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Long-Term Entecavir Treatment Reduces Hepatocellular Carcinoma Incidence in Patients With Hepatitis B Virus Infection

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Chronic hepatitis B virus (HBV) infection leads to cirrhosis and hepatocellular carcinoma (HCC). Antiviral agents are thought to reduce HCC development, but agents such as lamivudine (LAM) have a high rate of drug resistance. We compared the incidence of HCC in 472 entecavir (ETV)-treated patients and 1,143 nontreated HBV patients (control group). Propensity score matching eliminated the baseline differences, resulting in a sample size of 316 patients per cohort. The drug mutation resistance was 0.8% (4/472) in the ETV group. The cumulative HCC incidence rates at 5 years were 3.7% and 13.7% for the ETV and control groups, respectively ($P < 0.001$). Cox proportional hazard regression analysis, adjusted for a number of known HCC risk factors, showed that patients in the ETV group were less likely to develop HCC than those in the control group (hazard ratio: 0.37; 95% confidence interval: 0.15-0.91; $P = 0.030$). Both cohorts were applied in three previously reported risk scales and risk scores were generated based on age, gender, cirrhosis status, levels of alanine aminotransferase, hepatitis B e antigen, baseline HBV DNA, albumin, and bilirubin. The greatest HCC risk reduction occurred in high-risk patients who scored higher on respective risk scales. In sub analyses, we compared treatment effect between nucleos(t)ide analogs, which included matched LAM-treated patients without rescue therapy (n = 182). We found HCC suppression effect greater in ETV-treated ($P < 0.001$) than nonrescued LAM-treated ($P = 0.019$) cirrhosis patients when they were compared with the control group. *Conclusion:* Long-term ETV treatment may reduce the incidence of HCC in HBV-infected patients. The treatment effect was greater in patients at higher risk of HCC. (HEPATOLOGY 2013;58:98-107)

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More than 2 billion people worldwide have been exposed to hepatitis B virus (HBV) and about 350 million people are chronically infected, the majority of whom are in Asia (75%). The prevalence of HBV in Japan is 0.8%, which is lower than other Asian countries such as Taiwan (>10%) and China.[1-3] As chronic HBV infection leads to cirrhosis and hepatocellular carcinoma (HCC), published studies have shown that up to 25% of chronically infected patients eventually die of liver cirrhosis or HCC.[4]

A large-scale longitudinal epidemiologic study has shown that a patient's baseline HBV DNA level is an independent predictor for the development of HCC.[5] Studies have begun to show that treatment to decrease HBV DNA reduces the risk of HCC development in HBV patients with cirrhosis or advanced fibrosis or in chronic HBV patients.[6,7]

Within the past 10 years, new antiviral therapies, including nucleos(t)ide analogs (NAs), have been approved and were successful in suppressing circulating serum viral loads. Studies that have examined the relationship between NA therapy and HCC almost exclusively used older drugs such as lamivudine and/or adefovir. Although results of long-term studies showed the importance of antiviral suppression, HCC risk among patients treated by newer NAs remains inconclusive. Entecavir (ETV) is a relatively new antiviral NA that has proved effective in suppressing HBV

Abbreviations: ALT, alanine aminotransferase; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; ETV, entecavir; HBeAg, hepatitis B e antigen; HBV DNA, hepatitis B virus deoxyribonucleic acid; HR, hazard ratio; NA, nucleos(t)ide analogs; PS, propensity score; ROC, receiver operating characteristic curve.

