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

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)	0.556
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

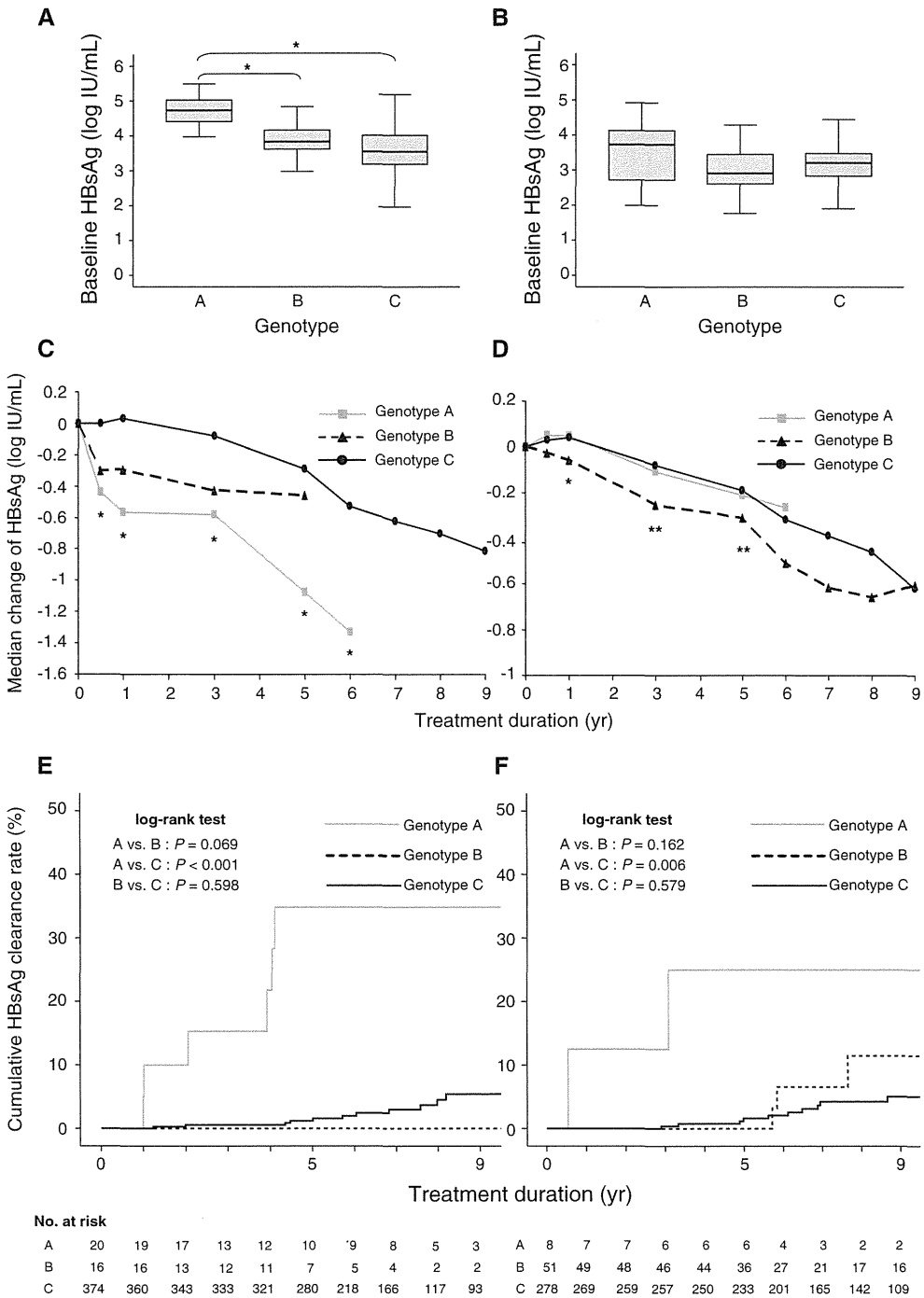

Fig. 2 **a** Box plot of baseline HBsAg levels in patients with different HBV genotypes (HBeAg+ cohort). The *asterisk* (*) indicates a statistical significance of $P < 0.001$, as determined by the Mann–Whitney U test and Bonferroni correction. **b** Box plot of baseline HBsAg levels in patients with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

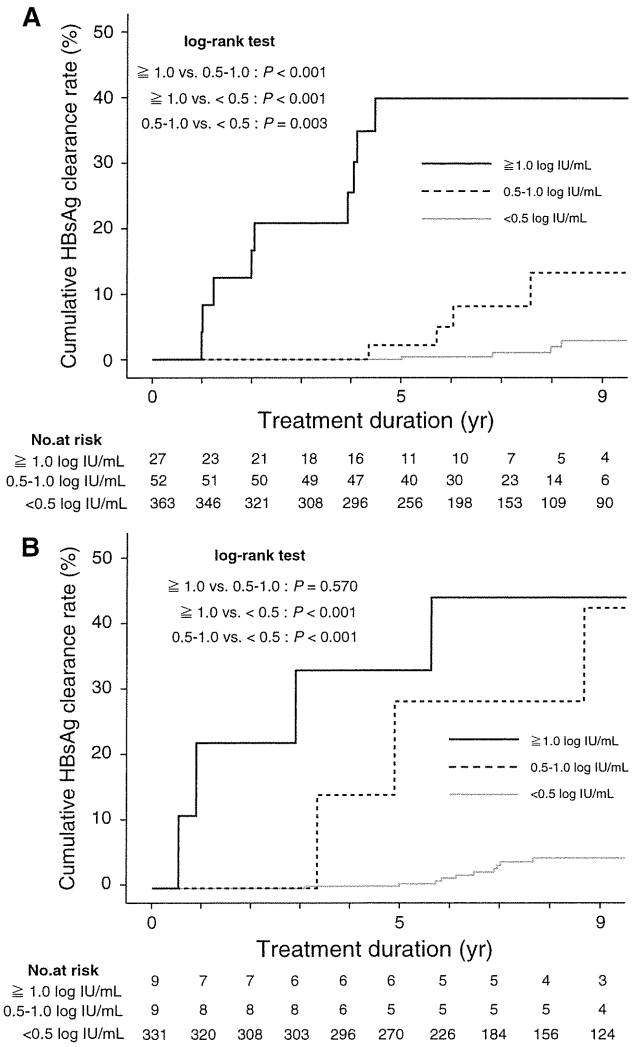

Fig. 3 **a** Kaplan–Meier life table showing cumulative HBsAg clearance rates in patients with varying rates of HBsAg decline within the first six months (HBeAg+ cohort). Clearance rates were highest in the rapid decline group, followed by the intermediate decline group and the slow or steady group (log-rank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

