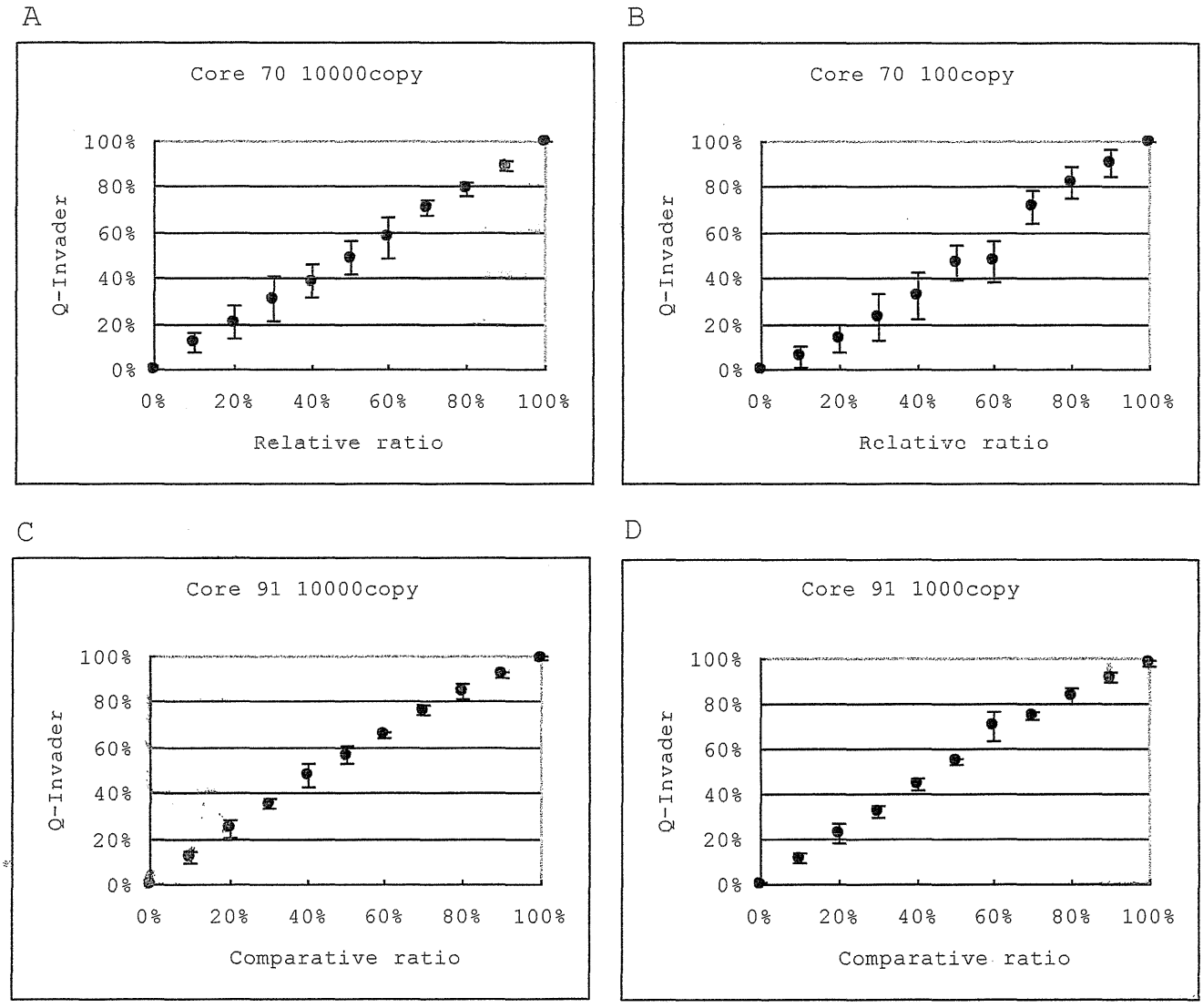
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Figure 5.

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ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

Clearance of hepatitis B surface antigen during long-term nucleot(s)ide analog treatment in chronic hepatitis B: results from a nine-year longitudinal study

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Abstract
Background Clearance of hepatitis B surface antigen (HBsAg) is considered the ultimate goal in chronic hepatitis B treatment. One treatment option is long-term nucleot(s)ide analog (NA) therapy. We followed a group of long-term NA therapy patients to evaluate the efficacy of this treatment in promoting clearance and longitudinal declines of HBsAg.
Method The study included 791 NA therapy patients who received lamivudine as their first drug. At the baseline, 442 patients were hepatitis B e antigen (HBeAg)+ and 349 were HBeAg−. All analyses were performed after separating the HBeAg+ and HBeAg− cohorts. Cox proportional hazards models were used to determine which factors were associated with HBsAg clearance.
Results HBsAg clearance was observed in 18 (4.1 %) of the HBeAg+ patients and 20 (5.7 %) of the HBeAg− patients at baseline, giving seroclearance rates of 6.4 and 6.9 %, respectively, over the nine-year study period. HBsAg clearance was influenced by several independent factors that varied according to HBeAg cohort. For HBeAg+ patients, these included previous interferon therapy, infection with hepatitis B virus (HBV) genotype A, a ≥0.5 log IU/mL decline in HBsAg level within six months, and clearance of HBeAg at six months. For HBeAg− patients, these included infection with HBV genotype A, decline in HBsAg at six months, and a baseline HBsAg level of <730 IU/mL.
Conclusion This study suggests that both direct antiviral potential and host immune response are needed to achieve HBsAg clearance by NA therapy. Viral genotype strongly influenced HBsAg clearance during NA therapy.

Keywords Hepatitis B surface antigen · Nucleot(s)ide analog · Lamivudine · Interferon

Introduction

Worldwide, an estimated 400 million people are infected with hepatitis B virus (HBV) persistently, and one million people die of decompensated cirrhosis and/or hepatocellular carcinoma (HCC) annually [1, 2]. Recently, oral nucleot(s)ide analogs (NAs) have been used as a mainstay therapeutic strategy against chronic hepatitis B. Five such antiviral agents—lamivudine (LAM), entecavir (ETV), telbivudine, adefovir dipivoxil (ADV), and tenofovir disoproxil fumarate—which inhibit viral replication [e.g., hepatitis B virus DNA (HBV DNA) priming, reverse transcription of negative-stranded HBV DNA, and synthesis of positive-stranded HBV DNA] have been approved; these NAs vary in both the strength and the rapidity with which they suppress HBV DNA [3–10]. Sustained viral suppression by NA therapy can improve liver fibrosis and clinical outcomes of patients [11, 12]. LAM was the first NA to be approved to treat chronic hepatitis B in Japan, followed by ADV and ETV.

Responses to antiviral treatments can be evaluated by monitoring serum HBV DNA levels, hepatitis B e antigen (HBeAg) and antibody levels, and hepatitis B surface

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Antiviral therapy and drug resistance

All 791 patients received 100 mg LAM daily as an initial therapy, but a LAM-resistant rtM204I/V mutation developed in 439 (55 %) of these patients. Over time, 334 (42 %) individuals experienced an increase in HBV DNA (≥1 log copies/mL) [e.g., virological breakthrough (VBT)] and, as a result, 299 (98.5 %) individuals were also provided with ADV treatment (10 mg) added onto LAM as a rescue therapy. The remaining patients continued to receive LAM monotherapy and were lost to follow-up before the administration of ADV because of the lack of approval for ADV administration in Japan at the time. The resistant mutation for rtM204I/V was detected in 312 of 334 patients who experienced VBT using a commercial kit (as described below). Patients who had achieved an optimal or suboptimal virological response or who wished to participate in the clinical trial of ETV for LAM-refractory patients (ClinicalTrials.gov: NCT 1037166)—152 and 17 patients, respectively—switched from LAM to ETV (0.5 mg/day). Additionally, patients in whom subsequent ADV- or ETV-resistant mutants emerged received an optimal rescue therapy with other NAs (ETV + ADV combination for ADV resistance, and LAM + ADV combination for ETV resistance).

