Table 2 Factors influencing the seroclearance of HBsAg in 2,112 patients evaluated by time-dependent uni- and multivariate analyses

Factors	Univariate analysis HBsAg clearance Relative risk (95 % CI)	*p* value	Multivariate analysis HBsAg clearance Relative risk (95 % CI)	*p* value
Age ≥50 years	1.06 (0.64–1.76)	0.824		
Male gender	1.15 (0.69–1.90)	0.594		
No HBV infection in family	1.55 (0.93–2.57)	0.092		
Treatment	1.26 (0.72–2.19)	0.413		
Cirrhosis	2.40 (1.20–4.83)	0.014		
AST ≥50 IU/L	1.30 (0.66–2.57)	0.454		
ALT ≥50 IU/L	1.81 (0.89–3.70)	0.104		
γ-GTP ≥20 IU/L	1.26 (0.72–2.23)	0.418		
Total bilirubin ≥1 mg/dL	1.39 (0.69–2.79)	0.358		
Albumin ≥4 g/dL	1.03 (0.58–1.81)	0.927		
Platelets >150 × 10^3/mm^3	1.22 (0.68–2.18)	0.501		
α-Fetoprotein ≤10 μg/L	1.06 (0.59–1.89)	0.845		
Genotype A or B, C	1.55 (0.86–2.76)	0.142		
HBeAg-negative status	3.01 (0.79–2.07)	0.001	1.81 (1.30–2.77)	<0.001
HBV DNA ≥5 log copies/mL	1.17 (0.64–2.15)	0.612		
HBsAg ≤2,000 IU/mL	2.13 (1.27–3.56)	0.004	2.60 (1.94–3.50)	<0.001
HBcrAg ≥4 log U/mL	1.11 (0.61–2.03)	0.731		
Wild-type precore sequence	0.98 (0.59–1.53)	0.964		
Wild-type core promoter sequence	2.74 (0.80–9.30)	0.104		

Wild-type precore sequence, G1896; wild-type core promoter sequence, A1762/G1764

AST aspartate aminotransferase, *ALT* alanine aminotransferase, *γ-GTP* γ-guanosine triphosphate, *HBeAg* hepatitis B e antigen, *HBsAg* hepatitis B surface antigen, *HBcrAg* hepatitis B core-related antigen

[median 0.5 vs. 0.7 mg/dL ($p < 0.001$)]; albumin [median 4.4 vs. 4.3 g/dL ($p < 0.001$)]; platelets [median 202 vs. 181 × 10^3/mm^3 ($p < 0.001$)]; α-fetoprotein [median 4 vs. 4 μg/L ($p < 0.001$)]; HBeAg-negative status [75.8 vs. 31.8 % ($p < 0.001$)]; HBsAg levels [median 2,240 vs. 5,270 IU/mL ($p < 0.001$)]; HBcrAg [median 3.6 vs. >6.8 log U/mL ($p < 0.001$)]; distribution of genotypes A/B/C/others (5.7/20.0/72.6/1.7 vs. 3.4/11.1/84.9/0.5 %, $p < 0.001$); and HBV DNA [median 4.7 vs. 8.0 log copies/mL ($p < 0.001$)].

The rate of HBsAg seroclearance in treated patients was 8 % in 5 years, 20 % in 10 years, 28 % in 15 years, 41 % in 20 years, 49 % in 25 years, and 49 % in 30 years, with an annual HBsAg seroclearance rate of 2.05 % (Fig. 2). The rate in untreated patients was 9 % in 5 years, 18 % in 10 years, 26 % in 15 years, 33 % in 20 years, 42 % in 25 years, and 56 % in 30 years, with an annual HBsAg seroclearance rate of 1.65 %. No differences in the annual HBsAg seroclearance rate were noted between treated and untreated patients ($p = 0.289$).

HBsAg seroclearance in untreated patients

In the 1,130 untreated patients, HBsAg persisted in 930 (82.3 %), whereas HBsAg seroclearance occurred in 200 (17.7 %). In the baseline characteristics, significant differences were found for age ($p < 0.001$), male gender ($p = 0.003$), chronic hepatitis ($p = 0.020$), γ-GTP ($p < 0.001$), albumin ($p = 0.004$), HBV genotypes ($p < 0.001$), HBeAg-negative status ($p < 0.001$), HBV DNA ($p < 0.001$), HBsAg level ($p < 0.001$), HBcrAg ($p < 0.001$), precore wild-type ($p < 0.001$), and core promoter wild-type ($p = 0.001$) (Table 4).

Factors contributing to HBsAg seroclearance in untreated patients

In the 1,130 untreated patients, factors influencing HBsAg seroclearance in univariate analysis by the Cox regression analyses were age ≥50 [RR 1.63 ($p = 0.002$)]; no family history in third-degree or closer relatives [RR 1.38 ($p = 0.037$)]; and HBsAg ≤2,000 IU/mL [RR 1.87 ($p < 0.006$)].

In multivariate analyses, only 2 factors contributed to HBsAg seroclearance: age ≥50 [RR 1.61 ($p = 0.018$)] and HBsAg ≤2,000 IU/mL [RR 1.77 ($p = 0.014$)] (Table 5).

HBsAg seroclearance in treated patients

In the 982 treated patients, HBsAg persisted in 833 (84.8 %). HBsAg seroclearance occurred in 149 (15.2 %). In the baseline characteristics, significant difference were found for male gender ($p = 0.004$), no family history in third-degree or closer relatives ($p = 0.010$), chronic hepatitis ($p = 0.001$), AST ($p = 0.010$), γ-GTP ($p = 0.023$), platelet counts ($p < 0.001$), HBeAg-negative status

Table 3 Baseline characteristics in untreated and treated patients

Features at the baseline	Untreated (n = 1,130)	Treated (n = 982)	Differences p value
Age (years)	31 (1–81)	36 (6–75)	<0.001
Men	705 (62.4 %)	726 (71.9 %)	<0.001
Chronic hepatitis	1,094 (96.8 %)	937 (96.4 %)	0.079
Cirrhosis	36 (3.2 %)	45 (3.6 %)	
AST (IU/L)	27 (3–1,776)	56 (6–2,192)	<0.001
ALT (IU/L)	28 (2–3,020)	96 (8–2,740)	<0.001
γ-GTP (IU/L)	20 (4–1,494)	45 (4–1,278)	<0.001
Total bilirubin (mg/dL)	0.5 (0.1–20.1)	0.7 (0.2–21.2)	<0.001
Albumin (g/dL)	4.4 (2.2–5.8)	4.3 (1.1–5.4)	<0.001
Platelets ($\times 10^3/mm^3$)	202 (40–443)	181 (40–483)	<0.001
α-Fetoprotein (μg/L)	4 (1–2,060)	4 (1–1,610)	<0.001
HBeAg-negative status	857 (75.8 %)	312 (31.8 %)	<0.001
HBsAg (IU/mL)	2,240 (0.06–141,000)	5,270 (0.09–277,000)	<0.001
HBcrAg (log U/mL)	3.6 (<3.0 to >6.8)	> 6.8 (<3.0 to >6.8)	<0.001
Genotypes [A/B/C/others (%)]	5.7/20.0/72.6/1.7	3.4/11.1/84.9/0.5	<0.001
HBV DNA (log copies/mL)	4.7 (<2.1 to >9.1)	8.0 (<2.1 to >9.1)	<0.001

Median values with the range in parentheses or numbers with the percentage in parentheses are given

AST aspartate aminotransferase, *ALT* alanine aminotransferase, *γ-GTP* γ-guanosine triphosphate, *HBeAg* hepatitis B e antigen, *HBsAg* hepatitis B surface antigen, *HBcrAg* hepatitis B core-related antigen

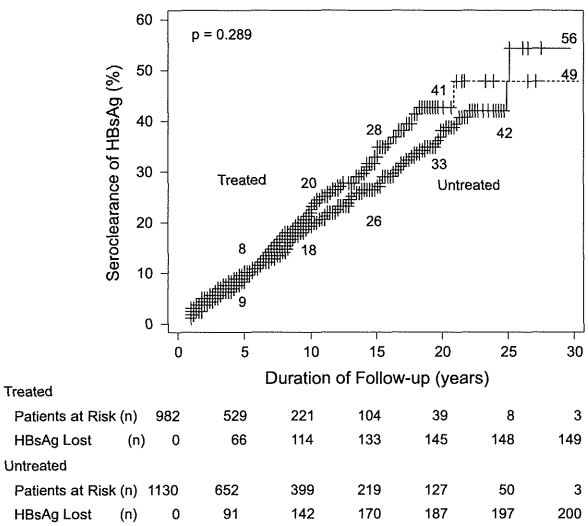

Fig. 2 Comparison of HBsAg seroclearance rates between 982 treated and 1,130 untreated patients. Numbers of patients at risk and those of patients who lost HBsAg are indicated below each time point

($p < 0.001$), HBV DNA ($p = 0.002$), HBsAg ($p < 0.001$), HBcrAg ($p = 0.003$), and precore wild-type ($p = 0.013$) (Table 6).

Factors contributing to HBsAg seroclearance in treated patients

In the 982 treated patients, factors influencing HBsAg seroclearance in univariate analysis by the Cox regression analyses were age ≥50 [RR 1.91 ($p = 0.001$)]; male gender [RR 2.14 ($p = 0.001$)], no family history in third-degree or closer relatives [RR 1.58 ($p = 0.005$)]; previous treatment with interferon [RR 2.13 ($p < 0.001$)]; chronic hepatitis [RR 3.12 ($p < 0.001$)]; AST ≥50 IU/L [RR 1.47 ($p = 0.031$)]; γ-GTP ≥20 IU/L [RR 1.87 ($p = 0.001$)]; platelets $\leq 150 \times 10^3/mm^3$ [RR 2.10 ($p < 0.001$)]; HBeAg-negative status [RR 2.53 ($p < 0.00$)]; HBV DNA ≤5 log copies/mL [RR 2.07 ($p = 0.001$)]; HBsAg ≤2,000 IU/mL [RR 2.29 ($p < 0.001$)]; HBcrAg ≤4 log U/mL [RR 2.28 ($p = 0.003$)]; and the wild-type precore sequence [RR 2.04 ($p = 0.011$)].

