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

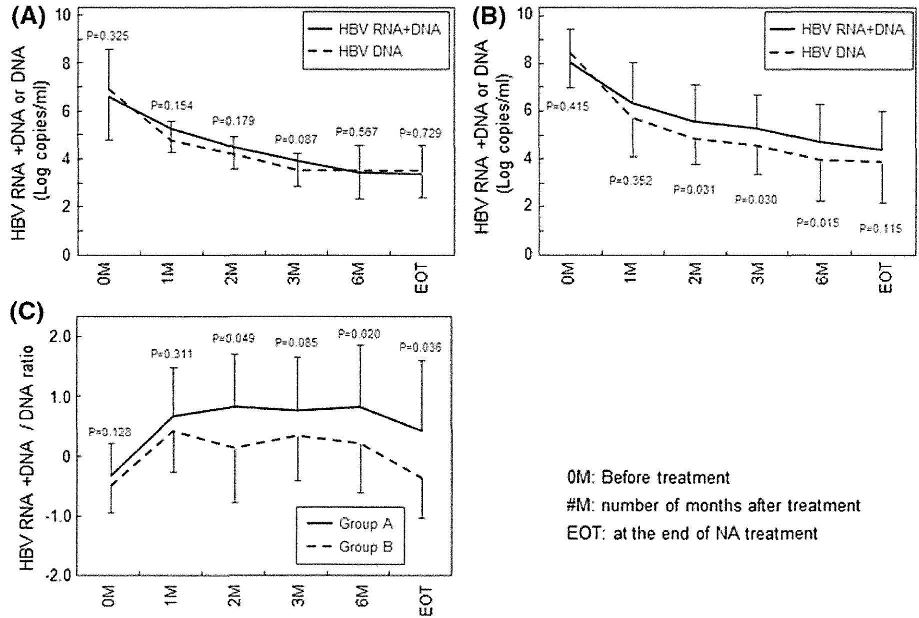
The rebound of HBV DNA after discontinuation of NA therapy was determined based on two criteria: (1) when the HBV DNA reached >4.0 log copies/mL after discontinuation of NA therapy in patients whose HBV DNA titers became negative (<2.6 log copies/mL) at the end of NA therapy; (2) when the HBV DNA increased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

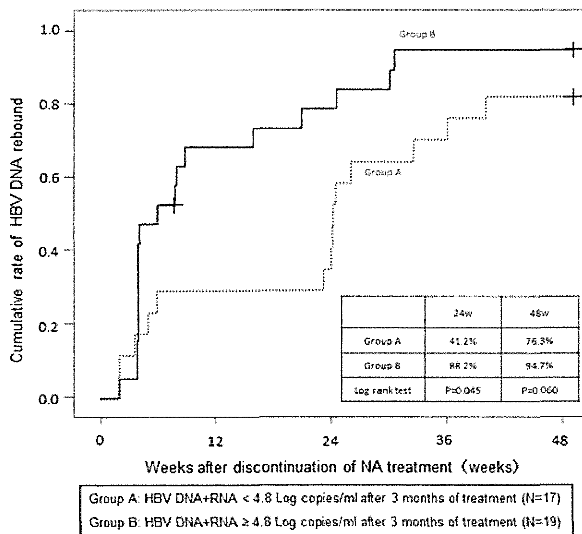
Alanine aminotransferase (ALT) rebound after discontinuation of NA therapy was defined using the following criteria: (1) when ALT reached >50 IU/L after discontinuation of NA therapy in those patients whose ALT levels had normalized (≤35 IU/L) at the end of NA therapy; (2) when ALT increased by >80 IU/L (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





	24w	48w
Group A	41.2%	76.3%
Group B	88.2%	94.7%
Log rank test	P=0.045	P=0.060

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

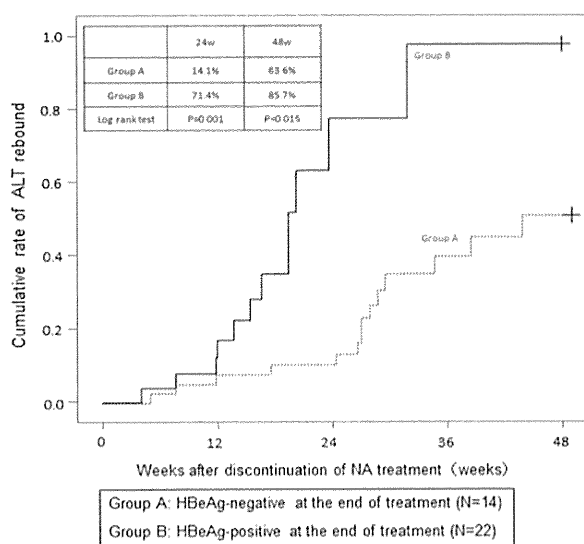


Fig. 4 Cumulative rate of ALT rebound after discontinuation of NA treatment. Fourteen patients who were hepatitis B virus e antigen (*HBeAg*) negative at the end of treatment were assigned to group A; the other 22 patients, who were positive to HBeAg at the end of treatment, were assigned to group B. 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