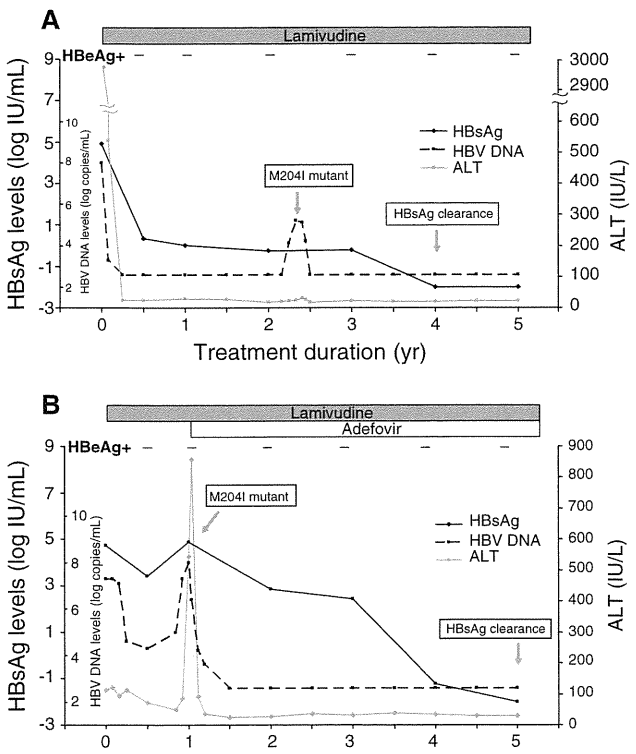

Fig. 4 Case presentation of the typical clinical and virological courses of two representative patients who achieved HBsAg clearance after VBT occurred. **a** Patient 1, a 45-year-old man who was HBeAg+ at baseline and had genotype A. **b** Patient 2, a 38-year-old man who was HBeAg+ at baseline and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

Long-term efficacy and emergence of multidrug resistance in patients with lamivudine-refractory chronic hepatitis B treated by combination therapy with adefovir plus lamivudine

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Abstract
Background Few studies have investigated the emergence of multidrug resistance to adefovir dipivoxil (ADV) plus lamivudine (LAM) combination therapy for patients with LAM-refractory chronic hepatitis B (CHB). In this retrospective study, we investigated the long-term clinical course of these patients with or without multidrug resistance mutations.
Methods We analyzed 406 Japanese patients with LAM-refractory CHB treated with combination therapy with follow-up for a median of 5.4 (0.5–9.5) years. Multidrug resistance of hepatitis B virus (HBV) DNA was analyzed using direct sequencing or cloning methods at baseline and viral breakthrough or insufficient decline during combination therapy.
Results Ratio of patients with undetectable serum HBV DNA levels (<2.6 log copies/mL) during combination therapy was 63, 72, 75, 79, 82, 80 and 85 % at years 1 through 7, respectively. Substitutions associated with multidrug resistance were identified in 11 patients (2.7 %) at baseline, and in 12 patients (3 %) during therapy. HBV DNA levels of patients with rtA181S mutation at baseline and emergence of rtA181T + rtN236T double mutation or a wide variety of mutations during combination therapy could not be suppressed. Moreover, using ultra-deep sequencing, rtA181T/V mutations were detected at baseline in 7 of 10 patients with emergent multidrug resistance during combination therapy, although 6 of these 7 patients had very low frequency (<1 %) variants.
Conclusion Long-term ADV plus LAM combination therapy is effective in LAM-refractory patients. However, HBV DNA levels of the patients with multidrug resistance at baseline or during combination therapy sometimes could not achieve complete suppression or were re-elevated after a decrease.

Keywords Adefovir dipivoxil · Lamivudine · Hepatitis B virus · Ultra-deep sequence · Multidrug resistance

Abbreviations

HBV	Hepatitis B virus
IFN	Interferon
NA	Nucleoside/nucleotide analogues
LAM	Lamivudine
ADV	Adefovir dipivoxil
ETV	Entecavir
TDF	Tenofovir disoproxil fumarate
CHB	Chronic hepatitis B
HBeAg	Hepatitis B e antigen
ALT	Alanine aminotransferase
HBsAg	Hepatitis B surface antigen
PCR	Polymerase chain reaction
CLEIA	Chemiluminescent enzyme immunoassay
rt	Reverse transcriptase

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VBT Viral breakthrough
AST Aspartate aminotransferase
CI Confidence interval
Pt Patient

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 (NA) such as lamivudine (LAM), adefovir dipivoxil (ADV), entecavir (ETV), and tenofovir disoproxil fumarate (TDF) are currently approved for treatment of chronic hepatitis B (CHB) in most countries [2–8]. Successful treatment of CHB with clearance of hepatitis B e antigen (HBeAg), reduction in serum HBV DNA levels, and normalization of alanine aminotransferase (ALT) levels are associated with favorable long-term outcomes, independent of the antiviral drug used [9–11].

LAM is effective in suppressing HBV replication, improving transaminase levels and liver histology, and enhancing the rate of loss of HBeAg. A major problem with the long-term use of lamivudine, however, is its potential to induce viral resistance, with associated increases in HBV DNA and serum transaminases [3, 12, 13]. ADV is reportedly effective in suppressing HBV replication and is approved as a standard therapy in LAM-resistant patients in Japan [14, 15]. However, data concerning the long-term efficacy of ADV treatment in LAM-resistant CHB patients remain limited.

Although both experimental and clinical studies have shown that ADV suppresses not only wild-type but also LAM-resistant strains, the potential for ADV-resistance mutation has emerged. Selection of the rtA181V/T or rtN236T mutant was associated with ADV [13, 16]. Moreover, we previously reported that the emergence of ADV-resistant mutations before and during combination therapy for a period of 2 years was rare [17]. However, ADV-resistant mutations emerging before and during combination therapy might be caused by a poor response to therapy. Moreover, long-term clinical and virological data concerning ADV- or ETV-resistant mutations in LAM-resistant CHB patients receiving long-term ADV plus LAM combination therapy are limited.

The aims of this study were to evaluate the long-term efficiency of ADV plus LAM combination therapy based on virological response (VR), HBeAg clearance, and Hepatitis B surface antigen (HBsAg) clearance, and to investigate the emergence of ADV-, ETV-, or TDF-resistant (or multidrug resistant) mutations before and during combination therapy, and the clinical course of these patients.