In multivariate analysis, only 3 factors contributed to HBsAg seroclearance: no family history in third-degree or closer relatives [RR 2.22 ($p = 0.006$)]; previous treatments with interferon [RR 3.15 ($p < 0.001$)]; and HBeAg-negative status [RR 3.75 ($p < 0.001$)] (Table 7).

Discussion

In Japan, perinatal materno-fetal transmission was the main route of HBV infection, but this transmission has been prevented since 1986 by the national campaign to prevent it by immunoprophylaxis with combined passive-active immunization of babies born to HBeAg-positive carrier mothers. However, HCC develops in about 10 % of the patients who have established chronic HBV infection by materno-fetal infection or through child-to-child transmission. Hence, HBsAg seroclearance is crucially required for preventing the development of cirrhosis followed by HCC.

In the present study, we analyzed 2,112 patients with persistent HBV infection to establish the factors

Table 4 Differences between the baseline characteristics of 917 untreated patients in whom HBsAg persisted and 213 those who lost HBsAg

Features at the baseline	HBsAg persisted ($n = 917$)	HBsAg lost ($n = 213$)	Differences p value
Age (years)	37 (1–81)	44 (0–80)	<0.001
Men	553 (60.3 %)	152 (71.4 %)	0.003
HBV in family members	349 (38.1 %)	76 (35.7 %)	0.509
Chronic hepatitis	893 (97.4 %)	201 (94.4 %)	0.020
AST (IU/L)	27 (3–1,144)	25 (6–1,776)	0.283
ALT (IU/L)	28 (6–1,960)	27 (6–3,020)	0.389
γ-GTP (IU/L)	22 (1–1,494)	29 (4–1,092)	<0.001
Total bilirubin (mg/dL)	0.6 (0.2–20.1)	0.7 (0.1–4.0)	0.257
Albumin (g/dL)	4.3 (2.0–5.3)	4.4 (1.6–5.7)	0.004
Platelets ($\times 10^3$/mm^3)	203 (40–443)	203 (33–417)	0.473
α-Fetoprotein (μg/L)	3 (1–2,060)	1 (1–478)	0.373
Genotypes [A/B/C/others (%)]	5.7/19.0/73.3/1.9	5.5/24.7/69.2/0.7	<0.001
HBeAg-negative status	663 (72.3 %)	194 (91.1 %)	<0.001
HBV DNA (log copies/mL)	4.9 (<2.1 to >9.1)	3.8 (<2.1 to >9.1)	<0.001
HBsAg (IU/mL)	3,100 (1.94–141,000)	149 (0.06–88,800)	<0.001
HBcrAg (log U/mL)	3.9 (<3.0 to >6.8)	2.9 (<3.0 to >6.8)	<0.001
Wild-type precore sequence	441 (48.1 %)	160 (75.0 %)	<0.001
Wild-type core promoter sequence	320 (34.9 %)	47 (22.0 %)	0.001

Wild-type precore sequence, G1896; wild-type core promoter sequence, A1762/G1764

AST aspartate aminotransferase, *ALT* alanine aminotransferase, *γ-GTP* γ-guanosine triphosphate, *HBeAg* hepatitis B e antigen, *HBsAg* hepatitis B surface antigen, *HBcrAg* hepatitis B core-related antigen

Table 5 Factors influencing the seroclearance of HBsAg in untreated patients evaluated by time-dependent uni- and multivariate analyses

Factors	Univariate analysis HBsAg clearance Relative risk (95 % CI)	p value	Multivariate analysis HBsAg clearance Relative risk (95 % CI)	p value
Age ≥50 years	1.63 (1.19–2.23)	0.002	1.61 (1.09–2.37)	0.018
Male gender	1.08 (0.79–1.48)	0.618		
No HBV infection in family	1.38 (1.02–1.86)	0.037		
Cirrhosis	1.19 (0.73–1.93)	0.484		
AST ≥50 IU/L	1.01 (0.70–1.45)	0.979		
ALT ≥50 IU/L	0.93 (0.68–1.27)	0.633		
γ-GTP ≥20 IU/L	1.17 (0.85–1.61)	0.330		
Total bilirubin ≥1 mg/dL	1.41 (0.80–2.49)	0.239		
Albumin ≥4 g/dL	0.78 (0.51–1.18)	0.239		
Platelets >150 × 10^3/mm^3	0.99 (0.67–1.46)	0.946		
α-Fetoprotein ≤10 μg/L	0.84 (0.48–1.47)	0.543		
Genotype A or B	1.17 (0.81–1.69)	0.410		
HBeAg-negative status	0.78 (0.79–2.07)	0.314		
HBV DNA ≥5 log copies/mL	0.84 (0.58–1.24)	0.383		
HBsAg ≤2,000 IU/mL	1.87 (1.19–2.91)	0.006	1.77 (1.12–2.77)	0.014
HBcrAg ≥4 log U/mL	0.85 (0.50–1.45)	0.555		
Wild-type precore sequence	0.99 (0.60–1.52)	0.967		
Wild-type core promoter sequence	0.78 (0.35–1.73)	0.538		

Wild-type precore sequence, G1896; wild-type core promoter sequence, A1762/G1764

AST aspartate aminotransferase, *ALT* alanine aminotransferase, *γ-GTP* γ-guanosine triphosphate, *HBeAg* hepatitis B e antigen, *HBsAg* hepatitis B surface antigen, *HBcrAg* hepatitis B core-related antigen

contributing to HBsAg seroclearance. The overall rate of HBsAg seroclearance was 1.75 % annually. The annual seroclearance rates of HBsAg are reported to be 1.7 % in Korea [14] and 1.6 % in Taiwan [15–17], as well as 2.5 % in Goto Islands of Japan, where HBV infections are very prevalent [18]. In 1,271 natives in Alaska, the rate of HBsAg seroclearance was 0.7 % annually [19]. These differences could be ascribed, in part, to HBV genotypes distinct among Asian countries and Alaska. Since treatment with IFN and/or nucleot(s)ide analogues has suppressive effects on the development of HCC [6, 20], they may influence HBsAg seroclearance.

Table 6 Differences in baseline characteristics between the 833 treated patients in whom HBsAg persisted and 149 those who lost HBsAg

Features at the baseline	HBsAg persisted (n = 833)	HBsAg lost (n = 149)	Differences p value
Age (years)	41 (13–88)	43 (17–71)	0.285
Men	601 (72.2 %)	124 (83.2 %)	0.004
HBV in family members	496 (59.6 %)	72 (48.3 %)	0.010
Chronic hepatitis	802 (96.3 %)	134 (89.9 %)	0.001
AST (IU/L)	54 (6–2,192)	78 (7–888)	0.010
ALT (IU/L)	93 (8–2,740)	118 (8–1,700)	0.117
γ-GTP (IU/L)	44 (4–1,278)	46 (4–1,278)	0.023
Total bilirubin (mg/dL)	0.7 (0.2–21.2)	0.7 (0.3–8.4)	0.273
Albumin (g/dL)	4.3 (1.1–5.4)	4.5 (1.4–5.3)	0.281
Platelets ($\times 10^3$/mm^3)	182 (40–483)	171 (50–391)	<0.001
α-Fetoprotein (μg/L)	4 (1–1,610)	4 (1–765)	0.682
Genotypes [A/B/C/others (%)]	3.2/10.7/85.1/1.0	5.1/12.4/81.6/0.9	0.565
HBeAg-negative status	230 (27.6 %)	79 (53.0 %)	<0.001
HBV DNA (log copies/mL)	7.8 (<2.1 to >9.1)	8.3 (<2.1 to >9.1)	0.002
HBsAg (IU/mL)	7,880 (0.04–277,000)	1,380 (0.04–188,000)	<0.001
HBcrAg (log U/mL)	6.9 (<3.0 to >6.8)	5.9 (<3.0 to >6.8)	0.003
Wild-type precore sequence	554 (66.6 %)	61 (41.2 %)	0.013
Wild-type core promoter sequence	274 (32.9 %)	67 (45.0 %)	0.836

Wild-type precore sequence, G1896; wild-type core promoter sequence, A1762/G1764

AST aspartate aminotransferase, *ALT* alanine aminotransferase, *γ-GTP* γ-guanosine triphosphate, *HBeAg* hepatitis B e antigen, *HBsAg* hepatitis B surface antigen, *HBcrAg* hepatitis B core-related antigen

Table 7 Factors influencing the seroclearance of HBsAg in treated patients evaluated by time-dependent uni- and multivariate analyses

Factors	Univariate analysis HBsAg clearance Relative risk (95 % CI)	p value	Multivariate analysis HBsAg clearance Relative risk (95 % CI)	p value
Age ≥50 years	1.91 (1.32–2.77)	0.001		
Male gender	2.14 (1.37–3.33)	0.001		
No HBV infection in family	1.58 (1.15–2.19)	0.005	2.22 (2.32–3.94)	0.006
Treatments (interferon vs. others)	2.13 (1.53–2.98)	<0.001	3.15 (1.69–5.87)	<0.001
Chronic hepatitis	3.12 (2.05–4.74)	<0.001		
AST ≥50 IU/L	1.47 (1.04–2.09)	0.031		
ALT ≥50 IU/L	1.29 (0.82–1.92)	0.201		
γ-GTP ≥20 IU/L	1.87 (1.30–2.70)	0.001		
Total bilirubin ≥1 mg/dL	1.35 (0.87–2.08)	0.179		
Albumin ≥4 g/dL	1.11 (0.66–1.86)	0.688		
Platelets ≤150 × 10^3/mm^3	2.10 (1.49–2.96)	<0.001		
α-Fetoprotein ≤10 μg/L	1.33 (0.92–1.92)	0.136		
Genotype A or B vs. others	1.16 (0.74–1.82)	0.529		
HBeAg-negative status	2.53 (1.83–3.50)	<0.001	3.75 (2.09–6.74)	<0.001
HBV DNA ≤5 log copies/mL	2.07 (1.37–3.13)	0.001		
HBsAg ≤2,000 IU/mL	2.29 (1.52–3.47)	<0.001		
HBcrAg ≤4 log U/mL	2.28 (1.31–3.97)	0.003		
Wild-type precore sequence	2.04 (1.18–3.55)	0.011		
Wild-type core promoter sequence	1.18 (0.63–2.21)	0.608		

Wild-type precore sequence, G1896; wild-type core promoter sequence, A176.2/G1764

AST aspartate aminotransferase, *ALT* alanine aminotransferase, *γ-GTP* γ-guanosine triphosphate, *HBeAg* hepatitis B e antigen, *HBsAg* hepatitis B surface antigen, *HBcrAg* hepatitis B core-related antigen

Therefore, we went on to extend our analysis to untreated patients and those treated with IFN or nucleotide analogues separately. Criteria for upper or lower levels of each parameter were set, taking into consideration the median value or a cutoff value with the lowest p value of the entire 2,112-patient cohort (Table 1), and unified for untreated and treated patients (Tables 5, 7).

