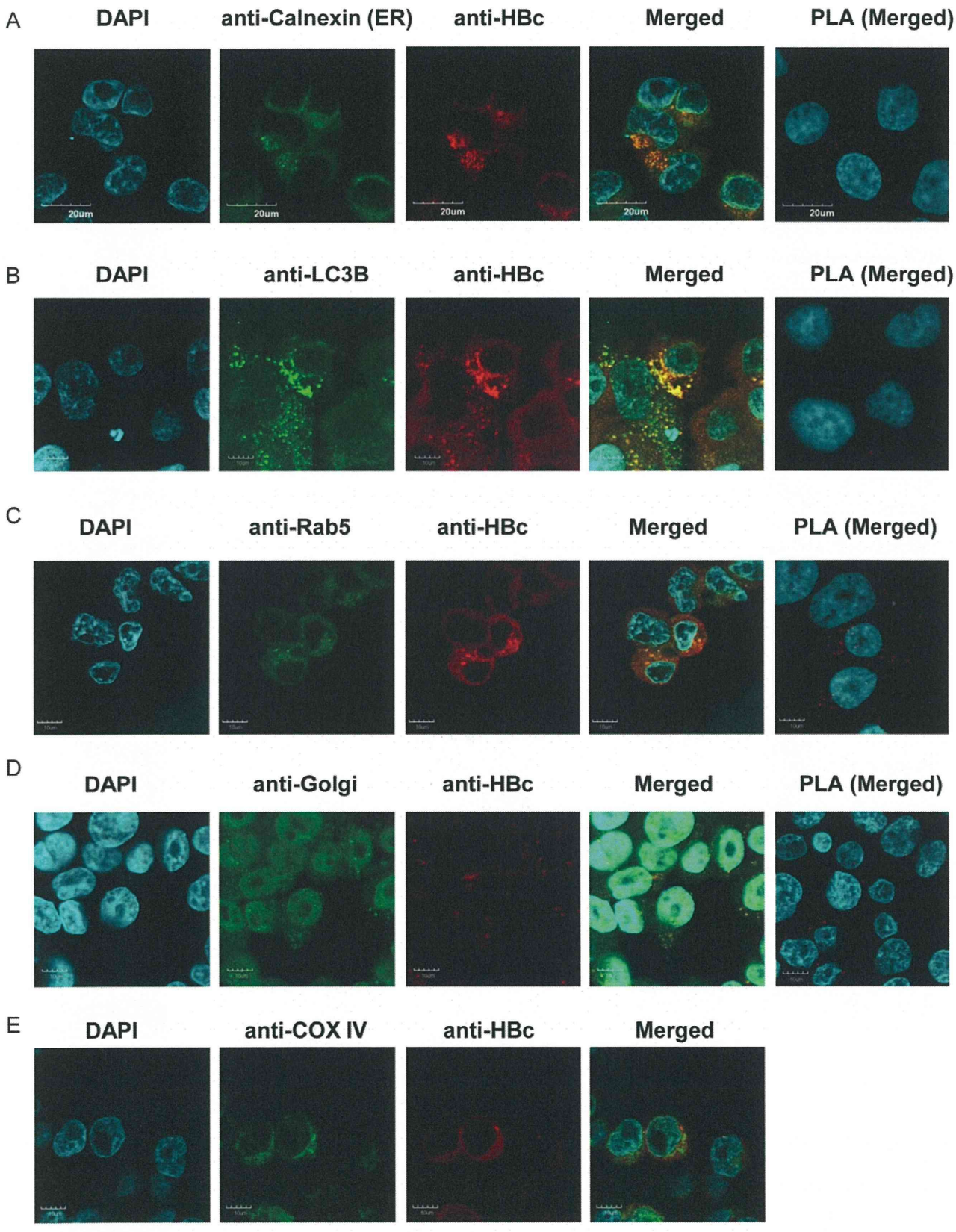


Figure 2. Interactions between HBc and HBs. A) Co-localization of anti-HBc and anti-Calnexin staining by immunocytochemistry and PLA analysis indicate that HBc probably localizes in the ER. Overlap with B) anti-LC3B, C) anti-Rab5, and D) anti-Golgi staining suggests that HBc probably also localizes in autophagosomes, endosomes, and Golgi, respectively. E) However, no overlap was observed with anti-COX IV staining, indicating that HBc probably does not localize at mitochondria.
doi:10.1371/journal.pone.0047490.g002