Patients and methods

Patients

A total of 406 consecutive adult Japanese patients with chronic HBV infection were treated with ADV in addition to ongoing LAM treatment from 2002 at Toranomon Hospital (Table 1). Several of these patients were included in previous reports [14, 15, 17, 18]. Enrollment in this study and the start of ADV treatment were determined by the following criteria. First, an increase in serum HBV DNA levels of $\geq$1 log copies/mL during LAM treatment compared with the nadir of initial antiviral efficacy on at least two consecutive occasions, or a serum HBV DNA level of $\geq$5 log copies/mL after 1 year of LAM monotherapy; and second, no history of treatment with other NAs such as ETV or TDF. Exclusion criteria were a serum creatinine level $\geq$1.2 mg/dL; coinfection with hepatitis C virus or HIV; and history of other liver diseases, such as autoimmune hepatitis, alcoholic liver disease, or metabolic liver disease. The study was conducted in accordance with the ethical principles of the Declaration of Helsinki and was approved by the Toranomon Hospital Ethical

Table 1 Characteristics of patients at the commencement of adefovir dipivoxil plus lamivudine combination therapy

Demographic data	
Total number	406
Sex (female/male)	86/320
Age, years (range)	48 (25–78)
Duration of treatment, years (range)	5.4 (0.5–9.5)
History of IFN therapy (+/−)	157/249
Laboratory data	
Aspartate aminotransferase, IU/L (range)	54 (12–1413)
Alanine aminotransferase, IU/L (range)	76 (9–1563)
Bilirubin, mg/dL (range)	0.7 (0.2–15.5)
Albumin, g/dL (range)	3.9 (1.9–4.7)
Platelets, $\times 10^3/\mu$L (range)	160 (28–452)
Staging of liver histology (CH/LC)	325/81
Serum HBV DNA, log copies/mL (range)	6.7 (<2.6 to >7.6)
HBeAg, positive/negative/unknown	208/193/5
HBV genotype (A/B/C/D/F)	14/25/364/2/1
rtM204 mutant (%)	365 (90 %)

Values are expressed as the median and range in parentheses, or number and percentage in parentheses

IFN interferon, *HBV* hepatitis B virus, *CH* chronic hepatitis, *LC* liver cirrhosis, *HBeAg* hepatitis B e antigen

Committee (approval no. 714). Informed consent was obtained from all patients.

Patients received a single daily oral administration of ADV 10 mg, in addition to ongoing LAM treatment (100 mg/day). The dosing interval of ADV was modified by the attending physician when serum creatinine level increased to >1.2 mg/dl. Liver cirrhosis was defined by the presence of stage 4 fibrosis on histopathological examination and/or clinical evidence of portal hypertension.

Blood tests and serum viral markers

Routine biochemical tests were performed using standard procedures before and during therapy at least once every 3 months. Levels of HBsAg, HBeAg, and anti-HBe were determined using radioimmunoassay kits (Abbot Diagnostics, Chicago, IL, USA) or Chemiluminescent enzyme immunoassay (CLEIA; Lumipulse System, Fujirebio, Inc. Tokyo, Japan). Serum HBV DNA was quantified using the polymerase chain reaction (PCR)-based Amplicor HBV Monitor assay (Roche Diagnostics, Indianapolis, IN; lower limit of detection, 2.6 log copies/mL).

Determination of nucleotide sequences of HBV DNA

DNA was extracted from 100 μL of serum. PCR reactions for detection of the reverse transcriptase (rt) region (nt 130–1161) of HBV DNA were performed in two parts. The first and second PCR reactions for detection of the first part of the rt region were performed using primers BGF1 (sense; 5′-CTGTGGAAGGCTGGCATTCT-3′) and BGR2 (antisense; 5′-GGCAGGATAGCCGCATTGTG-3′), and PreSBamH1 (sense; 5′-CTTGGGATCCAGAGCTAC AGCATGG-3′) and BR112 (antisense; 5′-TTCCGTCG ACATATCCCATGAAGTTAAGGGA-3′), respectively, under conditions of initial denaturation for 4 min, 35 cycles of amplification with 94 °C for 1 min, 55 °C for 2 min, 72 °C for 3 min, and a final extension at 72 °C for 7 min. The first and second PCR reactions for detection of the second part of the same region were performed using primer pairs B11F (sense; 5′-GGCCAAGTCTGTACAA CATC-3′) and B12R (antisense; 5′-TGCAGAGGTG AAGCGAAGTG-3′), and B11F and B14R (antisense; 5′-GATCCAGTTGGCAGCACACC-3′), respectively, under the same conditions. The amplified PCR products were used for direct sequencing or cloning methods as previously described [19, 20]. When mutations as a mixed viral population with the wild type sequence for direct sequencing were present, PCR was performed using a cloning method. Sequences of 9–26 independent clones from the sample were determined and analyzed. Measurement of sequences in the rt region was performed at the start of ADV treatment, and on viral breakthrough (VBT) during ADV plus LAM combination therapy. VBT was defined as any increase in serum HBV-DNA by >1 log copies/mL from the nadir or redetection of serum HBV-DNA at levels tenfold the lower limit of detection of the HBV-DNA assay after having an undetectable result. Moreover, sequences for serum HBV DNA level of ≥4 log copies/mL after 1 or 2 years of ADV plus LAM combination therapy were also measured.

Measurement of LAM-, ADV-, ETV- and TDF-resistant variants using ultra-deep sequencing