Firstly, in the univariate analysis, age, no family history of HBV infection in third-degree or closer relatives, and decreased HBsAg levels lowered the annual rate of HBsAg seroclearance significantly. In multivariate analysis, age $\geq$50 years (RR 1.61, $p = 0.018$) and HBsAg $\leq$2,000 IU/mL (RR 1.77, $p = 0.014$) decreased the annual rate of HBsAg seroclearance significantly. Kato et al. [18] reported high HBsAg seroclearance rates in patients over 40 or over 50 years; in our patients, also, age $\geq$50 years increased RR to 1.61 ($p = 0.018$). As for HBsAg and HBV DNA, low HBsAg and HBV DNA levels increased the HBsAg seroclearance rate to 37.7 %, and therefore, low HBsAg levels are an important factor. In actuality, HBsAg levels $\leq$2,000 IU/mL increased the rate of HBsAg seroclearance with RR 1.77 ($p = 0.014$).

In treated patients, by contrast, age, the male gender, no HBV infections in third-degree or closer relatives, treatment with IFN, chronic hepatitis, high AST levels, high γ-GTP levels, low platelet counts, HBeAg-negative status, low HBsAg levels, low HBcrAg levels and the wild-type precore sequence were significant factors in univariate analysis. In multivariate analysis, no HBV infections in third-degree or closer relatives (RR 2.22, $p = 0.006$), interferon treatments (RR 3.15, $p < 0.001$), and HBeAg-negative status (RR 3.75, $p < 0.001$) were significant factors.

Thus, there were differences in factors predictive of the HBsAg loss between untreated and treated patients. Remarkably, age and HBsAg titer were independent factors in untreated patients, whereas family history and negative HBeAg were independent factors in treated patients. Since this work studied patients who were followed for a long time (>15 years), age and HBsAg titer were factors for clearance of HBsAg in untreated patients. Treated patients, in contrast, would have included more patients with HBeAg, with a good response to antiviral treatment, as well as those without family history who would have been infected with HBV with a sorter duration than those with family history. In other words, most untreated patients were those with favorable clinical course, in whom HBsAg titer gradually decreased and eventually lost it with time. In fact, there would be many such patients, the majority of whom do not visit hospitals and are unaware of HBV infection, who may have unapparent liver disease. Treated patients, on the other hand, would have had higher risks for cirrhosis and HCC, owing to elevated ALT/AST levels; this risk is especially high for patients with a family history of HBV [21]. Therefore, patients with family history would not be able to easily lose HBsAg.

In treated patients, IFN led to HBsAg loss more effectively than other treatments [RR 2.13, $p < 0.001$ (Table 7)]. The immunomodulatory activity of IFN, which is not shared by nucleot(s)ide analogues, would have accelerated the immune response to HBV required for the seroclearance of HBsAg. Of the 333 patients who received IFN, 190 (57 %) were treated with IFN multiply. In them, seroclearance of HBsAg was achieved in 49 of the 190 (26 %) patients with multiple IFN treatments in comparison with 41 of the 143 (29 %) with single IFN treatment. Owing to indications for IFN, patients who received IFN tended to be younger, without previous treatments and higher HBV DNA as well as ALT levels. They might have increased the rate of HBsAg loss that was higher with IFN than other treatments.

Since this is a retrospective cohort study of patients visiting our hospital for more than 15 years, and there has been so much innovation in the treatment of chronic hepatitis B during that period, treated and untreated patients have different backgrounds at the baseline. Hence, treated patients had higher ALT and HBV DNA levels with severer liver disease than untreated patients (Table 3). This might have been responsible, at least in part, for the failure in finding differences in the rate of HBsAg loss between untreated and treated patients (Fig. 2). Future studies will be aimed at analyzing contributing factors in treated and matched controls. This will allow us to analyze factors contributing to HBsAg seroclearance in the treatment of patients with chronic hepatitis B.

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Conflict of interest These authors disclose the following: Dr. Kumada reports having received investigator, lecture, and consulting fees from Dainippon Sumitomo Pharma Co., MSD KK, Bristol-Myers Squibb, Pharma International, Dentsu Sudler, and Hennessey Inc. Dr. Ikeda reports having received investigator, lecture, and consulting fees from Dainippon Sumitomo Pharma Co. No other potential conflicts of interest relevant to this article were reported.

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HLA-DP genes polymorphisms associate with hepatitis B surface antigen kinetics and seroclearance during nucleot(s)ide analogue therapy

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Keywords
hepatitis B surface antigen – *HLA-DP* – lamivudine – nucleot(s)ide analogue

Abbreviations
ADV, adefovir dipivoxil; ALT, alanine aminotransferase; ETV, entecavir; GWAS, genome-wide association study; HBeAg, hepatitis B e antigen; HBsAg, hepatitis B surface antigen; HBV DNA, hepatitis B virus deoxyribonucleic acid; HBV, hepatitis B virus; HLA, human leucocyte antigen; HR, hazard ratio; IFN, interferon; LAM, lamivudine; NA, nucleos(t)ide analogues; SNP, single nucleotide polymorphism; VBT, virological breakthrough; VR, virological response.

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Abstract

Background & Aims: Genome-wide association studies (GWAS) recently indicated that polymorphisms in the human leucocyte antigen *(HLA)-DP* genes were associated with risk of persistent hepatitis B virus (HBV) infection and clearance of HBV, but the effect of *HLA-DP* gene polymorphisms on the effect of antiviral therapy was unknown. We here investigated whether such polymorphisms were associated with decreases in HBsAg levels and seroclearance in patients who received long-term lamivudine (LAM) treatment. *Methods:* Japanese patients (202) who were hepatitis B e antigen positive at baseline, received LAM as first-line treatment, and consented to *HLA-DP* genotyping (*HLA-DPA1* rs3077 and HLA-DPB1 rs9277535) were categorized into two cohorts, viz., a cohort who achieved virological response without rescue therapy (cohort 1) and those who did so with rescue therapy (cohort 2). *Results:* Serum HBsAg levels declined significantly between year 3 and 9 from baseline among cohort 1 patients possessing ≥2 A-alleles at rs3077 and rs9277535. The percentages of such patients in cohort 1 patients with decreases in HBsAg ≥0.5 log IU/ml were higher than those with <2 A-alleles (71.8% [28/39] vs. 38.9% [23/59]; $P = 0.004$). However, there was no significant difference in cumulative HBsAg seroclearance rates between patients with ≥2 and those with <2 A-alleles in cohort 1. In cohort 2, HBsAg seroclearance rates were higher in patients with ≥2 A-alleles than in those with <2 A-alleles ($P = 0.003$). *Conclusion:* We found an association between *HLA-DP* polymorphisms and decreases in HBsAg levels and seroclearance among HBeAg-positive patients treated with LAM.

More than 2 billion people worldwide have been exposed to hepatitis B virus (HBV) and about 350 million people are chronically infected, the majority of whom are in Asia (75%). The prevalence of HBV in Japan is 0.8%, which is lower than that in other Asian countries, such as Taiwan (>10%) and China (1–3). Recently, oral nucleot(s)ide analogues (NAs) have been used as a mainstay therapeutic strategy against chronic hepatitis B. Such antiviral agents, including lamivudine (LAM), entecavir (ETV), telbivudine, adefovir dipivoxil (ADV) and tenofovir disoproxil fumarate, inhibit viral replication. These NAs vary in both the strength and the rapidity with which they suppress HBV DNA (4–10). Sustained viral suppression by NA therapy can improve liver fibrosis and clinical outcomes of patients (11, 12). LAM was the first NA to be approved for treating chronic hepatitis B in Japan, followed by ADV and ETV.

Responses to antiviral treatments can be evaluated by monitoring serum HBV DNA levels, and hepatitis B e (HBeAg) and hepatitis B surface antigen (HBsAg) and antibody levels. Serum HBsAg levels have been recognized as one of the predictive markers of the prognosis and effect of antiviral therapy. Some studies recently reported the rates of HBsAg seroclearance and HBsAg

kinetics after pegylated interferon (PegIFN) and/or NAs therapy (13–15). These studies demonstrated that HBsAg seroclearance is associated with HBV genotype, baseline HBsAg levels, and the decrease in HBsAg early during treatment. However, it remains unclear whether host factors are associated with treatment-related HBsAg seroclearance.

Genome-wide association studies (GWAS) have been well applied in the field of viral hepatitis, and several studies have reported that the human leucocyte antigen (*HLA*)-*DP* locus, located on chromosome 6, is associated with the risk of persistent infection with HBV (16). A few studies have reported that the *HLA-DP* locus is also associated with HBV clearance (17–20). Two single nucleotide polymorphisms (SNPs) in a region including *HLA-DPA1* and *HLA-DPB1* are strongly associated with persistent HBV infection (*HLA-DPA1* rs3077 and *HLA-DPB1* rs9277535). The minor alleles (A-alleles) of both rs3077 and rs9277535 seem to have protective effects against chronic hepatitis B (16). Although there have been two reports on the association between the *HLA-DP* locus and antiviral therapy for chronic hepatitis B, further investigation of association of *HLA-DP* SNPs with the effect of antiviral therapy is warranted (21, 22).

