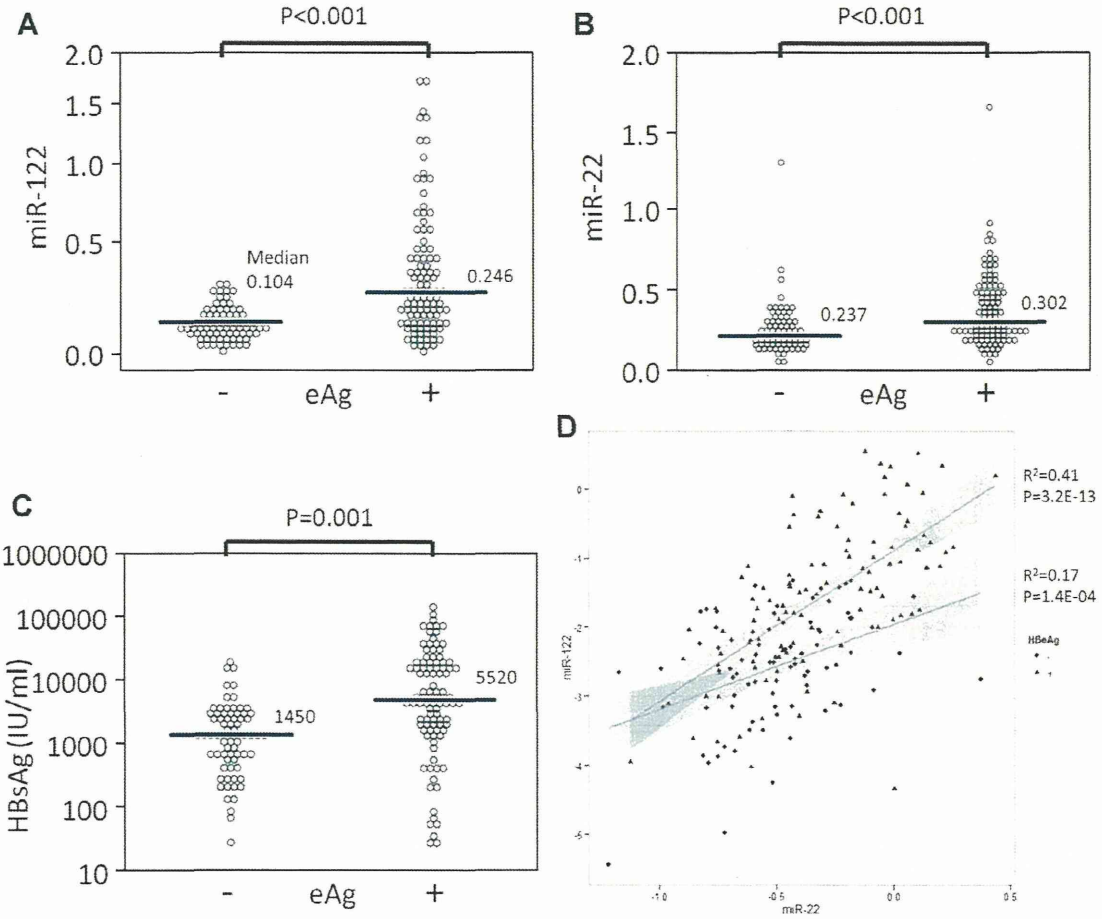

Fig. 3. Comparison of miR-122 and miR-22 with HBsAg levels between patients positive or negative for HBeAg. Serum levels of miR-122 (**A**), miR-22 (**B**), and HBsAg (**C**) were analyzed using the Mann–Whitney U test. Bars indicate median values.