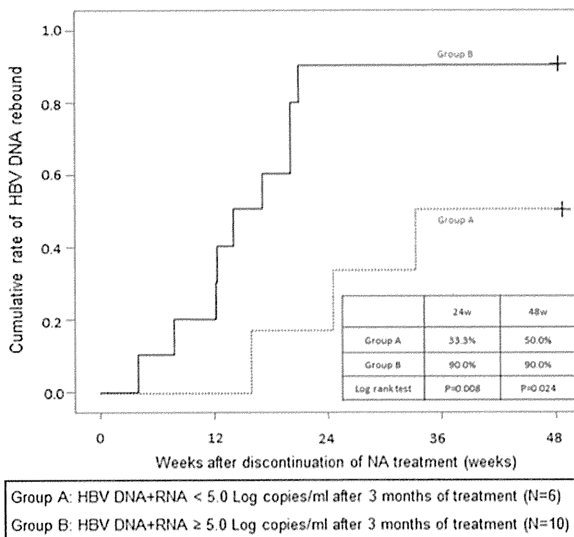


Fig. 5 Cumulative rate of ALT rebound after discontinuation of NA treatment in HBeAg-positive chronic hepatitis B patients. Six patients whose HBV DNA + RNA titers reached <5.0 log copies/mL after 3 months of treatment were assigned to group A; the other ten patients, whose HBV DNA + RNA titers were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

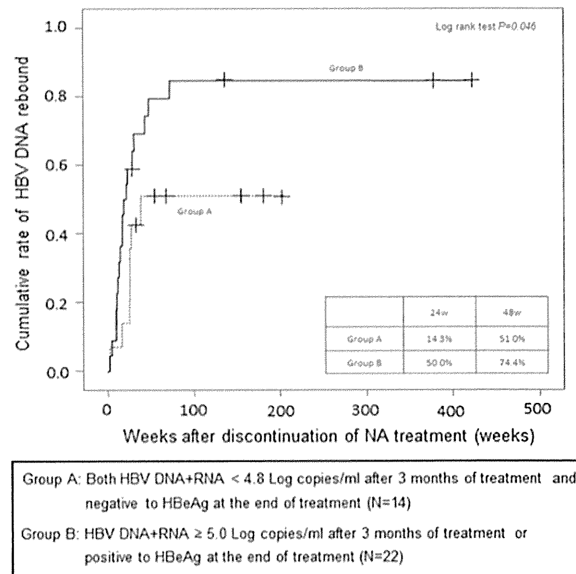


Fig. 6 Cumulative rate of ALT rebound after discontinuation of NA treatment by using combined criteria. The subjects were divided using combined criteria. Fourteen patients whose HBV DNA + RNA titers reached <5.0 log copies/mL after 3 months of treatment and who were HBeAg negative at the end of NA treatment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

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

Although the presence of HBeAg before treatment, HBV DNA and DNA + RNA titers after 3 months of treatment, and the presence of HBeAg, HBsAg titer, and HBV DNA + RNA titer at the end of treatment were all significantly associated with ALT rebound in the univariate analysis, only the presence of HBeAg at the end of treatment was identified as an independent predictive factor for ALT rebound following multivariate analysis (Table 4). HBeAg is commonly strongly associated with the activity of HBV replication, and HBV DNA levels are high in HBeAg-positive HBV carriers. Thus, HBe seroconversion usually indicates suppression of HBV activity, and the absence of HBeAg is thought to indicate the inactivation of HBV replication.

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

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

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

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

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

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

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Conflict of interest None to declare.

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Hepatitis B Virus-Specific miRNAs and Argonaute2 Play a Role in the Viral Life Cycle

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Abstract

Disease-specific serum miRNA profiles may serve as biomarkers and might reveal potential new avenues for therapy. An HBV-specific serum miRNA profile associated with HBV surface antigen (HBsAg) particles has recently been reported, and AGO2 and miRNAs have been shown to be stably associated with HBsAg in serum. We identified HBV-associated serum miRNAs using the Toray 3D array system in 10 healthy controls and 10 patients with chronic hepatitis B virus (HBV) infection. 19 selected miRNAs were then measured by quantitative RT-PCR in 248 chronic HBV patients and 22 healthy controls. MiRNA expression in serum versus liver tissue was also compared using biopsy samples. To examine the role of AGO2 during the HBV life cycle, we analyzed intracellular co-localization of AGO2 and HBV core (HBcAg) and surface (HBsAg) antigens using immunocytochemistry and proximity ligation assays in stably transfected HepG2 cells. The effect of AGO2 ablation on viral replication was assessed using siRNA. Several miRNAs, including miR-122, miR-22, and miR-99a, were up-regulated at least 1.5 fold ($P<2E-08$) in serum of HBV-infected patients. AGO2 and HBcAg were found to physically interact and co-localize in the ER and other subcellular compartments. HBs was also found to co-localize with AGO2 and was detected in multiple subcellular compartments. Conversely, HBx localized non-specifically in the nucleus and cytoplasm, and no interaction between AGO2 and HBx was detected. SiRNA ablation of AGO2 suppressed production of HBV DNA and HBs antigen in the supernatant.

Conclusion: These results suggest that AGO2 and HBV-specific miRNAs might play a role in the HBV life cycle.

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Introduction

Hepatitis B virus (HBV) is a partially double-stranded DNA virus in the Hepadnaviridae family [1]. New therapies are urgently needed for the 350 million chronically infected individuals who face a significantly elevated lifetime risk of cirrhosis and hepatocellular carcinoma [2,3]. Recent insight into the role of non-coding RNAs in the liver has highlighted potential applications of microRNAs (miRNAs) in HBV diagnosis and treatment [4,5,6,7,8,9].