Ultra-deep sequencing was performed using the Ion Personal Genome Machine (PGM) Sequencer (Life Technologies), as described previously [21]. An Ion Torrent adapter-ligated library was prepared using an Ion Xpress Plus Fragment Library Kit (Life Technologies). Briefly, 100 ng of fragmented genomic DNA was ligated to the Ion Torrent adapters P1 and A. The adapter-ligated products were nick-translated and PCR-amplified for a total of eight cycles. Subsequently, the library was purified using AM-Pure beads (Beckman Coulter, Brea, CA) and the concentration was determined using the StepOne Plus Real Time PCR (Life Technologies) and Ion Library Quantitation Kit in accordance with the manufacturer's instructions. Emulsion PCR was performed using Ion OneTouch (Life Technologies) in conjunction with an Ion OneTouch 200 Template Kit v2 (Life Technologies). Enrichment for templated ion spheres particles (ISPs) was performed using the Ion OneTouch Enrichment System (Life Technologies) in accordance with the manufacturer's instructions. Templated ISPs were loaded onto an Ion 314 chip and subsequently sequenced using 130 sequencing cycles in accordance with the Ion PGM 200 Sequencing Kit user guide. Total output read length per run is over 10 M base (0.5 M-tag, 200 base read). The results were analyzed with the CLC Genomics Workbench software (CLCbio, Aarhus, Denmark). A control experiment was included to validate the error rates in ultra-deep sequencing of the viral genome. In this study, amplification products of the second-round PCR were ligated with plasmid and transformed in Escherichia coli in a cloning kit (TA Cloning; Invitrogen, Carlsbad, CA). A plasmid-derived rt sequence was determined as the template by the control experiment. Coverage per position for aa180, aa181, aa184, aa194, aa202, aa204, aa233, aa236 and aa250 in the rt region was 63320, 63890, 67737, 49273, 57410, 57211, 40155, 34801 and 42914, respectively. Thus, using the control experiment based on the plasmid encoding rt sequence, amino acid mutations were defined as amino acid substitutions at a ratio of more than 0.25 % of total coverage. This frequency ruled out putative errors caused by the deep sequence platform used in this study.

HBV genotype

The major genotypes of HBV were determined using the enzyme-linked immunosorbent assay (ELISA, Institute of Immunology, Tokyo, Japan) or the PCR-invader assay (BML, Inc, Tokyo, Japan) according to the method described by Usuda et al. [22] or Tadokoro et al. [23].

Statistical analysis

Differences between groups were examined for statistical significance using the χ^2 or Fisher's exact test where appropriate. Independent risk factors predicting the achievement of HBeAg seroclearance were studied using stepwise Cox regression analysis. The following 14 potential predictors of HBeAg seroclearance were assessed in this study: age, sex, pretreatment with IFN, severity of liver disease (CH or liver cirrhosis), duration from LAM to ADV, substitution of rtM204, HBV genotype, and levels of aspartate aminotransferase (AST), ALT, bilirubin, albumin, γ-glutamyl transpeptidase, platelets, and HBV DNA. Each was 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 ($P < 0.10$) were tested in the multivariate Cox proportional hazards model, and hazard ratios and 95 % confidence intervals (CIs) were calculated to assess the relative risk confidence. The above calculations were performed using the Windows IBM SPSS version 19.0.0 software (IBM Corp., Armonk, NY, USA). A Kaplan–Meier estimate was also performed using the SPSS software.

Results

Study population

Clinical and virological profiles of the 406 patients at the start of ADV plus LAM combination therapy are shown in Table 1. At the start of combination therapy, 81 patients (20 %) had cirrhosis and 208 (51 %) were positive for HBeAg. Fourteen (3 %), 25 (6 %), 364 (90 %), 2 (0.5 %), and 1 (0.2 %) patients were infected with HBV genotypes A, B, C, D, and F, respectively. During the clinical course, 48 of 406 patients (12 %) showed an elevation in serum creatinine >1.2 mg/dL, and their ADV dose was accordingly reduced to 10 mg every second day.

Response to ADV plus LAM combination therapy

The ratio of patients with undetectable serum HBV DNA levels (<2.6 log copies/mL) was 63 % (231/367), 72 % (254/352), 75 % (249/331), 79 % (235/297), 82 % (210/256), 80 % (137/171), and 85 % (94/110) at years 1 through 7, respectively (Fig. 1a). Among HBeAg-positive patients at baseline, undetectable rates of serum HBV DNA levels gradually increased from 1 to 7 years (42, 57, 65, 70, 76, 75, 83 % at years 1 through 7, respectively; $n = 208$). In contrast, ratios in HBeAg-negative patients at baseline were >80 % at all points (86, 89, 88, 90, 91, 87, 89 % at years 1 through 7, respectively; $n = 193$). The undetectable rates of serum HBV DNA in HBeAg-negative patients

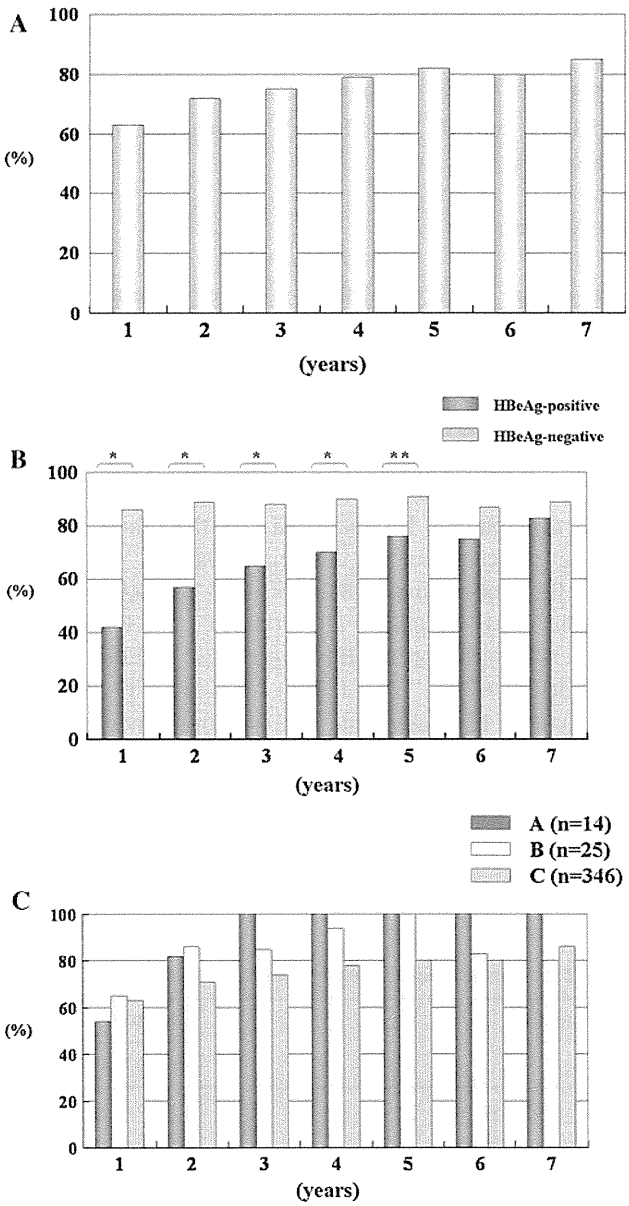

Fig. 1 Undetectable serum HBV DNA levels (<2.6 log copies/mL) in years 1 through 7, respectively. **a** All patients. **b** HBeAg status. A *single asterisk* indicates a statistical significance of $P < 0.0001$ and a *double asterisk* indicates $P = 0.0044$, as determined at the χ^2 test. **c** Genotypes A, B and C

were significantly higher than those in HBeAg-positive patients at years 1 through 5 ($P < 0.0001$ at years 1 through 4, and $P = 0.0044$ at year 5) (Fig. 1b).