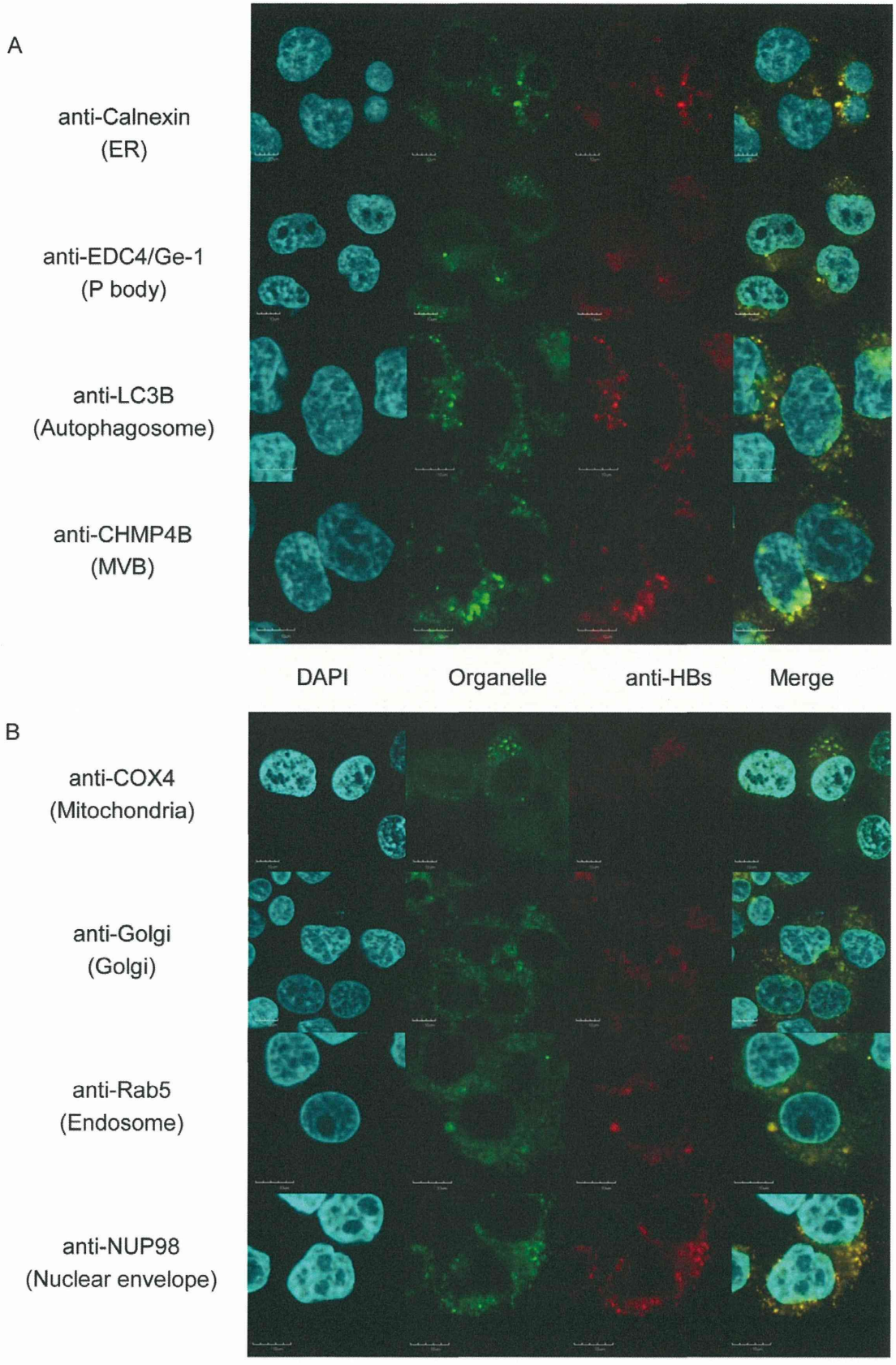
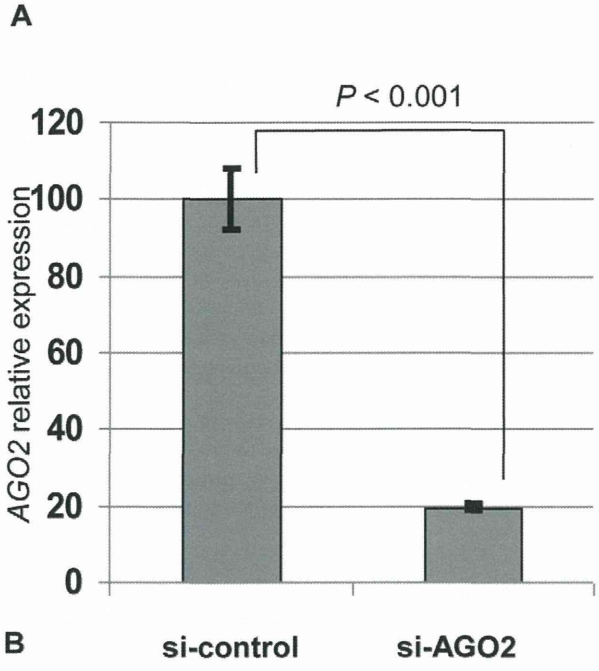