MiRNAs are a class of short non-coding RNAs involved in post-transcriptional gene regulation of multiple pathways [10]. In contrast to messenger RNAs, exosome-free extracellular miRNAs may be nuclease-resistant and remain in circulation for long periods of time by being stably bound to AGO2, a component of the RNA-induced silencing complex [11]. The origin and function of these extracellular miRNAs is unclear, but they may serve as biomarkers for liver injury and cancer [4]. Elucidating the function of hepatic miRNAs in HBV infection is important in the development of strategies to eradicate the virus and assess the risk of HCC. A number of miRNAs have been shown to be up- or down-regulated in HBV infection [4,12,13]. Noting that the defective hepatitis delta virus co-opts HBsAg subviral particles for export, Novellino et al. hypothesized that HBsAg subviral particles might also sequester miRNAs from the liver [5]. Using HBsAg immunoprecipitation, they identified a set of liver-specific and immune regulatory AGO2-bound miRNAs associated with HBsAg.

These reports suggest that AGO2 and a specific subset of miRNAs may participate in HBV replication, either as part of a host anti-HBV defense or as viral strategy to exploit or evade the RISC machinery. In this study, we examined serum miRNA expression in chronic HBV and healthy individuals and found a specific subset of miRNAs that are over-expressed in HBV-positive

patients and in which miR-122 was strongly up-regulated. To determine whether components of the miRNA system are associated with other HBV components, we performed subcellular localization experiments with viral proteins and AGO2.

Materials and Methods

Study Subjects

We performed a series of experiments to compare miRNA profiles of healthy and HBV-infected individuals in serum and liver tissue. All patients had chronic hepatitis B and agreed to provide blood samples for a viral hepatitis study. Patient profiles are shown in Table 1. Histopathological diagnosis was made according to the criteria of Desmet et al. [14]. The study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki, and all patients provided written informed consent. This study was approved a priori by the ethical committee of Hiroshima University.

miRNA Expression Levels in Serum

miRNA expression in serum samples was measured using the Toray Industries miRNA analysis system, in which serum miRNA samples were hybridized to 3D-Gene human miRNA ver12.1 chips containing 900 miRNAs (Toray Industries, Inc., Tokyo, Japan). MiRNA gene expression data were scaled by global normalization, and differential expression was analyzed using the limma package in the R statistical framework. Serum was collected from 20 patients with high HBV DNA and HBsAg levels and with either high (>42 IU/l) or low (≤42 IU/l) ALT levels. Serum from the 10 low ALT patients was analyzed as a mixture, whereas serum from each of the 10 high ALT patients was analyzed both separately and as a mixture. For comparison with healthy controls we collected separate mixtures of serum from 10 healthy females and 12 healthy males. Serum samples from each healthy female were also measured separately. All healthy controls were negative for HBsAg, HBcAb, and HCV Ab. For comparison with miRNA expression in hepatocytes, miRNA expression was measured in non-tumor biopsy tissue from an HBV-infected patient and compared to non-cancerous liver tissue samples from two patients without HBV or HCV infection.

Table 1. Clinical characteristics of chronic hepatitis B virus patients (n = 248).

Factor	Value
Age	44 (15–76)
Sex (male/female)	169/77
Alanine aminotransferase (IU/l)	56 (10–1867)
Aspartate aminotransferase (IU/l)	43.5 (15–982)
HBV DNA (IU/ml)	6.3 (1.8–9.1)
Liver fibrosis (1/2/3/4)	69/102/46/26
Necroinflammatory activity (0/1/2/3/4)	1/70/127/45/0
γ-glutamyl transpeptidase (IU/l)	43 (9–459)
Alpha-fetoprotein (μg/l)	6.15 (0–9400)
Promthrombin time (s)	93 (0–146)
Albumin (g/dl)	4.4 (0–5.2)
Platelets (x10^4/mm^3)	16.75 (1–36)
HBsAg (IU/l)	2765 (0.05–239000)
HBeAg (−/+)	115/127
HBeAb (−/+)	113/128

Continuous variables are shown as median and range, and categorical variables are shown as counts.
Fibrosis and necroinflammatory activity were scored according to the criteria of Desmet et al. [14].
doi:10.1371/journal.pone.0047490.t001

Quantitative Real-time Polymerase Chain Reaction miRNA Analysis

Using real-time polymerase chain reaction (RT-PCR) we measured the expression of 19 miRNAs in serum from 248 patients with chronic HBV infection and from 10 healthy females and 12 healthy males. Circulating microRNA was extracted from 300 μl of serum samples using the mirVana PARIS Kit (Ambion, 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, 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. MiRNAs were amplified using primers and probes provided by Applied Biosystems 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 transcription product and 10.25 μl of nuclease free water in a total volume of 25 μl. 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 miRNA was used to generate a standard curve that permitted absolute quantification of molecules.

Pathway Analysis

Target genes of differentially expressed miRNAs were predicted based on agreement among three miRNA prediction tools, miRanda, miRBase, and TargetScan. Gene Set Enrichment Analysis (http://www.broadinstitute.org/gsea) was used to identify significantly over-represented gene ontology (GO) terms among the predicted targets.

Plasmid Construction

The construction of wild-type HBV 1.4 genome length, pTRE-HB-wt, was described previously [15]. We used pTRE2 vector without pTet-off vector and doxycycline because a sufficient amount of HBV transcript was produced from internal HBV promoters, and transcription from the pTRE2 promoter is negligible under these conditions. The nucleotide sequence of the HBV genome that we cloned into plasmids pTRE-HB-wt was deposited into GenBank under accession number AB206817.

