT (IU/L) <sup>a</sup>	135 (22–780)	192 (94–296)	0.450
HBsAg (IU/mL) <sup>a</sup>	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) <sup>a</sup>	5.4 (3.4–8.8)	6.8 (5.4–7.9)	0.330
HBV DNA (log copies/mL) <sup>a</sup>	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) <sup>a</sup>	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) <sup>a</sup>	2,195 (48–16,301)	533 (<1.1–9,680)	0.105
HBeAg (+:–)	13:18	1:4	0.628 <sup>b</sup>
HBcrAg (log U/mL) <sup>a</sup>	4.7 (3.0–8.2)	4.6 (3.6–6.6)	0.657
HBV DNA (log copies/mL) <sup>a</sup>	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 <sup>b</sup>
Duration of treatment (weeks) <sup>a</sup>	36 (24–221)	86 (24–304)	0.278

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

<sup>a</sup> Median (range) univariate analysis was performed with Mann-Whitney *U* test

<sup>b</sup> 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.

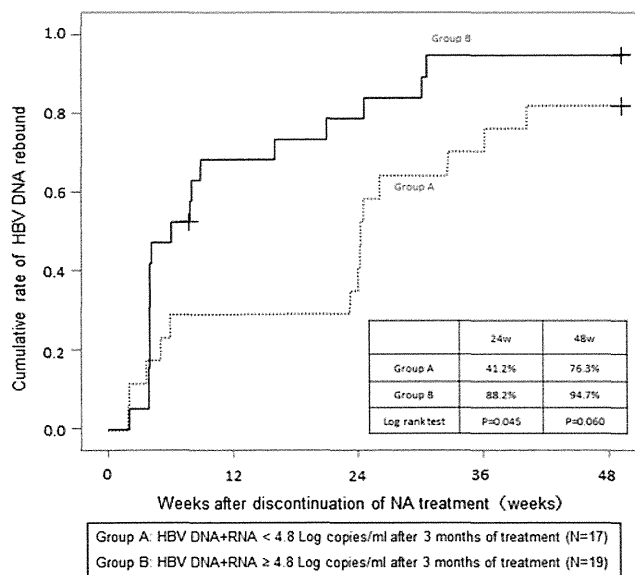
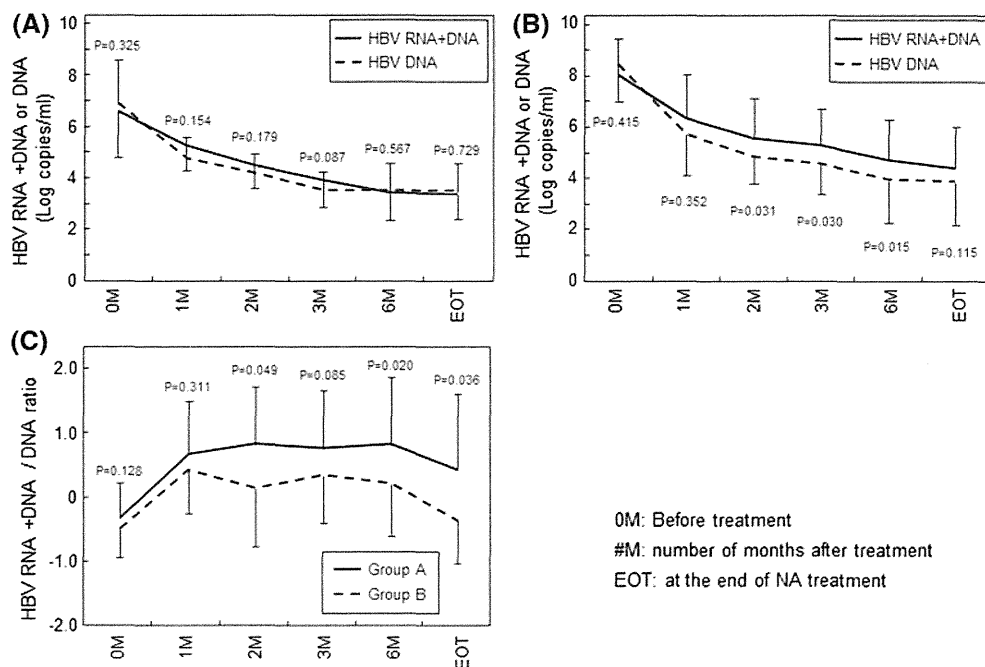
Alanine aminotransferase (ALT) rebound after discontinuation of NA therapy was defined using the following criteria: (1) when ALT reached  $>50$  IU/L after