NA treatment was continued as a rule; median NA treatment duration was 75 months (25th–75th percentile, 55–102) in the HBeAg+ cohort and 92 months (67–119) in the HBeAg− cohort. Ultimately, 55 (7 %) of the 791 patients discontinued treatment; 16 of these individuals terminated treatment after achieving HBsAg seroclearance. Follow-ups were conducted for all patients, regardless of length of treatment, for as long as possible.

Clinical data collection and follow-ups

Data on patient characteristics, biochemistry, hematology, virology, histology, and previous treatments were collected and registered in our institute's database at the time of patient enrollment. Prior to beginning LAM, all patients were surveyed about the presence of a family history of HBV infection. Data on treatment dose and duration of previous IFN therapy were collected from our hospital's IFN therapy database or requested from other hospitals as necessary. Complete details on the previous treatment were lacking for 29 (9.7 %) of 297 patients who received IFN therapy before starting LAM.

At least every 1–3 months, liver function and virological markers of HBV infection were measured in all patients. All serum HBsAg titers were measured from frozen serum samples collected at six months, one year, three years, five years, and once annually for 6–10 years, and then stored at −80 °C. The day of HBsAg clearance was defined by the measurement in consecutive available serum samples before it was undetected in subsequent samples. A genotypic analysis of drug resistance was performed in cases of insufficient virological response or VBT, defined as an increase in serum HBV DNA levels ≥1 log above the nadir measured after the initial virological response. Cirrhosis was diagnosed by laparoscopy, liver biopsy, or clinical data such as imaging modalities and portal hypertension. The primary outcome for this study was HBsAg clearance. The endpoint of the follow-up was HBsAg clearance or last visit before January 2011.

Markers of HBV infection

Serum HBsAg titers were measured using ARCHITECT HBsAg QT assay kits (Abbott Laboratories, Tokyo, Japan), which have a lower limit of detection of 0.05 IU/mL and an upper limit of detection of 250 IU/mL. To expand the upper range from 250 to 125,000 IU/mL, serum samples, going off the scale, were diluted stepwise to 1:20 and 1:500 with ARCHITECT diluents as the product document described. HBeAg was determined by enzyme-linked immunosorbent assay with a commercial kit (HBeAg EIA; Institute of Immunology, Tokyo, Japan). HBV DNA was quantified using the Amplicor monitor assay (Roche Diagnostics, Tokyo, Japan), which has a dynamic range of 2.6–7.6 log copies/mL, or COBAS TaqMan HBV v.2.0 (Roche Diagnostics, Tokyo, Japan), which has a dynamic range of 2.1–9.0 log copies/mL. A commercial kit (HBV Genotype EIA; Institute of Immunology) was used to serologically determine HBV genotypes using the combination of epitopes expressed on the pre-S2 region product, which is specific to each of the seven major genotypes (A–G). YMDD mutants (rt M204I/V) were determined by polymerase chain reaction-based enzyme-linked mini-sequence assay with a commercial kit (Genome Science Laboratories, Tokyo, Japan).

Statistical analyses

Categorical data were compared between groups using chi-square or Fisher's exact tests. Continuous variables with a nonparametric distribution were analyzed with Mann–Whitney *U* tests, while those with a parametric distribution were analyzed with Student's *t* tests. When appropriate, Kruskal–Wallis tests were used to conduct pairwise comparisons of specific variables. Cox regression analyses were used to assess which variables were significantly associated with HBsAg clearance. Cut-off values were provided using the area under the receiver operating characteristic curve (ROC) only after rejecting the null hypothesis for the ROC curve. All baseline factors that were found to be significantly associated with HBsAg clearance by univariate analysis

antigen (HBsAg) and antibody levels. Serum HBsAg levels appear to reflect the amount of intrahepatic covalently closed circular DNA (cccDNA), which acts as a template for the transcription of viral genes [13–15]. Previous studies have shown that both interferon (IFN) and NA therapy result in a reduction of intrahepatic cccDNA [16, 17], suggesting that these treatments may be helpful in achieving the ultimate therapeutic goal of antiviral therapy for chronic hepatitis B (i.e., total clearance of HBsAg).

Very low rates of HBsAg clearance have been reported in the past [18–22]. Recent work has shown that over a one-year period, pegylated (PEG)-IFN therapy is more successful than ETV at reducing serum HBsAg [23]; furthermore, PEG-IFN therapy has also been reported to promote the complete clearance of HBsAg [24–27]. Several studies have detailed similar successes achieved by NA therapy but over relatively short (<5 years) treatment durations [18–20, 22, 28, 29]. The kinetics of HBsAg during long-term (>5 years) treatment remain unknown. NA therapy leads to time-dependent decreases in intrahepatic cccDNA and serum HBsAg levels if sustained viral suppression is longer term, and may therefore increase the rates of HBsAg clearance.

In order to evaluate this possibility empirically, we conducted a ten-year-long study in which we followed patients who received NA therapy initiated by the administration of LAM. We evaluated the resulting clearance and longitudinal declines of HBsAg using highly sensitive assays. Our aim was to determine whether long-term NA therapy can lead to HBsAg clearance, as suggested; if so, we also wished to elucidate the factors associated with its success.

Methods

Study population

Over a period of 12 years (September 1995 to September 2007), 949 consecutive patients who were chronically monoinfected with HBV (confirmed HBsAg positivity for at least six months), were treated with LAM monotherapy at the Department of Hepatology, Toranomon Hospital, Metropolitan Tokyo. The indication for antiviral therapy was abnormal ALT levels accompanying the increase in HBV DNA (over 4 log copies/mL) as a rule. However, in cases where ALT levels were normal, patients with advanced fibrosis were administered LAM. We did not treat patients without fibrosis who had low HBV DNA and normal ALT levels as a rule. We selected 791 patients for the final study after we had excluded all those who had been treated with LAM for <6 months, were co-infected with hepatitis C virus, had not provided sufficient serum samples, and/or had insufficient clinical records (Fig. 1). No patient was co-infected with human immunodeficiency virus in this cohort. Seven hundred ninety-one patients were enrolled in this cohort study. Of these 791 patients, 442 were HBeAg+ and 349 were HBeAg− at baseline. All analyses were performed after separating the HBeAg+ and HBeAg− cohorts. 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. This study has been registered in the University Hospital Medical Information Network Clinical Trials Registry (UMIN CTR) as the number UMIN000007993.

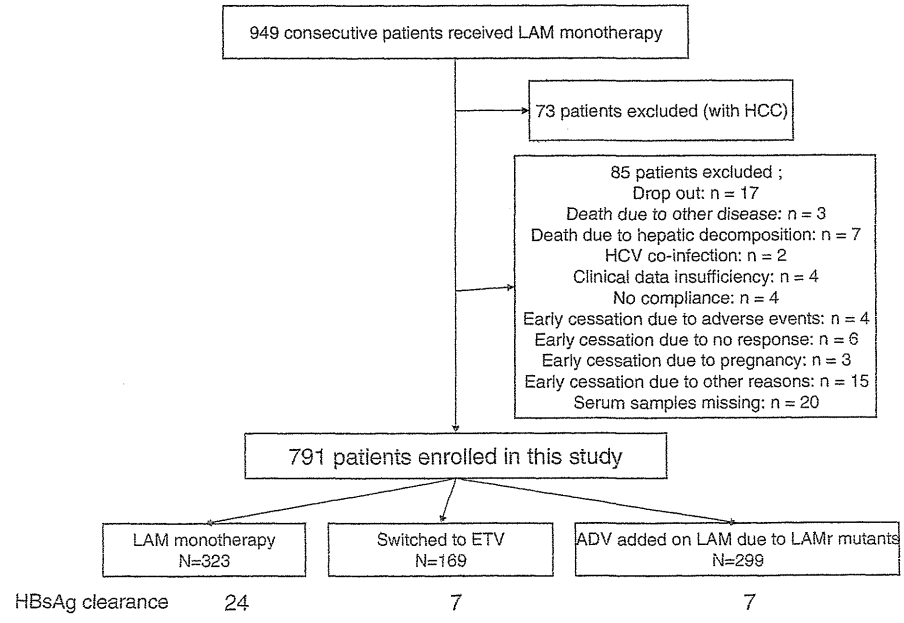

Fig. 1 Schematic of study protocol. *LAM* lamivudine, *HCC* hepatocellular carcinoma, *HCV* hepatitis C virus, *ETV* entecavir, *ADV* adefovir dipivoxil, *HBsAg* hepatitis B surface antigen