Cell Culture

HepG2 cells, derived from a human hepatoma cell line, were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum at 37°C and under 5% CO_2. For the production of stably transfected cell lines, HepG2 cells were transfected with 20μg of the plasmid pTRE-HB-wt by calcium precipitation and the transfected cells were selected with

400μg/ml hygromycin-included DMEM. Sixty colonies were isolated, and clones that were positive for both HBs and HBe antigens were selected. Finally, one cell line named T23 was selected and used for further experiments. T23 cells continuously produced more than 6 log copies/ml of HBV DNA in supernatant over more than 12 months (data not shown).

Immunocytochemistry

Co-localization between AGO2 and several HBV proteins (HBc, HBs, and HBx) was analyzed using immunocytochemistry, followed by cellular localization assays using antibodies targeting various sub-cellular compartments. HepG2 or T23 cells were seeded in 2-well chamber plates and harvested 48 hours after seeding. The cells were washed with PBS and fixed with 4% (v/v) paraformaldehyde. After fixation, the cells were stained with several primary antibodies (Table S1). The bound antibodies were detected with an Alexa 488-conjugated antibody against rabbit IgG (1:2000) or Alexa 568-conjugated antibody against mouse IgG (1:2000), respectively (Molecular Probes, Eugene, OR). Nuclei were counterstained with 6-diamidino-2-phenylindole (DAPI) (Vector laboratories, Burlingame, CA). The stained cells were examined with a Fluoview FV10i microscope (Olympus, Tokyo, Japan).

In situ Proximity Ligation Assay

We used proximity ligation assays (PLA) to determine whether AGO2 and HBc physically interact. PLA is a recent method to detect protein-protein interactions using protein-DNA conjugates that can be detected using fluorescence microscopy [16]. PLA improves on traditional immunoassays by directly detecting even weak or transient protein interactions [16]. HepG2 and T23 cells were seeded in 2-well chamber plates and harvested 48 hours after seeding. The cells were washed with PBS and fixed with 4% (v/v) paraformaldehyde. After fixation, the cells were stained with primary antibodies. The primary antibodies used are listed in Table S1. After overnight incubation with primary antibody at 4°C, PLA was performed using Duolink II PLA probe anti-rabbit plus and anti-mouse minus and Duolink II Detection Reagents Orange (Olink, Uppsala, Sweden) following the manufacturer's protocol. Nuclei were counterstained with DAPI. Imaging was performed using a Fluoview FV10i microscope.

Analysis of Supernatant HBV Production by RNA Interference Against AGO2

To investigate the necessity of AGO2 for HBV production, we performed RNA interference assay using T23 cells that are HepG2 cells stably transfected with the plasmid pTRE-HB-wt. We used Silencer Select Pre-designed siRNA small interfering RNA targeting *AGO2* (#s25932, Ambion, Austin, TX) and Silencer Select Negative Control #1 siRNA for control (Ambion). T23 cells were transfected with one of the siRNA oligonucleotides (10 nM) using Lipofectamine RNAiMAX (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. To examine the knockdown effect of siRNAs against *AGO2* by real-time quantitative RT-PCR, T23 cells transfected with siRNAs were harvested 72 hours after transfection. Total RNA was isolated using the QuickGene RNA cultured cell kit S (Fujifilm, Tokyo, Japan). One μg of each RNA sample was reverse transcribed with the SuperScript VILO cDNA Synthesis kit (Invitrogen). First-strand complementary DNA (cDNA) was amplified with specific primers for the coding sequence of *AGO2*. The primers were as follows: forward, 5′-CCAGCATACTACGCTCACCT-3′; reverse, 5′-CAGAGTGTCTTGGTGAACCTG-3′. We quantified *AGO2* mRNA with EXPRESS SYBR Green ER qPCR Supermix Universal (Invitrogen) according to the manufacturer's instructions. Amplification and detection were performed using the Mx3000P Multiplex quantitative PCR system (Stratagene, La Jolla, CA). Results were normalized to the transcript levels of the housekeeping reference gene glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*). Three to seven days after transfection, the culture media were collected to examine HBV production in supernatant. HBs antigen was measured quantitatively using the Abbott chemiluminescence immunoassay kit (Abbott Japan, Tokyo, Japan). HBV DNA levels were determined by Cobas TaqMan HBV standardized real-time PCR assay (Roche Molecular Systems, Pleasanton, CA). Results are expressed in log10 international units/ml. We also evaluated viability of cells using the Cell Counting kit-8 (Dojindo Laboratories, Kumamoto, Japan) at 3, 5 and 7 days after transfection, according to the manufacturer's instructions. All assays were performed in triplicate, and the results are expressed as mean ± SD.

Statistical Analysis

All analyses were performed using the R statistical package (http://www.r-project.org). Continuous variables are reported using the median and range. Moderated t statistics or Mann Whitney U tests were used to detect significant associations, as appropriate, and P-values were adjusted for multiple testing based on the false discovery rate.

Results

MiRNA Microarray Results

We performed miRNA microarray analysis to identify HBV-associated differences in serum miRNA profiles between 10 chronic HBV patients and 10 healthy controls (Fig. S1). 26 miRNAs with an absolute log fold change greater than 1.5 were found to be significantly (P_{FDR} <0.05) up-regulated in serum of HBV patients, and 8 miRNAs were significantly down-regulated (Table 2). MiR-122, miR-22, and miR-99a levels were the most strongly up-regulated in serum of HBV-infected patients, and levels of miR-575, miR-125a-3p, and miR-4294 were the most down-regulated. We also examined miRNAs associated with presence of HBe antigen or HBe antibody, but no miRNAs were significant following correction for multiple testing (data not shown).

