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.

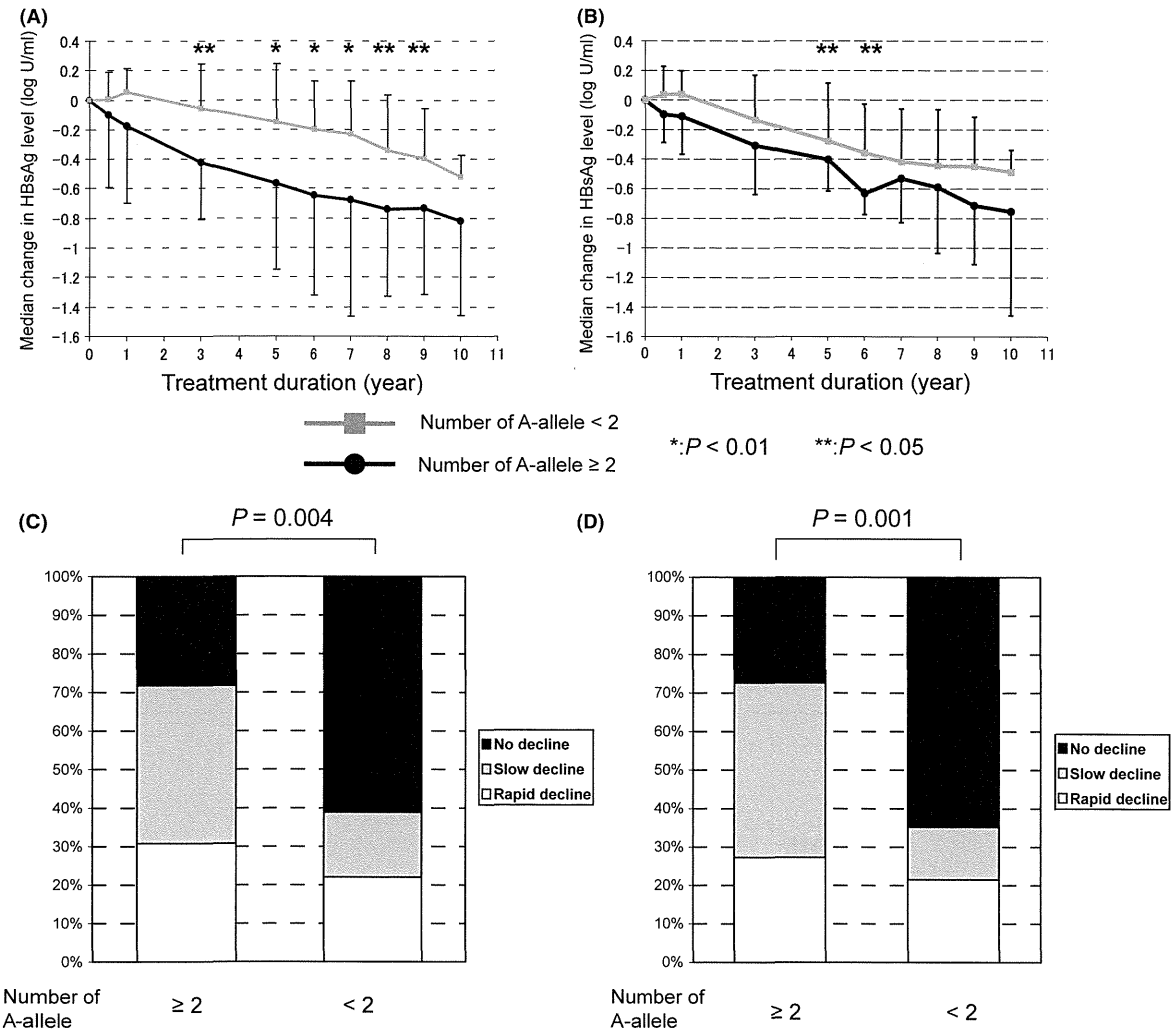

Fig. 2. (A) Median change in HBsAg level from baseline in patients with *HLA-DP* gene alleles (cohort 1; all patients). The asterisk (*) indicates a statistical significance of $P < 0.01$, and (**) indicates $P < 0.05$ as determined by the Mann–Whitney *U*-test. (B) Median change in HBsAg level from 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

