Table 2 Baseline and on-treatment response factors associated with HBsAg clearance, as determined by time-dependent univariate and multivariate analyses at year 9 (HBeAg+ cohort)

Variable	Univariate		Multivariate	
	HBsAg clearance rate ratio (95 % CI)	*P*	HBsAg clearance rate ratio (95 % CI)	*P*
Baseline factors				
Age (≥50 years)	1.36 (0.48–3.86)	0.564		
Gender (F)	0.51 (0.12–2.23)	0.371		
Family history of HBV infection	0.42 (0.16–1.09)	0.074		
Previous IFN therapy	**5.60 (1.61–19.5)**	**0.007**	**6.15 (1.69–22.4)**	**0.006**
Previous NA therapy	2.42 (0.55–10.6)	0.242		
Presence of cirrhosis	0.85 (0.52–1.40)	0.527		
HBV genotype (A)	**3.64 (2.21–5.99)**	**<0.001**	**3.18 (1.80–5.62)**	**<0.001**
HBV DNA (≥6.0 log copies/mL)	2.56 (0.34–19.3)	0.362		
HBsAg (<730 IU/mL)	1.57 (0.51–4.81)	0.432		
AST (≥4.5 × ULN)	**4.53 (1.68–12.2)**	**0.003**		
ALT (≥7.2 × ULN)	**3.56 (1.35–9.36)**	**0.010**		
Total bilirubin (≥1.5 mg/dL)	2.63 (0.92–7.46)	0.070		
Platelet count (<1.2 × 10^5/mm^3)	0.58 (0.13–2.59)	0.476		
On-treatment response factors				
Decline of HBsAg level (≥0.5 log IU/mL within six months)	**15.8 (5.14–48.5)**	**<0.001**	**18.6 (5.78–60.0)**	**<0.001**
HBeAg positive → clearance within six months	**4.33 (1.65–11.4)**	**0.003**	**2.95 (1.04–8.39)**	**0.042**
Undetectable HBV DNA (<400 copies/mL) at six months	**3.95 (1.14–13.7)**	**0.031**		
Emergence of rtM204I/V mutants[a]	0.88 (0.32–2.44)	0.802		
Viral breakthrough due to mutants[a]	**0.32 (0.10–1.00)**	**0.050**		
Breakthrough hepatitis due to mutants[a]	0.41 (0.13–1.31)	0.134		

[a] Time-dependent covariates. *Bold text* indicates statically significant *P* valuesVariables analyzed in multivariate analysis: previous IFN therapy, HBV genotype, ALT, decline of HBsAg levels, HBeAg clearance within six months, undetectable HBV DNA at six months, and viral breakthrough due to mutants (time-dependent covariate)

regression analysis identified four baseline characteristics and four on-treatment responses that were associated with HBsAg clearance in the HBeAg+ cohort (Table 2), and two baseline characteristics and two on-treatment responses in the HBeAg− cohort (Table 3). ROC curve analysis provided the optimal cut-off values and indices for the prediction of HBsAg clearance. ROC curve analysis confirmed cut-off indices of 4.5 × ULN for AST and 7.2 × ULN for ALT for HBsAg clearance in the HBeAg+ cohort [area under the curve = 0.677 (95 % CI 0.524–0.830) (AST) and 0.643 (95 % CI 0.503–0.783) (ALT)]. Meanwhile, ROC curve analysis confirmed a cut-off value of 730 IU/mL (2.86 log IU/mL) for HBsAg for HBsAg clearance in the HBeAg− cohort [area under the curve = 0.696 (95 % CI 0.556–0.836)]. Time-dependent multivariate Cox regression analysis identified two significant baseline characteristics and two on-treatment responses related to HBsAg clearance: previous IFN therapy, infection with HBV genotype A, a decline in HBsAg level of ≥0.5 log IU/mL within six months, and HBeAg clearance within six months in the HBeAg+ cohort (Table 2). In the HBeAg− cohort, two baseline characteristics and one on-treatment response were identified in multivariate analysis: infection with HBV genotype A, HBsAg level of <730 IU/mL (2.86 log IU/mL), and a decline in HBsAg level of ≥0.5 log IU/mL within six months (Table 3).

Association between HBV genotype and HBsAg clearance

We performed a detailed analysis of the association between HBV genotype and HBsAg clearance in patients treated with NAs. Median baseline HBsAg levels were 4.7 log IU/mL (25th–75th percentile, 4.4–5.1) among patients with genotype A, 3.8 (3.5–4.2) among patients with genotype B, and 3.5 (3.2–4.0) among patients with genotype C in the HBeAg+ cohort (Fig. 2a); and 3.7 (2.5–4.1) in patients with genotype A, 2.9 (2.6–3.5) in patients with genotype B, and 3.2 (2.8–3.5) in patients with genotype C in the HBeAg− cohort (Fig. 2b). HBeAg+ patients with genotype A had higher baseline HBsAg levels than those with genotypes B or C ($P < 0.001$) (Fig. 2a). There were no significant differences in baseline HBsAg levels between the genotypes in the HBeAg− cohort.

Table 3 Baseline and on-treatment response factors associated with HBsAg clearance, as determined by time-dependent univariate and multivariate analyses at year 9 (HBeAg− cohort)

Variable	Univariate		Multivariate	
	HBsAg clearance rate ratio (95 % CI)	*P*	HBsAg clearance rate ratio (95 % CI)	*P*
Baseline factors				
Age (≥50 years)	1.39 (0.54–3.60)	0.498		
Gender (F)	0.98 (0.28–3.40)	0.971		
Family history of HBV infection	0.49 (0.19–1.27)	0.140		
Previous IFN therapy	0.88 (0.32–2.38)	0.797		
Previous NA therapy	2.41 (0.32–18.2)	0.394		
Presence of cirrhosis	0.71 (0.43–1.16)	0.173		
HBV genotype (A)	**2.79 (1.33–5.85)**	**0.007**	**2.73 (1.29–5.81)**	**0.009**
HBV DNA (≥6.0 log copies/mL)	1.16 (0.43–3.14)	0.772		
HBsAg (<730 IU/mL)	**3.91 (1.59–9.52)**	**0.003**	**4.90 (1.85–10.6)**	**0.001**
AST (≥4.5 × ULN)	1.76 (0.57–5.40)	0.324		
ALT (≥7.2 × ULN)	1.89 (0.62–5.81)	0.265		
Total bilirubin (≥1.5 mg/dL)	1.18 (0.27–5.20)	0.825		
Platelet count (<1.2 × 10^5/mm^3)	0.77 (0.17–3.55)	0.733		
On-treatment response factors				
Decline of HBsAg level (≥0.5 log IU/mL within six months)	**11.5 (4.24–31.0)**	**<0.001**	**16.9 (5.89–48.4)**	**<0.001**
Undetectable HBV DNA (<400 copies/mL) at six months	2.78 (0.37–20.8)	0.322		
Emergence of rtM204I/V mutants[a]	0.64 (0.23–1.79)	0.392		
Viral breakthrough due to mutants[a]	0.72 (0.23–2.29)	0.581		
Breakthrough hepatitis due to mutants[a]	0.65 (0.21–2.06)	0.465		

[a] Time-dependent covariates. *Bold text* indicates statically significant *P* values

Variables analyzed in multivariate analysis: HBV genotype, baseline HBsAg, decline of HBsAg levels

HBsAg kinetics over time in the HBeAg+ and − cohorts are shown in Fig. 2c, d, respectively. Among patients with genotype A in the HBeAg+ cohort, the median HBsAg change from baseline was −0.44 log IU/mL at six months, −0.56 at one year, −0.58 at three years, −1.08 at five years, and −1.33 at six years. Among patients with genotype B in the HBeAg+ cohort, median changes were −0.30 log IU/mL at six months, −0.30 at one year, −0.43 at three years, and −0.46 at five years. Kinetics were not calculated for some groups (genotype A at seven years, genotype B at six years) because the number of patients was too small. Finally, among patients with genotype C in the HBeAg+ cohort, median changes were 0.00 log IU/mL at six months, 0.03 at one year, −0.08 at three years, −0.29 at five years, −0.53 at six - years, −0.62 at seven years, −0.70 at eight years, and −0.82 at nine years. Genotype had a significant effect on the slopes between data collection points at six months and six years. In the HBeAg+ cohort, declines were faster in patients with genotype A than in those with genotypes B or C. HBeAg− patients with genotype A displayed a median HBsAg change from baseline of 0.05 log IU/mL at six months, 0.05 at one year, −0.11 at three years, −0.21 at five years, and −0.26 at six years. Among patients with genotype B in the HBeAg− cohort, median changes were −0.03 log IU/mL at six months, −0.06 at one year, −0.25 at three years, −0.31 at five years, −0.51 at six years, −0.62 at seven years, −0.66 at eight years, and −0.61 at nine years. Among patients with genotype C in the HBeAg− cohort, median changes were 0.03 log IU/mL at six months, 0.04 at one year, −0.08 at three years, −0.19 at five years, −0.32 at six years, −0.39 at seven years, −0.46 at eight years, and −0.62 at nine years. The decline was slightly faster in patients with genotype B than in those with genotypes A and C in the HBeAg− cohort.

We investigated whether HBsAg clearance were influenced by genotype or baseline HBeAg. Cumulative HBsAg clearance rates in the HBeAg+ cohort were as follows: 15 % at year 3, and 35 % at year 5 in patients with genotype A; 0 % over all years in patients with genotype B; and 0.6 % at year 3, 1.2 % at year 5, and 5.4 % at year 9 in patients with genotype C (Fig. 2e). In the HBeAg− cohort, clearance rates were 12 % at year 3, and 25 % at year 5 in patients with genotype A; 0 % at year 3, 0 % at year 5, and 11.5 % at year 9 in patients with genotype B; and 0.4 % at year 3, 1.6 % at year 5, and 5.1 % at year 9 in