We therefore hypothesized that the minor alleles of *HLA-DPA1* rs3077 and *HLA-DPB1* rs9277535 may have an impact on HBsAg kinetics and seroclearance during NA therapy.

In this study, we investigated whether polymorphisms in *HLA-DP* genes are associated with reduction in HBsAg titres and seroclearance in chronic HBeAg-positive hepatitis B patients who received long-term LAM treatment and subsequently achieved favourable sustained viral responses.

Patients & methods

Study population

Over a period of 12 years (September 1995 to September 2007), 949 consecutive patients, chronically mono-infected with HBV (confirmed HBsAg positivity over a period of at least 6 months), were treated with LAM monotherapy at the Department of Hepatology, Toranomon Hospital, Metropolitan Tokyo. The indication for antiviral therapy was abnormal ALT levels accompanying the increase in HBV DNA levels (over 4 log copies/ml). However, in cases where ALT levels were normal, patients with advanced fibrosis were also administrated LAM. We selected 791 patients as study subjects after we excluded all those who had been treated with LAM for <6 months, were co-infected with hepatitis C virus, had not provided sufficient serum samples, and/or had insufficient clinical records. No patient was co-infected with human immunodeficiency virus in this cohort. Of these 791 patients, 441 were HBeAg positive and 350 were HBeAg negative at baseline.

HLA-DP SNPs were analysed in 253 of 441 patients who are HBeAg positive. Ninety eight of 253 patients achieved viral response (VR: HBV DNA <600 copies/ml) and subsequently maintained low viral load (HBV DNA <1 log copies/ml from nadir; cohort 1). Over time, 136 of these 253 individuals experienced an increase in HBV DNA (≥1 log copies/ml; e.g. because of virological breakthrough [VBT]), and as a result, 133 (98%) individuals were provided with ADV treatment (10 mg) added to LAM, as a rescue therapy. Of the 133, 104 patients achieved VR with rescue therapy and subsequently maintained a low viral load (HBV DNA <1 log copies/ml from nadir; cohort 2). Thus, in total, 202 patients were enrolled in this retrospective cohort study (Fig. 1). All of these patients are Japanese. Written informed consent was obtained from each patient. This study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki and was approved *a priori* by the institution's human research committee.

Clinical data collection and follow-ups

Data on patient characteristics, biochemistry, haematology, virology, histology and previous treatments were collected and registered in our institute's database at the time of patient enrolment. Prior to beginning LAM therapy, the presence of family history of HBV infection was surveyed in all patients. Data on the treatment dose and duration of previous IFN therapy were collected from our hospital's IFN therapy database or requested from other hospitals as necessary.

At least every 1–3 months, liver function and virological markers of HBV infection were measured in all patients. All serum HBsAg titres were measured from frozen serum samples collected at 6 months, 1, 3, 5 years, and once annually for 6–10 years, and then stored at −80°C. The time-point of HBsAg clearance was defined by the measurement in consecutive available serum samples before HBsAg undetected. A genotypic analysis of drug resistance was performed in cases of insufficient virological response or VBT, defined as an increase in serum HBV DNA levels ≥1 log above the nadir measured after the initial virological response. Cirrhosis was diagnosed by laparoscopy, liver biopsy, or clinical data, such as imaging modalities and portal hypertension. The primary outcome for this study was seroclearance and significant reduction in HBsAg. The endpoint of the follow-up was HBsAg clearance or last visit before March 2013.

Markers of HBV infection

Serum HBsAg titres were measured using ARCHITECT HBsAg QT assay kits (Abbott Laboratories, Tokyo, Japan), which have a lower limit of detection of 0.05 IU/ml and an upper limit of detection of 250 IU/ml. To expand the upper range from 250 to 125 000 IU/ml, serum samples exceeding the scale were

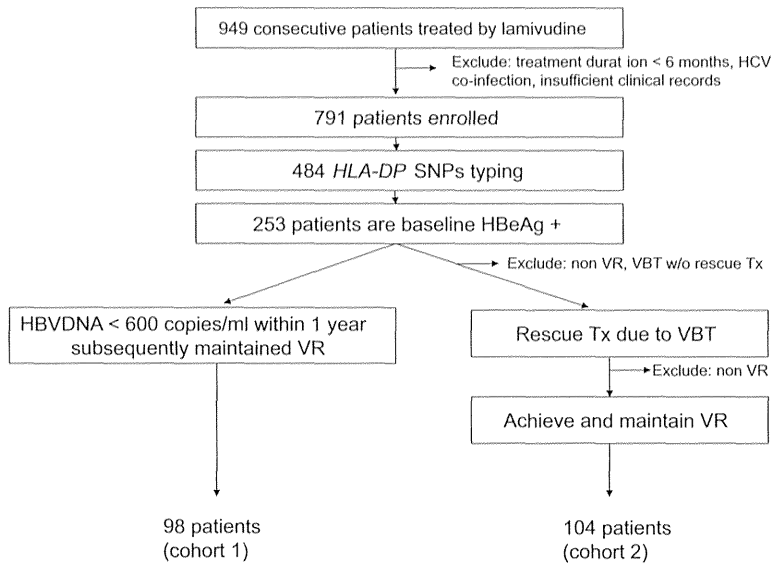

Fig. 1. Schematic of study protocol. HBeAg, hepatitis B e antigen; HCV, hepatitis C virus; HLA, human leucocyte antigen; SNP, single nucleotide polymorphism; VBT, virological breakthrough; VR, virological response.

diluted stepwise to 1:20 and 1:500 with ARCHITECT diluents as the product document described. HBeAg was determined by enzyme-linked immunosorbent assay with a commercial kit (HBeAg EIA; Institute of Immunology, Tokyo, Japan). HBV DNA was quantified using the Amplicor monitor assay (Roche Diagnostics, Tokyo, Japan), which has a dynamic range over 2.6–7.6 log copies/ml, or COBAS TaqMan HBV v.2.0 (Roche Diagnostics), which has a dynamic range over 2.1–9.0 log copies/ml. A commercial kit (HBV Genotype EIA; Institute of Immunology) was used to determine HBV genotypes serologically by using the combination of epitopes expressed on the pre-S2 region product, which is specific for each of the seven major genotypes (A–G). YMDD mutants (rt M204I/V) were determined by polymerase chain reaction-based enzyme-linked mini-sequence assay with a commercial kit (Genome Science Laboratories, Tokyo, Japan).

HLA-DP SNPs typing

SNPs in *HLA-DPA1* (rs3077) and *HLA-DPB1* (rs9277535), located on chromosome 6, were genotyped by TaqMan assay or Invader assay, as previously described (16). *IL28B* genotype was assayed for the rs8099917 SNP using TaqMan assay or Invader assay.

Statistical analyses

Categorical data were compared between groups using chi-square or Fisher's exact tests. Continuous variables with a non-parametric distribution were analysed with Mann–Whitney *U*-tests, while those with a parametric distribution were analysed with Student's *t*-tests. Cox regression analyses were used to assess which variables were significantly associated with HBsAg clearance. All baseline factors that were found to be significantly associated with HBsAg clearance by univariate analysis were entered into a multivariate analysis. Independent baseline factors associated with clearance of HBsAg were calculated using a stepwise Cox regression analysis. Cumulative HBsAg clearance rates were analysed using the Kaplan–Meier method; differences in the resulting curves were tested using log-rank tests. Cochran–Armitage trend tests were performed for the association between HBsAg seroclearance and an increase in the number of A-alleles. Significance was defined as $P < 0.05$ for all two-tailed tests. Data analysis was performed with IBM SPSS version 19.0 software (IBM Corp., Armonk, NY, USA) and R software version 2.13 (R Foundation for Statistical Computing, Vienna, Austria; www.r-project.org).

Results

Patient characteristics and clinical course

Eighteen of 202 patients successfully cleared HBsAg. Of these, 11 belonged to cohort 1, and seven to cohort 2. Table 1 provides a comparison of the baseline characteristics between patients who were and were not able to successfully clear HBsAg (all patients, cohort 1 and cohort 2).

In cohort 1, baseline characteristics that were significantly associated with HBsAg clearance included HBV genotype and high HBV DNA levels; in cohort 2, such

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Table 1. Baseline and demographic characteristics of patients with and without HBsAg seroclearance