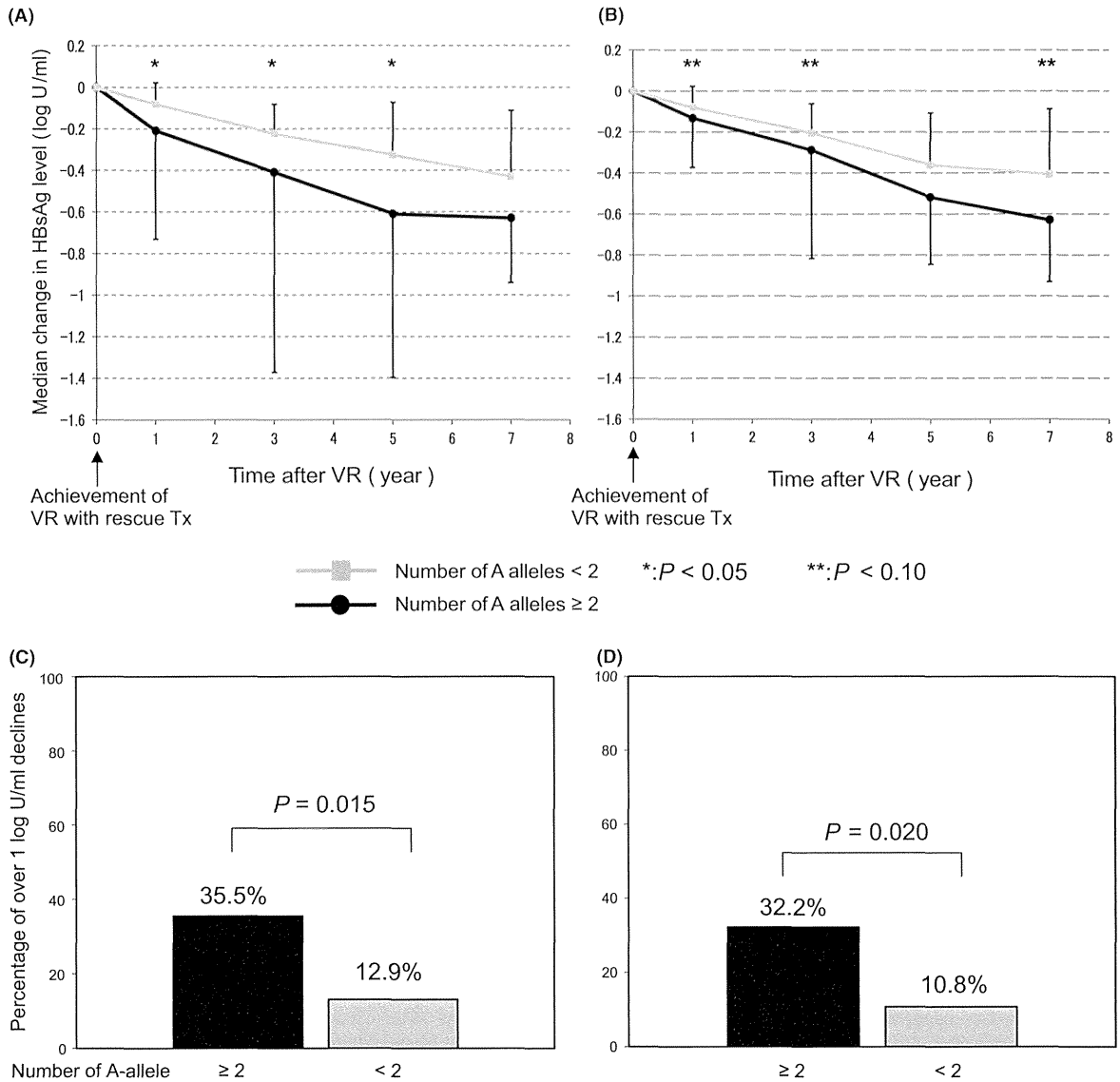

Fig. 3. (A) Median change in HBsAg level from VR in patients with *HLA-DP* gene alleles (cohort 2). The asterisk (*) indicates a statistical significance of $P < 0.05$ as determined by the Mann–Whitney *U*-test. VR, virological response. (B) Median change in HBsAg level from VR in patients with *HLA-DP* 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.