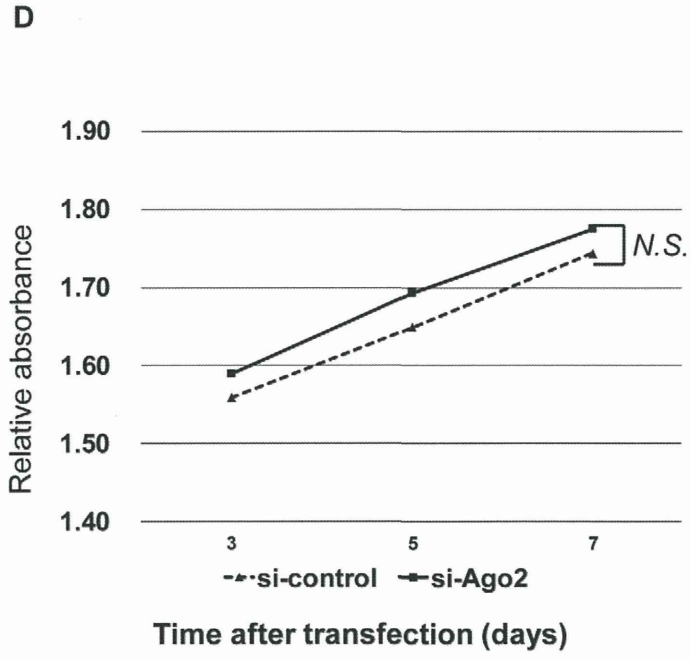
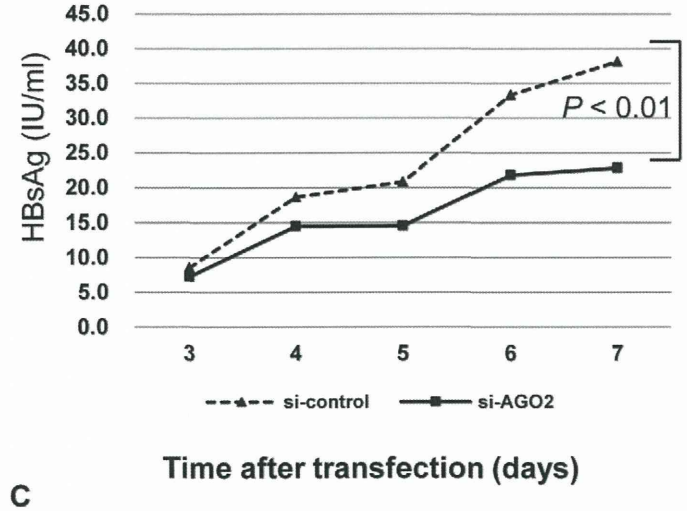