By genotype, serum HBV DNA levels were undetectable after 3 years in 100 % of those with genotype A (54, 82, 100, 100, 100, 100, 100 % at years 1 through 7, respectively; $n = 14$), and in >80 % after 2 years in those with genotype B (65, 86, 85, 94, 100, 83, 80 %, at years 1 through 7, respectively; $n = 25$). In contrast, ratios in patients with genotype C gradually increased from 1 to 7 years (63, 71, 74, 78, 80, 80, 86 %, at years 1 through 7, respectively; $n = 364$) (Fig. 1c).

Moreover, the ratio of patients with ALT normalization (≤30 IU/L) was 66 % (250/380), 73 % (262/358), 78 % (255/327), 77 % (226/292), 77 % (194/251), 76 % (125/165), and 77 % (81/105) at years 1 through 7, respectively.

HBeAg clearance

Eighty-four of 208 HBeAg-positive patients (40 %) achieved seroclearance of HBeAg. Cumulative HBeAg seroclearance rates from the commencement date of ADV plus LAM combination therapy were 13 % at 1 year, 24 % at 3 years, 35 % at 5 years, and 52 % at 7 years (Kaplan–Meier method; Supplementary Figure). No patients experienced the reappearance of HBeAg after seroclearance. Six factors found to be associated with the achievement of HBeAg seroclearance in univariate analysis were: AST upper limit of normal (ULN: 30 IU/L)×2<($P = 0.017$), bilirubin 1.1 < mg/dL ($P = 0.020$), ALT ULN×3 <($P = 0.040$), history of IFN therapy ($P = 0.068$), platelets 150<×10^3 μL ($P = 0.074$), and non C genotype ($P = 0.081$). In multivariate analysis, independent factors predicting the achievement of HBeAg seroclearance were history of IFN therapy ($P = 0.009$), AST ($P = 0.016$), bilirubin ($P = 0.030$), and genotype ($P = 0.042$) (Table 2).

HBsAg clearance

Eight of 406 patients (1.9 %) achieved seroclearance of HBsAg (Supplementary Table). All patients were older than 40 years, and all but one was male. Three, two, and three patients were infected with HBV genotypes A, B, C, respectively; two patients were HBeAg-positive at baseline of combination therapy; and five patients had a history of IFN therapy. The duration of HBsAg seroclearance was 2.1–6.8 years.

Genotypic analysis of ADV- and ETV-resistant mutants at baseline of combination therapy and clinical course

Genotypic resistance to LAM, ADV, ETV or TDF was analyzed in baseline samples before the start of ADV plus LAM combination therapy. Substitutions were assessed by direct sequencing or cloning, namely those at rtL180 or rtM204 associated with LAM resistance; rtA181, rtI233, or rtN236 associated with ADV resistance; rtT184, rtS202, or rtM250 associated with ETV resistance; and rtA194 associated TDF resistance. At baseline, substitutions associated with resistance to ADV or ETV were identified in 11 patients (2.7 %) (Table 3). RtA181S/T mutations without substitution at rtM204 were identified in four patients, whereas rtA181T mutation with substitution at rtM204 on the same clones was identified in three patients. RtA181T mutation and rtM204V/I mutation, which existed together on other clones, was identified in two patients. Substitutions related with ETV resistance were identified in the remaining two patients. All but one (Pt. 11) patient was HBeAg-positive and most were younger (<40 years old) and had a high viral load at baseline of LAM therapy. In the remaining 395 patients, rtM204 mutations without substitutions associated with resistance to ADV, ETV or TDF were identified in 358 patients, whereas 37 patients had no substitutions associated with resistance to LAM, ADV, ETV or TDF.

Table 2 Factors associated with HBeAg seroclearance due to ADV plus LAM combination therapy on univariate and multivariate analyses

Parameter	Univariate analysis		Multivariate analysis	
	Hazard ratio (95 % CI)	*P*	Hazard ratio (95 % CI)	*P*
AST (≤UNL×2/UNL×2<)	1.717 (1.102–2.676)	0.017	1.750 (1.112–2.754)	0.016
Bilirubin (≤1.1/1.1<)	1.783 (1.095–2.903)	0.020	1.743 (1.056–2.876)	0.030
ALT (≤UNL×3/UNL×3<)	1.577 (1.008–2.468)	0.040		
History of IFN therapy (−/+)		0.068	1.824 (1.164–2.857)	0.009
Platelets (≤150 × 10^3/150 × 10^3<)		0.074		
Genotype (C/non C)		0.081	2.096 (1.025–4.274)	0.042

HBeAg hepatitis B e antigen, *ADV* adefovir dipivoxil, *LAM* lamivudine, *CI* confidence interval, *AST* aspartate aminotransferase, *UNL* upper limit of normal: 30 IU/L, *ALT* alanine aminotransferase, *IFN* interferon

Table 3 Characteristics of patients with resistance to ADV, ETV or TDF at baseline of ADV plus LAM combination therapy

No.	Baseline of LAM therapy					Baseline of ADV plus LAM combination therapy	
	Age	Sex	Genotype	HBeAg	HBV DNA level	Mutation type (rt region)	Duration from start of LAM to emergence of mutation (years)
1	29	M	C	+	7.6<	A181S	3.3
2	32	M	C	+	7.6<	A181T	1.3
3	23	M	C	+	7.6	A181T	2
4	34	M	C	+	nd	A181T	5
5	35	M	C	+	7.6<	A181T (17/19), L180M + M204V (2/19)	1
6	37	M	C	+	6.5	A181T (7/24), M204I (15/24), L180M + M204V (2/24)	1.3
7	51	M	C	+	7.4	A181T + M204I	1.3
8	38	F	C	+	nd	A181T + M204I (7/13), M204I (6/13)	4
9	33	M	C	+	nd	A181T + M204I (10/21), A181T + M204V(1/21), M204I (10/21)	1.3
10	25	F	D	+	nd	L180M + S202G + M204V	5
11	31	F	C	−	7.6<	L180M + M204V + M250L	6