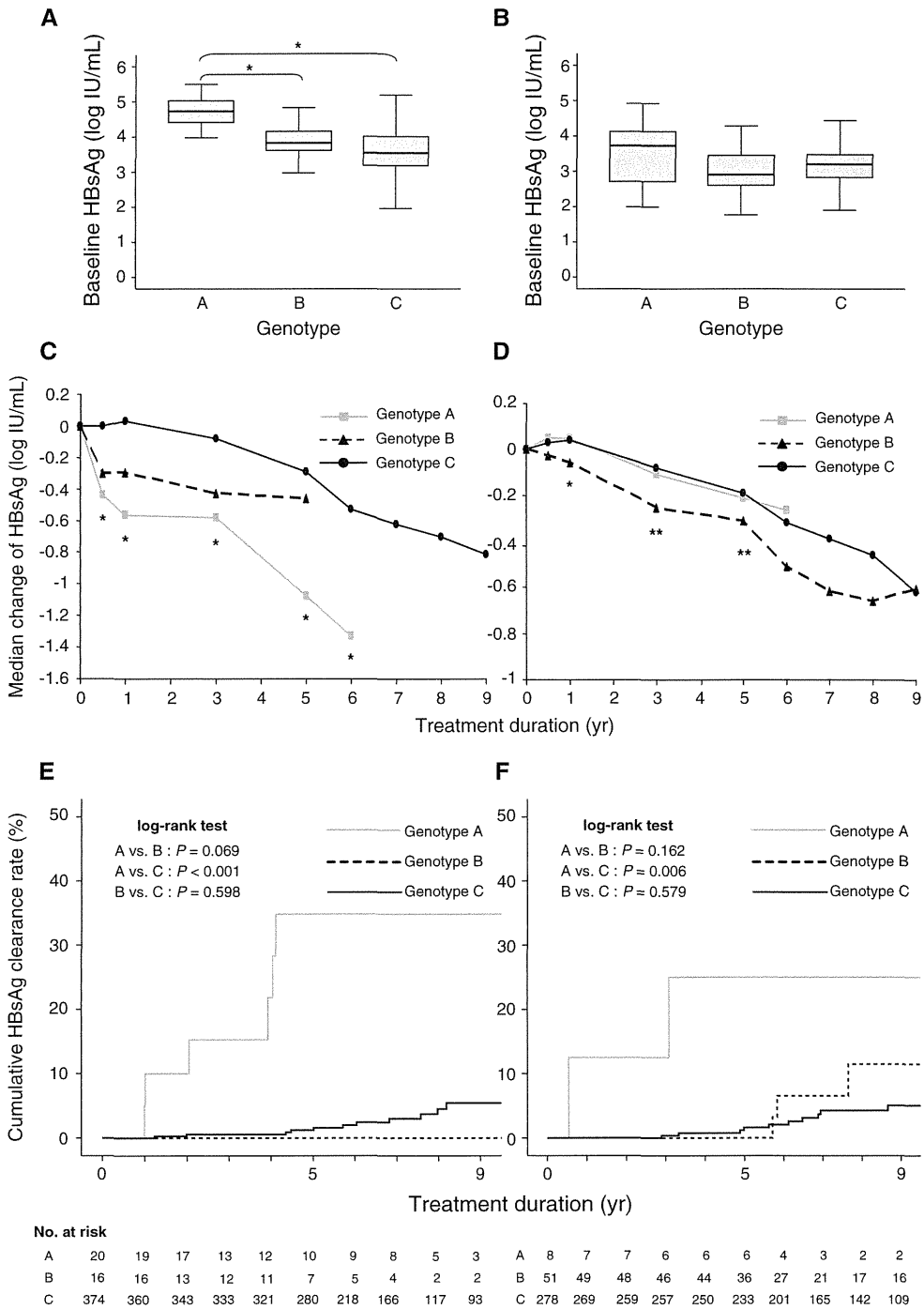

Fig. 2 **a** Box plot of baseline HBsAg levels in patients with different HBV genotypes (HBeAg+ cohort). The *asterisk* (*) indicates a statistical significance of $P < 0.001$, as determined by the Mann–Whitney U test and Bonferroni correction. **b** Box plot of baseline HBsAg levels in patients with different HBV genotypes (HBeAg− cohort). **c** Median change in HBsAg level from baseline in patients with different HBV genotypes (HBeAg+ cohort). A *single asterisk* (*) indicates $P < 0.001$, as determined by the Kruskal–Wallis test. **d** Median change in HBsAg level from baseline in patients with different HBV genotypes (HBeAg− cohort). A *single asterisk* (*) indicates $P < 0.001$ and a *double asterisk* (**) indicates $P < 0.02$, as determined by the Kruskal–Wallis test. **e** Kaplan–Meier life table showing cumulative HBsAg clearance rates in patients with different HBV genotypes (HBeAg+ cohort). Cumulative HBsAg clearance rates were significantly higher among patients with genotype A (log-rank test; A vs. B: $P = 0.069$, A vs. C: $P < 0.001$, B vs. C: $P = 0.598$, after Bonferroni correction). **f** Kaplan–Meier life table showing cumulative HBsAg clearance rates in patients with different HBV genotypes (HBeAg− cohort). Cumulative HBsAg clearance rates were significantly higher among patients with genotype A (log-rank test; A vs. B: $P = 0.169$, A vs. C: $P = 0.006$, B vs. C: $P = 0.579$, after Bonferroni correction)

patients with genotype C (Fig. 2f). Clearance rates were significantly higher in patients with genotype A than in those with genotype C ($P < 0.001$ in the HBeAg+ cohort, $P = 0.006$ in the HBeAg− cohort).

Association between on-treatment response and subsequent HBsAg clearance

We stratified patients into three groups according to the amount of HBsAg decline within the first six months of treatment; this allowed us to evaluate the impact of on-treatment response factors on the clearance of HBsAg. The stratifications were as follows: rapid decline (≥1.0 log IU/mL), intermediate decline (0.5–1.0 log IU/mL), and slow decline or steady (<0.5 log IU/mL). Cumulative HBsAg clearance rates in the HBeAg+ cohort were 11 % at year 3, and 40 % at year 5 in the rapid decline group; 0 % at year 3, 2.2 % at year 5, and 13 % at year 9 in the intermediate decline group; and 0 % at year 3, 0 % at year 5, and 2.9 % at year 9 in the slow decline or steady group (Fig. 3a).

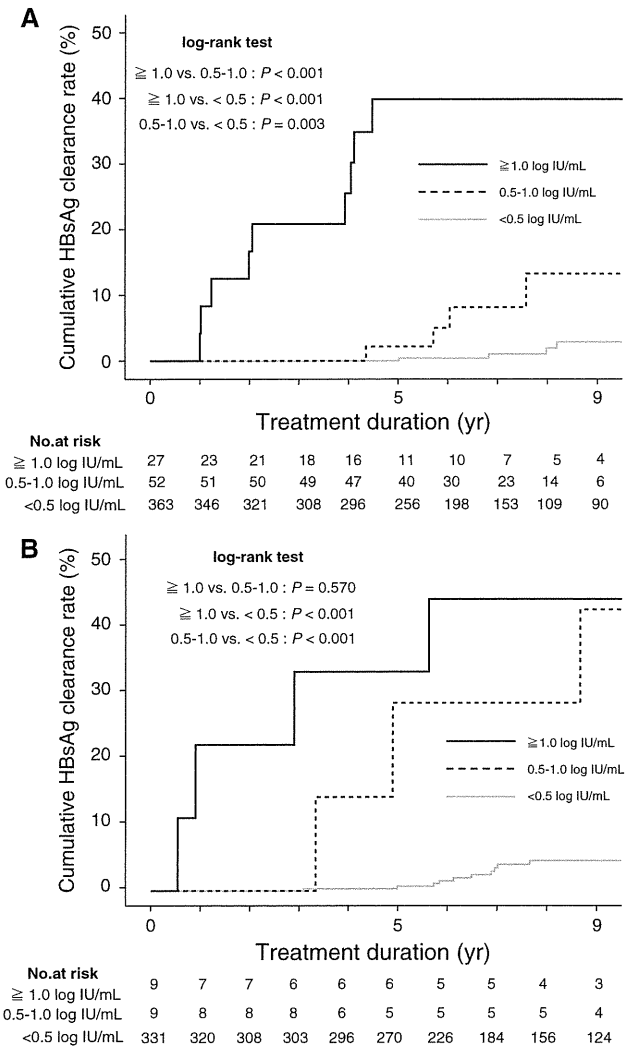

Fig. 3 **a** Kaplan–Meier life table showing cumulative HBsAg clearance rates in patients with varying rates of HBsAg decline within the first six months (HBeAg+ cohort). Clearance rates were highest in the rapid decline group, followed by the intermediate decline group and the slow or steady group (log-rank test; rapid vs. intermediate: $P < 0.001$, rapid vs. slow: $P < 0.001$, intermediate vs. slow: $P = 0.003$, after Bonferroni correction). **b** Kaplan–Meier life table showing cumulative HBsAg clearance rates in patients with varying rates of HBsAg decline within the first six months (HBeAg− cohort). Clearance rates were highest in the rapid decline group, followed by the intermediate decline group and the slow or steady group (log-rank test; rapid vs. intermediate: $P = 0.570$, rapid vs. slow: $P < 0.001$, intermediate vs. slow: $P < 0.001$, after Bonferroni correction)

Cumulative HBsAg clearance rates in the HBeAg− cohort were 33 % at year 5, and 44 % at year 7 in the rapid decline group; 0 % at year 3, 29 % at year 5, and 43 % at year 9 in the intermediate decline group; and 0.3 % at year 3, 0.7 % at year 5, and 4.6 % at year 9 in the slow decline or steady group (Fig. 3b). Clearance rates were highest in the rapid decline group, followed by the intermediate decline group and the slow or steady group in both the HBeAg+ and HBeAg− cohorts. The decline of HBsAg within the first six months was a strong predictor of HBsAg clearance.

Viral breakthrough and subsequent HBsAg clearance

Although VBT was not associated with HBsAg clearance in the multivariate model, as described above, HBsAg clearance was observed in ten patients who experienced VBT (five patients in the HBeAg+ cohort and five in the HBeAg− cohort). All ten patients achieved clearance of HBsAg after VBT occurred. Six of these patients received ADV added on to LAM for VBT, and subsequently achieved clearance of HBsAg (five patients in the HBeAg+ cohort and one in the HBeAg− cohort). The other four patients spontaneously recovered from VBT while continuing to receive LAM monotherapy, and subsequently achieved clearance of HBsAg (one patient in the HBeAg+ cohort and three in the HBeAg− cohort). LAM-resistant mutant strains (M204I/V mutants) were detected in nine patients in whom VBT occurred. HBV DNA negativity continued for the follow-up period after HBsAg clearance in these ten patients. The typical clinical and virological courses of two representative patients who achieved HBsAg clearance after VBT are shown in Fig. 4a, b.

Virological courses after discontinuation of NAs

Sixteen (42.1 %) of 38 patients with HBsAg clearance discontinued NA treatment due to HBsAg clearance. Median interval between HBsAg clearance and discontinuation of NAs was nine months (range 2–29 months). Median follow-up period after discontinuation of NAs was 24 months (range 7–171) in these patients. No relapses of serum HBsAg or HBV DNA were observed during the follow-up period. Serum anti-HBs appeared in 12 (75 %) of the 16 patients who discontinued NAs. Median time to the appearance of anti-HBs after HBsAg clearance was 16 months (range 2–92) in patients who discontinued NAs. Two of 22 patients who continued NAs with HBsAg clearance had the appearance of anti-HBs, and median time to the appearance of anti-HBs after HBsAg clearance was two and seven months in these two patients, respectively.