were entered into a multivariate analysis. Independent baseline factors associated with clearance of HBsAg were calculated using a stepwise Cox regression analysis. We then performed a time-dependent Cox regression to analyze independent factors associated with HBsAg while adjusting for on-treatment factors and independent baseline factors. Three covariates of the on-treatment response factors—emergence of rtM204I/V mutants, VBT, and biochemical breakthrough—were set as the time-dependent covariates. Cumulative HBsAg clearance rates were analyzed using the Kaplan–Meier method; differences in the resulting curves were tested using log-rank tests. We performed Cox regression analysis, Kaplan–Meier curve analysis, and HBsAg kinetics analysis for no more than nine years, as the number of patients with a long-term follow-up of over ten years was too small to permit analysis [30]. Bonferroni adjustments were used to correct for the number of different ways a single predictor variable can be split. Significance was defined as $P < 0.05$ for all two-tailed tests. Data analysis was performed with IBM SPSS version 19.0 software (IBM Corp., Armonk, NY, USA).

Results

Patient characteristics

Thirty-eight (4.8 %) of 791 patients successfully cleared HBsAg. Of these, 24 had received LAM, 7 had switched to ETV treatment, and 7 had been treated with both LAM and ADV (Fig. 1). Of the 38 patients who achieved HBsAg clearance, 18 were HBeAg+, whereas 20 were HBeAg− at baseline. Table 1 provides a comparison of the baseline and on-treatment characteristics between patients who were and were not able to successfully clear HBsAg (all patients, HBeAg+ and − cohorts, respectively). In the HBeAg+ cohort, baseline characteristics that were significantly associated with HBsAg clearance included previous IFN therapy, HBV genotype, HBV DNA, and AST and ALT levels; in the HBeAg− cohort, significant characteristics included HBV genotype and HBsAg levels. Significant on-treatment characteristics in the HBeAg+ cohort included decline in HBsAg, clearance of HBeAg, and decline in HBV DNA to <2.6 log copies/mL at six months;

Table 1 Baseline, demographic, and on-treatment characteristics of patients with and without HBsAg seroclearance

Characteristics	All patients (n = 791)	HBeAg+ at baseline (n = 442)			HBeAg− at baseline (n = 349)		
		Persistently HBsAg+ (n = 424)	HBsAg seroclearance (n = 18)	P	Persistently HBsAg+ (n = 329)	HBsAg seroclearance (n = 20)	P
Baseline							
Age[a] (years) (SD)	43 (11.1)	41 (11.2)	44 (10.5)	0.177	47 (10.3)	46 (10.3)	0.899
Gender (male:female)	627:164	329:95	16:2	0.385	265:64	16:4	1.000
Race				0.446			
Japanese	768 (97)	411 (97)	17 (94)		320 (97)	20 (100)	1.000
Non-Japanese (%) (Asian:Caucasian)	23 (3) (21:2)	13 (3) (20:2)	1 (3) (1:0)		9 (3) (20:2)	0 (3) (1:0)	
Family history of HBV infection	539 (68)	311 (73)	10 (56)	0.107	208 (63)	10 (50)	0.238
Previous IFN therapy	297 (38)	167 (39)	15 (83)	**<0.001**	106 (32)	9 (45)	0.326
IFN duration (weeks)	27 (20–58)	26 (18–53)	52 (21–79)	0.214	32 (22–89)	23 (14–72)	0.457
Duration from the end of IFN to start of lamivudine (weeks)	50 (3–189)	26 (7–124)	37 (2–89)	0.505	119 (3–316)	102 (18–289)	0.746
Previous NA therapy	34 (4)	21 (5)	2 (11)	0.239	10 (3)	1 (5)	0.483
Presence of cirrhosis	169 (21)	76 (18)	2 (11)	0.752	87 (26)	4 (20)	0.610
HBV genotype				**<0.001**			**<0.001**
A	28 (3.5)	14 (3.3)	6 (33)		6 (1.8)	2 (10)	
B	67 (8.5)	16 (3.8)	0 (0)		48 (14.6)	3 (15)	
C	664 (83.9)	374 (88.2)	12 (67)		265 (80.5)	13 (65)	
D	3 (0.4)	2 (0.4)	0 (0)		0 (0)	1 (5)	
F	2 (0.3)	2 (0.4)	0 (0)		0 (0)	0 (0)	
Unclassified/missing	27 (3.4)	16 (3.8)	0 (0)		10 (3.0)	1 (5)	

Table 1 continued

Characteristics	All patients (n = 791)	HBeAg+ at baseline (n = 442)			HBeAg− at baseline (n = 349)		
		Persistently HBsAg+ (n = 424)	HBsAg seroclearance (n = 18)	P	Persistently HBsAg+ (n = 329)	HBsAg seroclearance (n = 20)	P
Baseline HBV DNA (log copies/mL)	7.0 (5.8–8.0)	7.6 (6.7–8.2)	8.0 (7.5–8.4)	**0.027**	6.3 (5.2–7.2)	6.1 (5.0–7.0)	0.652
Baseline HBsAg level (IU/mL)	2530 (907–6590)	3910 (1690–12300)	5280 (943–67600)	0.331	1590 (599–3050)	529 (58–1610)	**0.004**
Baseline AST level (IU/L)	74 (48–135)	81 (52–165)	201 (78–666)	**0.011**	66 (42–113)	57 (39–96)	0.694
Baseline AST level (×ULN)	2.2 (1.5–4.1)	2.5 (1.6–5.0)	6.1 (2.3–20.2)	**0.011**	2.0 (1.3–3.4)	1.7 (1.2–2.9)	0.736
Baseline ALT level (IU/L)	115 (63–252)	130 (72–290)	326 (104–775)	**0.021**	101 (56–194)	101 (55–215)	0.904
Baseline ALT level (×ULN)	3.0 (1.7–6.4)	3.5 (1.9–7.8)	7.8 (2.5–20.3)	**0.040**	2.6 (1.4–5.2)	2.6 (1.4–5.2)	0.955
Baseline total bilirubin level (mg/dL)	0.8 (0.6–1.1)	0.8 (0.5–1.1)	0.9 (0.6–1.9)	0.117	0.7 (0.6–1.0)	0.8 (0.6–0.9)	0.536
Platelet count[a] (10^5/mm^3) (SD)	16.1 (5.7)	16.5 (6.1)	14.7 (3.5)	0.221	15.6 (5.1)	17.7 (6.9)	0.216
On-treatment response							
Decline of HBsAg level (≥0.5 log IU/mL within six months)	97 (1)	67 (16)	13 (72)	**<0.001**	11 (3)	6 (30)	**<0.001**
HBeAg positive → clearance within six months	109 (14)	94 (22)	10 (56)	**0.005**	NA	NA	
Undetectable HBV DNA (<400 copies/mL) at six months	532 (67)	221 (52)	15 (83)	**0.014**	277 (84)	19 (95)	0.330
Emergence of rtM204I/V mutants	439 (55)	251 (59)	9 (50)	0.469	170 (52)	9 (45)	0.646
Viral breakthrough due to mutants	334 (42)	216 (51)	5 (28)	0.055	108 (33)	5 (25)	0.473
Biochemical breakthrough due to mutants	318 (40)	200 (47)	5 (28)	0.146	108 (33)	5 (25)	0.473

Except where marked with a superscript letter a, values are expressed as the median and 25th–75th percentiles (parenthetically), or number and percentage (parenthetically). ULN; AST = 33 IU/L, ALT = 42 IU/L (male), and 27 IU/L (female). *Asterisks* indicate data displayed as mean values and standard deviations. *Bold text* indicates statistically significant P values

the only significant characteristic in the HBeAg− cohort was a decline in HBsAg within six months. ROC curve analysis confirmed a cut-off value of 0.5 log IU/mL for a decline in HBsAg level within six months in the HBeAg+ and − cohorts [area under the curve = 0.810 (95 % CI 0.673–0.947) (HBeAg+ cohort) and 0.760 (95 % CI 0.611–0.909) (HBeAg− cohort)].