No. of clones with combined mutations in rt region/total clones are shown in parentheses

ADV adefovir dipivoxil, *ETV* entecavir, *TDF* tenofovir disoproxil fumarate, *LAM* lamivudine, *HBV* hepatitis B virus, *HBeAg* hepatitis B e antigen, *nd* not done, *rt* reverse transcriptase, *M* male, *F* female

Following ADV plus LAM combination therapy, HBV DNA levels of four patients (Pt. 5, 6, 8, 10) were undetectable (<2.6 log copies/mL) (Fig. 2a), while those of the remaining seven were ≥2.6 log copies/mL. One patient (Pt. 7) achieved HBeAg clearance at 2 weeks, while HBeAg reappeared in a second patient (Pt. 11) at 40 weeks. Ratios of patients with undetectable levels of HBV DNA were 9 % (1/11) at 1 year, 22 % (2/9) at 2 years and 50 % (4/8) at 3 years. Three patients (Pt. 1, 2, 9) received TDF plus LAM or TDF plus ETV therapy after ADV plus LAM combination therapy due to insufficient virological response. Mutations of rtA181T + rtM204I, rtA181T + rtM204V and rtM204I in Pt. 9 changed to rtA181T + rtN236T and rtL180V + rtM204V after 3 years of combination therapy, and HBV DNA level was again thereafter elevated.

Genotypic analysis of ADV- and ETV-resistant mutants during combination therapy and clinical course

Genotypic resistance to ADV, ETV or TDF was analyzed during ADV plus LAM combination therapy in 395 patients without ADV- or ETV-resistant mutants at baseline. During combination therapy, substitutions associated with resistance to ADV or ETV were identified in 12 patients (3 %) (Table 4). All patients were genotype C and had a high viral load (>5.0 log copies/ml) at baseline of combination therapy. Substitutions of rtM204 were identified in all but one patient (Pt. 19) at baseline. RtA181V/S/

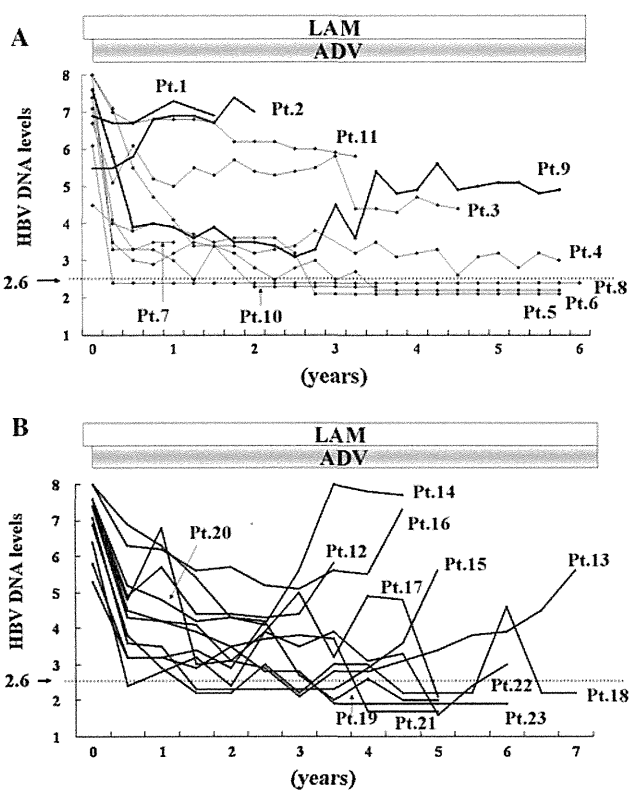

Fig. 2 Clinical course (HBV DNA load) of patients. **a** Patients with resistance associated with ADV or ETV at baseline of ADV plus LAM combination therapy. **b** Patients with resistance associated with ADV or ETV during ADV plus LAM combination therapy

Table 4 Characteristics of patients with emergence of resistance to ADV, ETV or TDF during ADV plus LAM combination therapy

No.	Baseline of ADV plus LAM combination therapy						During ADV plus LAM combination therapy	
	Age	Sex	Genotype	HBeAg	HBV DNA level	rtM204 mutant type	Mutation type(rt)	Duration from start of ADV + LAM to emergence of mutations (years)
12	32	M	C	+	7.6	M204I/V	A181T + N236T (12/16), L180M + N236T (1/16), A181T (1/16), L180M + A181T + M204V (1/16), L180M + M204V + N236T (1/16)	1
13	29	M	C	+	7.6	M204I/V	A181T + M204I + M250L (13/18), L180M + M204V + M250L (2/18), L180M + T184I + M204I + M250L(1/18), L180M + M204I + M250L(1/18), A181T + M204I (1/18)	5
14	58	M	C	+	7.6 <	M204I	L180M + T184I + M204I + M250L(16/26), L180M + T184I + M204I(6/26), A181T(4/26),	3.5
15	49	M	C	+	5.1	M204I/V	A181V + M250L	5
16	46	M	C	+	7.6	M204V	A181T + N236T	3
17	30	F	C	+	7.4	M204I	A181T	0.2
18	40	M	C	+	6.9	M204I	A181S	4
19	40	M	C	+	5.3	M204	A181S	2.3
20	49	M	C	+	7.6	M204V	A181V	0.1
21	63	M	C	−	5.8	M204I	A181T(10/11), A181T + M204I(1/11)	2
22	56	M	C	−	6.4	M204V	A181S	0.6
23	36	M	C	+	7.4	M204I	M180M + A181T(5/9), L180M + A181T + M204I + M250I (3/9), L180M + M204I + M250I(1/9)	1

No. of clones with combined mutations in the rt region/total clones are shown in parentheses

ADV adefovir dipivoxil, *ETV* entecavir, *TDF* tenofovir disoproxil fumarate, *LAM* lamivudine, *HBV* hepatitis B virus, *HBeAg* hepatitis B e antigen, *rt* reverse transcriptase, *M* male, *F* female

T mutation with or without substitution at rtM204 was identified in all patients, whereas rtT184I or rtM250I/L mutation with or without substitution at rtM204 was identified in 4 patients. Moreover, rtA181T + N236T double mutation related with ADV resistance was identified in two patients (Pt. 12 and 16). Interestingly, substitutions of rtM204 were not detected in five patients (Pt 15, 17, 18, 19, 22) when these ADV- or ETV-related mutations emerged.