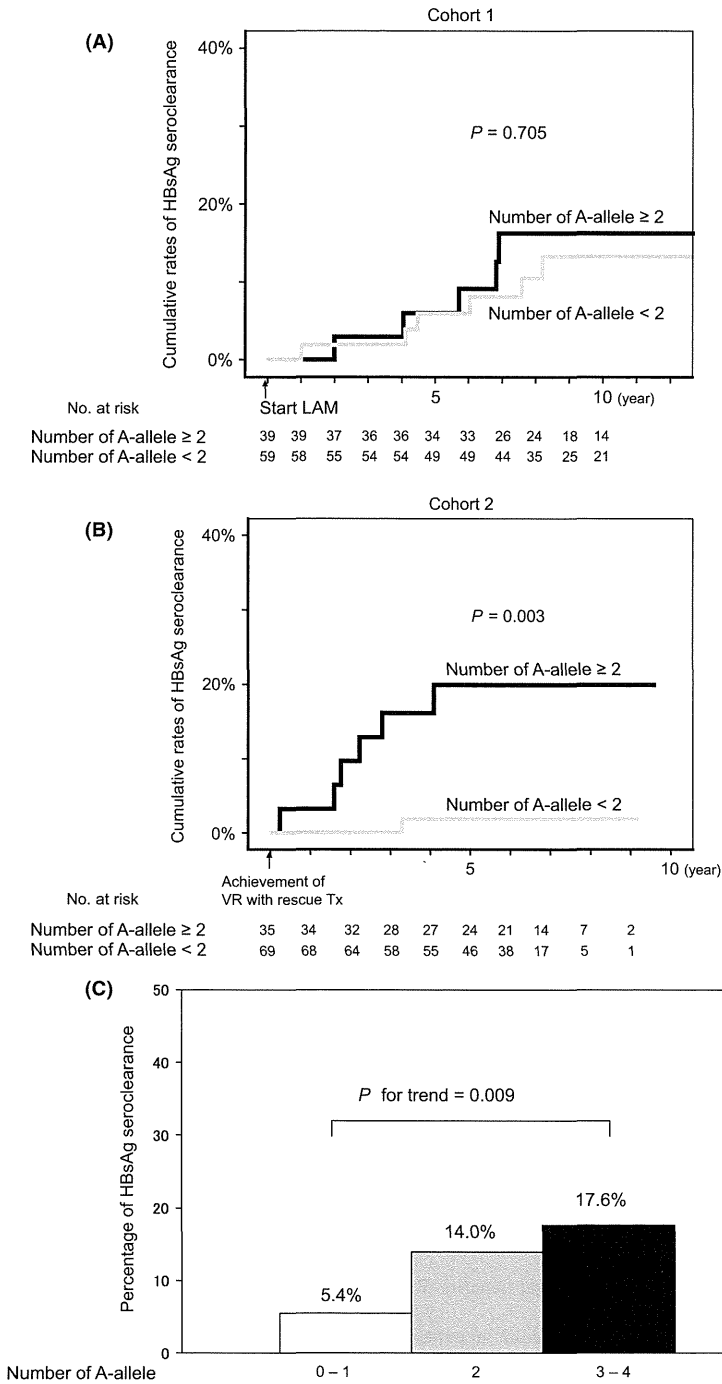

Fig. 4. (A) Kaplan–Meier life table showing cumulative HBsAg clearance rates by the number of A-alleles in rs3077 and rs9277535 (cohort 1). (B) Kaplan–Meier life table showing cumulative HBsAg clearance rates after achievement of VR with rescue therapy by the number of A-alleles (cohort 2). (C) Percentage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

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ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

Seroclearance rate of hepatitis B surface antigen in 2,112 patients with chronic hepatitis in Japan during long-term follow-up