LAM-resistant rtM204I/V mutants were detected in 439 (55.5 %) of 791 patients. Of these, 334 (42.2 % of all patients) also developed VBT accompanied by an increase in HBV DNA (≥1 log copies/mL). The rate of VBT was marginally significantly lower in the HBsAg clearance group in the HBeAg+ cohort (Table 1).

Factors associated with HBsAg clearance

The overall cumulative rates of HBsAg clearance were 0.2 % at one year, 1.2 % at three years, 2.6 % at five years, 4.2 % at seven years, and 6.4 % at nine years in the HBeAg+ cohort; and 0.6 % at one year, 0.9 % at three years, 2.2 % at five years, 5.2 % at seven years, and 6.9 % at nine years in the HBeAg− cohort. Univariate Cox

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

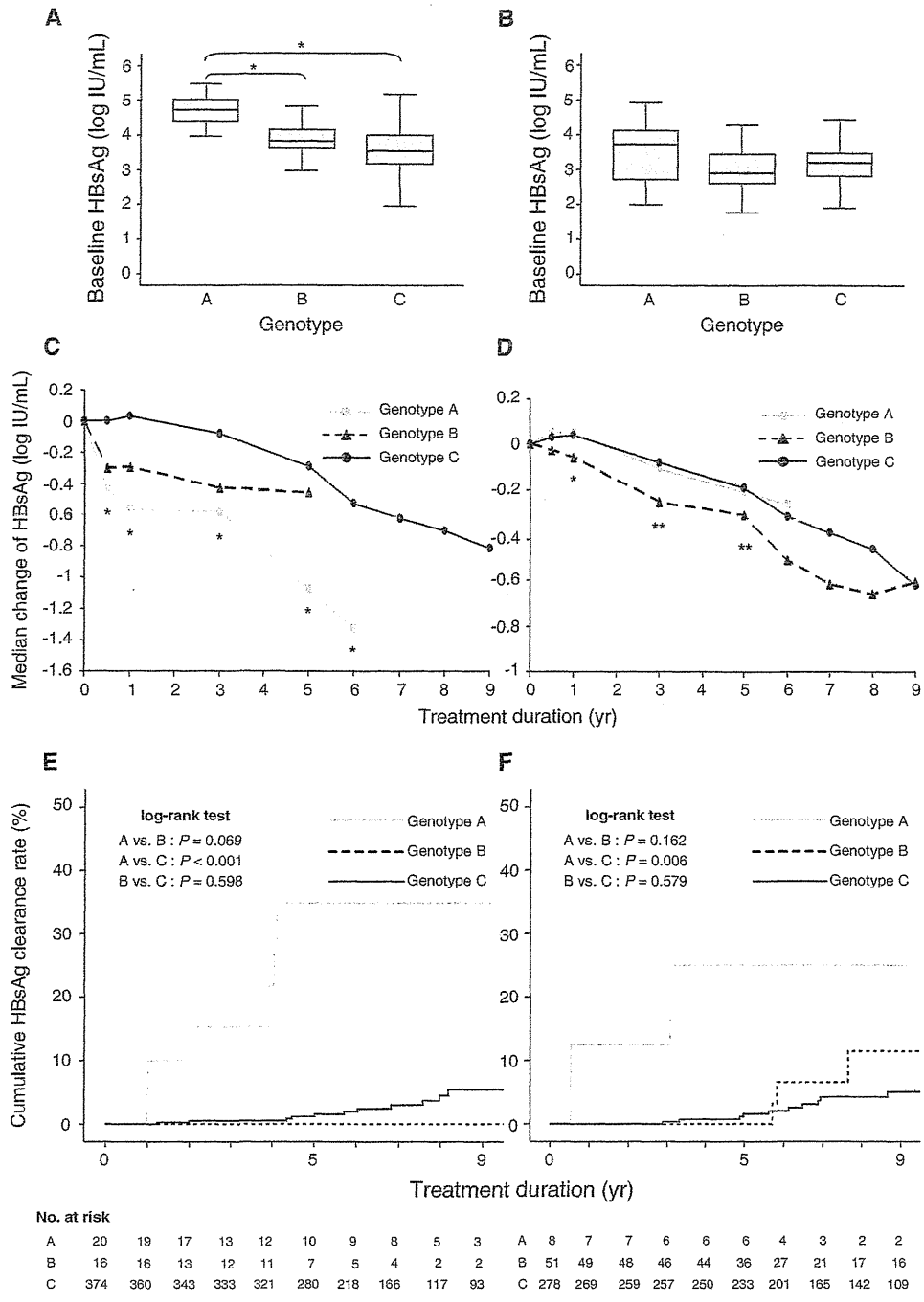

Fig. 2 **a** Box plot of baseline HBsAg levels in patients with different HBV genotypes (HBeAg+ cohort). The *asterisk* (*) indicates a statistical significance of $P < 0.001$, as determined by the Mann–Whitney U test and Bonferroni correction. **b** Box plot of baseline HBsAg levels in patients with 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).

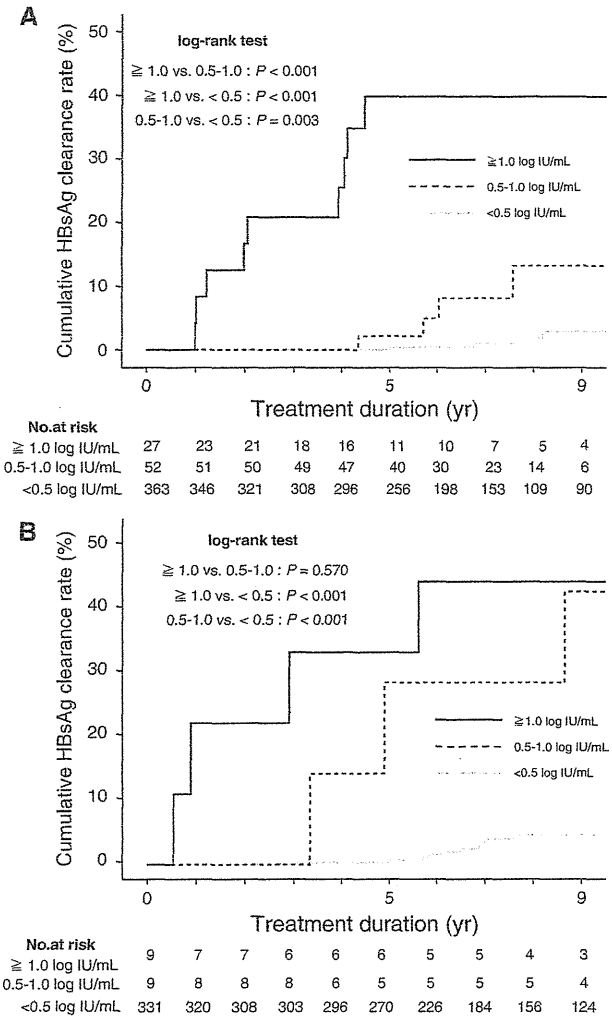

Fig. 3 **a** Kaplan–Meier life table showing cumulative HBsAg clearance rates in patients with varying rates of HBsAg decline within the first six months (HBeAg+ cohort). Clearance rates were highest in the rapid decline group, followed by the intermediate decline group and the slow or steady group (log-rank 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