Characteristics	All patients (n = 202)	LAM VR cohort (without rescue Tx) (cohort 1)			LAM add-on rescue Tx cohort (cohort 2)		
		Persistently HBsAg positive (n = 87)	HBsAg seroclearance (n = 11)	P	Persistently HBsAg positive (n = 97)	HBsAg seroclearance (n = 7)	P
Baseline							
*Age (y) (SD)	43 (11.5)	41 (11.9)	45 (12.6)	0.553	43 (11.3)	44 (6.3)	0.590
Gender (male:female)	151:51	60:27	9:2	0.499	75:22	7:0	0.341
Family history of HBV infection	139 (69)	57 (66)	6 (60)	0.737	71 (73)	4 (57)	0.395
Previous IFN therapy	100 (49)	44 (51)	9 (82)	0.060	42 (43)	5 (71)	0.240
Pre-existing cirrhosis	42 (21)	16 (18)	1 (9)	0.685	23 (24)	2 (29)	0.676
HBV genotype				**0.016**			**1.65×10^{-6}**
A	8 (4.0)	3 (3.4)	3 (27)		0 (0)	2 (28.6)	
B	9 (4.5)	6 (6.9)	0 (0)		2 (2.1)	1 (14.3)	
C	180 (89.1)	76 (87.4)	8 (73)		92 (94.8)	4 (57.1)	
D	1 (0.5)	0 (0)	0 (0)		1 (1.0)	0 (0)	
Unclassified/missing	4 (1.9)	2 (2.3)	0 (0)		2 (2.1)	0 (0)	
Baseline HBV DNA (log copies/ml)	7.6 (6.7–8.1)	7.4 (6.4–8.0)	8.0 (7.6–8.6)	**0.036**	7.7 (6.7–8.1)	7.9 (7.2–8.0)	0.693
Baseline HBsAg level (IU/ml)	3070 (1290–10 800)	2350 (1040–6650)	5660 (773–52 500)	0.168	3370 (1720–12 100)	2300 (946–66 600)	0.948
Baseline AST level (IU/L)	86 (60–174)	105 (60–244)	229 (64–1170)	0.109	77 (60–130)	88 (49–218)	0.829
Baseline ALT level (IU/L)	149 (80–337)	173 (94–441)	480 (79–1024)	0.132	125 (71–226)	106 (96–152)	0.953
Baseline total bilirubin level (mg/dl)	0.8 (0.6–1.1)	0.8 (0.6–1.3)	0.8 (0.6–7.4)	0.409	0.8 (0.5–1.1)	0.8 (0.6–1.1)	0.799
*Platelet count (10^5/mm^3) (SD)	16.1 (5.6)	16.9 (6.4)	14.2 (3.1)	0.201	15.6 (5.3)	13.3 (2.9)	0.252
HLA-DPA1 (rs3077)				0.949			**0.001**
GG	125 (61.9)	51 (58.6)	6 (54.6)		67 (69.1)	1 (14.3)	
GA	65 (32.2)	30 (34.5)	4 (36.3)		27 (27.8)	4 (57.1)	
AA	12 (5.9)	6 (6.9)	1 (9.1)		3 (3.1)	2 (28.6)	
HLA-DPB1 (rs9277535)				0.288			**0.039**
GG	117 (57.9)	50 (57.5)	5 (45.4)		61 (62.9)	1 (14.3)	
GA	71 (35.2)	29 (33.3)	6 (54.5)		31 (32.0)	5 (71.4)	
AA	14 (6.9)	8 (9.2)	0 (0)		5 (5.1)	1 (14.3)	
Number of A-alleles ≥2 (rs3077, rs9277535)	74 (36.6)	34 (39.1)	5 (45.4)	0.750	29 (29.9)	6 (85.7)	**0.006**
Treatment duration	9.0 (7.3–11.2)	9.0 (7.2–11.8)	6.5 (2.5–9.6)	0.084	9.4 (8.0–11.2)	6.5 (3.8–11.7)	0.132

Except where marked with an asterisk (*), values are expressed as the median and 25th–75th percentile (parenthetically), or number and percentage (parenthetically). Asterisks indicate data displayed as mean values and standard deviation. Bold text indicates statistically significant P-values.

significant characteristics included HBV genotype and *HLA-DP* SNPs. Ninety-one of 98 patients in cohort 1 sustained VR (HBV DNA <600 copies/ml) during NA treatment. Within 1 year, HBV DNA levels have increased minimally by <1 log copies/ml from the nadir in the other seven patients. Forty-four patients switched from LAM to ETV (0.5 mg/day) in cohort 1 because of favourable viral suppression. Viral suppression was subsequently continued after switching from LAM to ETV in cohort 1 patients. In cohort 2, the median duration from the start of rescue therapy to VR was 24 weeks.

Baseline characteristics and early virological response by *HLA-DP* gene polymorphisms

The genotypic distributions of rs3077 and rs9277535 genotypes were in Hardy–Weinberg equilibrium ($\chi^2 = 0.671$, $P = 0.714$ and $\chi^2 = 0.513$, $P = 0.774$ respectively). The minor allele frequencies (MAF) of rs3077 and rs9277535 were 0.220 and 0.245 respectively (minor allele = A).

Table 2 shows baseline characteristics and early virological response stratified by *HLA-DP* gene genotype. There were no differences in the distribution of baseline characteristics by rs3077, rs9277535 or the number of A-alleles at rs3077 and rs9277535. There were no differences in the early virological response (decline of HBsAg level [≥0.5 log IU/ml within 6 months], HBeAg seroclearance within 6 months, and undetectable HBV DNA [<400 copies/ml at 6 months]) by *HLA-DP* gene polymorphisms. There were no differences in the distribution of baseline characteristics, and in the early virological response by rs8099917 of *IL28B* gene (Supplementary Table).

Association between *HLA-DP* gene polymorphisms and HBsAg kinetics in cohort 1

HBsAg kinetics in cohort 1 is shown in Fig. 2A. Among patients with A-alleles ≥2 at the *HLA-DP* polymorphisms (rs3077, rs9277535), the median HBsAg change from baseline was −0.36 log IU/ml at 3 years, −0.49 at 5 years, −0.60 at 7 years and −0.73 at 9 years. Among patients with the number of A-alleles <2, the median changes were −0.06 log IU/ml at 3 years, −0.15 at 5 years, −0.23 at 7 years and −0.38 at 9 years. *HLA-DP* gene polymorphisms had a significant effect on the slopes between data collection points at 3 and 9 years. Moreover, we subanalysed HBsAg kinetics only in patients with HBV genotype C because about 90% of this cohort had genotype C. The results were similar to those of all genotypes. *HLA-DP* gene polymorphisms had a significant effect on the slopes between data collection points at 5 and 7 years (Fig. 2). The significant differences in HBsAg decline were not observed according to *IL28B* polymorphism.

We categorized the slopes of HBsAg kinetics from baseline to last visit into three groups as follows: no decline, <0.5 log IU/ml decrease or increase, slow decline, 0.5–0.99 log IU/ml decrease, and rapid decline, over 1 log IU/ml decline. The percentages of patients in which the number of A-alleles at the *HLA-DP* polymorphisms ≥2 were 30.8% (12/39) in the rapid decline group, 41.0% (16/39) in the slow decline group, and 28.2% (11/39) in the no decline group (Fig. 2C). The percentages of patients with <2 A-alleles were 22.0% (13/59) in the rapid decline group, 16.9% (10/59) in the slow decline group, and 61.0% (36/59) in the no decline group (Fig. 2C). There were significant differences in the HBsAg decline patterns according to *HLA-DP* polymorphisms ($P = 0.004$). The results were similar in HBV genotype C subpopulation. There were significant differences in the HBsAg decline patterns according to *HLA-DP* polymorphisms as shown in Fig. 2D ($P = 0.001$).

Association between *HLA-DP* gene polymorphisms and HBsAg kinetics in cohort 2

Because the timing of VBT in cohort 2 patients varied, it was difficult to analyse the kinetics of changes in HBsAg levels. Therefore, we examined HBsAg kinetics after the achievement of VR by rescue therapy (Fig. 3A). Among patients with A-alleles ≥2, the median HBsAg change from VR with rescue therapy was −0.15 log IU/ml at 1 year, −0.31 at 3 years, −0.53 at 5 years and −0.63 at 7 years, and among patients with A-alleles <2, the median changes were −0.08 log IU/ml at 1 year, −0.21 at 3 years, −0.37 at 5 years and −0.43 at 7 years. *HLA-DP* gene polymorphisms had a significant effect on the slopes of VR between 1 and 5 years. Although the tendency of HBsAg change was observed in HBV genotype C subpopulation, *HLA-DP* gene polymorphisms had only a marginally significant effect on the slopes of VR (Fig. 3B). The significant differences in HBsAg decline were not observed according to *IL28B* polymorphism. The percentages of VR patients with A-alleles ≥2 with ≥1 log IU/ml declines in HBsAg levels were significantly higher than those with <2 A-alleles (Fig. 3C). Moreover, the results were similar in genotype C subpopulation (Fig. 3D).

We evaluated whether ALT flare-up before starting ADV, HBeAg loss before starting ADV, and HBsAg levels at the start of ADV affected subsequent HBsAg seroclearance in cohort 2 because the phenomenon in which virological response and breakthrough by LAM resistance resulted might affect clinical courses. Median peek ALT levels before ADV were 234 IU/L (interquartile range, IQR: 23–385) in patients with HBsAg seroclearance, and 132 IU/L (IQR: 62–308) with persistent HBsAg positivity. There was no significant difference in peek ALT levels before ADV ($P = 0.851$). Thirty-one patients (29.8%) achieved HBeAg loss during LAM monotherapy before ADV added-on LAM. Four of 31 patients (12.9%) with HBeAg loss, and 3 of 73 patients (4.1%) without HBeAg loss achieved HBsAg

Table 2. Baseline, demographic and on-treatment characteristics according to *HLA-DP* genotypes