Figure 3. HBsAg localization. A) Co-localization of anti-HBs suggests that HBs localizes in the ER, processing bodies, autophagosomes, and multivesicular bodies, B) and more diffusely in mitochondria, Golgi, endosomes, and at the nuclear envelope.
doi:10.1371/journal.pone.0047490.g003


A
P < 0.001
AGO2 relative expression
120
100
80
60
40
20
0
si-control
si-AGO2


D
1.90
1.80
1.70
1.60
1.50
1.40
Relative absorbance
N.S.
3
5
7
si-control
si-Ago2
Time after transfection (days)


B
45.0
40.0
35.0
30.0
25.0
20.0
15.0
10.0
5.0
0.0
HBsAg (IU/ml)
P < 0.01
3
4
5
6
7
si-control
si-AGO2
Time after transfection (days)

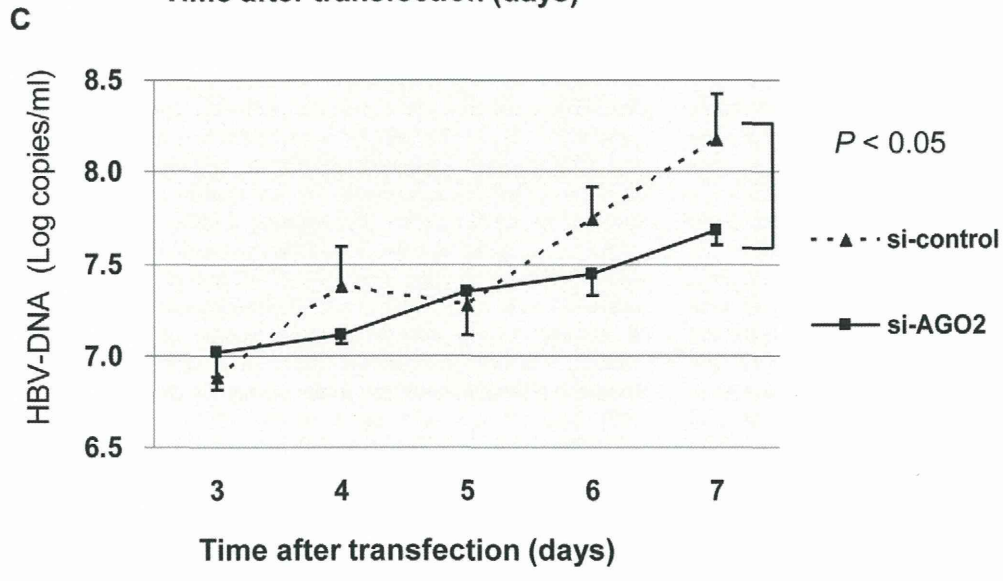

C
8.5
8.0
7.5
7.0
6.5
HBV-DNA (Log copies/ml)
P < 0.05
si-control
si-AGO2
3
4
5
6
7
Time after transfection (days)

Figure 4. siRNA knock down of AGO2 expression. A) Knock down of *AGO2* expression in T23 cells by specific siRNAs for *AGO2* or control siRNAs, confirmed by real-time quantitative RT-PCR analysis. B) Supernatant HBs antigen, and C) HBV-DNA were measured. Both were higher in supernatant of cells transfected with si-control than in cells transfected with si-*AGO2*. D) There was no significant difference in cell viability between cells transfected with si-control compared to those with si-*AGO2*.
doi:10.1371/journal.pone.0047490.g004

controls. Mir-122, miR-22, miR-99a, and miR-125b in particular, were significantly elevated in serum of HBV patients. We also showed that AGO2, an essential component of the RNA silencing complex, co-localizes with both HBc and HBs proteins. HBc and/or HBs localize to several organelles associated with protein synthesis, processing, and degradation, including the ER, Golgi, endosomes, autophagosomes, processing bodies, and multivesicular bodies. Although we expected that depletion of AGO2 would relieve inhibition of HBV replication, we found instead that knockdown of AGO2 appears to inhibit HBV replication, implying that HBV may require AGO2 during its life cycle.

The role of AGO2 is unclear, but viruses have previously been shown to interfere with elements of the RNA-induced gene silencing pathway [17]. HCV core protein and the HIV-1 Tat protein suppress gene silencing by inhibiting Dicer, a cytoplasmic protein that processes pre-microRNA [18]. HBV down-regulates expression of Drosha, the nuclear protein involved in the first step of miRNA processing, which might globally suppress miRNA expression levels [19]. Viruses also influence expression of individual miRNAs [17].

Considering that miR-122 strongly suppresses HBV replication, it is curious that HBV is nonetheless often able to establish chronic infection in the liver [20,21,22]. In the case of HCV, miR-122/AGO2 binding stabilizes the HCV genome and prevents degradation, such that suppression of either miR-122 or AGO2 inhibits HCV replication [23,24,25]. In HBV, we also found that AGO2 knockdown suppresses replication, but Wang et al. demonstrated that anti-sense depletion of miR-122 promoted HBV replication instead of suppressing it [26]. MiR-122 suppresses HBV replication both through direct binding to HBV RNA as well as indirectly through cyclin G1-modulated p53 activity [20,27,28]. HBV might therefore be expected to down-regulate miR-122 levels to evade miR-122 binding and suppression. Wang et al. indeed found that miR-122 levels are significantly decreased in the liver of chronic HBV patient [26], whereas elevated miR-122 levels in the serum have been reported [4,29].