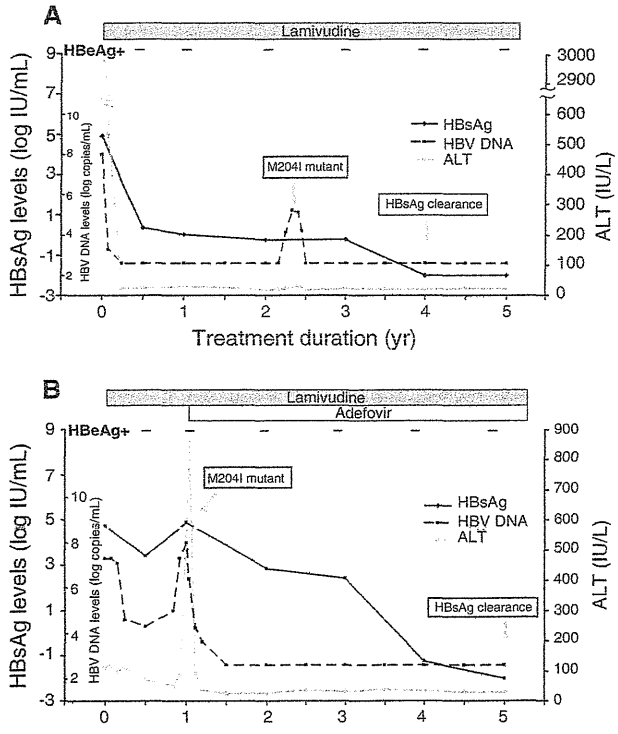

Fig. 4 Case presentation of the typical clinical and virological courses of two representative patients who achieved HBsAg clearance after VBT occurred. **a** Patient 1, a 45-year-old man who was HBeAg+ at baseline and had genotype A. **b** Patient 2, a 38-year-old man who was HBeAg+ at baseline and 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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Safety, pharmacokinetics and resistant variants of telaprevir alone for 12 weeks in hepatitis C virus genotype 1b infection

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SUMMARY. *Background:* Telaprevir in combination with peginterferon and ribavirin is a promising advancement in chronic hepatitis C treatment. However, the safety, tolerability, pharmacokinetics and antiviral profiles of telaprevir alone beyond 2 weeks have not been studied. *Methods:* In a phase 1b study in Japan, 10 treatment-naïve patients infected with hepatitis C virus genotype 1b with high viral load (>5 $\log_{10}$ IU/mL) received telaprevir 750 mg every 8 h (q8h) for 12 weeks. We examined the safety, tolerability, pharmacokinetics, hepatitis C virus (HCV) RNA levels and resistant variants of telaprevir. *Results:* Neither serious adverse events nor discontinuations of study drug owing to an adverse event occurred. The most common adverse drug reactions were rash (80%) and anaemia (70%). Telaprevir concentration reached its steady state within 2 days after the first administration without abnormal accumulation. Telaprevir alone provided potent antiviral activity: a median $\log_{10}$ decrease of 2.325 at 16 h and 5.175 on Day 14. During the treatment, HCV RNA levels at the nadir were below the limit of the quantification in seven patients and undetectable in three of 10 patients. Viral breakthrough associated with mainly Ala^{156}-substituted variants occurred in eight patients, and only one patient showed end-of-treatment response. The selected variants reverted to the wild-type during the 24-week follow-up period. *Conclusion:* Telaprevir alone was well tolerated at 750 mg q8h for up to 12 weeks. The safety profile and emergence of resistant variants of genotype 1b under telaprevir monotherapy for 12 weeks will become increasingly important in evaluating an oral combination of telaprevir with other direct-acting antiviral agents.

Keywords: genotype 1b, pharmacokinetics, resistant variants, telaprevir monotherapy, tolerability.

INTRODUCTION

Hepatitis C virus (HCV) infection often causes chronic hepatitis (CHC) that may result in life-threatening complications including cirrhosis and hepatocellular carcinoma (HCC) [1,2]. Thus, the development of medical agents or therapies that are highly effective against HCV has been eagerly sought for a long time. The current standard of care (SOC) for patients with hepatitis C, the concomitant administration of peginterferon (PEG-IFN) with ribavirin (RBV) for 48 weeks, is one such therapy, but it results in sustained virological response (SVR) in only about 45% of patients with genotype 1 HCV infection [3–5]. In addition to this low rate of SVR, another large problem of the SOC is that its practical use has been often interrupted or discontinued with several side effects including flu-like symptoms, depression, neutropenia and anaemia, and some patients are also excluded from SOC. Patients not eligible for SOC include many with comorbid conditions that often accompany HCV, including decompensated liver disease and renal failure. Thus, there is an unmet need for CHC therapies that are more effective and are better tolerated than what is presently available. Telaprevir, which is a novel peptidemimetic slow and tight-binding inhibitor of the HCV NS3-4A protease discovered using a structure-based drug design approach [6], has been intensively developed in the world as a member of a new class of direct-acting antivirals (DAAs) to improve SVR rates for genotype 1. In the first, phase 1 trial (VX04-950-101) in CHC patients, telaprevir was well tolerated and reduced HCV RNA in plasma by 2 $\log_{10}$ or greater after its consecutive administration for 14 days [7]. In a subsequent

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; CHC, chronic hepatitis C; DAA, direct acting antiviral; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; LLOQ, lower limit of quantification; LOD, limit of detection; PEG-IFN, peginterferon; q8h, every 8 h; RBV, ribavirin; SOC, standard of care; SVR, sustained virological response.

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phase 1 clinical trial (VX05-950-103), all eight patients given telaprevir alone had an initial, rapid and profound antiviral response, but the four patients with genotype 1a infection experienced a viral breakthrough, whereas the other four patients with genotype 1b infection had a continuous decline in viral load [8]. Because genotype 1b infection accounts for 70% of patients and genotype 1a is rarely met with in Japan [9], viral kinetics and emergence of resistant variants from telaprevir use alone beyond 2 weeks remain to be evaluated among patients with genotype 1b infection. Besides virological reasons, a safer therapy without concomitant administration of PEG-IFN or RBV is desirable if possible, because the majority of HCV carriers are of age >55 years whose tolerability is of concern in Japan [10]. Therefore, the purpose of this trial is to examine the safety, tolerability, antiviral effects and pharmacokinetics of monotherapy with telaprevir in 10 Japanese patients with genotype 1b infection for up to 12 weeks.

PATIENTS AND METHODS

Study design and organization

This single-arm, open-label study was conducted from December 2007 to October 2008 at the Department of Hepatology in the Toranomon Hospital in Metropolitan Tokyo in full compliance with the guideline of Good Clinical Practice and the Declaration of Helsinki (ClinicalTrials.gov Identifier: NCT00591214). Before the study started, the protocol and informed consent forms were reviewed and approved by the institutional review board. Informed consent was obtained from all patients in writing after sufficient explanation was given and before they participated in the study. For 12 consecutive weeks, all 10 patients received 750 mg telaprevir q8h under feeding conditions. Telaprevir was supplied as a 250-mg tablet.

Patients

Patients enrolled in this study were treatment-naïve, HCV-infected male or female participants with characteristics shown in Table 1, who met the following inclusion criteria: diagnosed with chronic hepatitis C; infected with HCV genotype 1b proved by phylogenetic analysis in the NS5B region; not received any prior antiviral therapy for hepatitis C; had HCV RNA level of 5 $\log_{10}$ IU/mL or more determined by the Roche COBAS TaqMan HCV test (Roche Diagnostics, Tokyo, Japan); belonged to Japanese race (Mongoloid) aged from 20 to 65 years at entry; and agreed birth control from the time of obtaining informed consent to 24 weeks after the completion of administration of the study drug. Patients were excluded from the study if they met any of the following criteria: diagnosed with decompensated liver cirrhosis and/or presence of hepatitis B surface antigen in serum; diagnosed with HCC or its history; previously treated for malignant neoplasm; diagnosed with autoimmune hepatitis, alcoholic liver disease, haemochromatosis, or chronic liver disease other than chronic hepatitis C; women who were pregnant, were breast-feeding, or who could become pregnant; had a history of alcohol addiction; and had complications of heart, kidney and lung disease.