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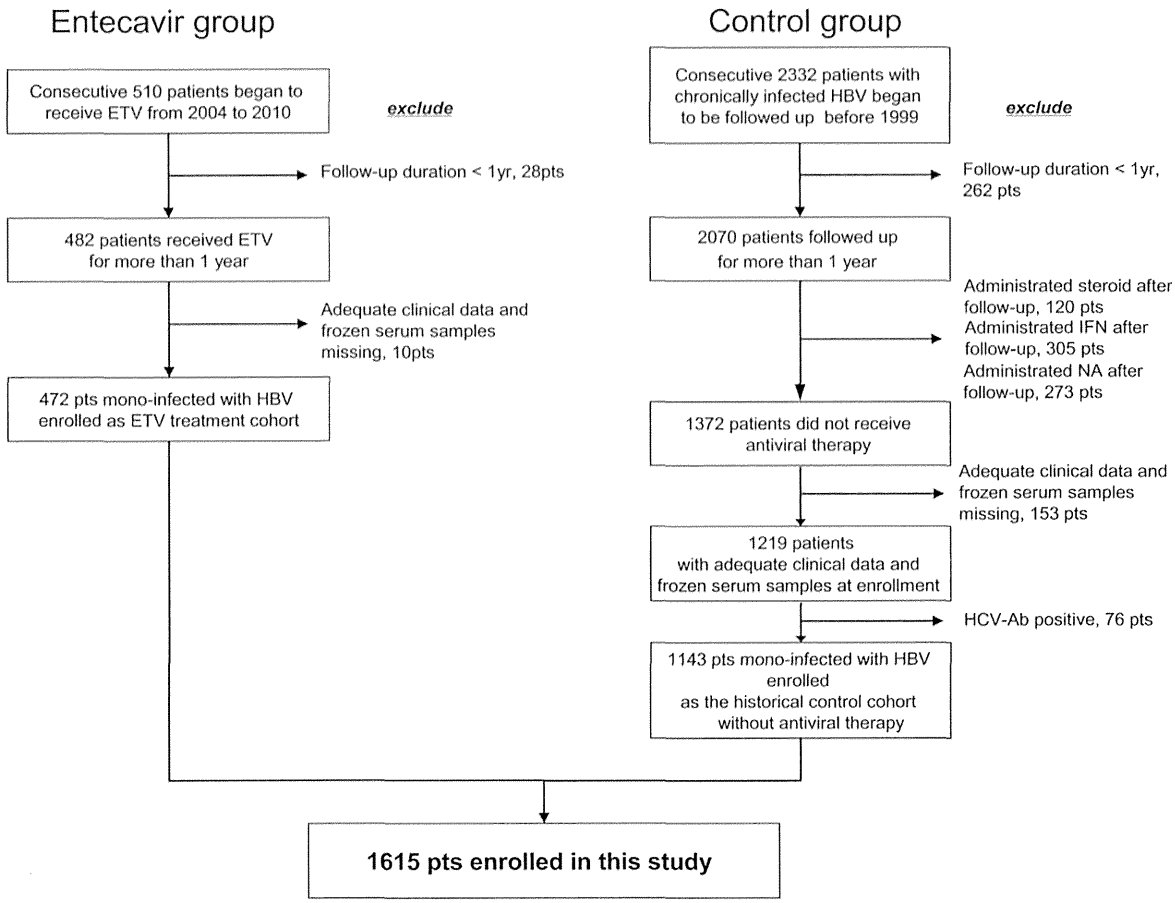

Fig. 1. Entecavir-treated and nontreated cohorts. ETV, entecavir; HBV, hepatitis B virus; IFN, interferon; NA, nucleos(t)ide; HCV-Ab, anti-hepatitis C virus antibody.

DNA replications with minimal drug resistance.[8,9] In this study we examined whether long-term ETV treatment would reduce HCC risk in HBV-infected patients when compared with NA-naïve patients.

Patients and Methods

Patients and Design. From 2004 to 2010, we consecutively recruited 510 patients treated with 0.5 mg ETV (ETV group); the ETV group was compared with a retrospective cohort of 2,332 NA-naïve, HBV-infected patients (control group).