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 ($R^2 = 0.694$, $P < 0.001$

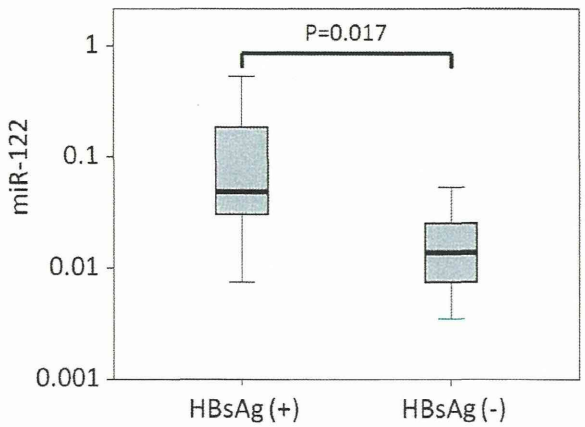

Fig. 4. miR-122 and HBsAg elimination. miR-122 levels before and after HBsAg elimination are shown for patients who became negative for HBsAg (n = 13). Bars represent median, minimum, and maximum levels, and boxes represent the 25th and 75th percentiles. Data were analyzed using the Mann–Whitney U test.

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 ($R^2 = 0.17$, $P = 1.4E{-}04$).