Analysis of Serum Sample Mixtures from HBV-infected Patients and Healthy Controls

In addition to individual serum samples, we also examined 4 pooled serum samples as follows: 10 healthy males, 10 healthy females, 10 HBV patients with low ALT levels, and 10 HBV patients with high ALT levels (Fig. S2). In agreement with results from individual analysis, miR-122 and miR-99 levels were significantly higher in serum from HBV serum samples compared to healthy control samples (Table 2). Corresponding results with a log change greater than 1.5 were found for several other miRNAs, including miR-22, miR-642b, miR-125b (up-regulated) and miR-575 and miR-4294 (down-regulated), but results were not significant following correction for multiple testing in the mixture samples due to the small number of samples compared.

RT-PCR Analysis

Serum levels of 19 miRNAs were analyzed using quantitative RT-PCR analysis of 250 chronic HBV patients and 20 healthy controls. Several miRNAs (miR-122, miR-22, miR-99a, miR-720, miR-125b, and miR-1275) were significantly up-regulated in

Table 2. Top 10 up- or down-regulated serum miRNAs associated with chronic HBV infection.

Sample	Direction	miRNA	logFC	AveExpr	t	P	P_{FDR}
Serum	Up	hsa-miR-122	5.97	9.09	12.84	3.27E−12	3.06E−09
		hsa-miR-99a	2.59	6.20	10.73	2.11E−10	2.19E−08
		hsa-miR-22	2.49	9.55	10.47	2.10E−10	2.19E−08
		hsa-miR-191	2.19	8.42	11.87	1.68E−11	3.93E−09
		hsa-miR-642b	2.03	10.07	9.93	5.92E−10	4.26E−08
		hsa-miR-125b	1.95	5.99	8.72	9.91E−09	4.21E−07
		hsa-miR-486-3p	1.79	9.09	8.01	3.19E−08	9.95E−07
		hsa-miR-378	1.78	5.97	9.94	9.00E−10	6.02E−08
		hsa-miR-320d	1.70	7.19	7.88	4.25E−08	1.21E−06
		hsa-miR-23b	1.69	8.99	7.62	7.64E−08	1.93E−06
	Down	hsa-miR-575	−2.10	8.35	−10.00	5.20E−10	4.05E−08
		hsa-miR-125a-3p	−1.99	7.22	−11.91	1.56E−11	3.93E−09
		hsa-miR-4294	−1.75	11.82	−11.37	4.07E−11	7.63E−09
		hsa-miR-92a-2*	−1.64	11.03	−7.70	6.36E−08	1.75E−06
		hsa-miR-1202	−1.59	8.60	−12.41	6.72E−12	3.14E−09
		hsa-miR-30c-1*	−1.31	6.29	−8.66	1.12E−08	4.35E−07
		hsa-miR-1275	−1.19	9.91	−7.50	1.00E−07	2.35E−06
		hsa-miR-3197	−1.05	11.46	−8.58	9.24E−09	4.21E−07
		hsa-miR-1908	−1.03	13.75	−9.05	3.49E−09	2.04E−07
Mixture	Up	hsa-miR-122	6.80	9.09	20.51	1.09E−06	0.001
		hsa-miR-99a	2.58	6.34	9.32	9.80E−05	0.037
		hsa-miR-22	2.07	8.60	3.16	0.020	0.528
		hsa-miR-125b	2.03	6.29	5.09	0.002	0.264
		hsa-miR-1915*	1.80	8.32	6.24	0.001	0.158
		hsa-miR-3648	1.69	14.16	5.06	0.002	0.264
		hsa-miR-642b	1.64	9.82	4.49	0.004	0.377
		hsa-miR-1288	1.39	6.43	3.56	0.012	0.528
		hsa-miR-325	1.30	4.91	2.87	0.047	0.586
		hsa-miR-486-3p	1.29	8.98	3.87	0.009	0.480
	Down	hsa-miR-575	−1.95	8.43	−6.38	0.001	0.158
		hsa-miR-4294	−1.79	11.95	−5.99	0.001	0.158
		hsa-miR-654-3p	−1.35	5.36	−2.99	0.042	0.569
		hsa-miR-1202	−1.24	8.52	−3.97	0.008	0.480
		hsa-miR-1237	−1.06	7.52	−3.10	0.022	0.531
		hsa-miR-744	−1.03	9.51	−2.91	0.028	0.545