discontinuation of NA therapy in those patients whose ALT levels had normalized ( $\leq 35$  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 <sup>a</sup>	DNA relapsed ( <i>n</i> = 5)	DNA non-relapsed ( <i>n</i> = 6)	Univariate <i>P</i> value <sup>b</sup>
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) <sup>c</sup>	41 (3–52)	54 (32–66)	0.119
Platelet ( $\times 10^4/\mu\text{L}$ ) <sup>c</sup>	18.8 (11.7–27.5)	14.8 (10.2–23.6)	0.221
ALT (IU/L) <sup>c</sup>	186 (79–303)	95 (48–270)	0.273
HBsAg (IU/mL) <sup>c</sup>	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) <sup>c</sup>	5.4 (5.0–7.8)	4.1 (3.4–7.9)	0.462
HBV DNA (log copies/mL) <sup>c</sup>	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) <sup>c</sup>	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) <sup>c</sup>	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) <sup>c</sup>	4.5 (3.6–4.9)	3.4 (3.0–5.6)	0.297
HBV DNA (log copies/mL) <sup>c</sup>	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) <sup>c</sup>	31 (24–175)	24 (24–110)	0.291

<sup>a</sup> Unless indicated otherwise, the values are given as the number (*n*) of patients

<sup>b</sup> Univariate analysis was performed with Mann-Whitney *U* test unless indicated otherwise

<sup>c</sup> 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 <sup>a</sup>	DNA relapsed (n = 16)	DNA non-relapsed (n = 9)	Univariate <i>P</i> value <sup>b</sup>	Multiple logistic regression <sup>c</sup>	
				<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) <sup>d</sup>	41 (25–59)	39 (30–62)	0.777		
Platelet ( $\times 10^4/\mu\text{L}$ ) <sup>d</sup>	17.4 (9.6–28.0)	14.7 (9.6–18.8)	0.183		
ALT (IU/L) <sup>d</sup>	148 (37–780)	118 (22–304)	0.610		
HBsAg (IU/mL) <sup>d</sup>	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) <sup>a</sup>	6.4 (4.8–8.8)	6.5 (3.7–7.4)	0.796		
HBV DNA (log copies/mL) <sup>d</sup>	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) <sup>d</sup>	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) <sup>d</sup>	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) <sup>d</sup>	5.1 (3.0–8.2)	5.1 (3.1–6.6)	1.000		
HBV DNA (log copies/mL) <sup>d</sup>	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) <sup>d</sup>	35 (24–221)	86 (24–304)	0.164		

<sup>a</sup> Unless indicated otherwise, the values are given as the number (*n*) of patients

<sup>b</sup> Univariate analysis was performed with Mann-Whitney *U* test unless indicated otherwise

<sup>c</sup> Multiple logistic regression analysis was performed using variables that were at least marginally significant (*P* < 0.10) in the univariate analysis

<sup>d</sup> 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 <sup>a</sup>	ALT relapsed ( <i>n</i> = 13)	ALT non-relapsed ( <i>n</i> = 23)	Univariate <i>P</i> value <sup>b</sup>	Multiple logistic regression <sup>c</sup>	
				<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) <sup>d</sup>	40 (25–59)	47 (29–66)	0.149		
Platelet ( $\times 10^4/\mu\text{L}$ ) <sup>d</sup>	19.1 (9.6–28.0)	14.8 (9.6–27.5)	0.205		
ALT (IU/L) <sup>d</sup>	35 (37–309)	143 (22–780)	0.795		
HBsAg (IU/mL) <sup>d</sup>	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) <sup>d</sup>	6.4 (5.5–8.8)	5.4 (3.4–7.9)	0.131		
HBV DNA (log copies/mL) <sup>d</sup>	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) <sup>d</sup>	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) <sup>d</sup>	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) <sup>d</sup>	5.4 (3.6–8.2)	4.3 (3.0–6.6)	0.085	0.264	
HBV DNA (log copies/mL) <sup>d</sup>	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) <sup>d</sup>	29 (24–221)	51 (24–304)	0.169		