Discussion

We found that three baseline factors and two on-treatment response factors are associated with HBsAg clearance in patients who begin treatment with LAM and continue with long-term NA therapy. HBV genotype and the decline in HBsAg over the first six months were associated with

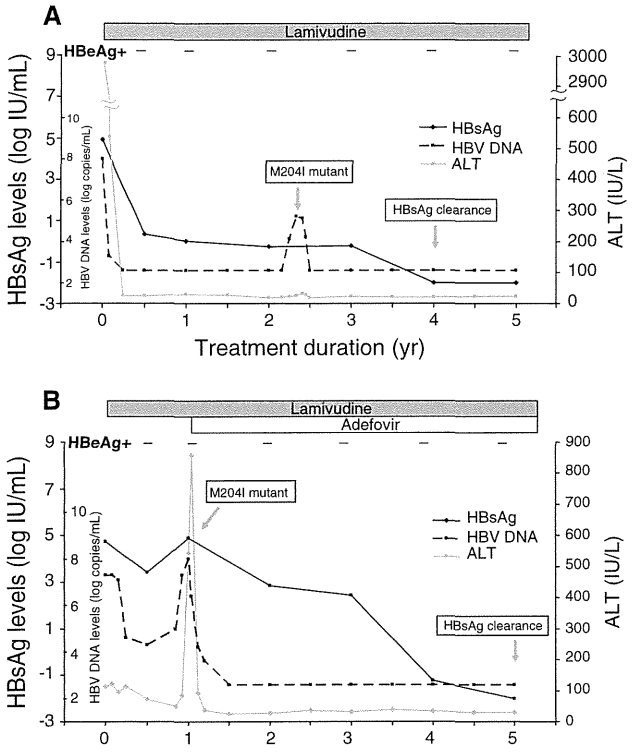

Fig. 4 Case presentation of the typical clinical and virological courses of two representative patients who achieved HBsAg clearance after VBT occurred. **a** Patient 1, a 45-year-old man who was HBeAg+ at baseline and had genotype A. **b** Patient 2, a 38-year-old man who was HBeAg+ at baseline and had genotype A. *VBT* virological breakthrough

HBsAg clearance in both the HBeAg+ and − cohorts, whereas the clearance of HBsAg was associated with previous IFN therapy and the clearance of HBeAg over the first six months only in the HBeAg+ cohort, and baseline HBsAg levels only in the HBeAg− cohort.

HBV genotype was recently reported to influence declines in and the clearance of HBsAg among patients who underwent PEG-IFN therapy [31]. In one study where negativity for serum HBV DNA and seroconversion of HBeAg represented the study end point, genotype was not found to influence response to NA therapy [31]. However, other reports have indicated that genotype does impact on declines in and the clearance of HBsAg [20, 29]. Heathcote et al. [20] reported that 20 HBeAg+ patients (8 %) who were treated with tenofovir achieved HBsAg clearance in three years. Twelve (60 %) of 20 patients were infected with genotype A and the others with genotype D. In this study, cumulative HBsAg clearance rates were 15 % at year 3 in HBeAg+ patients with genotype A. This result seems to be similar regardless of the antiviral potential. Previous studies with more ethnically diverse study populations than ours found that HBsAg clearance rates were highest in patients with genotype A. The similarity between those results and ours implies that the HBV genotype is more influential than ethnicity on HBsAg clearance during NA therapy. Of 28 genotype A patients in our population, the majority (79 %) did not have a family history of infection. Recent work has shown that sexual transmission of acute HBV genotype A infections is increasing in Japan, resulting in chronic HBV infection, especially in young adult patients [32, 33]. Cumulatively, these findings imply that HBsAg clearance is more likely in genotype A patients because they have been infected with HBV for a shorter period of time. Furthermore, Hou et al. [34] demonstrated that genotype A responded better than other HBV genotypes to IFN therapy. They revealed that a lower number of amino acid substitutions at baseline were associated with a better response to IFN therapy, and that this variable was linked with HBV genotype A, which had the lowest number of amino acid substitutions in the core gene among genotypes B, C, or D. Although amino acid substitutions in the core gene were not analyzed in this study, the relation between the core gene and treatment responses of NAs is necessary to be investigated in the future.

Although Gish et al. [19] reported that previous IFN therapy is not associated with HBsAg clearance in patients who are HBeAg+, the opposite was true in our HBeAg+ cohort. These contradictory findings may result from the fact that their patients received NA therapy over a much shorter time period (median duration 23 vs. 75 months, a 3.2-fold difference). We believe that there are two main reasons why HBsAg clearance rates were higher in patients who had previously received IFN therapy: the influence of AST/ALT flares after IFN therapy and changes in host immune response to HBV as a result of the immune-modulating activity of IFN. It has previously been shown that in patients with high baseline ALT levels, HBV DNA and HBeAg are likely to rapidly decrease during NA therapy [35, 36]. In this study, HBsAg clearance was likely to occur in patients who had high ALT levels at baseline, and in patients with previous IFN therapy (Table 2) in the HBeAg+ cohort. High virological responses have been reported in response to robust ALT flares induced by IFN therapy [37, 38]. Moreover, Wursthorn et al. [29] recently indicated that the antiviral potential of NAs and antiviral T cell reactivity are associated with HBsAg clearance in response to telbivudine treatment. These findings may be also associated with the achievement of HBsAg clearance after VBT occurs. Taken together, these results imply that both direct antiviral potential and host immune response are needed to achieve HBsAg clearance, especially in HBeAg+ patients.

We found that the initial HBsAg reduction was a strong predictor of subsequent HBsAg clearance during NA therapy, which supports a similar previous finding [29]. HBsAg reduction over the initial six months is important

for predicting the subsequent HBsAg kinetics in both HBeAg+ and HBeAg− patients. The novel finding in this study was that HBeAg− individuals achieved HBsAg clearance. We found that the median duration to HBsAg clearance was longer in patients with HBeAg− than in those who were HBeAg+ in this study (6.0 vs. 4.4 years). Manesis et al. [28] used modeling to determine that HBeAg− patients receiving LAM treatment would likely require >10 years to achieve HBsAg loss. Furthermore, baseline HBsAg titers were <730 IU/mL in 60 % (12/20) of HBeAg− patients who achieved HBsAg clearance. The only baseline predictive factor of HBsAg clearance was baseline HBsAg levels in HBeAg− patients, except for genotype. There was no difference in HBsAg clearance rates in HBeAg− patients with high- and low-baseline HBV DNA or ALT levels. We hypothesize that HBsAg clearance in these patients may result from long treatment duration and low HBsAg titers.

Our study was limited by the fact that it was a hospital-based retrospective analysis, which means there may be some bias associated with patient type and treatment selection. We were unable to compare HBsAg clearance rates obtained in our study with those of controls untreated with NA. Because all subjects in the study received LAM as an initial NA, and then received rescue therapy when drug-resistant mutations emerged, NA therapy regimens were not uniform across all patients, and there were variations in both treatment dose and duration of previous IFN therapy. We were not able to collect immunological data on our subjects. Finally, our results need to be validated by further studies investigating a large study population receiving long-term ETV or tenofovir with high antiviral potential and a high genetic barrier.

Despite these drawbacks, we were able to determine several factors associated with HBsAg clearance, including HBV genotype and a decline in HBsAg over the initial six months of treatment (HBeAg+ and − cohorts); previous IFN therapy and clearance of HBeAg over the initial six months of treatment (HBeAg+ cohort only); and HBsAg levels (HBeAg− cohort only). It seems that both direct antiviral potential and host immune response are needed to achieve HBsAg clearance by NA therapy. Future studies are needed to validate these findings and to develop treatment regimens for HBsAg clearance in patients with chronic hepatitis B.

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Conflict of interest Dr. Kumada reports having received investigator, lecture, and consulting fees from Bristol–Myers Squibb, Dainippon Sumitomo Pharma Co., MSD K.K., and Toray Co. 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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ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

Long-term efficacy of interferon therapy in patients with chronic hepatitis B virus infection in Japan

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Abstract
Background Few studies have investigated the long-term effects of interferon (IFN) therapy for chronic hepatitis B (CHB). In this retrospective study, we investigated the efficacy of and predictors of response to IFN therapy in CHB patients.
Methods We analyzed data for 615 Japanese CHB patients (hepatitis B e antigen [HBeAg]-positive 414, HBeAg-negative 201) treated with IFN, and conducted follow up for a median duration of 8.1 years (range 0.5–23.2). Responders were defined as patients who showed continuously normalized alanine transaminase (ALT) levels, HBeAg clearance, and low hepatitis B virus (HBV) DNA levels at 6 months post-treatment or for a span of more than 6 months until each test point at 1, 3, 5, and 10 years.
Results The IFN response rates of all patients were 21, 18, 21, 23, and 25% at 6 months and 1, 3, 5, and 10 years, respectively. On multivariate analysis, significant determinants of the outcome of IFN therapy were as follows: at 6 months and 1 year, young age, low HBV DNA levels, and long duration of treatment; at 3 years, long duration of treatment, young age, and high level of albumin; at 5 years, high level of albumin, female, and pretreated with IFN; and at 10 years, HBeAg-negative. Sixty-nine of the 615 patients (11%) achieved seroclearance of hepatitis B surface antigen (HBsAg). On multivariate analysis, age ≥30 years, HBV genotype A, and male were all independent factors predicting the achievement of HBsAg seroclearance.
Conclusion HBeAg, HBV DNA level, age, sex, albumin, duration of treatment, pretreatment with IFN, and HBV genotype were important factors in determining long-term response to IFN therapy.

Keywords Interferon · Hepatitis B virus · Chronic hepatitis B · Genotype · Hepatitis B surface antigen

Abbreviations

CHB	Chronic hepatitis B
HBV	Hepatitis B virus
IFN	Interferon
HBeAg	Hepatitis B e antigen
ALT	Alanine transaminase
MU	Million units
HBsAg	Hepatitis B surface antigen
CLEIA	Chemiluminescent enzyme immunoassay
bDNA	Branched-chain DNA probe assay
TMA-HPA	Transcription-mediated amplification and hybridization protection assay
PCR	Polymerase chain reaction
ELISA	Enzyme-linked immunosorbent assay
AST	Aspartate transaminase
AFP	α Fetoprotein
OR	Odds ratio
CI	Confidence interval
HCC	Hepatocellular carcinoma

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Introduction

Hepatitis B virus (HBV) infection is a common disease that can induce a chronic carrier state and is associated with the risk of developing progressive disease and hepatocellular carcinoma [1]. Interferon (IFN) and several nucleoside/nucleotide analogues such as lamivudine, adefovir dipivoxil, entecavir, and tenofovir disoproxil fumarate are currently approved as treatments for chronic hepatitis B (CHB) in most countries [2–5]. Successful treatment of CHB with clearance of hepatitis B e antigen (HBeAg), reduction in serum HBV DNA levels, and normalization of alanine transaminase (ALT) levels is associated with a favorable long-term outcome, independent of the antiviral drug used [6, 7].

A meta-analysis of IFN therapy published in 1993 reviewed 15 randomized controlled studies involving 837 adult patients who received IFN-α at doses of 5–10 million units (MU) administered at intervals ranging from daily to three times weekly for 4–6 months [8]. Clearance of HBeAg was noted in 33% of the treated patients compared with 12% of controls. Elimination of detectable HBV DNA and normalization of ALT levels were also more common in the treated patients than in the controls. The major pretreatment factors that correlated with a response were high ALT levels [9–11], low HBV DNA levels [9, 10], female sex, and elevated liver activity and fibrosis on liver biopsy [8]. Another recent meta-analysis of 24 randomized controlled trials concluded that the rates of persistent ALT normalization, clearance of HBeAg, and sustained elimination of HBV DNA (determined by hybridization) induced by IFN therapy were approximately 25% greater than the rates for controls. A more recent meta-analysis report showed that IFN increased the incidence of HBeAg and hepatitis B surface antigen (HBsAg) seroclearance after long-term follow up of 3–7 years [12].