Hepatitis C virus RNA measurement

Antiviral effects of telaprevir on HCV were assessed by measuring the serum HCV RNA levels using the COBAS

Table 1 Patient characterstics, treatment duration, and viral response

	Sex	Age	BMI (kg/m^2)	Baseline hepatitis C virus (HCV) RNA ($Log_{10}IU/mL$)	Treatment duration (day)	HCV RNA Nadir ($Log_{10}IU/mL$)	Viral response
1	M	31	29.1	7.10	58*	1.6	Breakthrough
2	M	64	30.7	6.70	50*	<1.2 detectable	Breakthrough
3	M	48	25.7	5.10	63*	Undetectable	Breakthrough
4	M	49	22.7	6.60	45*	3.0	Breakthrough
5	F	64	24.2	6.95	85 (completed)	1.2	Partial responder†
6	M	58	19.7	6.50	63*	<1.2 detectable	Breakthrough
7	F	63	22.8	6.40	58*	<1.2 detectable	Breakthrough
8	M	49	22.6	5.50	87 (completed)	Undetectable	Relapser
9	M	59	21.2	6.35	85 (completed)	Undetectable	Breakthrough
10	F	55	19.0	6.25	51*	<1.2 detectable	Breakthrough

Subjects whose viral level increased by 2 $Log_{10}IU/mL$ from nadir or more than 3 $Log_{10}IU/mL$ after reaching undetectable levels during treatment phase are defined to show breakthrough. *Subjects discontinued telaprevir due to viral breakthrough. †Subject who did not meet both criteria of breakthrough and relapse.

TaqMan HCV test (Roche Diagnostics). Blood samples were collected on Day-28, before dosing (0 h) and 2.5, 4, 8, 16 and 24 h after the first dosing on Day 1 and before dosing on Days 3, 8, 14, 29, 43, 57 and 86 and at Weeks 1, 2, 4, 8, 12, 16, 20 and 24 after the end of treatment. The linear dynamic range of the assay was 1.2 to 7.8 $\log_{10}$ IU/mL. The qualitative result below the lower limit of quantification (LLOQ) was also determined as positive (1.0) and negative (0.5).

Sequence analysis of the hepatitis C virus NS3 protease domain

Hepatitis C virus RNA was isolated from serum samples collected on Day-28, and Days 1, 3, 8, 14, 29, 43, 57 and 86 and at Weeks 1, 2, 4, 8, 12, 16, 20 and 24 after the end of treatment. The DNA fragment of 534 bp in length (181 amino acids) encompassing the NS3 protease domain was amplified by nested RT-PCR and cloned. At least 39 clones per specimen were sequenced bidirectionally. The limit of detection (LOD) for sequencing analysis was around 3 $\log_{10}$ IU/mL.

Safety assessments

Safety and tolerability of study treatments were assessed by clinical laboratory results, vital signs, 12-lead electrocardiograms (ECGs) and occurrence of adverse events. These safety parameters were recorded at regular intervals from Day-28 through the follow-up visits.

Determination of pharmacokinetic parameters

Blood samples were collected immediately before dosing (0 h) and 1, 2.5, 4, 6, 8, 12, 16 and 24 h after the first dosing on Days 1, 14 and 85 and before dosing on Days 3, 8, 29, 43 and 57. Plasma concentrations of telaprevir were determined using a high-performance liquid chromatographic apparatus fitted with mass spectrometry. Plasma concentrations and actual plasma-sampling times were used to calculate the area under the plasma concentration–time curve from 0 to 8 h ($AUC_{0-8\ h}$) and terminal half-life ($t_{1/2}$) by the noncompartmental method using WinNonlin software version 5.2.1. The maximum plasma concentration (C_{max}) and time to reach C_{max} (t_{max}) were directly determined from the observed values on Days 1, 14 and 85.

Statistical analysis

From the plasma concentrations of telaprevir, descriptive statistics were calculated. The number of patients with adverse events was summarized by MedDRA (version 11.1.) system organ class, preferred term, severity and relationship to study drug. All statistical analyses were performed using the validated version 9.1.3 of the SAS® System (SAS Institute Inc., Cary, NC, USA).

RESULTS

Baseline characteristics

A total of 10 Japanese patients, whose background characteristics are shown in Table 1, were enrolled in this study. Their median age was 56.5 years (range, 31–64), and 7 (70.0%) and 3 (30.0%) were men and women, respectively. Baseline HCV RNA levels of each subject were similar in the range 5.10 $\log_{10}$–7.10 $\log_{10}$ IU/mL (median: 6.450).

Safety and tolerability

There were neither serious adverse events nor discontinuations owing to an adverse event. In the present study, 75 adverse events and 66 adverse drug reactions, respectively, developed in nine of 10 patients (90.0%). An incidence of adverse events that developed in two or more patients by the preferred terms is shown in Table 2. The adverse events with the incidence of 30% or higher were rash developing in eight patients (80.0%) (if pruritic rash is included in rash, nine patients [90.0%]), anaemia in seven patients (70.0%), blood uric acid increased in five patients (50.0%), low-density lipoprotein increased in five patients (50.0%), stomach discomfort in four patients (40.0%), peripheral oedema was present in three patients (30.0%), blood triglycerides increased in three patients (30.0%), and pruritus was seen in

Table 2 Incidence of adverse events that occurred in two or more patients

	N = 10			
	Mild	Moderate	Severe	Total
	N (%)	*N* (%)	*N* (%)	*N* (%)
Subjects with adverse events	9 (90.0)	5 (50.0)	0 (0.0)	9 (90.0)
Rash	7 (70.0)	1 (10.0)	0 (0.0)	8 (80.0)
Anaemia	7 (70.0)	0 (0.0)	0 (0.0)	7 (70.0)
Blood uric acid increase	4 (40.0)	1 (10.0)	0 (0.0)	5 (50.0)
Low-density lipoprotein increase	4 (40.0)	1 (10.0)	0 (0.0)	5 (50.0)
Stomach discomfort	4 (40.0)	0 (0.0)	0 (0.0)	4 (40.0)
Blood triglycerides increase	3 (30.0)	0 (0.0)	0 (0.0)	3 (30.0)
Pruritus	3 (30.0)	0 (0.0)	0 (0.0)	3 (30.0)
Peripheral Oedema	2 (20.0)	1 (10.0)	0 (0.0)	3 (30.0)
Malaise	2 (20.0)	0 (0.0)	0 (0.0)	2 (20.0)
Pyrexia	2 (20.0)	0 (0.0)	0 (0.0)	2 (20.0)
Nasopharyngitis	1 (10.0)	1 (10.0)	0 (0.0)	2 (20.0)

three patients (30.0%). The moderate adverse events (one each) that developed in five patients were vertigo, peripheral oedema, nasopharyngitis, increase in blood uric acid and in low density lipoprotein, facial palsy and rash, whereas all other adverse events were mild. It is notable that although seven patients discontinued the therapy, none did so owing to adverse events.