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Abstract
Background Rate of hepatitis B surface antigen (HBsAg) seroclearance was determined in 2,112 Japanese patients with chronic hepatitis B who were followed up for at least 15 years.
Methods Patients had a median age of 37 years and included 1,431 (67.8 %) men. Median values were AST/ALT, 43/62 IU/L; platelet counts, 182 × 10^3/mm^3; HBsAg, 3,400 IU/mL; and hepatitis B virus (HBV) DNA, 6.2 log copies/mL. Factors influencing HBsAg seroclearance were evaluated by the Cox proportional model and annual rate of HBsAg seroclearance by the Kaplan–Meier life table method.
Results The overall annual rate of HBsAg seroclearance was 1.75 % in 2,112 patients; it was 1.65 % in 1,130 untreated and 2.05 % in 982 treated patients ($p = 0.289$). In untreated patients, seroclearance was influenced by age, no HBV infections in third-degree or closer relatives, and HBsAg levels in univariate analysis. Seroclearance was influenced by a median age ≥50 years [relative risk (RR) 1.61 ($p = 0.018$)] and HBsAg ≤2,000 IU/mL [RR 1.77 ($p = 0.014$)] in multivariate analysis. In treated patients, age, male gender, no HBV infections in third-degree or closer relatives, interferon therapy, chronic hepatitis, high AST and γ-GTP levels, low platelet counts, hepatitis B e antigen (HBeAg)-negative status, low HBsAg levels and the wild-type precore sequence significantly influenced HBsAg seroclearance. In multivariate analysis, no family history [RR 2.22 ($p = 0.006$)], interferon treatment [RR 3.15 ($p < 0.001$)], and HBeAg-negative status [RR 3.75 ($p < 0.001$)] significantly influenced HBsAg seroclearance.
Conclusions In this retrospective cohort study, the annual rate of HBsAg seroclearance was 1.65 % in untreated patients and 2.05 % in treated patients.

Keywords Seroclearance · Hepatitis B surface antigen · Hepatitis B virus · Chronic hepatitis B

Abbreviations

ALT	Alanine aminotransferase
AST	Aspartate aminotransferase
ETV	Entecavir
HBeAg	Hepatitis B e antigen
HBcrAg	Hepatitis B core-related antigen
HBV	Hepatitis B virus
HBV DNA	Hepatitis B virus DNA
HBsAg	Hepatitis B surface antigen
IFN	Interferon
LAM	Lamivudine

Introduction

Worldwide, an estimated 400 million people are infected with hepatitis B virus (HBV) persistently. HBV infection is a common disease that can induce a chronic carrier state

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and is associated with the risk of developing progressive disease and hepatocellular carcinoma (HCC) [1–5]. In regions highly endemic for HBV, such as Asia and Africa, the persistent carrier state is established by perinatal transmission or early in infancy. Carriers serve as the reservoir of HBV in the community and can spread the infection to susceptible individuals. The incidence of HCC is decreased extremely by eradicating HBV from the circulation that is responsible for liver damage [6–9]. In Japan, interferon (IFN) was introduced for the treatment of persistent HBV infections, and long-term IFN increased seroclearance of hepatitis B surface antigen (HBsAg) [10]. Since 2000, the effect of long-term nucleot(s)ide analogues, such as lamivudine [11, 12] and entecavir [13], on HBsAg seroclearance has been monitored in Japan.

In the current study, we followed untreated or treated patients for at least 15 years. We evaluated the seroclearance of HBsAg, achieved in both groups of patients, by using highly sensitive assays. Our aim was to determine factors that can lead to HBsAg seroclearance and to elucidate the factors associated with its success.

Patients and methods

Patients

During at least 15 years from 1968, 2,112 consecutive patients, chronically mono-infected with HBV (confirmed by HBsAg-positivity for at least 6 months) were followed at the Department of Hepatology, Toranomon Hospital, in Metropolitan Tokyo. Patients met the following inclusion and exclusion criteria: (1) negativity for hepatitis C antibody and/or hepatitis C virus RNA by polymerase chain reaction (PCR) in the serum; (2) no history of HCC; and (3) no history of autoimmune hepatitis, alcohol liver disease, hemochromatosis, or chronic liver disease other than chronic hepatitis B. Thus, the 2,112 patients were enrolled in this cohort study. A written informed consent was obtained from each patient. The 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.