However, specific data on the long-term effects of IFN therapy (median follow-up duration of 8.1 years), particularly among the Japanese, are limited. Moreover, few reports have investigated factors predicting the achievement of HBsAg seroclearance. To further evaluate factors influencing clinical outcome, we performed a retrospective cohort study on CHB patients treated with IFN in our hospital.

Patients and methods

Patients

We retrospectively examined 615 Japanese patients (151 females and 464 males) who commenced IFN treatment between June 1984 and April 2008 in the Department of Hepatology at Toranomon Hospital (Table 1). Several of the patients have been included in previous reports [13–15].

Table 1 Characteristics of patients at commencement of interferon therapy

Demographic data	
Total number	615
Sex, female/male	151/464
Age, years (range)	35 (15–68)
Previously treated with interferon	123 (20%)
Duration of treatment, weeks (range)	26 (4–981)
Follow-up period, years (range)	8.1 (0.5–23.2)
Laboratory data	
Aspartate transaminase, IU/L (range)	72 (18–990)
Alanine transaminase, IU/L (range)	138 (12–1578)
Bilirubin, mg/dL (range)	0.7 (0.2–8.8)
Albumin, g/dL (range)	3.9 (2.6–5.3)
Platelets, $\times 10^3/\mu L$ (range)	174 (48–500)
Staging of liver histology (F0/1/2/3/4/ND)	8/77/185/162/72/111
Serum HBV DNA, log copies/mL (range)	>7.6 (<2.6 to >7.6)
HBeAg (positive/negative)	414/201
HBV genotype (A/B/C/D/H/B + C/unknown)	24/37/504/1/1/1/47

Values are expressed as medians and ranges (in parentheses) or as numbers and percentages (in parentheses)

HBV hepatitis B virus, *HBeAg* hepatitis B e antigen, *ND* not done

All enrolled patients were followed up for a range of 0.5–23.2 years from completion of IFN treatment, with a median follow-up duration of 8.1 years. Before the commencement of IFN treatment, all patients had been positive for HBsAg in the serum for more than 6 months, and all were confirmed to have hepatitis caused by HBV and not by another vector, such as infection with hepatitis C virus or autoimmune hepatitis. None had a history of drug abuse or alcoholic hepatitis, and none had received nucleoside/nucleotide analogue therapy. The study was conducted in accordance with the ethical principles of the Declaration of Helsinki and was approved by the Toranomon Hospital Ethics Committee. Informed consent was obtained from each patient.

Interferon therapy and assessment of response to therapy

Patients received 3–12 MU of IFN-α or IFN-β (Sumiferon: Dainippon Sumitomo Pharma, Osaka, Japan; Canferon A: Takeda Chemical Industries, Osaka, Japan; Intron A: Schering-Plough MSD KK, Osaka, Japan; and Feron: Toray, Tokyo, Japan). The durations and regimens of treatment were as follows: 4 weeks (89 patients; daily for

4 weeks), 26 weeks (270 patients; daily for 4 weeks followed by 2 or 3 times a week), 52 weeks (103 patients; 2 or 3 times a week), 104 weeks (80 patients; 2 or 3 times a week), and more than 104 weeks (73 patients; 2 or 3 times a week). The median duration of treatment was 26 weeks (range 4–981).

The numbers of responders were evaluated at 6 months and 1, 3, 5, and 10 years after the completion of IFN therapy. In the baseline HBeAg-positive patients, responders were defined as patients who showed normalization of serum ALT level (normal level 6–30 IU/L), HBeAg clearance, and low HBV DNA level (<5 log copies/mL) at 6 months after completion of IFN therapy. In addition, baseline HBeAg-positive patients who showed continuous normalization of ALT levels, HBeAg clearance, and low HBV DNA level for more than 6 months until each test point at 1, 3, 5, and 10 years after completion of IFN therapy were also classified as "responders." In the baseline HBeAg-negative patients, responders were defined as those who showed sustained normalization of ALT level and low HBV DNA level (<4 log copies/mL) for more than 6 months until each test point after completion of IFN therapy.

All patients not considered to be responders were termed "non-responders." Patients receiving other therapies (IFN or nucleoside/nucleotide analogues) after the completion of IFN therapy were also termed non-responders.

Blood tests and serum viral markers

Routine biochemical tests were performed monthly via standard procedures during and for the first 12 months following the completion of IFN treatment and at least every 2 months thereafter. Levels of HBsAg, HBeAg, and anti-HBe were determined using radioimmunoassay kits (Abbott Diagnostics, Chicago, IL, USA) or a chemiluminescent enzyme immunoassay (CLEIA; Lumipulse System; Fujirebio, Tokyo, Japan). HBV DNA levels were measured using a branched-chain DNA probe assay (bDNA) (Chiron Laboratory Service, Van Nuys, CA, USA), a transcription-mediated amplification and hybridization protection assay (TMA-HPA) (Chugai Diagnostics Science, Tokyo, Japan), or a polymerase chain reaction (PCR)-based assay (COBAS Amplicor HBV Monitor Test or COBAS TaqMan HBV Test; Roche Diagnostics, Indianapolis, IN, USA).

HBV genotype

The major genotypes of HBV were determined using an enzyme-linked immunosorbent assay (ELISA; Institute of Immunology, Tokyo, Japan) or a PCR-invader assay (BML, Tokyo, Japan) according to the methods described by Usuda et al. [16] or Tadokoro et al. [17].

Statistical analysis

Differences between groups were examined for statistical significance using the χ^2 or Fisher's exact test and Mann–Whitney *U*-test where appropriate. Independent predictive factors associated with response to IFN treatment were determined using multivariate multiple logistic regression. The following 14 potential predictors of response to IFN treatment were assessed in this study: age, sex, pretreatment with IFN, duration of IFN treatment, severity of liver disease (CH or liver cirrhosis), HBV genotype, and levels of aspartate transaminase (AST), ALT, bilirubin, albumin, platelets, α fetoprotein (AFP), HBeAg, and HBV DNA. All factors found to be at least marginally associated with response to IFN therapy ($P < 0.10$) were entered into the multivariate multiple logistic regression analysis. The above calculations were performed using the Windows SPSS software package version 11.0.1 J (SPSS, Chicago, IL, USA).

The odds ratios (ORs) and 95% confidence intervals (CIs) were calculated to assess the relative risk. Independent risk factors predicting the achievement of HBsAg seroclearance were studied using stepwise Cox regression analysis. Potential factors predicting the achievement of HBsAg seroclearance assessed here were the above 14 variables, each transformed into categorical data consisting of two simple ordinal numbers for univariate and multivariate analyses. All factors found to be at least marginally associated with HBsAg seroclearance ($P < 0.10$) were tested in the multivariate Cox proportional hazard model. A Kaplan–Meier estimate was performed using the SPSS software, and *P* values were calculated using the Cox-Mantel log-rank test. A two-tailed *P* value of <0.05 was considered statistically significant.

Results

Study population

Twenty-four (4%), 37 (6%), 504 (82%), 1 (0.2%), 1 (0.2%), and 1 (0.2%) patients were infected with HBV genotypes A, B, C, D, H, and B + C, respectively. Genotype could not be measured in the remaining 47 patients. The baseline characteristics of the patients are shown in Table 1. Although few patients had genotypes A and B, the distribution of HBV genotype was similar to that in patients with CHB who had received care in our hospital, with a follow-up period of more than 2 years [18]. Twenty-two of 24 patients with genotype A, 14 of 37 with

genotype B, 342 of 504 with genotype C, 1 of 1 with genotype H, and 34 of 47 with unknown genotype were HBeAg-positive at the commencement of treatment. While we were able to measure HBV DNA levels in 254 patients at the commencement of IFN therapy, levels in the remaining 361 could not be measured owing to a lack of commercial kits before the bDNA assay was available. The numbers of patients receiving other additional therapies after the completion of IFN therapy were 111 (HBeAg-positive/-negative, 90/21), 92 (67/25), 34 (25/9), and 61 (39/22) at the 1-, 3-, 5-, and 10-year time points, respectively.

Response to interferon therapy in all patients

The IFN response rates in all patients were 21% (105/497), 18% (86/491), 21% (90/428), 23% (82/359), and 25% (59/235) at 6 months and 1, 3, 5, and 10 years, respectively, after completion of the IFN therapy (Fig. 1). In patients with genotype A, the response rate was highest at 6 months post-treatment and gradually decreased at subsequent time points from 1 to 10 years thence. In patients with genotype B, response rates were over 20% at all time points except for 6 months post-treatment, whereas rates in patients with genotype C were under 25% at all time points (Fig. 2a).

Evaluation of efficacy of IFN in relation to clinical factors in all patients

The data of all patients were subjected to univariate analyses to determine the clinical factors contributing to the efficacy of IFN at each time point. We then investigated the significance of response to IFN therapy using multivariate logistic regression analysis.

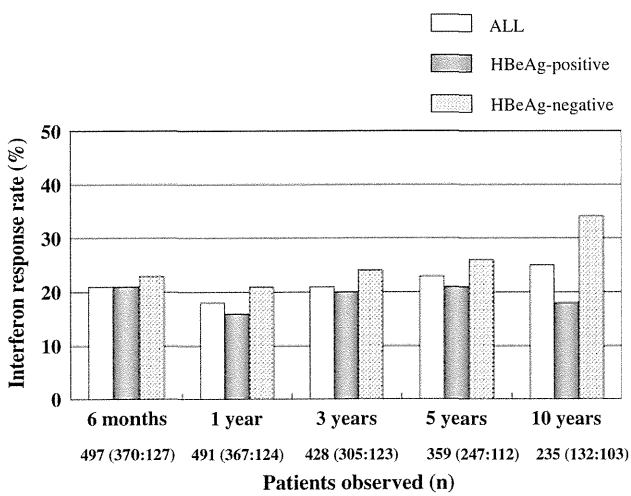

Fig. 1 Interferon response rates of all patients and hepatitis B e antigen (*HBeAg*)-positive and HBeAg-negative patients at 6 months and 1, 3, 5, and 10 years

Multivariate analyses including the variables noted above revealed several parameters that independently influenced the outcome of IFN therapy; namely, at 6 months: age ($P = 0.013$), HBV DNA level ($P = 0.019$), and duration of treatment ($P = 0.034$); at 1 year: HBV DNA level ($P < 0.001$) and age ($P = 0.001$); at 3 years: duration of treatment ($P < 0.001$), age ($P = 0.013$) and albumin level ($P = 0.013$); at 5 years: albumin level ($P = 0.004$), sex ($P = 0.005$), and pretreatment with IFN ($P = 0.039$); and at 10 years: HBeAg ($P < 0.001$) (Table 2).