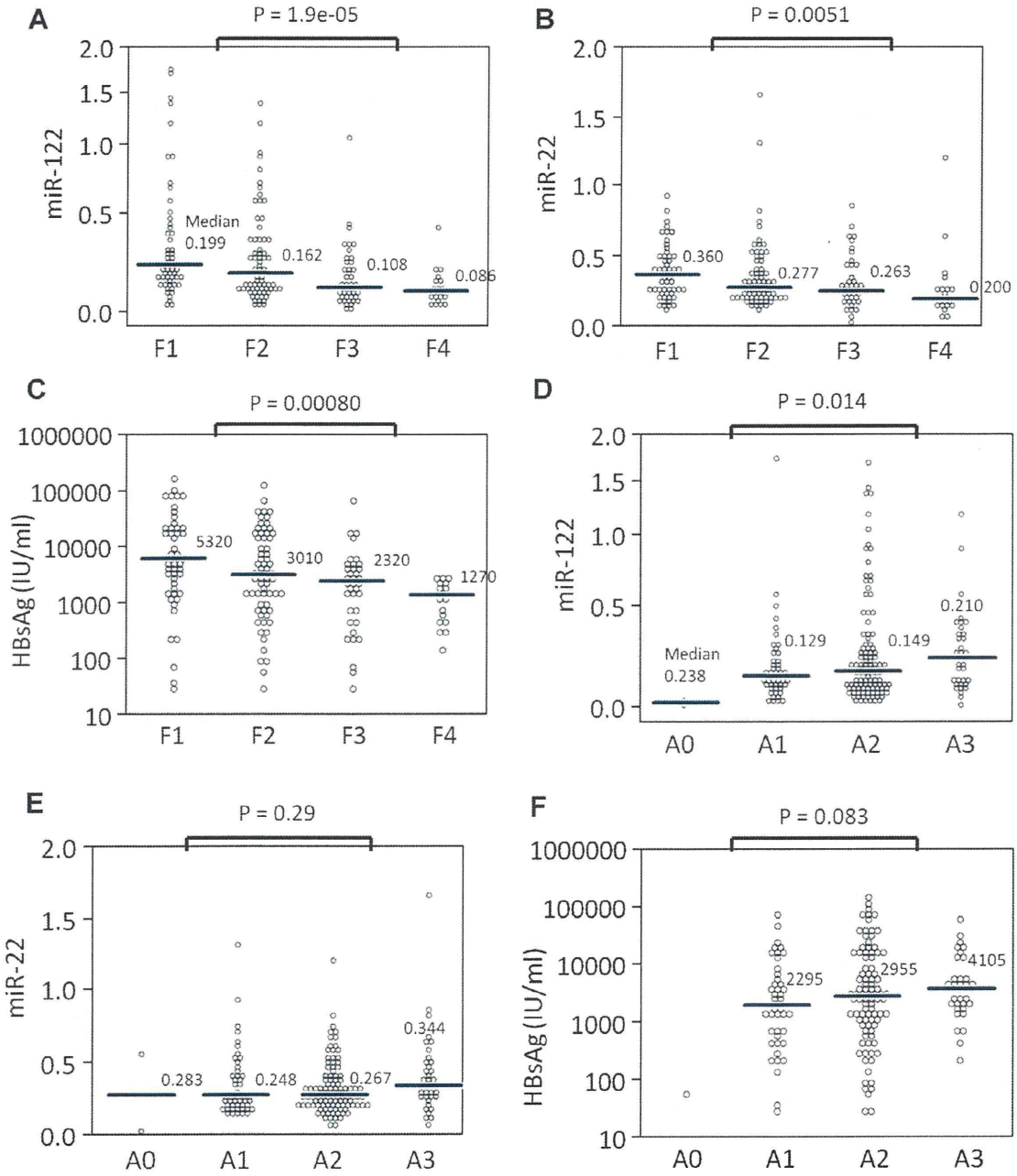

Fig. 5. Stage of fibrosis and histological inflammation activity by liver biopsy and miR-122, miR-22, and HBsAg levels. Serum levels of miR-122, miR-22, and HBsAg were plotted according to the stage of fibrosis (**A**, **B**, and **C**, respectively) and inflammation activity (**D**, **E**, and **F**). 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 ($P = 1.1E{-}67$), whereas serum AST levels were most strongly associated with miR-22 ($P = 4.7E{-}19$).

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

		Univariate			Multivariate	
MicroRNA	Variable	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