One explanation for the discrepancy between liver and serum miR-122 levels might be that HBV sequesters and expels AGO2-bound miR-122 inside of HBsAg particles, possibly along with other miRNAs that interfere with the viral life cycle. HBV vastly over-produces surface proteins that self-assemble into what were initially thought to be empty particles [30,31], but which may contain miRNAs stably bound to AGO2 [5]. Although HBV is a DNA virus, it relies on reverse transcription via an RNA intermediate in a way similar to retroviruses. Bouttier et al. showed that two unrelated retroviruses, HIV-1 and PFV-1, both require AGO2 interaction with viral RNA for assembly of viral particles. In these viruses, AGO2 is recruited to viral RNA and encapsidated along with it without impairing translation of viral RNA [32]. This suggests that some viruses may take advantage of another function of Argonaute, such as its role in the formation of P-bodies [33], although AGO2 possesses intrinsic exonuclease activity that must be countered. AGO2-mediated gene silencing requires recruitment of GW182 via multiple GW-rich regions [34]. While HIV-1 and PFV-1 encapsidate AGO2, they do not encapsidate GW182, which might provide a means to suppress AGO2 silencing. Some plant viruses use molecular mimicry to inhibit RISC activity by binding to Argonaute proteins through virally encoded WG/GW motifs [35]. Although HBV proteins appear to lack WG/GW motifs, the HBV core protein may use a similar mechanism to disrupt RISC activity while preserving other AGO2 functions. One possibility involves HSP90, a chaperone involved in maintenance of the polymerase/pgRNA complex. HSP90 binds to HBV core protein dimers and is internalized in capsids, but it also binds to the N-terminus of AGO2 and may be required for miRNA loading and targeting to P-bodies [36,37]. Co-localization studies with other proteins and analysis of bound miRNAs may be necessary to elucidate the role of AGO2 in HBV replication, but we speculate that HBV proteins might suppress miRNA activity by binding to and sequestering AGO2 and their bound miRNAs.

Pathway analysis of the predicted targets of the up-regulated serum miRNAs in HBV patients showed that genes involved in phosphatase activity were significantly over-represented. Each of several miRNAs, including miR-122, miR-125b, and miR-99a, was predicted to target a different phosphorylation-associated gene. Regulation of phosphorylation appears to be important in HBV replication, as phosphorylation of the C terminal domain of the HBV core protein is essential for pgRNA packaging and HBV capsid maturation [38]. Phosphorylation also inhibits AGO2 binding of miRNA [39] and is involved in localization to P-bodies [40]. Recent studies have demonstrated that HBV enhances and exploits autophagy via the HBx and small HBs proteins to promote viral DNA replication and envelopment without increasing the rate of protein degradation [41,42]. Sir et al suggested that autophagy may affect dephosphorylation and maturation of the core protein, which protects viral DNA during replication [43]. These reports suggest that HBV exploits multiple cellular pathways in order to establish an intracellular environment conducive to replication.

Although many HBV-associated miRNAs have been reported, the functions of only a few have been examined. MiR-122, miR-125a-5p, miR-199a-3p and miRNA-210 have all been reported to bind to and directly suppress HBV RNA [8,27,44], whereas other miRNAs have been shown to promote or suppress HBV replication indirectly. MiR-1 enhances HBV core promoter activity by up-regulating FXRα, a transcription factor essential for HBV replication [45], whereas miR-141 suppresses HBsAg production in HepG2 cells by down-regulating promoter activity via PPARA [46]. The role of miR-22 and miR-99a in HBV infection is less clear, but both are involved in regulation of cell fate and are implicated in development of HCC. MiR-99a is one of the most highly expressed miRNAs in normal liver tissue and is severely down-regulated in HCC and other cancers, suggesting a role as a tumor suppressor [47]. MiR-99a alters sensitivity to TGF-β activity by suppressing phosphorylation of SMAD3 [48], whereas the HBx protein disrupts TGF-β signaling by shifting from the pSmad3C pathway to the oncogenic pSmad3L pathway [49]. MiR-22 acts as a tumor suppressor by inducing cellular senescence and is down-regulated in several cancer lines [50]. However, over-expression of miR-22 in males is associated with down-regulation of ERα expression, which compromises the protective effect of estrogen and leads to up-regulation of IL-1α in hepatocytes under stress caused by reactive oxygen species, which is another hallmark of HBx interference [51]. Differences in

miRNA levels between hepatic and serum miRNA profiles may reveal miRNAs that play an essential role in the HBV life cycle, with potential application to miRNA-based diagnosis and therapy.