Response to interferon therapy and evaluation of efficacy of IFN in relation to clinical factors in HBeAg-positive patients

Response rates in baseline HBeAg-positive patients were 21% (76/370), 16% (60/367), 20% (61/305), 21% (53/247), and 18% (24/132) at 6 months and 1, 3, 5, and 10 years, respectively (Fig. 1). In patients with genotype A, the response rate was highest at 6 months post-treatment and the rate was roughly equivalent to the 6 months post-treatment rate at subsequent time points from 1 to 10 years. Response rates in patients with genotype B in particular were above 40% at all time points except at 6 months, although few patients had genotype B. On the other hand, response rates in patients with genotype C were under 20% at all time points (Fig. 2a).

In addition, multivariate analyses in HBeAg-positive patients also revealed several parameters that independently influenced the outcome of IFN therapy–at 6 months: duration of treatment ($P = 0.001$) and age ($P = 0.014$); at 1 year: age ($P = 0.011$) and HBV DNA level ($P = 0.027$); at 3 years: sex ($P = 0.008$), duration of treatment ($P = 0.019$), age ($P = 0.020$), pretreatment with IFN ($P = 0.029$), and albumin level ($P = 0.043$); at 5 years: sex ($P = 0.002$) and pretreatment with IFN ($P = 0.005$); and at 10 years, genotype ($P = 0.019$) and AST ($P = 0.035$) (Table 3).

Response to interferon therapy and evaluation of efficacy of IFN in relation to clinical factors in HBeAg-negative patients

Response rates in baseline HBeAg-negative patients were 23% (29/127), 21% (26/124), 24% (29/123), 26% (29/112), and 34% (35/103) at 6 months and 1, 3, 5, and 10 years, respectively (Fig. 1). Rates in patients with genotype C were gradually increased at subsequent time points, whereas those in patients with genotype B remained under 30% at all time points (Fig. 2b).

In addition, univariate and multivariate analyses in HBeAg-negative patients revealed that duration of treatment (≥1 year) independently influenced the outcome of

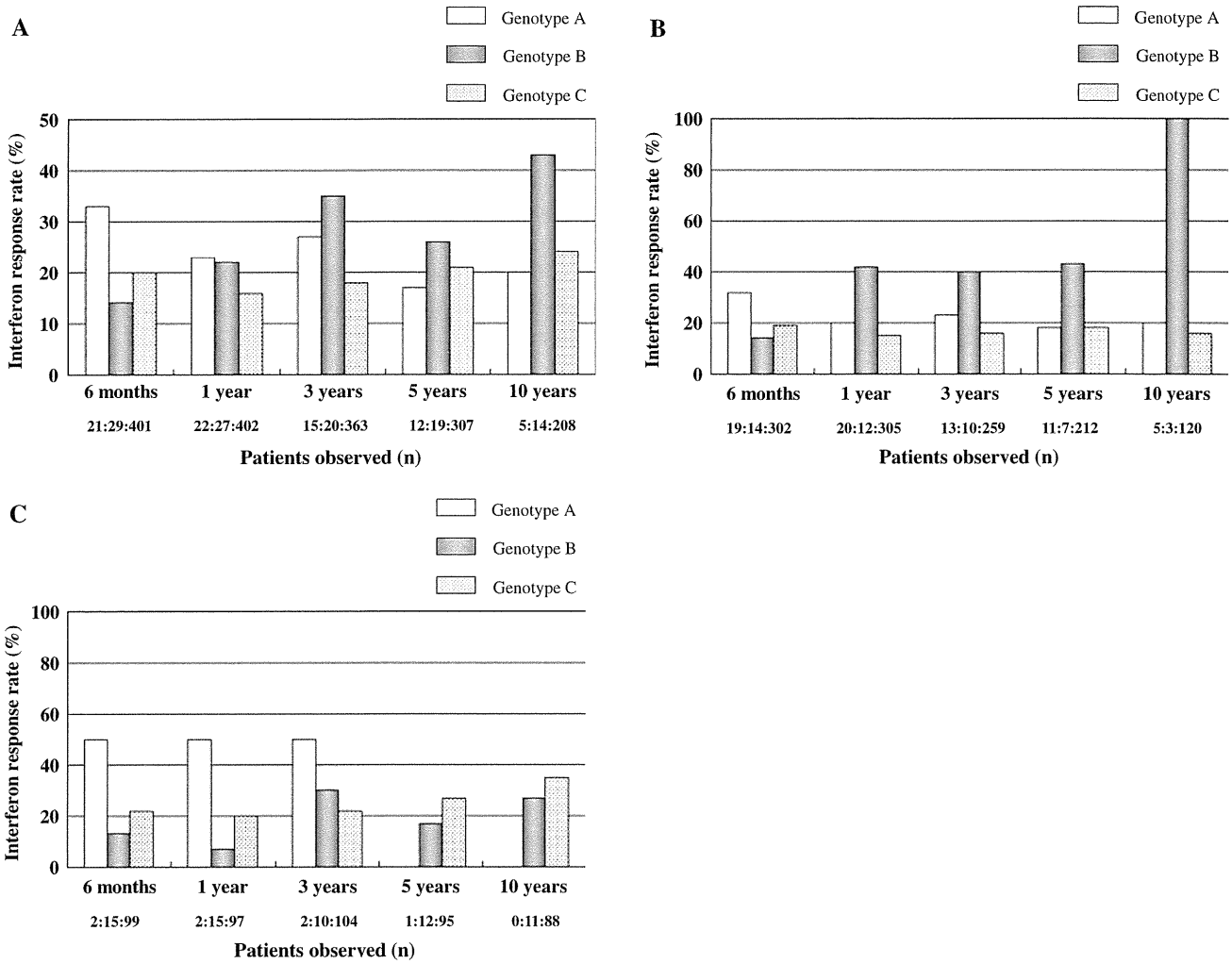

Fig. 2 Interferon response rates of patients with genotypes A, B, and C at 6 months and 1, 3, 5, and 10 years. **a** All patients, **b** HBeAg-positive patients, **c** HBeAg-negative patients

IFN therapy at 6 months, and at 1 and 3 years. No parameters independently influenced the outcome of IFN therapy at 5 or 10 years.

Evaluation of efficacy of IFN in relation to HBs antigen seroclearance

The HBsAg seroclearance rate in this study was obtained from patients who received IFN therapy alone; 69 of 615 patients (11%) achieved seroclearance of HBsAg. The cumulative HBsAg seroclearance rates in all patients from the commencement date of IFN therapy were 6.5% at 5 years, 15% at 10 years, 35% at 15 years, and 44% at 20 years (Kaplan–Meier method; Fig. 3a). No patients experienced the reappearance of HBsAg after seroclearance. Five factors found to be associated with achievement of HBsAg seroclearance on univariate analysis were: male sex ($P = 0.002$), age $\geq$30 years ($P = 0.011$), genotype A ($P = 0.038$), HBeAg-negativity ($P = 0.045$), and bilirubin $\leq$1.0 mg/dL ($P = 0.064$). On multivariate analysis, independent factors predicting the achievement of HBsAg seroclearance were: age $\geq$30 years, genotype A, and male sex (Table 4). The cumulative HBsAg seroclearance rate for genotype A patients was significantly higher than the rate for those with genotypes B or C ($P = 0.0116$) (Fig. 3b).

Relationship between the response to IFN and the development of hepatocellular carcinoma

Twenty-nine patients developed hepatocellular carcinoma (HCC) during the observation period, excluding 17 patients who received other additional therapies after the completion of IFN therapy and developed HCC thereafter. IFN response rates in the 29 patients who developed HCC were 5% (1/22), 5% (1/20), 10% (2/20), 13% (2/15), and 13% (2/16), respectively, at 6 months and 1, 3, 5, and 10 years after the completion of IFN. No patient developed HCC after HBsAg seroclearance.

Table 2 Factors associated with response to interferon therapy for all patients at 6 months and 1, 3, 5, and 10 years

Parameter	Univariate analysis		Multivariate analysis	
	OR (95% CI)	*P*	OR (95% CI)	*P*
6 Months after completion of IFN therapy ($n = 229$)				
Duration of treatment (≥1 year)	2.680 (1.724–4.166)	<0.001	2.107 (1.058–4.198)	0.034
HBV DNA level (≤7.0 log copies/mL)	2.165 (1.107–4.219)	0.026	2.309 (1.148–4.630)	0.019
Age (<30 years)		0.057	2.451 (1.209–4.950)	0.013
1 year after completion of IFN therapy ($n = 231$)				
Duration of treatment (≥1 year)	2.553 (1.588–4.104)	<0.001		
HBV DNA level (≤7.0 log copies/mL)	3.268 (1.597–6.667)	0.001	4.464 (2.058–9.709)	<0.001
Age (<35 years)	1.799 (1.125–2.874)	0.014	3.831 (1.718–8.547)	0.001
3 years after completion of IFN therapy ($n = 397$)				
Duration of treatment (≥1 year)	2.410 (1.495–3.885)	<0.001	2.739 (1.618–4.634)	<0.001
Age (<30 years)	2.070 (1.215–3.521)	0.009	2.110 (1.171–3.802)	0.013
Albumin (≥3.9 g/dL)	1.697 (1.045–2.757)	0.030	2.009 (1.158–3.486)	0.013
Genotype (non-C)	2.155 (1.033–4.504)	0.041		
5 years after completion of IFN therapy ($n = 356$)				
Albumin (≥3.9 g/dL)	1.869 (1.108–3.153)	0.017	2.321 (1.316–4.093)	0.004
Pretreatment with IFN (positive)	1.770 (1.016–3.084)	0.048	1.821 (1.029–3.222)	0.039
Sex (female)		0.060	2.381 (1.297–4.367)	0.005
Duration of treatment (≥1 year)		0.080		
10 years after completion of IFN therapy ($n = 234$)				
HBeAg (negative)	2.315 (1.269–4.219)	0.006	2.252 (1.230–4.115)	0.009
ALT (≥100 IU/L)	1.972 (1.053–3.690)	0.036		
Pretreatment with IFN (positive)		0.058		

ALT alanine transaminase, *IFN* interferon, *HBV* hepatitis B virus, *HBeAg* hepatitis B e antigen, *CI* confidence interval, *OR* odds ratio, *n* number submitted to multivariate analysis, including all factors found to be associated with response to IFN therapy

Discussion

Although IFN has been reported to exert beneficial effects in CHB patients, the response rate is not high. A meta-analysis published in 1993 reviewed 15 randomized controlled studies involving 837 adult patients who received IFN-α for 4–6 months, and elimination of HBeAg occurred in 33% of the treated patients [8]. In previous studies, we found the response rates among HBeAg-positive patients at 6 months after the completion of therapy to be 20 and 31% for 6 months and 1 year of IFN therapy, respectively [13, 15]. Although a recent meta-analysis reported that IFN increased the incidence of HBeAg and HBsAg seroclearance after long-term follow up of 3–7 years [12], the factors that influenced the clinical outcome were unclear.

In Japan, from 1988, 4-week IFN treatment was reimbursed by the healthcare system, and since 2002, 24-week IFN treatment has been conducted. In the present study, these two regimens were the major ones, and other regimens were used in clinical studies at our hospital (including previously reported studies [14, 15]). Although the durations of treatment differed, we analyzed the factors associated with long-term response to IFN therapy, including the factor of duration of treatment.