<sup>a</sup> Unless indicated otherwise, the values are given as the number (*n*) of patients

<sup>b</sup> Univariate analysis was performed with Mann-Whitney *U* test unless indicated otherwise

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

<sup>d</sup> 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 <sup>a</sup>	ALT relapsed ( <i>n</i> = 25)	ALT non-relapsed ( <i>n</i> = 11)	Univariate <i>P</i> value <sup>b</sup>	Multiple logistic regression <sup>c</sup>	
				<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) <sup>d</sup>	41 (25–64)	45 (29–66)	0.877		
Platelet ( $\times 10^4/\mu\text{L}$ ) <sup>d</sup>	15.6 (9.6–28.0)	16.5 (9.6–27.5)	0.768		
ALT (IU/L) <sup>d</sup>	143 (22–402)	118 (48–780)	0.945		
HBsAg (IU/mL) <sup>d</sup>	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) <sup>d</sup>	6.3 (4.0–8.8)	5.8 (3.4–7.9)	0.518		
HBV DNA (log copies/mL) <sup>d</sup>	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) <sup>d</sup>	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) <sup>d</sup>	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) <sup>d</sup>	5.1 (3.0–8.2)	3.9 (3.0–6.6)	0.291		
HBV DNA (log copies/mL) <sup>d</sup>	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) <sup>d</sup>	31 (24–221)	91 (24–304)	0.028	0.034	1.014 (1.001–1.027)

<sup>a</sup> Unless indicated otherwise, the values are given as the number (*n*) of patients

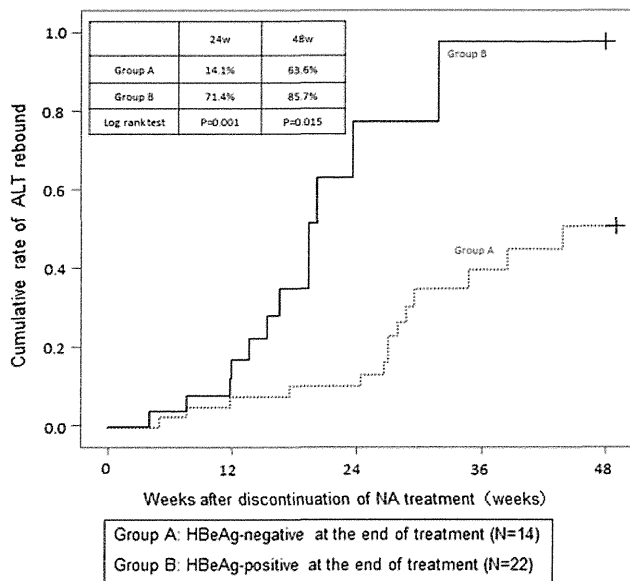
<sup>b</sup> Univariate analysis was performed with Mann-Whitney *U* test unless indicated otherwise

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

<sup>d</sup> 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 <sup>a</sup>	ALT relapsed ( <i>n</i> = 6)	ALT non-relapsed ( <i>n</i> = 19)	Univariate <i>P</i> value <sup>b</sup>	Multiple logistic regression <sup>c</sup>	
				<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) <sup>d</sup>	41 (31–59)	46 (29–66)	0.545		
Platelet ( $\times 10^4/\mu\text{L}$ ) <sup>d</sup>	20.3 (9.6–28.0)	14.7 (9.6–27.5)	0.484		
ALT (IU/L) <sup>d</sup>	161 (62–309)	118 (22–780)	0.750		
HBsAg (IU/mL) <sup>d</sup>	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) <sup>d</sup>	7.1 (6.5–7.8)	5.3 (3.4–7.9)	0.264		
HBV DNA (log copies/mL) <sup>d</sup>	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) <sup>d</sup>	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) <sup>d</sup>	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) <sup>d</sup>	4.1 (3.6–5.8)	3.7 (3.0–6.6)	0.406		
HBV DNA (log copies/mL) <sup>d</sup>	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) <sup>d</sup>	59 (25–221)	51 (24–304)	0.702		

<sup>a</sup> Unless indicated otherwise, the values are given as the number (*n*) of patients

<sup>b</sup> Univariate analysis was performed with Mann-Whitney *U* test unless indicated otherwise

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

<sup>d</sup> 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