Antiviral activity

Telaprevir rapidly decreased serum HCV RNA level in all patients enrolled in this study. The median serum HCV RNA level changed from 6.45 $\log_{10}$ IU/mL (range: 5.1–7.1) just before administration to 4.00 $\log_{10}$ IU/mL (range: 3.0–4.7) at 16 h after administration and 1.10 $\log_{10}$ IU/mL (range: 0.5–3.3) on Day 14 (Fig. 1). Telaprevir showed potent antiviral activity: a median $\log_{10}$ decrease of 2.325 at 16 h and 5.175 on Day 14. During the administration period of 12 weeks, HCV RNA levels decreased to less than the LLOQ of 1.2 $\log_{10}$ IU/mL in seven patients, and three patients achieved HCV RNA negativity on Day 14 or Day 29. After the decrease in serum HCV RNA, breakthrough occurred in eight patients, and seven of those patients discontinued the trial during the dosing period (from Day 45 to Day 63, Table 1). In addition, one of the remaining three patients who completed the administration of the study drug achieved virus negativity by the end of administration (Day 86), but relapsed 1 week after completion of drug therapy.

Hepatocyte injury markers

As shown in Fig. 2a, the alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels decreased during and after telaprevir treatment. Median changes from baseline (Day 1) in ALT and AST were −26.5 IU/L (range: −217–5, N = 10) and −8.5 IU/L (range: −118–2, N = 10) on Day 29, respectively. Fig. 2b shows total bilirubin levels. No clinically significant change in bilirubin was observed in all patients. These data indicate that long-term exposure to telaprevir caused neither damage nor injury in the liver.

Sequence analysis of hepatitis C virus NS3

Amino acid substitutions in the NS3 protease domain, which were selected by telaprevir administration, were examined in 39 clones or more for each sample (Table 3). The predominant variants detected during the early time points after administration (on Days 3 and 8) were V36G, T54A and A156V. Subsequently, these variants decreased below the LOD in nine patients, and the predominant variants detected at viral breakthrough after Week 6 of administration (Day 43–86) were single-substituted variants of A156F/T/V and multiple-substituted variants of T54S+A156T and

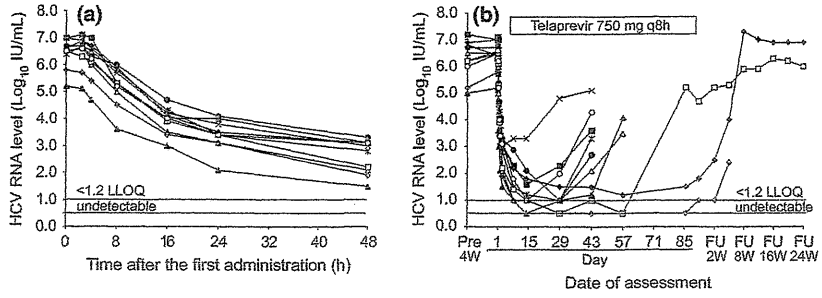

Fig. 1 Changes in patient hepatitis C virus (HCV) RNA level. For 12 consecutive weeks, all 10 patients received 750 mg telaprevir q8h under feeding conditions. <1.2 LLOQ, below lower limit of quantification of 1.2 $\log_{10}$ IU/mL; FU, follow-up.

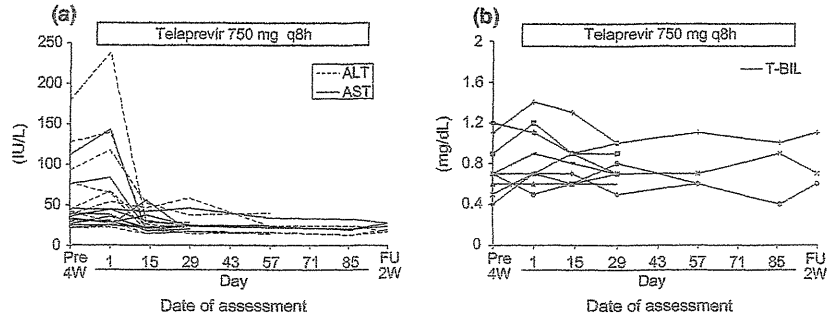

Fig. 2 Change in alanine aminotransferase, aspartate aminotransferase (a) and total bilirubin (b) levels. FU, follow-up.

Table 3 Means of hepatitis C virus (HCV) RNA levels and representation rates of variants in all subjects during and after telaprevir treatment

	Pre	Day 3	Day 8	Day 14	Day 29	Day 43	Day 50–60	Day 86	FU2W	FU4W	FU8W	FU12W	FU24W
N	10	10	10	10	10	10	9	3	3	3	2	2	2
Mean of HCV RNA level (log_{10} IU/mL)	6.35	2.61	1.84	1.45	1.56	2.53	3.22	2.40	2.90	4.13	6.60	6.45	6.45
HCV NS3 variants (%)													
Wild	100.0	40.0	0.2	–	0.2	0.1	–	–	–	18.3	86.4	51.8	97.8
V36A	–	–	–	–	–	–	0.3	–	–	23.8	3.4	38.5	1.1
V36G	–	10.0	0.4	–	2.4	0.8	–	–	–	–	–	–	–
T54A	–	–	9.4	9.5	4.7	0.1	–	–	–	17.9	1.1	5.0	–
A156F	–	–	–	–	–	10.0	25.5	0.8	–	–	–	–	–
A156T	–	–	–	0.5	–	7.5	16.6	31.1	16.3	–	–	1.2	–
A156V	–	–	30.0	–	1.1	15.9	2.3	–	–	–	–	–	–
T54S+A156S	–	–	–	–	–	–	–	–	–	19.2	3.4	1.3	–
T54S+A156T	–	–	–	–	–	9.6	11.4	1.5	16.3	15.0	–	–	–
A156T+V158I	–	–	–	–	–	3.3	10.1	–	–	–	–	–	–

–, not detected; FU, follow-up. Minor substitutions (maximum occupancy in a specimen was less than 10%): T54S, R155G, R155L, A156S, V36A+T54A, V36A+A156S, V36G+A156V, T54A+R155L, T54A+A156S, T54A+A156V, T54S+R155L, T54S+A156V, T54A+V132L, A156S+V132L, T54A+V163I, T54S+A156T+V158I, V36A+T54A+A156S

A156T+V158I; no wild-type virus was detected. In the three patients who completed the administration of telaprevir for 12 weeks, V36A, T54A and T54S+A156S/T were detectable after treatment. In the two patients followed up for 24 weeks, gradual enrichment of the wild-type viruses was observed.

Pharmacokinetics

The plasma concentration *vs* time curves on Days 1, 14 and 85 are shown in Fig. 3a and the C_{trough} on Days 1, 2, 3, 8, 14, 15, 29, 43, 57 and 85 in Fig. 3b. The pharmacokinetic parameters of telaprevir on Days 1, 14 and 85 are given in Table 4.

As the t_{max} were similar on Days 1, 14 and 85 with medians of 2.50, 2.49 and 2.72 h, respectively, the repeated administration under the present conditions was unlikely to cause any change in absorption. The pharmacokinetic parameters of C_{max}, $AUC_{0-8\ h}$ and C_{trough} were lower on Day 1 than those on Days 14 and 85; thus, on Days 1, 14 and 85, the mean values of C_{max} were respectively 2.24, 3.34 and 3.68 μg/mL, the mean values of $AUC_{0-8\ h}$ were respectively 11.60, 22.31 and 23.98 μg·h/mL, and the mean values of C_{trough} at 8 h after the first administration were respectively 1.462, 2.239 and 2.312 μg/mL. The plasma concentration of telaprevir reached steady state on Day 2.

DISCUSSION

During the past decade, the combined use of PEG-IFN and RBV has provided a significant therapeutic advance for

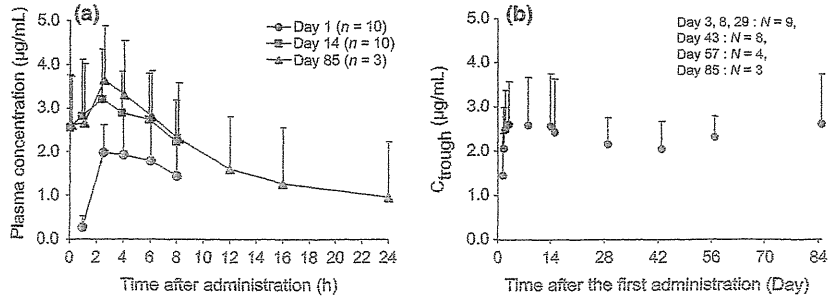

Fig. 3 Time course of plasma concentration (a) and C_{trough} (b) of telaprevir. Symbols and error bars indicate mean values and SD, respectively.