In the present study, response rates were low among HBeAg-positive patients and relatively high among HBeAg-negative patients at all time points. Approximately 20% of the HBeAg-positive patients had sustained a response at 6 months to 10 years of follow up. Long-term follow-up studies after a four- to six-month course of IFN therapy in HBeAg-positive patients in European and Taiwanese studies showed higher (33–75%) response rates (HBeAg loss) than our study [7, 19, 20]. The difference in response rates between our present study and previous studies in other countries may be due to differences in ethnicity or HBV genotype (mainly genotype C in Japan). Moreover, the low IFN response rates at 1, 3, 5, and 10 years in the HBeAg-positive patients in our study were likely due to the change in treatments (IFN or nucleoside/nucleotide analogues). On the other hand, the response rates of HBeAg-negative patients in the present study were about 20% at 6 months and gradually increased thereafter. The sustained response rate in HBeAg-negative patients was usually <30% in European studies [21–23]. The response

Table 3 Factors associated with response to interferon therapy for HBeAg-positive patients at 6 months and 1, 3, 5, and 10 years

Parameter	Univariate analysis		Multivariate analysis	
	OR (95% CI)	*P*	OR (95% CI)	*P*
6 months after completion of IFN therapy ($n = 279$)				
Duration of treatment (≥1 year)	2.449 (1.457–4.114)	0.001	2.801 (1.540–5.096)	0.001
Age (<35 years)	1.855 (1.112–3.096)	0.017	2.128 (1.164–3.891)	0.014
1 year after completion of IFN therapy ($n = 172$)				
Duration of treatment (≥1 year)	2.483 (1.407–4.380)	0.002		
HBV DNA level (≤7.0 log copies/mL)	3.509 (1.495–8.264)	0.005	3.003 (1.130–7.937)	0.027
Age (<35 years)	1.996 (1.133–3.521)	0.015	3.610 (1.351–9.615)	0.011
3 years after completion of IFN therapy ($n = 283$)				
Age (<35 years)	2.041 (1.155–3.597)	0.013	2.083 (1.122–3.861)	0.020
Duration of treatment (≥1 year)	2.055 (1.153–3.661)	0.016	2.130 (1.132–4.008)	0.019
Pretreatment with IFN (positive)	2.054 (1.050–4.019)	0.041	2.336 (1.091–4.998)	0.029
Albumin (≥3.9 g/dL)		0.055	1.974 (1.020–3.820)	0.043
Sex (female)		0.089	2.646 (1.284–5.464)	0.008
5 years after completion of IFN therapy ($n = 247$)				
Sex (female)	2.571 (1.328–4.975)	0.006	2.924 (1.477–5.814)	0.002
Pretreatment with IFN (positive)	2.460 (1.213–4.988)	0.015	2.870 (1.377–5.980)	0.005
10 years after completion of IFN therapy ($n = 122$)				
Genotype (non-C)	5.319 (1.222–23.26)	0.032	6.410 (1.364–30.30)	0.019
AST (≥100 IU/L)		0.081	2.932 (1.078–7.972)	0.035

AST aspartate transaminase, *IFN* interferon, *HBV* hepatitis B virus, *HBeAg* hepatitis B e antigen, *CI* confidence interval, *OR* odds ratio, *n* number submitted to multivariate analysis, including all factors found to be associated with response to IFN therapy

rates of HBeAg-negative patients in our present study and the studies in other countries [21–23] were similar.

Few reports have identified the factors associated with long-term virological response to IFN therapy. In our present study, HBeAg-negativity was the most important factor for predicting a long-term response (10 years). While the HBV DNA level was important for predicting the response at 6 months and 1 year for all patients and the response at 1 year for HBeAg-positive patients, other factors (age, sex, albumin level, AST, IFN pretreatment, and duration of treatment) were found to be important at some time points for all patients and for HBeAg-positive patients. The HBV DNA level may not have been associated with long-term response to IFN therapy because the follow-up period (median 5.7 years) in patients with an HBV DNA level measurable with commercial kits was significantly shorter than that in the other patients (median 11.2 years; $P < 0.001$).

Previous studies have reported that high ALT levels, low HBV DNA level, female sex, and elevated liver activity and level of fibrosis on liver biopsy were major pretreatment factors correlated with a response to IFN [8–11, 24]. However, in these studies the follow-up times for judging the response were short (typically 6 months to 1 year). Our present study has clarified that HBeAg, HBV DNA level, age, sex, IFN pretreatment, duration of treatment, and levels of albumin and AST are important factors in the long-term response to IFN. Further, non-C genotype was an important factor for long-term response in HBeAg-positive patients. Kao et al. [25] and Lin et al. [20] reported that HBV genotype B was associated with a higher response rate to IFN-α therapy than genotype C among CHB patients positive for HBeAg. Similarly, response rates among HBeAg-positive patients with genotype B in the present study were also higher than the response rates in those with genotype C in terms of long-term response (Fig. 2b). The long-term response rate among HBeAg-negative patients was relatively higher than that in HBeAg-positive patients. Previous reports have shown that response rates to a 6- to 12-month course of IFN-α in HBeAg-negative CHB patients range from 10 to 47% (average 24%) [26–29]. In addition, our previous report showed that 9 of 12 (75%) patients who received IFN-β twice per week for 24 weeks responded to the therapy [14]. However, the follow-up periods of these studies were short, and the long-term efficacy has not been clarified. While the efficacy of IFN in HBeAg-negative patients was high in the present study, the factors that might be useful in predicting a sustained response were less well-defined than those in HBeAg-positive patients, as previously reported [5].

A meta-analysis of IFN therapy published in 2010 reviewed 6 clinical controlled studies including 828 patients who received IFN [12]. The duration of follow-up

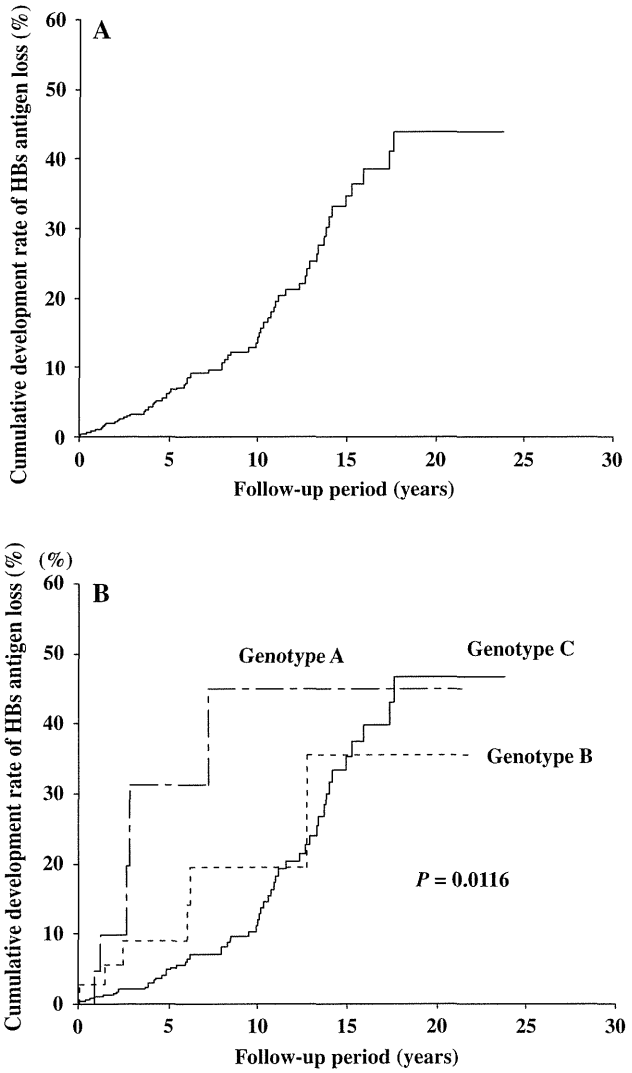

Fig. 3 Cumulative clearance of hepatitis B surface (*HBs*) antigen in patients treated with interferon (Kaplan–Meier method). **a** All patients, **b** patients stratified by genotypes A, B, and C

Table 4 Factors associated with HBsAg seroclearance by interferon therapy, determined by multivariate analysis

Parameter	Category	Hazard ratio	95% CI	*P*
Age	<30 years	1		0.002
	≥30 years	4.433	1.703–11.538	
Genotype	A	1		0.004
	B	0.296	0.087–1.005	
	C	0.199	0.075–0.528	
Sex	Female	1		0.005
	Male	2.962	1.387–6.327	

HBsAg hepatitis B surface antigen, *CI* confidence interval

ranged from 35.8 months to 7 years, and HBsAg seroclearance occurred in 9.5% (79/828). In the present study, we observed HBsAg seroclearance in 69 of 615 (11%) patients, with a median follow-up duration of 8.1 years. However, few reports have investigated factors predicting the achievement of HBsAg seroclearance. In our study, important factors for achieving HBsAg seroclearance were age ≥30 years, genotype A, and male sex. Patients with genotype A had primarily been infected during adulthood via sexual contact, and the average duration of infection was relatively short. In contrast, most Japanese carriers are infected perinatally and possess HBV genotype C, and therefore the efficacy of IFN therapy for patients with genotype C may be low. Male sex was also an important factor in determining potential to achieve HBsAg seroclearance, although female sex was an important factor in determining long-term response to IFN therapy. In our previous study of HBsAg seroclearance (mainly spontaneous seroclearance), we found that response rates were low among females (19%; 45/231) [30]. These present and previous findings indicate that male patients tended to achieve HBsAg seroclearance more frequently than females, although the reason is unclear. We previously reported that Kaplan–Meier analysis in 486 patients who received lamivudine therapy for 5 and 10 years showed an estimated loss of HBsAg in 3 and 13% of the patients, respectively, [31]. The cumulative clearance rates of HBsAg, also determined by Kaplan–Meier analysis, in patients treated with IFN were higher than those in the patients treated with lamivudine, albeit that there were differences in the baseline characteristics of the patients at the commencement of the respective therapies. The effects of IFN therapy in modulating the host immune response might induce HBsAg clearance.

In conclusion, we investigated the long-term efficacy of IFN therapy in Japanese CHB patients. Response rates were low among HBeAg-positive patients and relatively high among HBeAg-negative patients at all time points examined. HBeAg-negative status, HBV DNA level, age, sex, pretreatment with IFN, duration of treatment, and levels of albumin and AST were important factors in predicting long-term response for all patients and for HBeAg-positive patients. Age, genotype, and sex were important factors in predicting ability to achieve HBsAg seroclearance. Further studies exploring the efficacy of therapy over a longer duration may be necessary to confirm these findings and establish true response rates to IFN therapy, including treatment with pegylated IFN.

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Research Article

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Long-term continuous entecavir therapy in nucleos(t)ide-naïve chronic hepatitis B patients

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Background & Aims: We determined the antiviral potency and viral resistance rate after 4 years of continuous entecavir treatment in patients with chronic hepatitis B (CHB) infection.