In this study we demonstrated potential interactions between AGO2 and HBc and HBs, but not HBx, in stably transfected HepG2 cells. Suppression of HBV DNA and HBsAg in the supernatant following AGO2 knockdown and the presence of HBV-associated miRNAs in the serum may indicate a dependency on AGO2 during the HBV life cycle.

Supporting Information

Figure S1 Heat map of miRNA expression. Healthy controls and patients with chronic HBV clustered separately based on serum miRNA expression. "Healthy males" and "healthy females" refer to serum mixtures of 12 uninfected males and 10 uninfected females, respectively. "HBV low" and "HBV high" refer to serum mixtures from 10 patients with low (≤42 IU/l) ALT levels and 10 patients with high ALT levels (>42 IU/l), respectively.
(TIF)

Figure S2 Pairwise correlations among pooled serum miRNA samples. Pooled serum samples were collected from 10 healthy males, 10 healthy females, 10 HBV patients with low ALT levels, and 10 HBV patients with high ALT levels. Pairwise correlations in miRNA expression levels among all four pooled samples were strong (>0.90; P<0.001), but correlations were strongest between the healthy male and female samples (0.98) and between the low and high ALT HBV patients (0.98), suggesting that expression of a subset of miRNAs is altered during HBV infection.
(TIF)

Figure S3 Relationship between serum miRNAs and HBsAg levels in chronic HBV patients. Serum levels of several miRNAs were significantly correlated with HBsAg levels in patients with chronic HBV. MiR-99a, miR-122, and miR-125b levels were most strongly correlated with HBsAg levels, with R^2 of 0.69, 0.56, and 0.54, respectively.
(TIF)

Figure S4 Relationship between serum miRNAs and HBV DNA levels in chronic HBV patients. Serum levels of several miRNAs were significantly correlated with HBV DNA levels in patients with chronic HBV. MiR-122, miR-99a, and miR-125b levels were most strongly correlated with HBV DNA levels, with R^2 of 0.44, 0.43, and 0.39, respectively.
(TIF)

Figure S5 Relationship between serum miRNAs and ALT levels in chronic HBV patients. Serum levels of several miRNAs were significantly but somewhat diffusely correlated with ALT levels in patients with chronic HBV. MiR-122 and miR-22 levels were correlated with ALT levels with R^2 of 0.25 and 0.21, respectively.
(TIF)

Figure S6 Relationship between serum miRNAs and presence of HBe antigen in chronic HBV patients. Serum levels of miR-122, miR-99a, miR-720, and miR-125b were significantly elevated in patients positive for the HBe antigen.
(TIF)

Figure S7 Relationship between serum miRNAs and presence of HBe antibody in chronic HBV patients. Serum levels of miR-122, miR-99a, miR-720, and miR-125b were significantly elevated in patients negative for the HBe antibody.
(TIF)

Figure S8 Relationship between individual miRNAs in the liver and serum. Each point represents the level of a specific miRNA in non-cancerous liver tissue relative to serum in the same patient. Red points represent miRNA levels from a patient with chronic HBV, and blue and green points correspond to two different uninfected control subjects. Large red points and labels indicate the subset of miRNAs (Tables 2 and 3) that were significantly elevated in serum of chronic HBV patients. MiRNA expression levels were positively correlated ($R^2 = 0.57$; P<2.1E-16) between liver tissue and serum, suggesting that serum levels broadly reflect miRNA levels in the liver. There appears to be no clear discrepancy between liver and serum miRNA levels in the HBV-infected patient compared to the two uninfected patients.
(TIF)

Figure S9 Subcellular localization of HBx analyzed by immunocytochemistry. HBx localized non-specifically in the nucleus and cytoplasm, but we were unable to verify the sub-cellular location. Anti-Rab5 staining for endosomes is shown for illustration, but results were similar using antibodies against other compartments.
(TIF)

Table S1 Antibodies used for immunocytochemistry.
(DOC)

Table S2 Significantly up- or down-regulated miRNAs in liver samples from an HBV-infected patient compared to two non-HBV-infected patients.
(DOC)

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Author Contributions

Conceived and designed the experiments: KC CNH SA MT DM HAB HO NH. Performed the experiments: MT DM H. Abe NH MI SY H. Aikata TK YK RA KC. Analyzed the data: CNH SA MT DM HO KC. Contributed reagents/materials/analysis tools: CNH SA MT DM KC. Wrote the paper: CNH SA MT DM KC. Clinical data: KC MT DM HAB NH MI ST HAI TK YK WO. Obtained funding: KC MT DM. Critical review of the manuscript: CNH SA MT DM RA HAB HO NH MI ST HAI TK YK WO KC.