Following ADV plus LAM combination therapy, the ratio of patients with undetectable levels of HBV DNA was 0 % (0/12) at 1 year, 25 % (3/12) at 2 years, 27 % (3/11) at 3 years, and 20 % (2/10) at 4 years (Fig. 2b). The HBV DNA levels of five patients (Pt. 12–16) were re-elevated after a decrease, and these patients were then switched to a different treatment (TDF plus LAM or TDF plus ETV in four patients and ETV plus ADV in one). Two of these five patients (Pt. 12 and 16) had rtA181T + rtN236T double mutation-related ADV resistance, while three (Pt. 12–14) had a wide variety of mutations. In contrast, HBV DNA levels of patients who had HBeAg clearance (Pt. 17–19, 23) during ADV plus LAM combination therapy were sustained at ≤5 Log copies/mL after 1 year, and only three patients (Pt. 19, 21, 22) showed sustained levels of ≤2.6 Log copies/mL after 4 years.

Evolution of LAM-, ADV-, ETV- and TDF-resistant variants using ultra-deep sequencing

In 10 of 12 patients with emergent substitutions associated with resistance to ADV or ETV during combination therapy, LAM-, ADV-, ETV- and TDF-resistant variants were analyzed by ultra-deep sequencing at baseline (Table 5). Patients 13 and 20 could not be analyzed due to insufficient stored serum. RtA181T/V mutations were detected in all 7 patients by ultra-deep sequencing at baseline, although 6 of these 7 patients had very low frequency (<1 %) variants. Interestingly, rtA181S mutation in 3 patients could not be

Table 5 Detection of resistance to ADV, ETV or TDF by ultra-deep sequencing at baseline in patients with emergence of resistance during ADV plus LAM combination therapy

No.	Baseline of ADV plus LAM combination therapy (ultra-deep sequencing)									During therapy
	rtL180	rtA181	rtT184	rtA194	rtS202	rtM204	rtI233	rtN236	rtM250	Mutation type(rt)
12	L (50.7 %) M (49 %)	A (96.4 %) **T (3.5 %)**	T (99.9 %)	A (99.9 %)	S (99.9 %)	I (59.1 %) V (34.5 %)	I (99.8 %)	N (99.9 %)	M (99.8 %)	A181T, N236T
14	L (81.2 %) M (15.6 %)	A (99.4 %) **T (0.56 %)**	T (99.9 %)	A (99.7 %)	S (99.8 %)	I (99.6 %)	I (99.7 %)	N (99.8 %)	M (99.5 %) I (0.38 %)	A181T, T184I, M250L
15	L (75.3 %) M (24.4 %)	A (97.5 %) S (1.5 %) **V (0.75 %)**	T (99.7 %)	A (99.7 %)	S (99.7 %)	I (70.6 %) V (27.2 %)	I (99.7 %)	N (99.8 %)	M (99.6 %)	A181V, M250L
16	M (99.3 %) L (0.26 %)	A (99.7 %) **T (0.27 %)**	T (99.9 %)	A (99.7 %) T (0.27 %)	S (99.8 %)	V (99.5 %)	I (99.7 %)	N (99.8 %)	M (99.4 %) I (0.51 %)	A181T
17	L (99.8 %)	A (99.7 %) **T (0.25 %)**	T (99.9 %)	A (99.9 %)	S (99.9 %)	I (80.3 %) M (19.5 %)	I (99.7 %)	N (99.8 %)	M (99.7 %)	A181T
18	L (87.9 %) M (11.9 %)	A (98.7 %) T (1.3 %)	T (99.9 %)	A (99.4 %) T (0.55 %)	S (99.5 %)	I (98.2 %) V (1.7 %)	I (99.7 %)	N (99.8 %)	M (98.9 %) I (0.97 %)	A181S
19	L (99.8 %)	A (98.8 %) T (0.89 %)	T (99.9 %)	A (99.8 %)	S (99.8 %)	M (99.5 %)	I (99.6 %)	N (99.7 %)	M (99.6 %)	A181S
21	L (98.8 %) M (0.96 %)	A (98.2 %) V (0.99 %) S (0.48 %) **T (0.35 %)**	T (99.9 %)	A (99.8 %)	S (99.8 %)	I (72.3 %) M (27.0 %) V (0.49 %)	I (99.6 %)	N (99.7 %)	M (99.6 %)	A181T
22	M (99.4 %)	A (99.8 %)	T (99.8 %)	A (99.8 %)	S (99.8 %)	V (99.8 %)	I (99.6 %)	N (99.8 %)	M (99.6 %)	A181S
23	L (87.5 %) M (12.3 %)	A (99.1 %) **T (0.81 %)**	T (99.9 %)	A (99.9 %)	S (99.8 %)	I (99.4 %) M (0.48 %)	I (99.8 %)	N (99.8 %)	M (99.6 %) **I (0.31 %)**	A181T, M250I

Bold values indicate emergent substitutions during combination therapy

ADV adefovir dipivoxil, *ETV* entecavir, *TDF* tenofovir disoproxil fumarate, *LAM* lamivudine, *rt* reverse transcriptase

detected at baseline. In contrast, rtT184I, rtN236T or M250I/L mutations were detected in 1 of 4 patients with emergent mutations during combination therapy.

Discussion

Although ADV plus LAM combination therapy is a standard rescue treatment for patients with LAM-refractory HBV, the virological benefits of long-term therapy have not yet been fully assessed. Here, we evaluated the long-term efficacy of ADV plus LAM combination therapy in 406 LAM-refractory patients over a median follow-up period of 5.4 years. We also investigated baseline factors associated with HBeAg clearance and HBsAg clearance. We found long-term combination therapy produced a gradual virological improvement. In particular, virological response was higher in patients who were HBeAg-negative at baseline, and genotype A and B. Toyama et al. [24] recently evaluated the long-term (median 41 months, 158 patients) efficacy of add-on ADV treatment for patients with LAM-resistant HBV and reported a rate of virological response of 90.8 % at 4 years. Inoue et al. [25] reported that HBV-DNA levels were undetectable (<2.6 log copies/mL) on long-term ADV plus LAM combination therapy (median 47 months; 28 patients, including 7 genotype B) in 56, 80, 86, and 92 % of patients at 12, 24, 36, and 48 months, respectively, whereas Aizawa et al. [26] reported undetectable levels on the same long-term regimen (median 46 months, 72 patients) in 61, 74, 81, 84, and 85 % at 12, 24, 36, 48, and 60 months, respectively, a pattern of response that was similar to our present findings. These differences in virologic response among these Japanese studies might have been due to treatment duration, genotype, or number of patients. Nevertheless, all these long-term studies in Japanese showed a gradual increase in virological response rate for 7 years, and that combination therapy with ADV plus LAM was effective for LAM-refractory patients without multidrug-resistant HBV.