Methods: The cumulative rates of undetectable hepatitis B virus DNA (HBV DNA; <2.6 $\log_{10}$ copies/ml), hepatitis B e antigen (HBeAg) seronegativity, seroconversion, alanine aminotransferase (ALT) normalization, and entecavir signature mutations were calculated in 474 nucleos(t)ide-naïve CHB patients (HBeAg-positive: 47%) on continuous entecavir treatment for 4 years.

Results: Median age was 47 years and follow-up period was 2.4 years, with 403, 281, 165, and 73 patients followed-up for at least 1, 2, 3, and 4 years, respectively. Incremental increases were observed in the rates of undetectable HBV DNA, HBeAg seroclearance and seroconversion, and ALT normalization, reaching 96%, 42%, 38% and 93%, respectively, by the fourth year. In all, 100% and 93% of patients negative and positive for HBeAg, respectively, had undetectable HBV DNA at year 4. Of 165 patients, HBV DNA was detectable in nine patients after 3 years. Multivariate analysis identified HBV DNA level ($\leqslant$7.6 $\log_{10}$ copies/ml, OR = 15.8; 95% CI = 43.1–79.9, P = 0.001) as an independent predictor of undetectable HBV DNA at year 3. Five patients experienced virological breakthrough including two (0.4%) who developed entecavir-resistance mutations.

Conclusions: Continuous treatment of nucleos(t)ide-naïve CHB patients with entecavir over 4 years was associated with 96% chance of undetectable HBV DNA and only 0.4% chance of emerging entecavir-resistant mutations.

Keywords: Hepatitis B virus; Entecavir; Resistance; Virological breakthrough.
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Abbreviations: AFP, α fetoprotein; ALT, alanine aminotransferase; AST, aspartate transaminase; CHB, chronic hepatitis B; CIs, confidence intervals; HBeAg, hepatitis B e antigen; HBsAg, hepatitis B virus surface antigen; HBV, hepatitis B virus; HBV DNA, hepatitis B virus DNA; ORs, odds ratios; PCR, polymerase chain reaction; ULN, upper limit of normal; VBT, virologic breakthrough.

Introduction

Approximately 350–400 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 developing cirrhosis, liver failure, and hepatocellular carcinoma.

Recent investigations have shown that entecavir suppressed HBV DNA replication to undetectable levels and normalized alanine aminotransferase (ALT) levels in nucleos(t)ide-naive CHB patients in Japan and other countries [3–10]. In addition, genotypic resistance to long-term entecavir monotherapy remained rare [5,6,9,10]. To date, there are two 5-year studies [6,8] and two 3-year studies [7,9] of entecavir therapy for nucleos(t)ide-naïve patients. Both studies stemmed from extension studies with the original cohorts from two large-scale phase III trials of treatment-naïve patients [3,4]. In these trials, patients were administered 0.5 mg entecavir for 1 year and later divided into three categories: (i) complete responders, defined as patients with HBV DNA <7 $\times$ 10^5 copies/ml and ALT level <1.25 times the upper limit of normal (ULN) for hepatitis B e antigen (HBeAg)-negative patients and an additional loss of HBeAg for HBeAg-positive patients; (ii) non-responders, defined as HBV DNA $\geqslant$7 $\times$ 10^5 copies/ml; and, (iii) virological responders, defined as HBV DNA <7 $\times$ 10^5copies/ml and ALT >1.25$\times$ ULN regardless of HBeAg status or persistent HBeAg for HBeAg-positive patients. Treatment was terminated in the complete responders but continued in virological responders. Non-responders were provided additional therapy in a rollover study in which some patients were initially treated with a combination of 1 mg entecavir and lamivudine for several months before receiving 1 mg entecavir as monotherapy. Furthermore, a substantial proportion of complete responders relapsed after various intervals following cessation of therapy and they were also assigned to a rollover study receiving 1 mg entecavir monotherapy. Because of these strict protocols, the precise viral-suppression and drug-resistance data for treatment-naïve patients who were treated continuously with 0.5 mg entecavir daily (the recommended dosage) remain unavailable.

The aims of this cohort study were (1) to investigate the efficacy of entecavir in clinical practice beyond 4 years for nucleos(t)ide-naïve CHB and cirrhosis patients, (2) to explore baseline factors associated with virological response to entecavir,

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Journal of Hepatology **2012** vol. 57 | 508–514

and (3) to investigate virological breakthrough during long-term entecavir treatment.

Patients and methods

Study population

We performed a retrospective analysis of 474 CHB and cirrhosis patients who received entecavir treatment at the Department of Hepatology, Toranomon Hospital, Tokyo, from March 2004 to May 2011, and adhered to the treatment for more than 6 months (Table 1). All patients were negative for hepatitis C serological markers, but all had detectable HBV surface antigen (HBsAg) for at least 6 months prior to the start of entecavir therapy. Two patients received 0.01 mg entecavir and one patient received 0.1 mg entecavir for 24 weeks, prior to 0.5 mg/day from a phase II study ETV-047 in Japan [11]. The other patients received 0.5 mg entecavir. None had received other nucleos(t)ide analogs. The diagnosis of chronic hepatitis and cirrhosis was established by needle biopsy, peritoneoscopy, or clinically before treatment. The clinical criteria for chronic hepatitis included elevated ALT levels over 6 months and absence of clinical evidence of portal hypertension, such as esophageal varices, ascites, hepatic encephalopathy, and features suggestive of cirrhosis on ultrasonography. Chronic hepatitis and cirrhosis were diagnosed in 374 and 102 patients, respectively. Twenty-eight patients were lost to follow-up, including 10 patients who moved to other locations, seven who never visited the hospital again, two who became pregnant, four who died, four who had virological breakthrough (VBT), and one who showed disappearance of HBsAg. Moreover, 18 patients developed HCC during treatment and their data until loss to follow-up or diagnosis of HCC were analyzed. Informed consent was obtained from each patient enrolled in the study and the study protocol conformed to the ethical guidelines of the Declaration of Helsinki and was approved by the Toranomon Hospital Ethical Committee.

Analysis of treatment efficacy

The clinical efficacy of entecavir was assessed as the proportion of patients who achieved HBV DNA suppression to undetectable levels (<2.6 $\log_{10}$ copies/ml), and those who achieved ALT normalization (<1× ULN). HBV DNA was measured using the polymerase chain reaction (PCR)-based Amplicor HBV Monitor assay (Roche Diagnostics, Indianapolis, IN, lower limit of detection of 2.6 $\log_{10}$ copies/ml) [12]. HBeAg seroclearance and seroconversion were also analyzed. Measurements were made on stored samples taken at baseline and every year after that since entecavir treatment initiation.

Statistical analysis

Differences between groups were examined for statistical significance using the χ^2 test where appropriate. Spearman correlation coefficient (two-tailed) was used to evaluate the correlation between albumin and other factors. Independent predictive factors associated with response to entecavir treatment were determined using multivariate multiple logistic regression. The following 12 potential predictors of response to entecavir treatment were assessed in this study: age, sex, severity of liver disease (CH or cirrhosis), HBV genotype, as well as levels of aspartate transaminase (AST), ALT, bilirubin, albumin, platelets, α fetoprotein (AFP), HBeAg, and HBV DNA. All factors found to be at least marginally associated with undetectable levels of HBV DNA after 1–4 years (p <0.10) were entered into the multivariate multiple logistic regression analysis. The above calculations were performed using The Statistical Package for Social Sciences version 11.0.1J (SPSS Inc., Chicago, IL).

The odds ratios (ORs) and 95% confidence intervals (CIs) were calculated to assess the relative risk confidence. Independent risk factors predicting achievement of HBeAg seroclearance and seroconversion were analyzed using stepwise Cox regression analysis. Potential factors that could predict achievement of HBeAg seroclearance assessed here were the above 11 variables, each transformed into categorical data consisting of two simple ordinal numbers for univariate and multivariate analyses. All factors found to be at least marginally associated with HBeAg seroclearance and seroconversion (p <0.10) were tested in the multivariate Cox proportional hazard model. A Kaplan–Meier estimate was performed using the SPSS software, and p values were calculated using the Cox-Mantel log-rank test. The Mann–Whitney U test was used for comparison of HBV DNA levels in patients with seroconversion to those with seroclearance. A two-tailed p value <0.05 was considered statistically significant.

Table 1. Characteristics of patients at the start of entecavir therapy. Table data are number of patients or median (range).

Demography	
n	474
Sex, male/female	321/153
Age, yr	47 (17-82)
Family history of HBV	291 (61%)
Cirrhosis	102 (22%)
Median duration of treatment, yr (range)	2.37 (0.5-7.2)
Laboratory data	
AST, IU/L	52 (14-1595)
ALT, IU/L	70 (8-2121)
Bilirubin, mg/dl	0.7 (0.2-3.9)
γ-GTP, IU/L	38 (9-679)
Albumin, g/dl	3.9 (1.9-5.1)
Alpha fetoprotein, ng/ml	5 (1-379)
Viral load, $\log_{10}$ copies/ml	6.7 (<2.6->9.0)
HBeAg-positive	222 (47%)
HBV genotypes, A/B/C/H/unknown	12/67/336/2/57

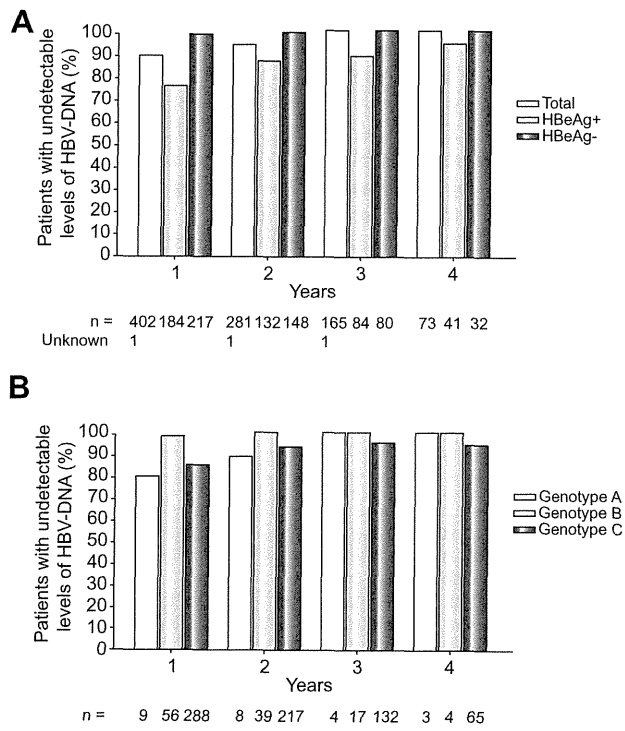

Fig. 1. Percentages of patients who had undetectable levels of HBV DNA between years 1 through 4. (A) HBeAg-positive and negative patients and (B) patients with genotype A, B, or C.

Table 2. Univariate and multivariate analyses of host and viral factors associated with undetectable levels of HBV DNA at year 1.