Expression levels were compared using moderated t-statistics, and P-values were corrected for multiple testing using the false discovery rate.
logFC: log2 fold-change between patients with chronic HBV infection relative to healthy individuals.
AveExpr: The average log2 expression level for each miRNA over all samples.
t: moderated t-statistic for patients with chronic HBV infection compared to healthy individuals P for each miRNA.
P: uncorrected P-value for t-test.
P_{FDR}: P-value adjusted for multiple testing based on the false discovery rate.
doi:10.1371/journal.pone.0047490.t002

serum from HBV-infected patients (Table 3). Agreement of microarray and RT-PCR results was strongest for up-regulation of miR-122, miR-22, and miR-125b in serum of HBV patients. To determine whether there is a linear relationship between HBV markers and HBV-associated miRNAs, we analyzed the correlation between HBsAg and 6 up-regulated miRNAs. MiR-122, miR-99a, and miR-125b levels were found to be significantly correlated with HBsAg levels with $R^2>0.5$ (Fig. S3). These three miRNAs were also significantly correlated with HBV DNA titers, with R^2 of about 0.4 (Fig. S4). MiR-122 and miR-22 were significantly but diffusely associated with serum ALT levels ($R^2>0.2$; Fig. S5). To identify miRNAs associated with different phases of HBV infection, we also analyzed the 6 significantly up-regulated miRNAs with respect to the presence of HBe antigen and antibody. MiR-122, miR-99a, miR-720, and miR-125b were each highly significantly elevated in chronic HBV patients who were positive for the HBe antigen ($P<4.0E-07$; Fig. S6). Similarly, each miRNA was significantly elevated in chronic HBV patients who were negative for the HBe antibody ($P<9.1E-05$; Fig. S7).

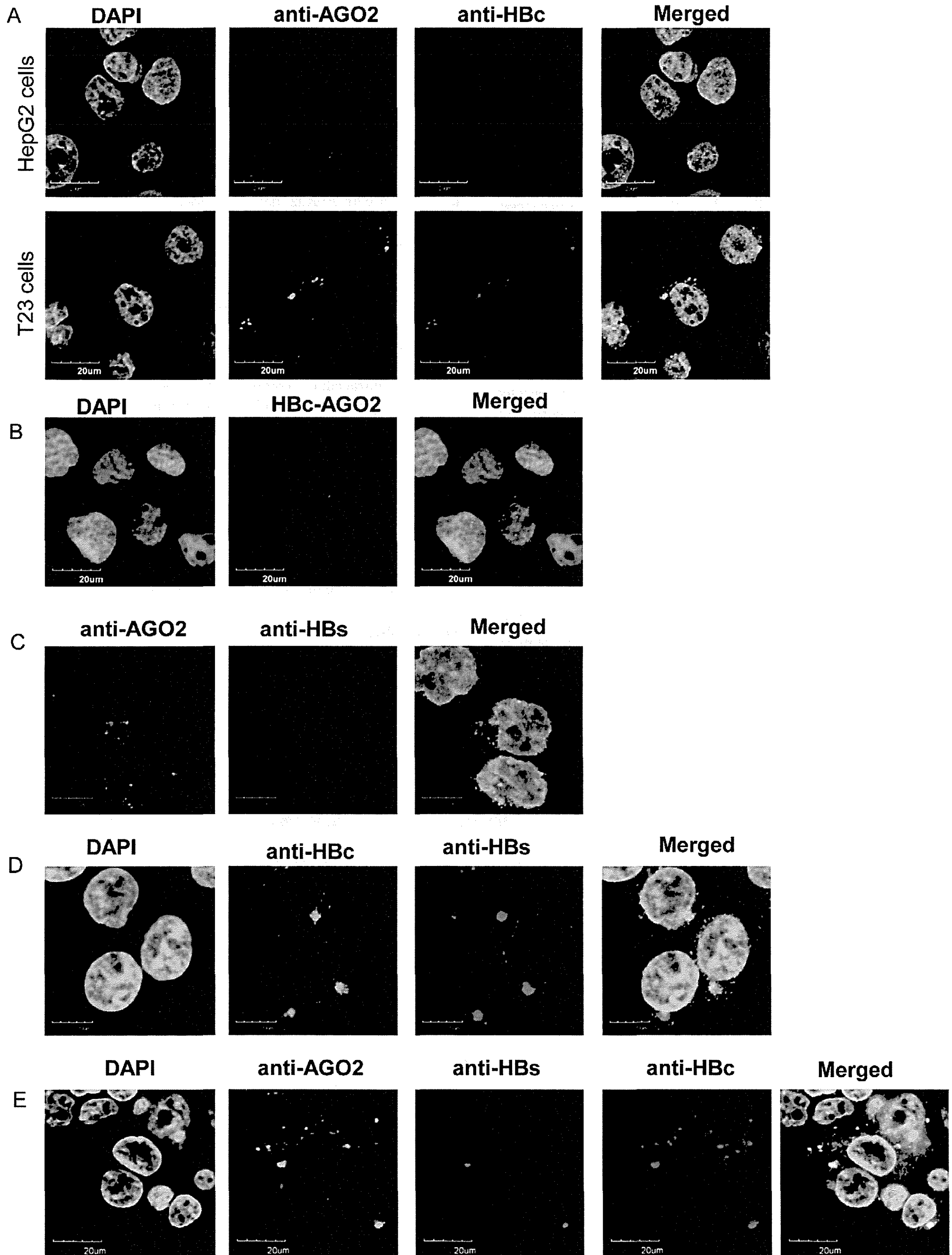
A
DAPI
anti-AGO2
anti-HBc
Merged
HepG2 cells
T23 cells
20um
B
DAPI
HBc-AGO2
Merged
C
anti-AGO2
anti-HBs
Merged
D
DAPI
anti-HBc
anti-HBs
Merged
E
DAPI
anti-AGO2
anti-HBs
anti-HBc
Merged