These patients were chronically monoinfected with HBV and were confirmed as hepatitis B s antigen (HBsAg)-positive for at least 6 months. As a general rule, ETV was initiated in a patient who had both abnormal alanine aminotransferase (ALT) levels (defined as ALT $\geq$45) and elevated HBV DNA levels of $\geq$4 log copies/mL. A patient with advanced fibrosis would be treated with ETV if the ALT level was normal; however, a patient without fibrosis or with a normal HBV DNA/ALT level would not be treated with ETV. Among the treated patients, 38 were excluded from the ETV group either because their follow-up period was less than 1 year (n = 28) or because the clinical data or serum samples were incomplete (n = 10). The remaining 472 ETV-treated patients were included in the analysis (Fig. 1). No patient in the ETV group received other NAs before ETV treatment.

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

The control group patients were recruited from 1973 to 1999. These patients were NA-naïve at baseline, as no NA therapy had yet been approved. Patients were excluded from the control group if (1) their follow-up duration was less than 1 year (n = 262); (2) corticosteroid withdrawal therapy (n = 120), IFN treatment (n = 305) or NA treatment (n = 273) was initiated during follow-up; (3) clinical data or serum samples were incomplete (n = 153); or (4) patients were found to be positive for anti-hepatitis C virus antibodies (HCV-Ab) (n = 76). The remaining 1,143 patients served as the control population (Fig. 1).

We also made subanalyses to examine the difference of HCC suppression effect between NAs. To make this comparison, we recruited a cohort of 949 consecutive patients from our hospital who were treated with lamivudine (LAM) (September 1995 to September 2007). LAM-treated patients who met the same inclusion criteria as the ETV group, who had no rescue therapy (LAM group, n = 492), were used in the comparison.

We received informed consent from each patient at their entry into the study. Informed consent for the clinical data collection and storage of serum samples were obtained from each patient in the historical control group. The study protocol was in accordance with the ethical guidelines of the Declaration of Helsinki and approved by the Toranomon Hospital Ethics Committee.

Clinical Data Collection and Follow-up. All ETV-treated and untreated patients were followed at 1- to 3-month intervals, during which biochemical and HBV virological markers, blood counts, tumor markers (e.g., alpha-fetoprotein and des-γ-carboxylprothrombin), and cirrhosis and HCC status were monitored. Viral response in the ETV group was defined as a reduction in HBV DNA levels to below 400 copies/mL. Cirrhosis was determined by laparoscopy, liver biopsy, imaging modalities, or portal hypertension. HCC was diagnosed predominantly via imaging, including dynamic computed tomography, magnetic resonance imaging, and/or digital subtraction angiography. When the hepatic nodule did not show typical imaging features, diagnosis was confirmed by fine-needle aspiration biopsy followed by histological examination. Patients were followed until any confirmed HCC diagnosis 1 year after the start of observation (primary outcome) or until the last visit before December 2011. All patients also underwent ultrasonography or helical dynamic computed tomography every 3 to 6 months (cirrhosis patients) or every 6 to 12 months (noncirrhosis patients).

HBV Infection Markers. HBV DNA levels were quantified using the COBAS Amplicor HBV Monitor Test (Roche Diagnostics, Tokyo, Japan), which has a dynamic range of 2.6 to 7.6 log copies/mL, or COBAS TaqMan HBV Test v2.0 (Roche Diagnostics) which has a dynamic range of over 2.1 to 9.0 log copies/mL. HBV DNA of the control group was measured from their stored frozen serum (−80°C) using COBAS TaqMan HBV v.2.0 once at the start of observation. Previous measurements were taken using the old DNA polymerase assay in the control group and thus were not used for comparisons. For the ETV group, drug-resistant mutations were determined from a nested polymerase chain reaction, using a primer specific at the polymerase region in patients who had an HBV DNA relapse of $\geq$1 log copies from nadir. Hepatitis B e antigen; (HBeAg) was determined by enzyme-linked immunosorbent assay with a commercial kit (HBeAg EIA; Institute of Immunology, Tokyo, Japan). 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 for each of the eight major genotypes (A to H).