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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 ($\times 10^4$/μL)[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

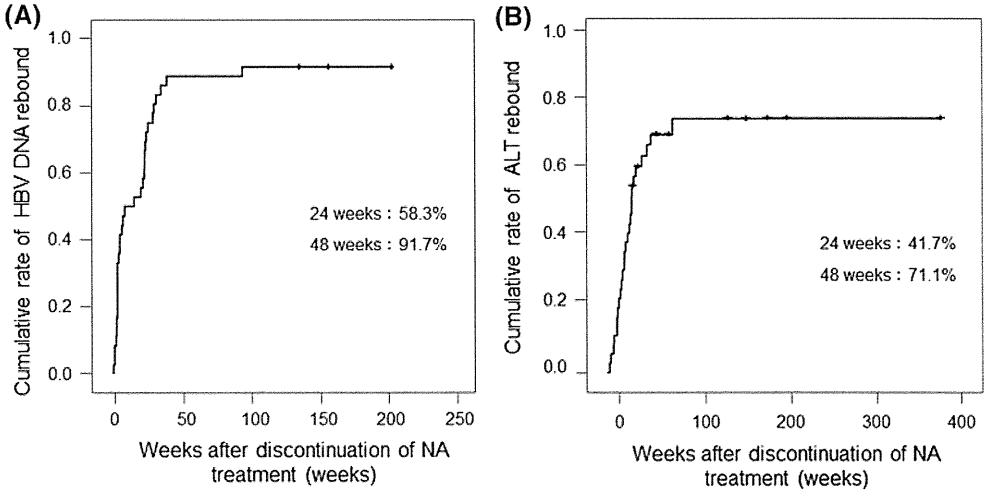

Fig. 1 Cumulative rate of hepatitis B virus (*HBV*) DNA (**a**) and alanine aminotransferase (*ALT*) rebound (**b**) in 36 chronic hepatitis B patients following discontinuation of nucleos(t)ide analogue (*NA*) therapy. Cumulative HBV DNA rebound rate and cumulative ALT rebound rate were analyzed using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

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

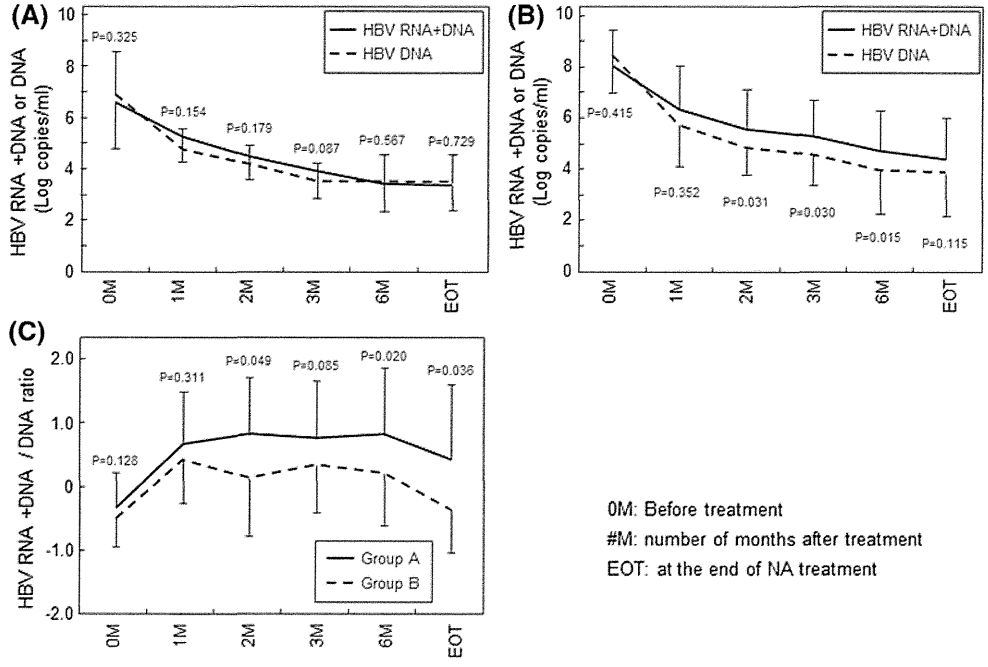

Fig. 2 Change in HBV DNA and HBV DNA + RNA titers during NA therapy. **a**, **b** HBV DNA + RNA titers and HBV DNA titers were compared at each time point for the DNA non-relapse group (**a**) and DNA relapse group (**b**). **c** Changes in the HBV RNA + DNA/HBV DNA ratio were compared with each group.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