Figure 1. Co-localization of HBcAg and HBsAg with AGO2 in stably transfected T23 cells. A) Anti-AGO2 and anti-HBc staining overlapped in stably transfected T23 cells, but not in HepG2 control cells, suggesting an interaction between HBc and AGO2. B) HBc-AGO2 was detected in T23 but not HepG2 cells using proximity ligation assays (PLA), suggesting a protein-protein interaction between HBcAg and AGO2. C) Overlap of anti-AGO2 and anti-HBs staining suggests co-localization of HBs and AGO2. D) Anti- HBc, and anti-HBs staining overlapped in T23 cells, which may indicate that HBc and HBs co-localize. E) Overlap of anti-AGO2, anti-HBc, and anti-HBs staining in T23 cells suggests that all three proteins may co-localize.
doi:10.1371/journal.pone.0047490.g001

Pathway Analysis

Predicted gene targets of up-regulated miRNAs were most strongly associated with the GO term PROTEIN_TYROSINE_PHOSPHATASE_ACTIVITY ($P = 5.24E-3$), and down-regulated miRNAs were associated with the term POSITIVE_REGULATION_OF_JNK_ACTIVITY (P 9.47e−4). Predicted target genes associated with phosphatase activity and dephosphorylation included MTMR3, PTPN18, DUSP5, PTPN2, DUSP2, and PPP1CA.

MiRNA Expression in Liver Biopsy Samples

We compared miRNA expression in non-cancerous liver biopsy samples from a patient with chronic HBV to two uninfected patients (Table S2, Fig. S8). MiRNA levels were highly correlated between liver tissue and serum in all patients ($P = <0.001$; $R^2 = 0.57$), including the top HBV-associated miRNAs identified by microarray and RT-PCR analysis in this study.

Co-localization of HBcAg and HBsAg with AGO2

Using immunocytochemistry and PLA analysis, we found that HBV core protein and AGO2 co-localized within T23 cells (Fig. 1A–B), suggesting a potential protein-protein interaction between HBcAg and AGO2. AGO2 also co-localized with HBs in T23 cells (Fig. 1C), indicating a potential interaction between HBs and AGO2. Overlap between anti-HBc and anti-HBs staining (Fig. 1D) and between anti-AGO2, anti-HBc, and anti-HBs (Fig. 1E) suggests that these three proteins may co-localize. No overlap was observed between anti-AGO2 and anti-HBx staining in HepG2 cells transfected with HBx expression plasmid (p3FLAG-HBx) nor in control cells, suggesting that HBx does not interact with AGO2 (data not shown).

Subcellular Localization

We also examined HBcAg sub-cellular localization using immunocytochemistry and PLA analysis and found that HBcAg localized to several intracellular compartments, including the ER, autophagosomes, endosomes, and Golgi (Fig. 2). No evidence was found for interaction with mitochondria (data not shown). Using immunocytochemistry, HBsAg was also found to localize diffusely to several intracellular compartments, including the ER, endosomes, autophagosomes, Golgi, mitochondria, processing bodies, multi-vesicular bodies, and the nuclear envelope (Fig. 3). HBx localized non-specifically in the nucleus and cytoplasm, and no sub-cellular location could be ascertained (Fig. S9).

RNA Interference against AGO2

Antisense RNA directed against AGO2 strongly suppressed AGO2 expression (Fig. 4A) and resulted in lower HBV DNA (Fig. 4B) and HBsAg (Fig. 4C) levels in the supernatant. Cell viability was not significantly reduced (Fig. 4D).

Discussion

In this study, we report a set of miRNAs that were up-regulated in serum of HBV infected individuals compared to healthy

Table 3. Quantitative RT-PCR results of selected miRNAs associated in serum of chronic HBV patients.

Factor	Total (n = 270)	HBV (n = 248)	Healthy (n = 22)	P
hsa-miR-122/cel-miR-238	0.1513 (0.0068–2.5)	0.1635 (0.0068–2.5)	0.02074 (0.013–0.04)	1.19E−13
hsa-miR-22/cel-miR-238	0.3 (0.06–1.7)	0.3028 (0.06–1.7)	0.2252 (0.11–0.48)	6.35E−03
hsa-miR-99a/cel-miR-238	0.09121 (0.0046–2.4)	0.102 (0.0086–2.4)	0.0136 (0.0046–0.051)	4.61E−12
hsa-miR-720/cel-miR-238	0.1206 (0.024–3.7)	0.1345 (0.031–3.7)	0.04274 (0.024–0.12)	8.93E−11
hsa-miR-125b/cel-miR-238	0.09732 (0.0066–3.1)	0.1131 (0.0066–3.1)	0.02255 (0.0066–0.05)	1.92E−11
hsa-miR-1275/cel-miR-238	0.4842 (0.099–1.6)	0.5046 (0.099–1.6)	0.4044 (0.24–0.6)	0.010781066
hsa-miR-1826/cel-miR-238	0.5023 (0.14–4.6)	0.5583 (0.26–4.6)	0.33 (0.14–1.4)	7.23E−03
hsa-miR-1308/cel-miR-238	2.831 (1.1–6.9)	2.578 (1.1–6.9)	3.113 (2.3–4.7)	0.223164946
hsa-miR-923/cel-miR-238	3.8 (1.8–9.6)	4.141 (1.8–9.6)	3.01 (2–5)	0.104331611
hsa-miR-1280/cel-miR-238	1.089 (0.36–5)	1.332 (0.6–5)	0.5275 (0.36–0.8)	1.06E−05
hsa-miR-26a/cel-miR-238	1.221 (0.34–3.4)	1.221 (0.34–3.4)	1.231 (0.82–2.4)	0.532171224
hsa-let-7a/cel-miR-238	0.9608 (0.2–2.5)	0.9211 (0.2–2.5)	1.074 (0.71–1.9)	0.235258945
hsa-let-7f/cel-miR-238	1.134 (0.052–2.6)	1.126 (0.052–2.6)	1.143 (0.8–1.7)	0.639411853
hsa-let-7d/cel-miR-238	1.147 (0.35–1.9)	1.106 (0.35–1.8)	1.231 (0.73–1.9)	2.88E−01
hsa-miR-638/cel-miR-238	1.23 (0.3–7)	1.082 (0.3–7)	1.366 (0.68–4)	0.288244047
hsa-miR-1908/cel-miR-238	1.369 (0.45–3.2)	1.357 (0.45–1.9)	1.447 (0.7–3.2)	0.370765019
hsa-miR-34a/cel-miR-238	0.07502 (0.013–1.2)	0.108 (0.026–1.2)	0.02738 (0.013–0.044)	1.41E−05
hsa-miR-886-5p/cel-miR-238	1.627 (0.54–3.6)	1.773 (0.54–3.6)	1.55 (0.97–2.7)	0.478520977