HCC Incidence by Risk Scores. To examine HCC incidence by risk scores, we applied published HCC risk scales, which are based on the natural course of HCC among HBV-positive patients, to our cohorts. We first searched Medline/PubMed using "hepatitis B," "cancer," and "risk score" as keywords and found four publications in English that used risk-score estimations.[10-13] One article was rejected because we were unable to compute the risk scores with our variables, and therefore we used only the scales indicated by the remaining three publications to generate the risk scores.[13] The risk scales were based on parameters such as age, gender, cirrhosis, levels of ALT, HBeAg, baseline HBV DNA, albumin, and bilirubin. The original risk score formula and the risk score distributions for our two cohorts derived from these formulas are shown in Supporting Table 1. The risk score cutoff points were determined from the following original articles. In Yang et al.'s article,[10] the risk score was derived from 17-point categories. When we applied the scores to our control group, we found that the 12-point scale was at best in detecting a difference in HCC incidence. With that, we examined the HCC suppression treatment effect by dividing the patients into equal halves with 12 points as the cutoff. Yuen et al.[11] divided their cohort in half and found risk scores of 82 as the optimal cutoff point. We also applied the same cutoff point to our cohorts. Wong et al.[12] used their risk scores to categorize their cohort into low-risk, medium-risk, and high-risk groups with respective cutoff points at $<$4, 4-19, $\geq$20. We also applied the same cutoff points to our cohorts to examine the treatment effect. Cumulative

HCC incidence rates were compared by these risk scores between the ETV and control groups.

Statistical Analysis. Categorical data were compared using chi-square or Fisher's exact tests. Continuous variables with normal distributions were compared using Student's t test, and those without normal distributions were compared using the Mann-Whitney U test. Cumulative HCC incidence rates were analyzed using the Kaplan-Meier method; patients followed beyond 5 years were censored to better compare the two cohorts because the ETV group had a shorter follow-up period when compared with the historical control group. We compared the cumulative incidence of HCC using the log-rank test, and Cox proportional hazard regression analysis, which was used to assess the variables that were significantly associated with the development of HCC. Deaths before HCC development were censored. Significance was defined as $P < 0.05$ for all two-tailed tests.

We used the propensity score (PS) matching method to reduce significant differences in demographics between the ETV and control groups.[14,15] Using multiple logistic regression analysis, a PS was estimated for all patients treated with ETV.[14] Variables used in the model included age, sex, presence of cirrhosis, HBeAg, HBV DNA< aspartate aminotransferase (AST), ALT, γ-glutamyl transpeptidase; (γ-GTP), bilirubin, albumin, and platelet counts. We performed caliper matching on the PS (nearest available matching). Pairs (ETV and the control group) on the PS logit were matched to within a range of 0.2 standard deviation (SD).[16,17] The PS logit distributions for each cohort showing the overlaps and SD ranges are shown in Supporting Fig. 1. The balance of covariates was measured by their standardized differences. A difference >10% of the absolute value was considered significantly imbalanced.[17] The cohorts were divided into five PS quintiles (Supporting Table 2). We also made subanalyses to examine the difference of HCC suppression effect between NAs by comparing the HCC incidence between propensity score matched ETV- and lamivudine (LAM)-treated patients without a rescue therapy. The LAM-treated patients were derived from consecutive sampling at our institution and were PS matched with ETV group according to the same method described above. Interaction of the subgroups by preexisting cirrhosis or risk scores and ETV treatment were evaluated. $P < 0.10$ was considered statistically significant. Data analysis was performed using IBM SPSS v. 19.0 software (Armonk, NY) and R software v. 2.13 (R Foundation for Statistical Computing, Vienna, Austria; www.r-project.org).