Characteristics	rs3077 GG (n = 125)	MAF = 0.220 GA (n = 65)	AA (n = 12)	P	rs9277535 GG (n = 117)	MAF = 0.245 GA (n = 71)	AA (n = 14)	P	Number of A-alleles <2 (n = 128)	≥2 (n = 74)	P
Baseline											
*Age (year) (SD)	44 (12.0)	41 (10.8)	43 (8.2)	0.309	44 (12.1)	42 (10.7)	42 (10.0)	0.536	44 (11.8)	42 (10.8)	0.304
Gender (male:female)	88:37	53:12	10:2	0.191	81:36	58:13	12:2	0.101	91:37	60:14	0.132
Family history of HBV infection	79 (69)	38 (68)	7 (70)	0.807	80 (68)	48 (68)	10 (71)	0.973	86 (67)	52 (70)	0.636
Previous IFN therapy	55 (44)	37 (57)	8 (67)	0.113	51 (44)	39 (55)	10 (71)	0.076	57 (45)	43 (58)	0.080
Pre-existing cirrhosis	25 (20)	13 (20)	4 (33)	0.564	23 (20)	16 (23)	3 (21)	0.898	26 (20)	16 (22)	0.858
HBV genotype				0.772				0.673			0.767
A	4 (3.2)	3 (4.6)	1 (8.3)		2 (1.7)	5 (7.0)	1 (7.1)		4 (3.1)	4 (5.4)	
B	5 (4.0)	3 (4.6)	1 (8.3)		4 (3.4)	4 (5.6)	1 (7.1)		5 (3.9)	4 (5.4)	
C	113 (90.4)	58 (89.2)	9 (75)		107 (91.4)	61 (86.0)	12 (85.7)		116 (90.6)	64 (86.5)	
D	1 (0.8)	0 (0)	0 (0)		1 (0.9)	0 (0)	0 (0)		1 (0.8)	0	
unclassified/missing	2 (1.6)	1 (1.7)	1 (8.3)		3 (2.7)	1 (1.4)	0 (0)		2 (1.6)	2 (2.7)	
Baseline HBV DNA (log copies/ml)	7.6 (6.7–8.0)	7.5 (6.8–8.1)	7.1 (5.9–8.6)	0.892	7.6 (6.7–8.1)	7.7 (6.8–8.2)	6.8 (5.7–7.8)	0.082	7.6 (6.7–8.1)	7.5 (6.7–8.0)	0.862
Baseline HBsAg level (IU/ml)	3180 (1350–12 600)	2910 (1270–5700)	2420 (1010–10 700)	0.582	3110 (1170–12 300)	3070 (1790–7250)	1690 (1200–10 500)	0.792	3120 (1300–12 700)	2930 (1280–6030)	0.455
Baseline AST level (IU/L)	83 (58–166)	100 (63–228)	82 (62–575)	0.343	85 (57–168)	88 (64–218)	97 (58–214)	0.637	84 (58–165)	93 (63–235)	0.178
Baseline ALT level (IU/L)	137 (76–268)	188 (84–375)	138 (91–861)	0.367	145 (71–297)	151 (82–371)	150 (72–462)	0.679	142 (77–263)	173 (86–380)	0.209
Baseline total bilirubin level (mg/dl)	0.8 (0.6–1.1)	0.8 (0.6–1.2)	0.8 (0.7–1.6)	0.567	0.8 (0.6–1.1)	0.8 (0.6–1.2)	0.8 (0.6–1.2)	0.769	0.8 (0.6–1.1)	0.8 (0.6–1.3)	0.229
*Platelet count (10^5/mm^3) (SD)	16.3 (5.5)	16.1 (6.3)	14.5 (4.1)	0.535	16.4 (5.6)	15.9 (6.1)	15.0 (3.8)	0.551	15.7 (6.0)	16.4 (5.5)	0.190
Early treatment response											
Decline of HBsAg level (≥0.5 log IU/ml within 6 months)	26 (21)	19 (29)	3 (25)	0.447	24 (21)	24 (28)	3 (21)	0.375	26 (20)	22 (30)	0.170
HBeAg positive→clearance within 6 months	41 (33)	19 (29)	6 (50)	0.389	40 (34)	20 (28)	6 (43)	0.477	42 (33)	24 (32)	1.000
Undetectable HBV DNA (<400 copies/ml) at 6 months	74 (59)	43 (66)	9 (75)	0.439	72 (62)	43 (58)	11 (79)	0.464	76 (59)	50 (68)	0.287

Except where marked with an asterisk (*), values are expressed as the median and 25th–75th percentile (parenthetically), or number and percentage (parenthetically). Asterisks indicate data displayed as mean values and standard deviation. MAF; minor allele frequency. Bold text indicates statistically significant P-values.

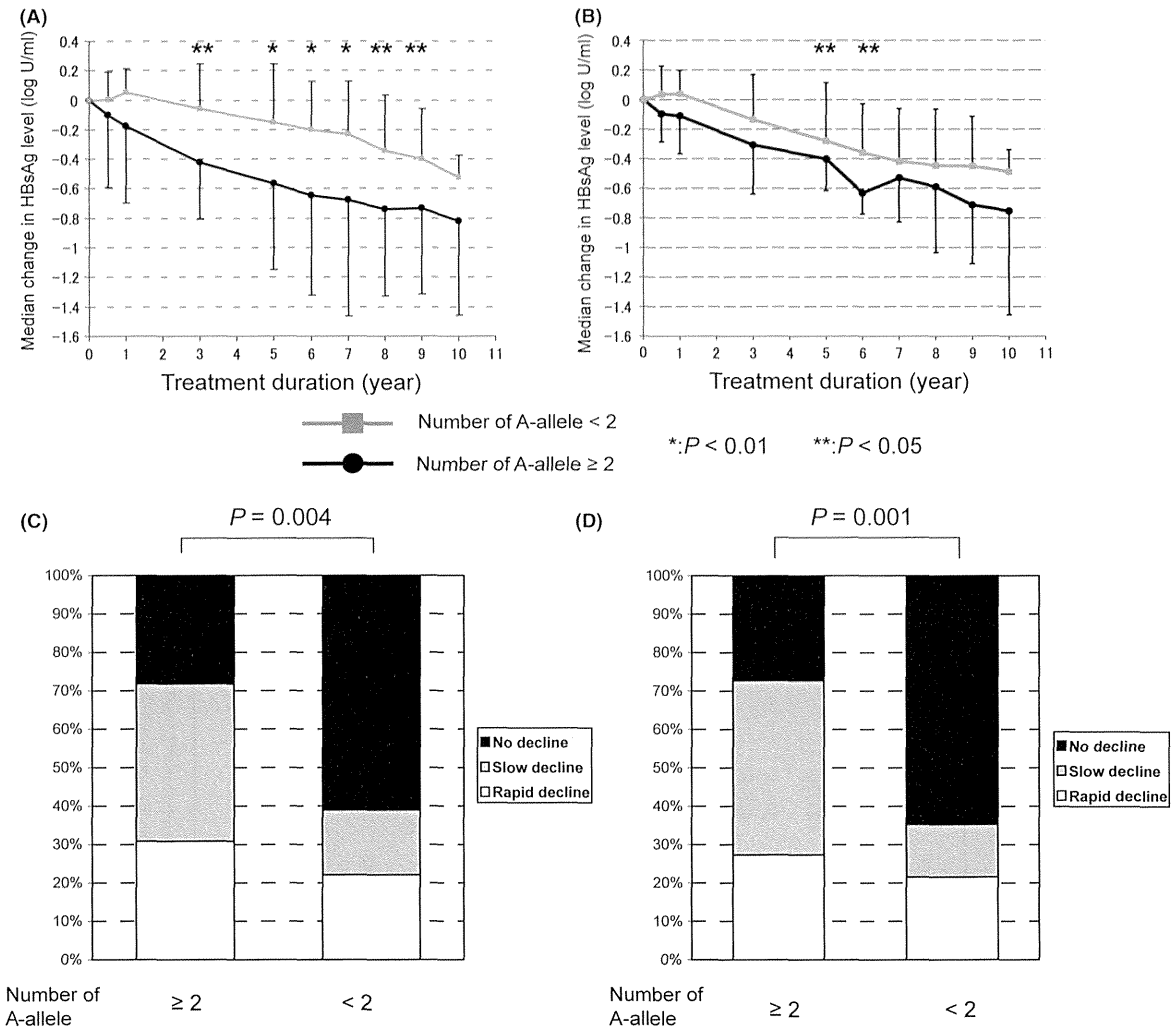

Fig. 2. (A) Median change in HBsAg level from baseline in patients with *HLA-DP* gene alleles (cohort 1; all patients). The asterisk (*) indicates a statistical significance of $P < 0.01$, and (**) indicates $P < 0.05$ as determined by the Mann–Whitney *U*-test. (B) Median change in HBsAg level from baseline in patients with *HLA-DP* gene alleles (cohort 1; HBV genotype C only). The asterisk (*) indicates a statistical significance of $P < 0.01$, and (**) indicates $P < 0.05$ as determined by the Mann–Whitney *U*-test. (C) Patterns of decrease in HBsAg in all cohort 1 patients with the number of A-alleles in rs3077 and rs9277535. No decline is defined as <0.5 log decrease or increase, slow decline as 0.5–0.99 log decline, and rapid decline as ≥1.0 log decline. (D) Patterns of decrease in HBsAg only in HBV genotype C cohort 1 patients with the number of A-alleles in rs3077 and rs9277535. No decline is defined as <0.5 log decrease or increase, slow decline as 0.5–0.99 log decline, and rapid decline as ≥1.0 log decline.

seroclearance after ADV added-on LAM. However, there was no significant difference in HBsAg seroclearance among patients with or without HBeAg loss ($P = 0.192$) before ADV because the number of patients with HBsAg seroclearance was small. There was also no significant difference in cumulative HBsAg seroclearance rates among patients with or without HBeAg loss ($P = 0.166$). Median HBsAg levels at the start of ADV were 1310 IU/ml (IQR: 6.64–44 200) in patients with HBsAg seroclearance, and 5850 IU/L (IQR: 2160–16 500) with persistent HBsAg positivity. There was no significant difference in HBsAg levels at the start of ADV ($P = 0.400$). Median peek ALT levels before ADV were 132 IU/L (IQR: 66–259) in patients with A-alleles <2, and 138 IU/L (IQR: 51–457) with A-alleles ≥2. Median HBsAg levels at the start of ADV were 5730 IU/ml (IQR: 2490–18 000) in patients with A-alleles <2, and 5450 IU/L (IQR: 1320–12 000) with A-alleles ≥2. There were no significant differences in peek ALT levels before ADV and HBsAg levels at the