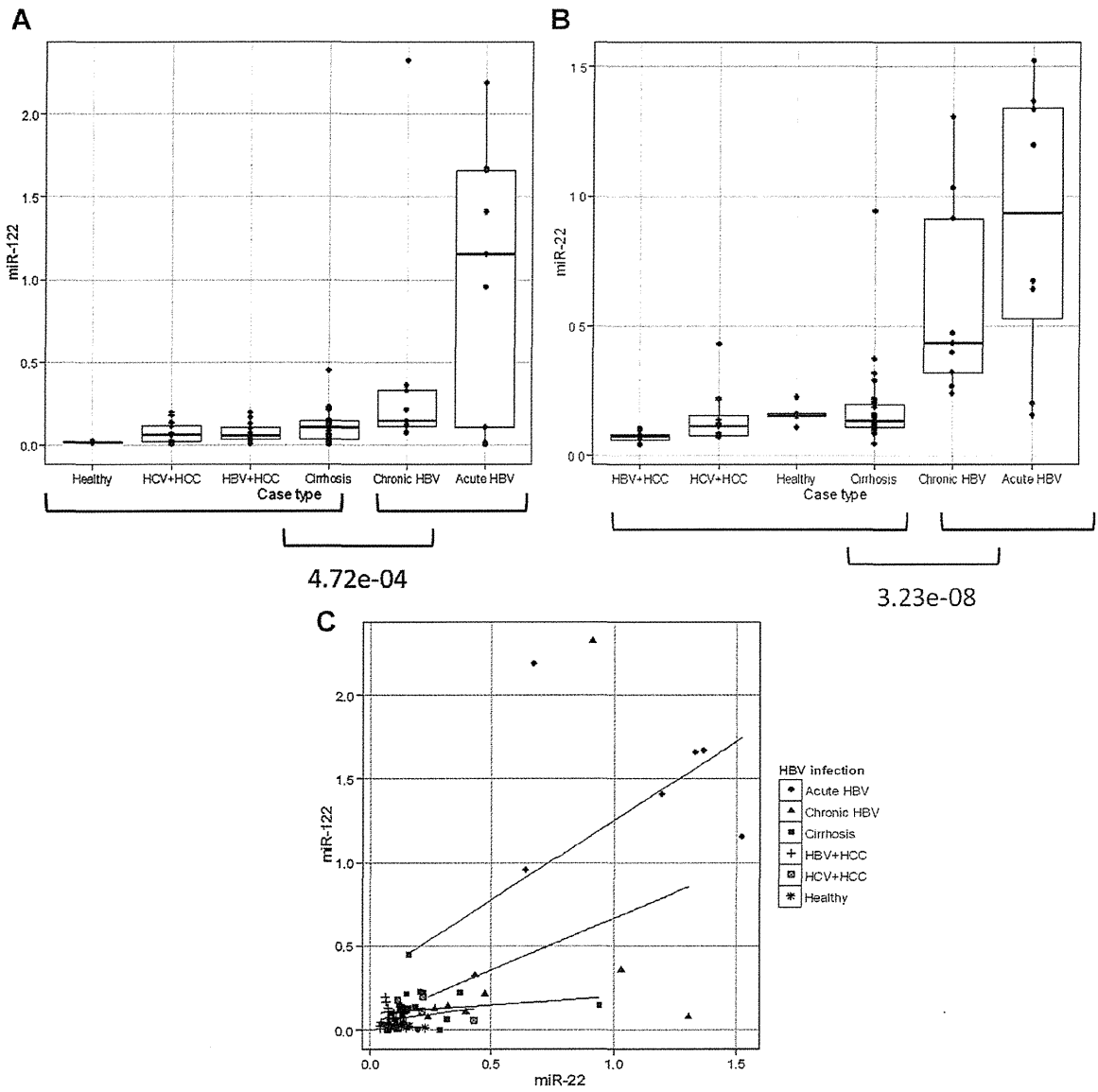

Fig. 6. miR-122 and miR-22 levels by case type. To examine the association of serum miR-122 and miR-22 with HBV infection, expression levels of miR-122 (**A**) and miR-22 (**B**) were compared among healthy controls, patients with acute or chronic HBV infection, liver cirrhosis, and HCC associated with 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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ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

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) 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× 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.

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 ($\times 10^4/\mu L$)[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)

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

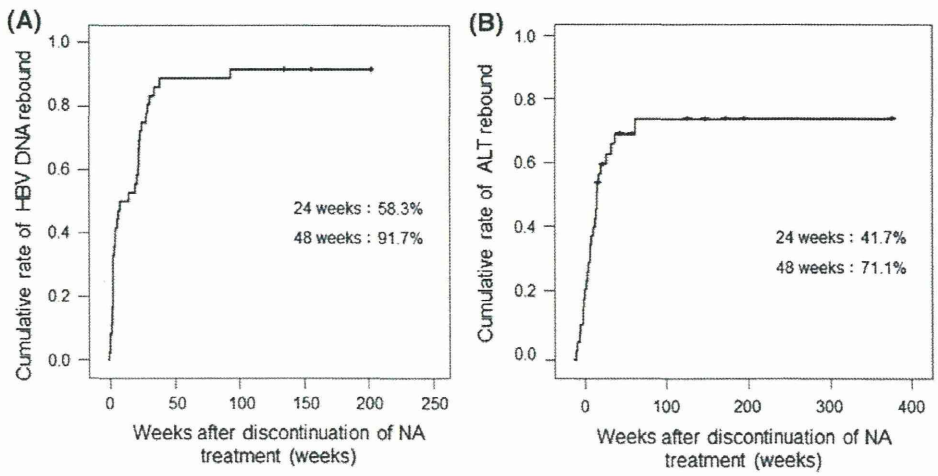

Fig. 1 Cumulative rate of hepatitis B virus (*HBV*) DNA (**a**) and alanine aminotransferase (*ALT*) rebound (**b**) in 36 chronic hepatitis B patients following discontinuation of nucleos(t)ide analogue (*NA*) therapy. Cumulative HBV DNA rebound rate and cumulative ALT rebound rate were analyzed using 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 $\log_{10}$(HBV DNA + RNA) to $\log_{10}$(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 P value[b]	Multiple logistic regression[c]	
				P 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 ($\times 10^4/\mu L$)[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)