Expression levels were compared using the Mann-Whitney U test.
doi:10.1371/journal.pone.0047490.t003

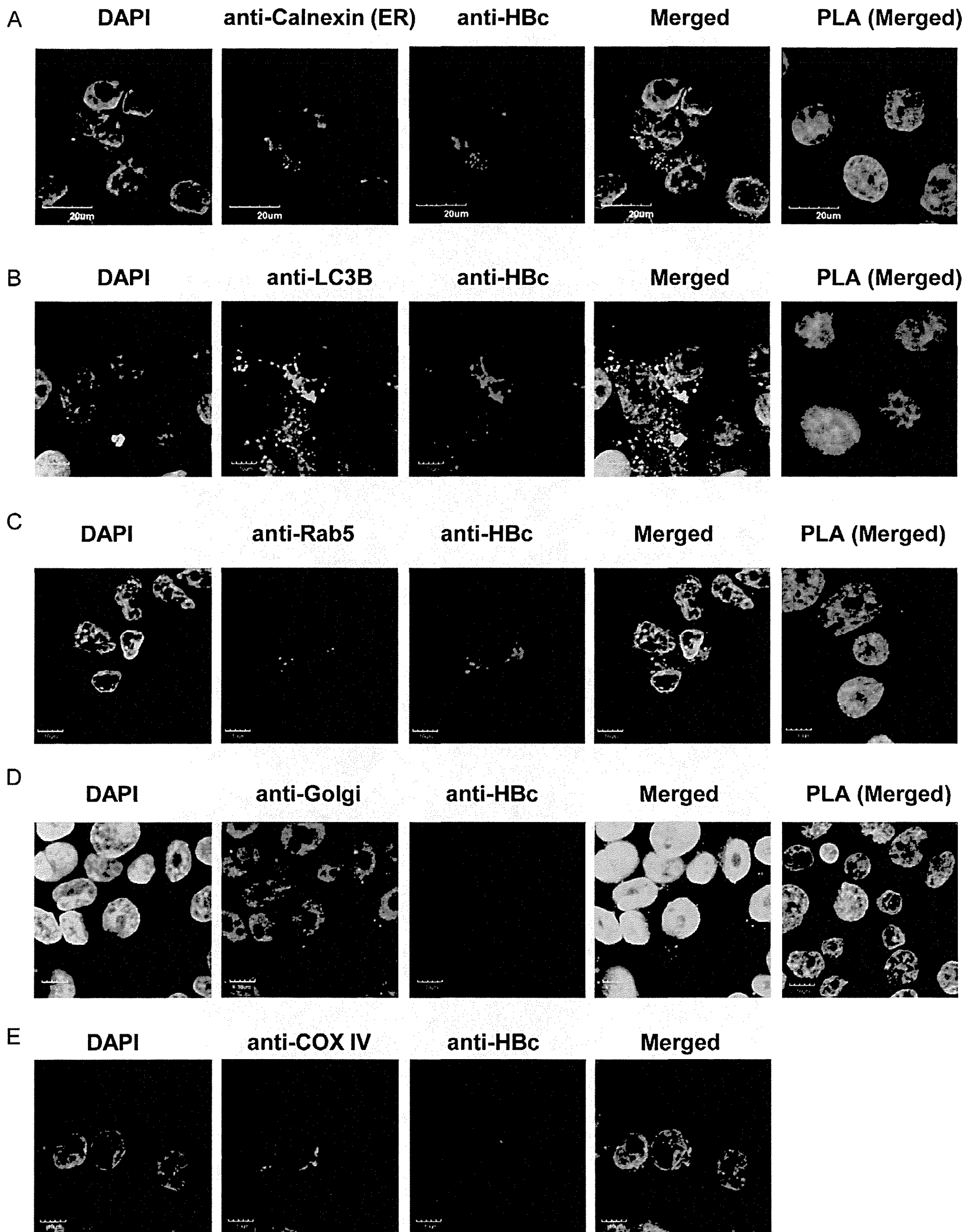

Figure 2. Interactions between HBc and HBs. A) Co-localization of anti-HBc and anti-Calnexin staining by immunocytochemistry and PLA analysis indicate that HBc probably localizes in the ER. Overlap with B) anti-LC3B, C) anti-Rab5, and D) anti-Golgi staining suggests that HBc probably also localizes in 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.

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