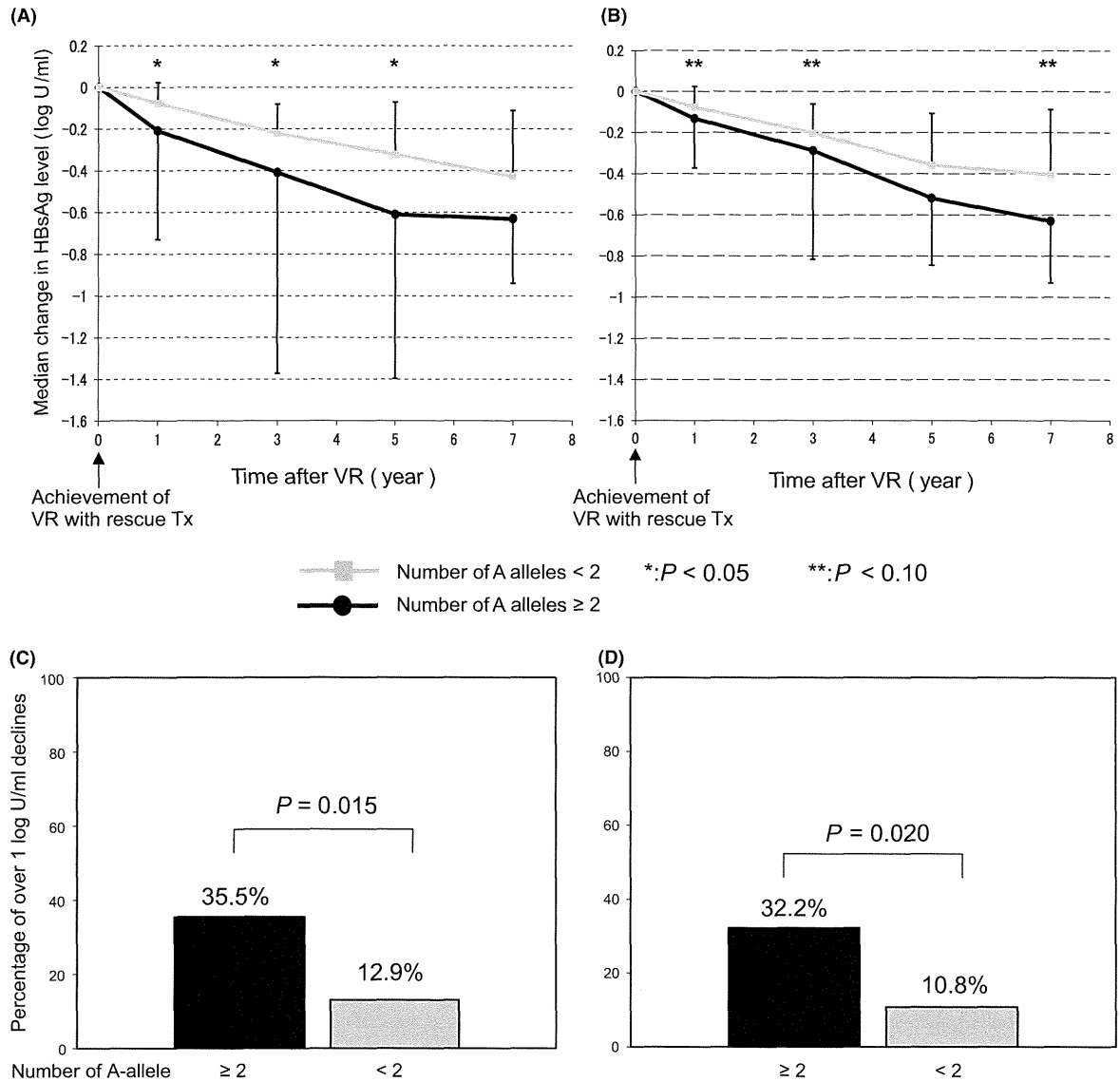

Fig. 3. (A) Median change in HBsAg level from VR in patients with *HLA-DP* gene alleles (cohort 2). The asterisk (*) indicates a statistical significance of $P < 0.05$ as determined by the Mann–Whitney *U*-test. VR, virological response. (B) Median change in HBsAg level from VR in patients with *HLA-DP* gene alleles (cohort 2; HBV genotype C only). The asterisk (**) indicates a marginal significance of $P < 0.10$ as determined by the Mann–Whitney *U*-test. VR, virological response. (C) Decreases of ≥1 log U/ml of HBsAg levels over time in all cohort 2 patients with the number of A-alleles in rs3077 and rs9277535. (D) Decreases of ≥1 log U/ml of HBsAg levels over time only in HBV genotype C cohort 2 patients with the number of A-alleles in rs3077 and rs9277535.

start of ADV according to the number of A-alleles (ALT; $P = 0.625$, HBsAg; $P = 0.320$).

Association between *HLA-DP* polymorphism and HBsAg seroclearance

We performed a detailed analysis of the association between *HLA-DP* gene polymorphisms and HBsAg seroclearance in patients treated with LAM. Cumulative HBsAg clearance rates from baseline in cohort 1 patients were as follows: 2.6% at 3 y, 5.3% at 5 y, and 14.4% at 7 y in patients with A-alleles ≥2; 1.7% at 3 y, 5.5% at 5 y, 7.4% at 7 y, and 12.4% at 9 y in patients with <2 A-alleles (Fig. 3A). There was no significant difference in HBsAg seroclearance rates between these two patient groups.

Cumulative HBsAg clearance rates from the achievement of VR with rescue therapy in cohort 2 patients were as follows: 2.9% at 1 y, 14.3% at 3 y, and 17.6% at 5 y in patients with A-alleles ≥2; 0% at 3 y and 1.7% at 5 y for patients with <2 A-alleles (Fig. 3B). HBsAg seroclearance rates from VR were significantly higher in cohort 2 patients with ≥2 A-alleles than in those with fewer A-alleles.

Multivariate Cox regression analysis identified four significant baseline characteristics related to HBsAg clearance: previous IFN therapy, infection with HBV genotype A, high total bilirubin levels and A-alleles ≥2 in the combined cohort (Table 3; model 1). However, in subanalysis among patients with genotype C, A-alleles ≥2 was not significantly associated with HBsAg clearance (Table 3; model 2). It seems to be that the reason is because the rates of HBsAg seroclearance was relatively low in genotype C subpopulation (Table 1). IL28B polymorphism was not associated with HBsAg seroclearance.

Number of A-alleles at rs3077 and rs9277535 and HBsAg seroclearance

We performed a detailed analysis of the association between the number of A-alleles and HBsAg clearance. Figure 4C shows the percentage of HBsAg seroclearance over time in all patients (cohort 1 + 2), stratified by the number of A-alleles. The percentage of HBsAg seroclearance over time was positively correlated with the number of A-alleles (P for trend = 0.009).

Discussion

We found that *HLA-DP* gene polymorphisms are associated with HBsAg kinetics in HBeAg-positive chronic hepatitis B patients who began treatment with LAM and continued with long-term NA therapy. *HLA-DP* gene polymorphisms were significantly associated with HBsAg seroclearance, and particularly in patients who received add-on rescue therapy. HBsAg kinetics and seroclearance during NA therapy were positively affected by a higher number (≥2) of A-alleles, i.e. the minor alleles at rs3077 and rs9277535.

Kamatani *et al.* first reported the association between the *HLA-DP* locus and chronic HBV infection, after GWAS in Japanese and Thai samples (16). Similar results have been reported in Chinese, Korean, German and other Japanese populations (17, 19, 20, 23, 24). The *HLA-DP* locus appears to be associated with natural HBV clearance. Kamatani *et al.* identified that two SNPs, viz., rs3077 and rs9277535, from a region including *HLA-DPA1* and *HLA-DPB1*, were strongly associated with chronic hepatitis B (16). Therefore, we here analysed the association between these two SNPs and HBsAg kinetics and seroclearance during NA therapy. Previous studies showed that the minor alleles (A) of rs3077 and rs9277535 protected against chronic HBV infection. We could also demonstrate that HBsAg levels decreased faster in patients with than those without A-alleles, as we had hypothesized. Although the reason for this finding is unclear, O'Brien *et al.* reported that the expression of *HLA-DPA1* and *HLA-DPB1* mRNA in normal human liver tissue increased in healthy donors with the presence of the minor allele of rs3077 and rs9277535 (18). They also showed that the order of expression levels of *HLA-DPA1* and *HLA-DPB1* was AA > AG > GG in both rs3077 and rs9277535, while the odds ratio for chronic HBV infection followed the opposite order. These findings support our finding that a larger number of A-alleles at rs3077 and rs9277535 were associated with a higher percentage of HBsAg seroclearance in patients receiving long-term NA therapy, as shown in Fig. 4C. Greater expression of *HLA-DPA1* and *HLA-DPB1* may facilitate HBsAg level decrease and seroclearance during NA therapy. Furthermore, previous studies reported that genetic variants in the antigen-binding region of *HLA-DQ* were also associated with persistent HBV infection (19, 23). Future studies should investigate the association between the combination of genetic variants at the *HLA-DP* and *HLA-DQ* loci and HBsAg kinetics.

In this study, besides the *HLA-DP* polymorphisms, HBsAg seroclearance was likely to occur in patients who had HBV genotype A, high bilirubin levels at baseline, and had previously undergone IFN therapy (Table 3). It has previously been reported that HBV genotype A is associated with HBsAg seroclearance during NA therapy (15, 25, 26). High ALT flares sometimes result in bilirubin flares and high virological responses have been reported in response to robust IFN therapy-induced ALT flares (27, 28). Moreover, Wursthorn *et al.* indicated that both antiviral potential of NAs and antiviral T-cell reactivity are associated with HBsAg clearance in response to telbivudine treatment (25). Although the treatment duration and timing of previous IFN were not associated with HBsAg seroclearance during LAM treatment as described in our previous paper (15), these results imply that both direct antiviral potential and host immune response are needed to achieve HBsAg seroclearance.