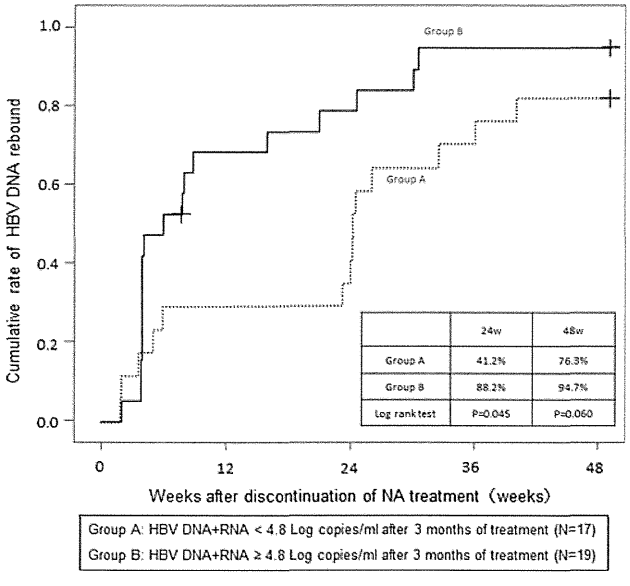

Fig. 3 Cumulative rate of HBV DNA rebound after discontinuation of NA treatment. Seventeen patients whose HBV DNA + RNA titers reached <4.8 log copies/mL after 3 months of treatment, were assigned to group A; the other 19 patients, whose HBV DNA + RNA titers were ≥4.8 log copies/mL after 3 months of 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 ($\times 10^4/\mu L$)[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 ($n = 16$)	DNA non-relapsed ($n = 9$)	Univariate P value[b]	Multiple logistic regression[c]	
				P 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 ($\times 10^4/\mu L$)[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 ($n = 13$)	ALT non-relapsed ($n = 23$)	Univariate P value[b]	Multiple logistic regression[c]	
				P 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 ($\times 10^4/\mu L$)[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 ($n = 25$)	ALT non-relapsed ($n = 11$)	Univariate P value[b]	Multiple logistic regression[c]	
				P 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 ($\times 10^4/\mu L$)[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

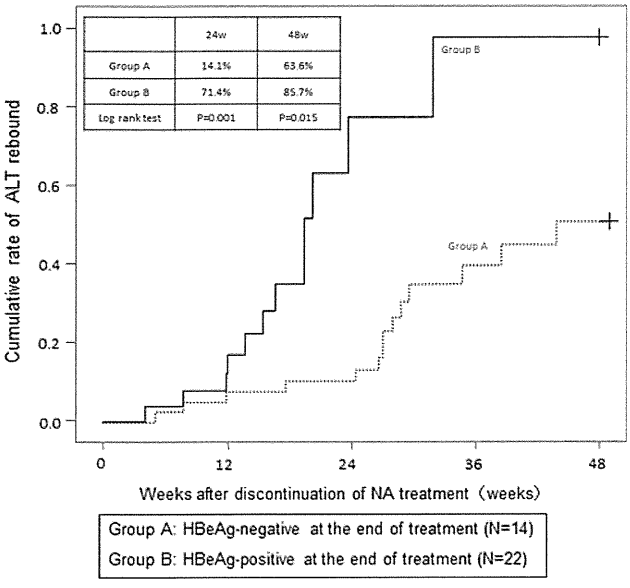

Fig. 4 Cumulative rate of ALT rebound after discontinuation of NA treatment. Fourteen patients who were hepatitis B virus e antigen (*HBeAg*) negative at the end of treatment were assigned to group A; the other 22 patients, who were positive to HBeAg at the end of treatment, were assigned to group B. 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 thanthat 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 ($n = 6$)	ALT non-relapsed ($n = 19$)	Univariate P value[b]	Multiple logistic regression[c]	
				P 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 ($\times 10^4/\mu L$)[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 ($n = 7$)	ALT non-relapsed ($n = 4$)	Univariate P 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 ($\times 10^4/\mu L$)[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 HBeAg-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 ($\times 10^4/\mu L$)[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		
HBeAg (+:−)	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