Treatment

Nine hundred and eighty-two patients received antiviral treatments. Of them, 156 patients received prednisolone (PSL) 40 mg daily for 1 week, 30 mg daily for 1 week, 20 mg daily for 1 week, and then 10 mg daily for 1 week until it was abruptly withdrawn (total 700 mg). A total of 428 patients received 100 mg lamivudine (LAM) daily as an initial therapy. In total, 333 patients received 3–12 MU of IFN-α or IFN-β. The durations and regimens of treatment were as follows: daily for 2 or 4 weeks and then 2 or 3 times per week for 26–104 weeks. The median duration of treatment was 26 weeks (range 4–981). There were 190 (57 %) patients who received multiple treatments of IFN.

LAM treatment was continued as a rule; median duration of LAM treatment was 75 months (55–102). LAM-resistant rtM204I/V mutants developed in 151 (35 %) of the 428 patients, and they were provided with adefovir dipivoxil (10 mg) added on LAM, as a rescue therapy. The remaining patients continued to receive LAM monotherapy. In addition, 65 patients received 0.5 mg entecavir (ETV) daily as an initial therapy. ETV treatment was continued as a rule, and median duration of ETV treatment was 45 months (1.0–104).

Markers of HBV infection

Serum HBsAg titers were determined annually 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 limit from 250 to 125,000 IU/mL, serum samples going off the scale were diluted stepwise to 1:20 and 1:500 with ARCHITECT diluents following instructions from the manufacturer.

Hepatitis B e antigen (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) with a dynamic range of 2.6–7.6 log copies/mL, or COBAS TaqMan HBV v.2.0 (Roche Diagnostics, Tokyo, Japan) with a dynamic range of 2.1–9.0 log copies/mL. Hepatitis B core-related antigen (HBcrAg) was determined by chemiluminescence enzyme immunoassay (CLEIA) with the HBcrAg assay kit (Fujirebio Inc., Tokyo, Japan). A commercial kit (HBV Genotype EIA; Institute of Immunology, Tokyo, Japan) was used to serologically determine HBV genotypes by the combination of epitopes expressed on the pre-S2 region product, which is specific for each of the 7 major genotypes (A–G).

Statistical analysis

Baseline data were obtained on the day of the first visit in untreated patients. In patients who received antivirals, baseline data were obtained at the start of the first day of treatment. Categorical data were compared between groups by chi-squared or Fisher's exact tests. Continuous variables with a nonparametric distribution were analyzed by Mann–Whitney *U* tests, whereas those with a parametric distribution were analyzed by the Student's *t* test. Cox

regression analyses were used to assess variables that were significantly associated with HBsAg seroclearance. All baseline factors that were found to be significantly associated with HBsAg seroclearance by univariate analysis were entered into a multivariate analysis. Independent baseline factors associated with the seroclearance of HBsAg were evaluated using a stepwise Cox regression analysis. We then performed a time-dependent Cox regression to analyze independent factors associated with HBsAg seroclearance while on-treatment factors and independent baseline factors had been adjusted.

Cumulative HBsAg seroclearance rates were analyzed using the Kaplan–Meier method; differences in the resulting curves were evaluated using log-rank tests. Significance was defined as $p < 0.05$ for all two-tailed tests. Data analysis was performed with the SPSS software package version 11.0.1 J (SPSS Inc., Chicago, IL, USA).

Results

Baseline characteristics in the 2,112 patients

The baseline characteristics of studied patients are shown in Table 1. They had a median age of 37 years (range 1–81), included 1,431 (67.8 %) men, and 2,031 (96.2 %) of them had chronic hepatitis. Their baseline values were AST/ALT, 43 (3–2,192)/62 (2–3,020 IU/L); γ-GTP, 27 (4–1,494) IU/L; platelet counts, 182 (40–483) × 10^3/mm^3; and HBV markers were HBsAg, 3,400 (0.06–27,700) IU/mL; and HBV DNA, 6.2 (<2.1 to >9.1) log copies/mL. HBeAg was not detectable in 5.4 % of studied patients, and the distribution of genotypes A/B/C/others was 4.5:15.6:79.6:0.3 %.