There were several limitations to our study. First, because LAM has a high potential for drug resistant mutations, many patients receiving LAM had experienced VBT and required rescue therapy (add-on ADV). Consequently, the study population available for this study had to be divided into a VR without rescue therapy cohort (cohort 1) and a LAM add-on rescue therapy cohort (cohort 2) to ensure a uniform treatment response, resulting in small cohort sizes. Second, because *HLA-DP* SNP analysis could not be conducted in all patients received LAM, there may have been a selection bias. However, the allele frequencies of rs3077 in *HLA-DPA1* and rs9277535 in *HLA-DPB1* in this study were similar to those observed in previous studies. Third, we were not able to collect immunological data

Table 3. Baseline factors associated with HBsAg clearance, as determined by univariate and multivariate analysis (cohort 1 + 2)

	Univariate		Multivariate (Model 1)		Multivariate (Model 2)	
Variable	HBsAg clearance rate ratio (95% CI)	*P*	HBsAg clearance rate ratio (95% CI)	*P*	HBsAg clearance rate ratio (95% CI)	*P*
Age (per year)	1.01 (0.97–1.06)	0.493				
Gender (F)	0.41 (0.09–1.78)	0.234				
Family history of HBV infection	0.61 (0.23–1.61)	0.318				
Previous IFN therapy	**3.47 (1.14–10.5)**	**0.028**	**3.14 (1.02–9.65)**	**0.045**	**5.51 (1.13–26.8)**	**0.035**
Pre-existing cirrhosis	0.91 (0.60–1.38)	0.645				
HBV genotype (A)	**16.0 (5.63–45.4)**	**1.88×10^{-7}**	**21.6 (7.05–66.3)**	**7.63×10^{-8}**		
HBV DNA (per log copies/ml)	1.47 (0.92–2.34)	0.104				
HBsAg (per log IU/ml)	1.71 (0.86–3.38)	0.123				
AST (per IU/L)	**1.001 (1.000–1.002)**	**0.018**				
ALT (per IU/L)	**1.001 (1.000–1.001)**	**0.044**				
Total bilirubin (per mg/dl)	**1.21 (1.03–1.43)**	**0.018**	**1.23 (1.02–1.48)**	**0.029**	**1.30 (1.05–1.61)**	**0.015**
Platelet count (per $1.0 \times 10^4/mm^3$)	0.93 (0.84–1.02)	0.132				
rs3077 (non-GG)	**2.69 (1.04–6.93)**	**0.041**				
rs9277535 (non-GG)	**2.78 (1.04–7.42)**	**0.041**				
Number of A-alleles among rs3077 and rs9277535 (≥2)	**2.81 (1.09–7.25)**	**0.033**	**2.88 (1.09–7.58)**	**0.044**		
IL28B rs8099917 (non-TT)	0.31 (0.04–2.36)	0.313				
Treatment group (without rescue Tx)	1.35 (0.84–2.17)	0.213				

Bold text indicates statistically significant *P*-values; ALT, alanine aminotransferase; AST, aspartate aminotransferase; HBsAg, hepatitis B virus surface antigen; HBV, hepatitis B virus; CI, confidence interval; IFN, interferon; Tx, treatment.

Model 1; including all patients. Model 2; including only patients with genotype C.

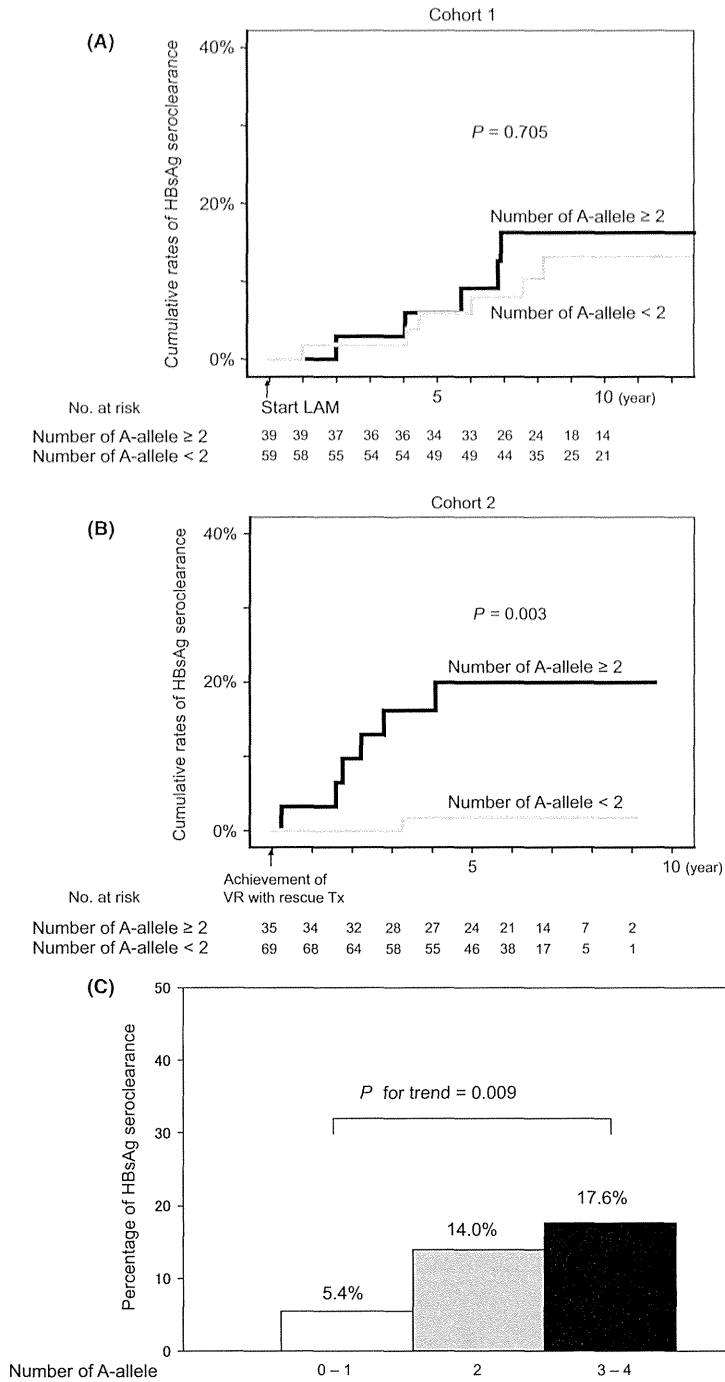

Fig. 4. (A) Kaplan–Meier life table showing cumulative HBsAg clearance rates by the number of A-alleles in rs3077 and rs9277535 (cohort 1). (B) Kaplan–Meier life table showing cumulative HBsAg clearance rates after achievement of VR with rescue therapy by the number of A-alleles (cohort 2). (C) Percentage of HBsAg seroclearance over time in all patients with the number of A-alleles in rs3077 and rs9277535.

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on our subjects. Fourth, we could not find association between the *HLA-DP* polymorphisms and HBsAg kinetics and seroclearance in HBeAg-negative patients receiving long-term LAM in our institute (data not shown). The reason for the results in HBeAg-negative patients remains unclear, but may be necessary to repeat this analysis in a larger population. Finally, our results should be validated by further studies investigating a large study population receiving long-term ETV or tenofovir with high antiviral potency and a high genetic barrier.

In our study, we observed an association between *HLA-DP* polymorphisms and declines in HBsAg levels and seroclearance among HBeAg-positive patients treated with LAM and who subsequently achieved favourable VR. HBsAg levels declined faster in patients with two or more A-alleles (minor alleles) at rs3077 and rs9277535, than those with fewer A-alleles. Although *HLA-DP* polymorphisms may not markedly affect the decision of the treatment choice, it will be helpful to identify the mechanism of HBsAg seroclearance among HBV-infected patients in future. Moreover, future studies should validate these findings in high antiviral treatment regimens among large cohorts of patients with chronic hepatitis B.

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Conflict of interest: These authors disclose the following: Dr. Kumada reports having received investigator, lecture, and consulting fees from Bristol-Myers Squibb, Dainippon Sumitomo Pharma Co., MSD KK, and Toray Co. Dr. Ikeda reports having received investigator, lecture, and consulting fees from Dainippon Sumitomo Pharma Co. The rest of the authors do not have any disclosures to report.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Table S1. Baseline, demographic and on-treatment characteristics according to *IL28B* genotypes

Hepatology Research

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Case Report

Sequential occurrence of acute hepatitis B among members of a high school Sumo wrestling club

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A 17-year-old male was admitted to our hospital and diagnosed with acute hepatitis B. Six weeks later, a 15-year-old male was admitted with acute hepatitis B as well. They were Sumo wrestling players in the same club. A detailed survey in the club revealed that a 28-year-old male coach was a hepatitis B surface antigen carrier with high-level viremia. The consistency of hepatitis B virus (HBV) DNA in the infected players was revealed by analyzing the complete HBV genome sequences. Sumo players are more likely to get injured, including cuts and bleeding, compared with players of other sports because of the characteristic wrestling style. Several past reports have suggested that highly viremic HBV carriers have high HBV DNA titers in both their blood and other body fluids such as sweat. In our cases, percutaneous HBV transmission through the bleeding wounds was the most probable infection route. We conclude that a universal HBV immunization program should be introduced urgently in Japan, similar to those implemented in other countries worldwide.

Key words: hepatitis B virus, horizontal transmission, Sumo, universal vaccination

INTRODUCTION

THE HORIZONTAL TRANSMISSION of hepatitis B virus (HBV) occurs in limited situations such as sexual intercourse with HBV positive partners, the transfusion of HBV-contaminated blood, and the re-use of needles and syringes used for the i.v. administration of drugs.[1–3] In addition, there are several reports of horizontal HBV transmission in elementary schools and day-care centers due to bites and scratches or exposure to blood or blood-contaminated fluids among children.[4–7] Although it is rare, HBV horizontal transmission has been reported in various sports as well, including Sumo wrestling and American football, because of contact with open wounds during training.[8,9]

In this paper, we report a sequential occurrence of acute hepatitis B in members of a high school Sumo wrestling club. After a detailed field survey, a 28-year-old male coach was determined to be a hepatitis B surface antigen (HBsAg) carrier with a high-level of viremia. This individual was identified as the source of transmission by analyzing the complete HBV genome sequences.

CASE REPORT

A 17-YEAR-OLD MALE (case 1) was admitted to our hospital with a 1-week history of jaundice and itching. He had no past medical history, except pediatric asthma, and was not taking any medications currently. There were no HBV carriers in his family. He reported no alcohol consumption, recent travel or sexual contact. He was a member of a high school Sumo wrestling club. On examination, the patient was slightly icteric with stable vital signs. Blood test results (Table 1) revealed the following: total bilirubin (T-Bil), 3.9 mg/dL; aspartate aminotransferase (AST), 1152 IU/L; alanine aminotransferase (ALT), 2856 IU/L; HBsAg, 12 229.87 IU/mL; hepatitis B e-antigen (HBeAg), 473.29 S/CO; anti-hepatitis B core (anti-HBc), 4.0 S/CO; immunoglobulin

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