Parameter	Univariate analysis		Multivariate analysis	
	OR (95% CI)	*p* value	OR (95% CI)	*p* value
Sex (female)	1.06 (0.56-2.02)	0.842		
Age (>40 yr)	1.85 (1.0-3.4)	0.047		
Cirrhosis (present)	2.39 (0.98-5.81)	0.048		
Albumin (>4 g/dl)	1.28 (0.63-2.62)	0.494		
Bilirubin (>1.2 g/dl)	1.63 (0.56-4.76)	0.366		
ALT (>5 x IU/L)	4.57 (1.38-15.1)	0.007	11.9 (3.3-41.7)	<0.001
AST (>5 x IU/L)	2.25 (0.67-7.53)	0.178		
γ-GTP (≤20 IU/L)	1.75 (0.60-5.08)	0.300		
AFP (>10 ng/ml)	1.63 (0.61-4.37)	0.328		
Platelets (≤10/mm^3)	2.39 (0.56-10.3)	0.288		
Genotype (B)	9.57 (1.29-70.92)	0.007		
HBeAg (negative)	23.78 (7.25-77.95)	<0.001	8.5 (2.3-31.2)	0.001
HBV DNA (≤7.6 $\log_{10}$ copies/ml)	16.5 (8.0-34.2)	<0.001	10.0 (4.3-23.1)	<0.001

Table 3. Univariate and multivariate analyses of host and viral factors associated with undetectable levels of HBV DNA at year 2.

Parameter	Univariate analysis		Multivariate analysis	
	OR (95% CI)	*p* value	OR (95% CI)	*p* value
Sex (male)	0.524 (0.169-1.627)	0.257		
Age (>40 yr)	2.825 (1.1-7.25)	0.025		
Cirrhosis (present)	3.06 (0.69-13.5)	0.173		
Albumin (≤3.5 g/dl)	4.64 (0.603-35.73)	0.134		
Bilirubin (≤0.5 g/dl)	2.80 (0.79-9.93)	0.126		
ALT (>5 x IU/L)	5.35 (0.7-40.9)	0.054	16.7 (2.0-136.8)	0.009
AST (>5 x IU/L)	2.62 (0.34-20.3)	0.298		
γ-GTP (≤100 IU/L)	1.79 (0.557-5.73)	0.304		
AFP (>15 ng/ml)	2.12 (0.27-16.95)	0.699		
Platelets (≤12/mm^3)	4.74 (0.619-36.31)	0.136		
Genotype (B)	1.082 (1.042-1.123)	0.076		
HBeAg (negative)	23.21 (3.05-176.46)	<0.001		
HBV DNA (≤7.6 $\log_{10}$ copies/ml)	39.91 (8.912-178.76)	<0.001	121.7 (15.3-965.9)	<0.001

Results

Study population

Of the 474 subjects in this study, 68% were males, and the mean age was 47 years. The mean HBV DNA level was 6.7 $\log_{10}$ copies/ml, mean ALT level was 70 IU/L, and 47% of patients were HBeAg-positive. At baseline, there were 12, 67, and 336 patients of genotype A, B, and C, respectively, and among the patients belonging to these genotypes, 4, 11, and 188, respectively, were HBeAg-positive.

Virological response

Undetectable levels of HBV DNA were identified at years 1 through 4 in 88% (353/402), 93% (262/281), 95% (156/165), and 96% (70/73) of patients, respectively (Fig. 1A). Among the HBeAg-positive patients at baseline, 75% (138/184), 86% (114/132), 89% (75/84), and 93% (38/41), and among the HBeAg-negative patients at baseline, 99% (214/217), 99% (147/148), 100% (80/80), and 100% (32/32) had undetectable levels of HBV DNA at years 1 through 4, respectively.

Among the patients with genotype A, 78% (7/9), 88% (7/8), 100% (4/4), and 100% (3/3) of patients had undetectable levels of HBV DNA at years 1 through 4, respectively (Fig. 1B). Among the HBeAg-positive patients with genotype A at baseline, 50% (2/4), 67% (2/3), 100% (2/2), and 100% (2/2) had undetectable levels of HBV DNA at years 1 through 4, respectively. Among patients with genotype B, 98% (55/56), 100% (39/39), 100% (17/17), and 100% (4/4) had undetectable levels at years 1 through 4, respectively (Fig. 1B). Among the HBeAg-positive patients with genotype B at baseline, 88% (7/8), 100% (5/5), and 100% (3/3) had

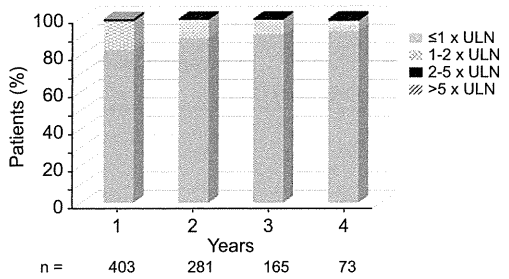

Fig. 2. Percentages of patients with ALT level <1× upper limit of normal level (ULN), <1–2× ULN, 2–5× ULN, and 5× ULN.

undetectable levels of HBV DNA at years 1 through 3, respectively. None of the patients with genotype B at baseline were HBeAg-positive at year 4. Among the patients with genotype C, 85% (246/288), 93% (201/217), 95% (125/132), and 95% (62/65) had undetectable levels of HBV DNA at years 1 through 4, respectively (Fig. 1B), and of these, 74% (116/156), 87% (102/117), 91% (67/74), and 92% (35/38), respectively, were HBeAg-positive.

Factors associated with detectable levels of HBV DNA at years 1, 2, and 3

Of the 402 patients, 353 had no detectable HBV DNA after 1 year. At the start of treatment, factors associated with undetectable levels of HBV DNA in the first year were age (>40 years, $p = 0.047$), cirrhosis (present, $p = 0.048$), ALT (>5× ULN, $p = 0.007$), genotype (B, $p = 0.007$), HBeAg (negative, $p < 0.001$), and HBV DNA level (<7.6 $\log_{10}$copies/ml, $p < 0.001$), by univariate analysis (Table 2). Multivariate analysis identified three parameters, namely ALT (>5× ULN, OR = 11.9; 95% CI = 3.3–41.7, $p < 0.001$), HBeAg (negative, OR = 8.5; 95% CI = 2.3–31.2, $p = 0.001$), and HBV DNA level (<7.6 $\log_{10}$ copies/ml, OR = 10.0; 95% CI = 4.3–23.1, $p < 0.001$).

Of 281 patients, HBV DNA was undetectable in 262 patients in the second year, with univariate analysis identifying the following associated factors: age (>40 years, $p = 0.025$), ALT (>5× ULN, $p = 0.054$), HBeAg (negative, $p < 0.001$), and HBV DNA level (⩽7.6 log copies/ml, $p < 0.001$). Of these, multivariate analysis identified ALT (>5× ULN, OR = 16.7; 95% CI = 2.0–136.8, $p = 0.009$) and HBV DNA level (⩽7.6 $\log_{10}$ copies/ml, OR = 121.7; 95% CI = 15.3–965.9, $p < 0.001$) as significant factors (Table 3).

Of 165 patients, HBV DNA was undetectable in 156 patients in the third year, with univariate analysis identifying the following associated factors at the start of treatment: Gender (male, $p = 0.04$), HBeAg (negative, $p = 0.002$) and HBV DNA level (⩽7.6 log copies/ml, $p < 0.001$). Multivariate analysis identified only HBV DNA level as significant (⩽7.6 $\log_{10}$ copies/ml, OR = 15.8; 95% CI = 43.1–79.9, $p = 0.001$).

Biochemical response

The percentages of patients with normal ALT levels (<1x ULN) at years 1, 2, 3, 4 were 83% (336/403), 89% (251/281), 92% (151/165), and 93% (68/73), respectively (Fig. 2). In HBeAg-positive patients at baseline, those who achieved normal ALT levels at years 1, 2, 3, 4 were 81% (148/183), 88% (116/132), 90% (76/84), and 95% (39/41), respectively. The respective data for HBeAg-negative patients at baseline were 85% (187/219), 91% (134/148), 93% (74/80), and 91% (29/32).

HBeAg seroclearance and seroconversion

HBeAg positivity at baseline was detected in 222 patients (47%) (Table 1), and Fig. 3A shows the cumulative clearance of HBeAg calculated with the Kaplan–Meier method. The percentages of patients with seroclearance were 16%, 24%, 37%, and 42% at years 1 through 4, respectively. Univariate analysis identified the following HBeAg seroclearance-associated factors at the start of treatment: age (>40 years, $p = 0.052$), platelet count (<12 × 10^4/mm^3, $p = 0.028$), and HBV DNA (<7.0 log copies/ml, $p = 0.006$). Multivariate analysis identified HBV DNA (<7 $\log_{10}$ copies/ml, RR = 1.9; 95% CI = 1.2–3.1, $p = 0.007$) as the only significant determinant of seroclearance. Of 70 patients who achieved anti-HBe seroclearance, 52 patients achieved anti-HBe seroconversion. Fig. 3B shows the cumulative seroconversion rate of HBeAg calculated by the Kaplan–Meier test. The proportions of patients who showed seroconversion were 12%, 18%, 29%, and 38% at years 1 through 4, respectively. Univariate analysis demonstrated that age (>40 years, $p = 0.020$), albumin (<3.5 g/dl, $p = 0.021$) and platelet count (<20 × 10^4/mm^3, $p = 0.067$) correlated with HBeAg seroconversion at the start of treatment. Multivariate analysis that included the above factors identified serum albumin as the only significant determinant of seroconversion (<3.5 g/dl, RR = 2.0; 95% CI = 1.1–3.6, $p = 0.019$). One patient achieved anti-HBe seroconversion at 25 months but became positive again at 28 months. Other patients who achieved anti-HBe seroconversion did not show HBeAg reversion. One patient achieved anti-HBe seroconversion but remained HBV DNA positive (Table 4, Patient 5). Another patient remained positive for HBV

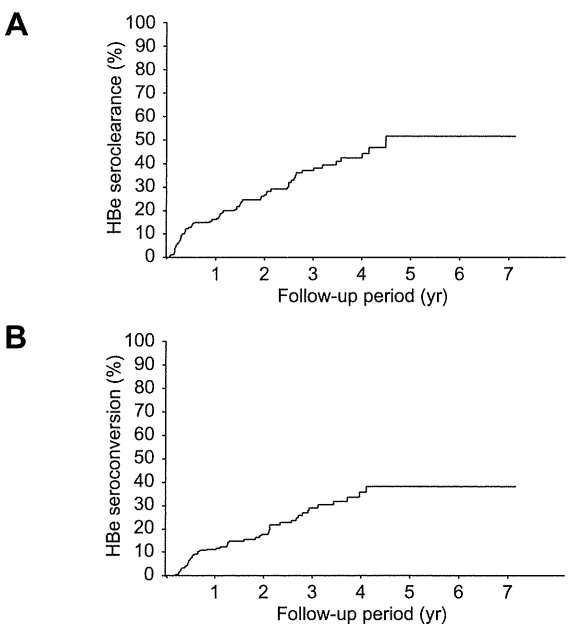

Fig. 3. Change of the HBeAg status during follow-up. Cumulative rates of (A) HBe seroclearance and (B) HBe seroconversion in HBeAg-positive patients, analyzed with the Kaplan–Meier test.