The HBsAg seroclearance rate analyzed by the Kaplan–Meier method was 9 % in 5 years, 17 % in 10 years, 27 % in 15 years, 35 % in 20 years, 44 % in 25 years, and 54 % in 30 years. The annual rate of HBsAg seroclearance was 1.75 % during 20 years (Fig. 1).

In the 2,112 patients, factors influencing HBsAg seroclearance in univariate analysis by the Cox regression analyses were cirrhosis [relative risk (RR) 2.40 ($p = 0.014$)]; HBeAg negative [RR 3.01 ($p = 0.001$)]; and HBsAg ≤2,000 IU/mL [RR 2.13 ($p = 0.004$)]. In multivariate analyses, only 2 factors contributed to HBsAg seroclearance: HBeAg negative [RR 1.81 ($p < 0.001$)]; and HBsAg ≤2,000 IU/mL [RR 2.60 ($p < 0.001$)] (Table 2).

Untreated patients and treated patients

Differences in the baseline characteristics between 1,130 untreated and 982 treated patients are shown in Table 3: age [31 years vs. 36 ($p < 0.001$)]; male gender [62.4 vs. 71.9 % ($p < 0.001$)]; AST [median 27 vs. 56 IU/L ($p < 0.001$)]; ALT [median 28 vs. 96 IU/L ($p < 0.001$)]; γ-GTP [median 20 vs. 45 IU/L ($p < 0.001$)]; total bilirubin

Table 1 Baseline characteristics 2,112 patients infected with HBV followed for longer than 15 years

Features at the baseline	Patients (n = 2,112)
Demographic data	
Age (years)	37 (1–81)
Men	1,431 (67.8 %)
Liver disease	
Chronic hepatitis	2,031 (96.2 %)
Cirrhosis	81 (3.8 %)
Laboratory data	
AST (IU/L)	43 (3–2,192)
ALT (IU/L)	62 (2–3,020)
γ-GTP (IU/L)	27 (4–1,494)
Total bilirubin (mg/dL)	0.7 (0.1–21.2)
Albumin (g/dL)	4.3 (1.1–5.8)
Platelets (×10^3/mm^3)	182 (40–483)
α-Fetoprotein (μg/L)	4 (1–2,060)
HBV markers	
HBeAg-negative status	1,169 (55.4 %)
HBsAg (IU/mL)	3,400 (0.06–277,000)
HBcrAg (log U/mL)	5.4 (<3.0 to >6.8)
Genotypes (A/B/C/others)	4.5 %/15.6 %/79.6 %/0.3 %
HBV DNA (log copies/mL)	6.2 (<2.1 to >9.1)

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

HBV hepatitis B virus, *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

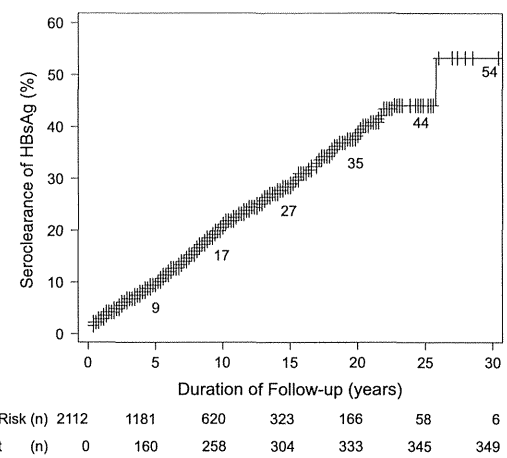

Fig. 1 Seroclearance of HBsAg in the 2,112 patients studied. Numbers of patients at risk and those of patients who lost HBsAg are indicated below each time point

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

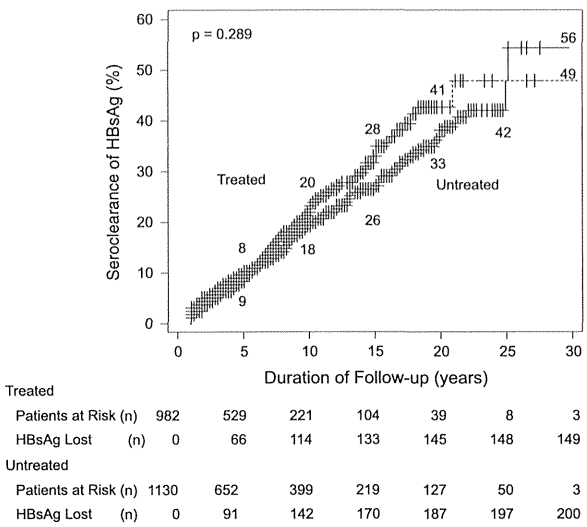

Fig. 2 Comparison of HBsAg seroclearance rates between 982 treated and 1,130 untreated patients. Numbers of patients at risk and those of patients who lost HBsAg are indicated below each time point