Table 4 Pharmacokinetic parameters of plasma telaprevir

	N	C_{max} (μg/mL)	t_{max} (h)*	$AUC_{0-8\ h}$ (μg·h/mL)	C_{trough} (μg/mL) †	$t_{1/2}$ (h)
Day 1	10	2.24 ± 0.93	2.50 (2.30–7.92)	11.60 ± 4.74	1.462 ± 0.949	5.57 ± 2.67‡,§
Day 14	10	3.34 ± 1.11	2.49 (0.98–5.97)	22.31 ± 8.29	2.239 ± 0.953	9.64 ± 6.14‡,¶
Day 85	3	3.68 ± 1.29	2.72 (2.68–4.00)	23.98 ± 9.45	2.312 ± 1.265	18.35 ± 22.91**

Mean value ± SD. *Median (minimum value to maximum value). †C_{trough} at 8 h after the first administration. ‡Calculated from measured values at 8 h after the first administration. §$N = 7$. ¶$N = 8$. **Calculated from measured values at 24 h after the first administration.

patients with CHC. Approximately 50% of patients infected with genotype 1 HCV do not, however, achieve SVR with this SOC [3–5]. On the contrary, the treatment with telaprevir-based triple regimen significantly improved SVR rates in patients with genotype 1 HCV. The PROVE 1 and 2 studies of telaprevir use with PEG-IFN and RBV in treatment-naïve patients achieved SVR rates of 61% and 69% (placebo: 41–46%) [11,12]. The Japanese study of the telaprevir-based triple regimen also showed high SVR rates [13–15]. However, the key safety concerns with the telaprevir-based triple regimen were anaemia, rash and IFN-induced systemic symptoms, all of which were most likely caused by the PEG-IFN/RBV treatment. In Japan, there are currently a large number of aged people with genotype 1b HCV and high viral loads, which is one of the most intractable HCV genotypes. As a result of advanced age, many subjects could not tolerate the adverse drug reactions in the telaprevir-based regimen, which was also observed with PEG-IFN/RBV therapies [13,15]. This observation prompted us to re-examine the safety profiles and pharmacokinetics of monotherapy with telaprevir for 12 weeks in Japanese patients.

In this study, 10 treatment-naïve patients with genotype 1b CHC and a high median viral load of 6.45 $\log_{10}$ IU/mL (range: 5.10–7.10) (Table 1) took 750 mg of telaprevir q8h for 12 weeks under feeding conditions. The plasma concentrations of telaprevir reached steady state within 2 days after the initiation of administration in the 750-mg q8h regimen as is shown by the constant C_{trough} from Day 2 to Day 85; hence, all the patients enrolled in this study were sufficiently exposed to telaprevir during treatment (Fig. 3b). These results demonstrate that the plasma concentrations of telaprevir were manageable even during the long-term repeated administration. There were no clinically significant events, although the incidence of some events exceeded 20.0% (Table 2). Notably, mild anaemia developed in seven patients (70%) and its occurrence was consistent with the decrease in haemoglobin values, although gradual, during the first 29 days after administration of telaprevir. The incidence of rash, which is reported to develop with a high incidence and high severity in the clinical trials of co-administration of telaprevir with PEG-IFN and RBV [11,12], was also high but its severity was mild in this study. Although exposure to telaprevir was sufficient to eliminate the virus, neither serious adverse events nor discontinuations because of adverse events occurred during the study period. The results confirmed the high tolerability of telaprevir alone after long-term administration. Although there has been no direct comparison of telaprevir monotherapy and telaprevir-based triple therapy, based on these results, the severe adverse drug reactions reported for telaprevir-based triple therapy including anaemia and rash were likely to be ascribed to the synergistic and/or additive effects of the three drugs, i.e., telaprevir, PEG-IFN, and RBV. The safety information under telaprevir monotherapy described here is very important to understand the aspects of adverse drug reactions, especially anaemia and rash, in telaprevir-based triple therapy. In addition, compared to baseline, the ALT and AST levels were significantly lower during the treatment in all patients, indicating that telaprevir was unlikely to cause direct liver damage or injury even after long-term use.

Although there is a report on HCV RNA mutation after monotherapy with a protease inhibitor for 14 days [16], no information about the selective pressure of such protease inhibitors administered alone for a longer period is available at present. During the treatment period in this study, HCV RNA levels were below the LLOQ in seven patients and undetectable in three patients. Importantly, one patient showed an end-of-treatment response. Viral breakthrough resulting from the selection of Ala^{156}-substituted variants with high-level resistance to telaprevir [16] occurred in eight patients. It has been reported that high-level resistance was absent, low-level resistance was minimized, and the majority of the viral population reverted to the wild-type by 3–7 months after telaprevir dosing for 14 days [16]. In the two patients who were studied up to the last visit, enrichment of the wild-type viruses was observed at Week 24 of the follow-up period. It is thus clear that the variants that appeared during prolonged administration of telaprevir for 12 weeks could be replaced by or could revert to the wild-type viruses. This study also provides new knowledge about a selective pathway of the NS3 protease domain of HCV genotype 1b during long-term telaprevir administration (Table 3). It is notable that the wild-type viruses were eliminated promptly by Day 3 of telaprevir monotherapy in all cases, but variants with amino acid substitutions such as V36G, A156V and T54A still remained on Days 3 and 8.

From Day 50 to Day 99, A156T was the predominant variant after viral breakthrough. On Day 43, several substitutions that are rarely reported were found: a single substitution of A156F and multiple substitutions of T54S+A156T and A156T+V158I. In the clonal sequencing analysis in this trial, the observed T54S and V158I substitutions were mostly associated with the A156S/T substitution, and enrichment of multiple-substituted variants was observed under prolonged telaprevir treatment (Fig. S1). A phenotypic enzyme assay suggested that the solo T54S substitution did not change the inhibitory concentration of telaprevir (data not shown). It has also been reported that the T54S and V158I substitutions were also positively selected in the clinical trials of boceprevir, but the solo V158I substitution did not confer telaprevir resistance [17]. Therefore, these two substitutions may be a secondary resistance-associated variant of genotype 1b. Moreover, we could speculate that these variants are susceptible to PEG-IFN and RBV, because the viral variants emerging after the longer selective pressure with telaprevir monotherapy were decreased rapidly by switching the treatment with telaprevir to that with PEG-IFN and RBV [18]. Although it was reported that one patient with low viral load achieved SVR in the treatment regimen in which 750 mg telaprevir was administered q8h for 24 weeks [19], no patients with high viral load achieved SVR in this study. As discussed earlier, PEG-IFN and RBV-free therapy is an unmet and strong medical need in Japan. Therefore, an oral cocktail therapy for HCV genotype 1b infection using telaprevir and different types of DAAs, for example HCV NS5A or NS5B polymerase inhibitors, would be warranted to improve efficacy and reduce adverse drug reactions of the telaprevir, PEG-IFN and RBV triple therapy.

In conclusion, the results of this study indicate that telaprevir is well tolerated at 750 mg q8h for 12 weeks in Japanese patients with HCV genotype 1b infection. The data obtained in this study on telaprevir monotherapy demonstrate that the severe side effects, rash and anaemia observed in the telaprevir-based triple regimen were likely to be attributable to the additive and/or synergistic effect of telaprevir, PEG-IFN and RBV, and this consideration has encouraged us to evaluate telaprevir in a combination therapy with a different class of DAAs in future.

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DISCLOSURE

The others have nothing to declare.

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