The rate of HBeAg clearance at the end of follow-up in our study of 40 % was compatible with previous reports [13, 24]. The strongest predictor of HBeAg clearance on multivariate analysis was IFN history, as in a previous report [24]. Moreover, we recently reported that HBsAg clearance during NA therapy in patients with HBeAg was influenced by previous IFN therapy and HBV genotype [27]. These results suggest that previous IFN therapy might have an immunomodulatory effect on NA therapy. In addition, baseline levels of AST and bilirubin were also significantly associated with HBeAg clearance in this study. Our results agree with those of many clinical studies that have shown baseline transaminase levels to be the strongest predictor of HBeAg seroconversion in response to both IFN [11] and NA therapy [6, 28]. On the other hand, the rate of HBsAg clearance at the end of follow-up in the present study was only 1.9 %. As mentioned above, we reported that HBsAg clearance during NA therapy was influenced by previous IFN therapy and HBV genotype as well as HBsAg level at baseline or by a decrease in HBsAg level within 6 months [27]. That study [27] included patients originally treated with LAM monotherapy or ETV therapy who switched to LAM monotherapy along with ADV plus LAM combination therapy. In this regard, further study to evaluate factors affecting HBsAg clearance in ADV plus LAM combination therapy is necessary.

We previously reported the emergence of ADV-resistant mutations (rtA181T, rtA181S and rtA181T + rtN236T) in 3 of 132 patients at baseline and in 2 during subsequent combination therapy for a period of 2 years [17]. Moriconi et al. [29] reported that rtA181S and rtT184S mutations, either alone or with rtM204 mutation, at baseline in combination therapy in patients with viral breakthrough during LAM monotherapy correlated negatively with virologic response. Moreover, Heo et al. [30] reported that the presence of the rtA181V/T mutation at baseline was associated with a decreased rate of virologic response at 12 months of combination therapy. In the present study, we analyzed more patients with multidrug resistance during combination therapy over a longer clinical course. Substitutions associated with resistance to ADV or ETV were identified at baseline in 11 of 406 patients (2.7 %), most of whom were HBeAg-positive, of younger age, and had a high viral load. Moreover, a virological response during combination therapy was obtained in only four patients. On this basis, substitution of rtA181 without rtM204 mutation might correlate with a poor virological response in combination therapy. In contrast, virological response rate in patients with mutations associated with ETV (Pt. 10 and 11) was 50 %. Inoue et al. [25] detected ETV-resistant mutations of rtT184S and rtS202C during ADV plus LAM combination therapy, and noted that these patients also showed an ADV resistance profile on in vitro analysis. Moreover, a previous report showed that A181S, A181S + M204I, and L180M + T184S + M204V/I mutations were associated with a poor response to ADV plus LAM combination therapy [29]. In light of these results, A181S mutation and A181T without rtM204I/V mutation at baseline might be associated with multidrug resistance.

On the other hand, substitutions associated with resistance to ADV or ETV were identified in 12 of 395 patients (3 %) during combination therapy. Two patients (Pt. 12 and 16) in this group and a patient (Pt. 9) with rtA181T + M204V/I mutations at baseline developed rtA181T + rtN236T double mutation-related ADV resistance. Considering our clinical study, rtA181T + rtN236T

double mutation correlated with a poor virological response. Moreover, a wide variety of mutations (Pt. 12–14) might be correlated with a poor virological response. Inoue et al. reported that 1 of 28 patients developed virologic breakthrough after combination therapy and sequence analysis identified a wide variety of mutations, including L180M + A200V + M204V + N236T, L180M + A200V + M204V, L180M + M204V, L180M + T184S + M204V and L180M + S202C + M204V [25]. The replication capacity of each clone differed [25], and accordingly a wide variety of mutations might be associated with the development of multidrug resistance. Although rtA181S mutation emerged in three patients (Pt. 18, 19, 22), their HBV DNA level was sustained below 5 log copies/mL. This might be explained by the fact that two of these patients (Pt. 18, 19) had HBeAg clearance during combination therapy while the third (Pt. 22) was HBeAg-negative at baseline. In contrast, Lampertico et al. [31] reported that 9 of 145 (6 %) LAM-resistant patients developed rtA181T/V mutation before and during combination therapy for 4 years, but that HBV DNA levels progressively declined to become undetectable in 7 (78 %). In that report, however, rtA181T and rtA181V mutations were detected as a mixed population together with the wild-type sequence rtA181 in all serum samples. In our study, in contrast, rtA181S/T/V mutations were the major population and may accordingly have influenced the poor virologic response. In any case, response to combination therapy may be influenced by amino acid substitutions other than the well-known mutations associated with LAM, ADV, or ETV resistance, and further in vivo and in vitro studies are required.

Moreover, rtA181T/V mutations were detected by ultra-deep sequencing at baseline in 7 of 10 patients with emergent substitutions associated with resistance to ADV or ETV during combination therapy. It was possible that these mutant viruses increased during combination therapy. However, rtA181S, rtT184I or rtN236T or M250L were not detected at baseline. These data indicate that resistant variants of a minor population increased in some cases, whereas de novo resistant variants emerged during combination therapy in others. However, the number of patients analyzed by ultra-deep sequencing in this study was small; and we did not obtain data from patients without emergent substitutions associated with resistance during combination therapy. Further studies should be performed to interpret the significance of the presence of low frequency variants detected by ultra-deep sequencing.

In conclusion, this study shows that long-term ADV plus LAM combination therapy is effective for LAM-refractory patients. A history of IFN therapy, AST, bilirubin, and genotype were important factors in predicting HBeAg seroclearance. However, some patients did not achieve complete viral suppression of HBV DNA level (<2.6 Log copies/mL). We speculate that incomplete suppression might favor further selection of drug-resistant mutants, albeit that the frequency of multidrug resistance in the present study (5.7 %, 23/406) was low. Moreover, the presence of rtA181S mutation at baseline and emergence of rtA181T + rtN236T double mutation or a wide variety of mutations during combination therapy might be associated with a poor virological response. Several recent reports have indicated the effectiveness of TDF for ADV- or ETV-refractory patients [32–34]. Where indicated, HBV DNA and virological analysis should be carefully monitored.

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Conflict of interest The authors declare that they have no conflict of interest.

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