($p < 0.001$), 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 ≤150 × 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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Long-term entecavir therapy results in falls in serum hepatitis B surface antigen levels and seroclearance in nucleos(t)ide-naïve chronic hepatitis B patients

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SUMMARY. Entecavir (ETV) is reported to result in suppression of hepatitis B virus DNA (HBV DNA) replication with minimal drug resistance. However, information on the long-term effect of such therapy on serum hepatitis B surface antigen (HBsAg) level and elimination of HBsAg is not available. ETV therapy was started in 553 nucleos(t)ide-naïve patients with chronic hepatitis B infection (HBeAg positive: 45%) in our hospital. Serum HBsAg levels were measured serially by the Architect assay. The median baseline HBsAg was 2180 IU/mL (0.12–243 000 IU/mL), and median follow-up period was 3.0 years, with 529, 475, 355, 247 and 163 patients followed-up for 1, 2, 3, 4 and 5 years, respectively. At year 5, the mean log HBsAg decline from baseline was −0.48 log IU/mL, and the cumulative HBsAg clearance rate was 3.5%. Multivariate analysis identified HBV DNA level at baseline (<3.0 log copies IU/mL, odd ratio = 10.2; 95% confidence interval = 1.87–55.5, $P = 0.007$) and HBsAg level (<500 IU/mL, odd ratio = 29.4; 95% confidence interval = 2.80–333, $P = 0.005$) as independent predictors of HBsAg seroclearance. These results indicate that although serum HBsAg level declines gradually during ETV therapy, HBsAg seroclearance remains a rare event.

Keywords: chronic hepatitis, entecavir, hepatitis B surface antigen, hepatitis B virus.

INTRODUCTION

Approximately 400 million people worldwide have chronic hepatitis B (CHB) infection, the majority of whom live in the Asia-Pacific region [1,2]. CHB patients with elevated viral load are at risk of cirrhosis, liver failure and hepatocellular carcinoma. Within the past 10 years, nucleos(t)ide analogs (NAs) have been approved in Japan for the treatment of CHB, and recent investigations have shown that entecavir (ETV) effectively suppresses hepatitis B virus DNA (HBV DNA) replication with minimal drug resistance [3–5].

Quantification of serum hepatitis B surface antigen (HBsAg) has been recently advocated as a marker of disease activity in CHB, and the correlation between HBV DNA and HBsAg level disappears after ETV therapy [6,7]. Very low rates of HBsAg clearance by antiviral therapies such as NAs have been reported in the past [4,8–13]. Other groups have also shown that serum HBsAg level can accurately predict the outcome of pegylated interferon therapy in CHB [14,15]. In this regard, pegylated interferon therapy is more successful than ETV at reducing serum HBsAg [16]. Nonetheless, the duration of follow-up period in the majority of the above studies is relatively short. On the other hand, the kinetics of serum HBsAg measurement during long-term NAs therapy remains unknown. Recent studies showed that serum HBsAg levels fall gradually during lamivudine (LAM) therapy [13]. However, little is known about serum HBsAg kinetics during long-term ETV therapy in CHB patients. In the present study, we assessed serum HBsAg kinetics, including the rate of HBsAg clearance, during long-term ETV treatment of NA-naïve CHB patients.

Abbreviations: ALT, alanine aminotransferase; AST, aspartate transaminase; CHB, chronic hepatitis B; CIs, confidence intervals; ETV, entecavir; HBeAg, hepatitis B e antigen; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; HBV-DNA, hepatitis B virus DNA; ORs, odds ratios; PCR, polymerase chain reaction; ULN, upper limit of normal.

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