Results

Patient Characteristics. The patient characteristics at the baseline, before PS matching are shown in Table 1. The ETV group was followed for an average of 3.2 years (1,561 person-years), whereas the control group was followed for an average of 9.5 years (12,381 person-years). Before matching, patients in the ETV group and the control group differed significantly in age, gender, genotype, baseline HBV DNA level, and other clinical data. In the ETV group, 421 patients (89%) had HBV DNA (<400 copies/mL) at year 1. Not all patients in the control group were tested for HBV DNA level during follow-up. The drug mutation resistance was 0.8% (4/472). The four patients who had drug mutation did not develop HCC. During follow-up, 12 patients (2.54%) in the ETV group and 144 patients (12.60%) in the control group developed HCC. The incidence rates of HCC for the ETV and the control groups were 76/10,000 patient-years and 116/10,000 patient-years, respectively. During this period, 21 patients in the control group developed liver cirrhosis while no patient developed liver cirrhosis in the ETV group. During the same observation period, there were four deaths in the ETV group and 10 deaths in the control group. We took competing risk into account[18,19] and compared incidence of non-HCC deaths between the cohorts and the results were not different. However, because there were only four patients in the non-HCC deaths in the ETV group (two patients in the PS matched cohort) and 10 patients in the control group (six patients in the PS matched cohort), we considered that it was not meaningful to apply competing risk analysis in our cohorts.

Factors Associated with HCC and Effect of ETV Treatment on HCC Development. To allow a common ground for comparison between the two cohorts, we used PS matching with selected key characteristics and compared the two groups within the same time period of 5 years. The PS matching process resulted in a matched sample size that consisted of 316 patients in each group (Table 1). The PS matching reduced the significant variability of the two cohorts. While five (42%) of the 12 covariates varied by >10% before matching, all covariates differed by <10% of the absolute value after matching (Supporting Fig. 2). In the PS score matched cohort, 10 out of the 231 noncirrhosis patients progressed to liver cirrhosis within the 5 years of observation. The cumulative incidence rates of HCC in the matched ETV groups were 0.7% at year 2, 1.2% at year 3, 2.5% at year 4, and 3.7% at year 5. The cumulative incidence rates of HCC in the

Table 1. Patient Characteristics and Demographics

Characteristics	Entire Cohort				Propensity Score Matched Cohort		
	All Patients (n = 1,615)	Entecavir (n = 472)	Control (n = 1,143)	P	Entecavir (n = 316)	Control (n = 316)	P
Age (y)†	42 (13.5)	47 (12.4)	39 (13.1)	<0.001	46 (12.1)	46 (13.5)	0.907
Gender (male:female)	1,035:580	315:157	720: 423	0.171	210:106	210:106	1.000
Alcohol consumption (>200kg)	355 (22)	97 (20.5)	288 (25.1)	0.013	62 (20)	105 (33)	<0.001
Cigarette smoking	443 (27)	157 (33.2)	286 (25.0)	0.005	110 (35)	110 (35)	1.000
Preexisting cirrhosis	311 (19)	116 (25)	195 (17)	0.001	79 (25)	85 (29)	0.324
HBV genotype	–	–	–	<0.001	–	–	0.843
A	53 (3.3)	12 (2.5)	41 (3.6)	–	8 (2.5)	9 (2.8)	–
B	254 (15.7)	66 (14.0)	188 (16.4)	–	49 (15.5)	50 (15.8)	–
C	1,135 (70.3)	344 (72.9)	791 (69.2)	–	225 (71.2)	226 (71.5)	–
D	1 (0.06)	0	1 (0.09)	–	0	0	–
F	1 (0.06)	0	1 (0.09)	–	0	0	–
H	2 (0.1)	2 (0.4)	0	–	0	0	–
Unclassified / missing	169 (10.4)	48 (10.2)	121 (10.5)	–	34 (10.7)	31 (9.8)	–
Baseline HBeAg positive	617 (38)	219 (46)	398 (35)	<0.001	135 (43)	133 (42)	0.936
Baseline HBV DNA (log copies/mL)	6.0 (4.3-7.7)	6.7 (5.3-8.0)	5.8 (4.0-7.5)	<0.001	6.3 (5.2-7.9)	6.6 (4.5-7.8)	0.795
Baseline AST level (IU/L)	35 (22-63)	53 (35-95)	28 (20-50)	<0.001	45 (32-70)	49 (27-98)	0.956
Baseline AST level (x ULN)	1.1 (0.7-1.9)	1.6 (1.1-2.9)	0.8 (0.6-1.5)	<0.001	1.4 (1.0-2.1)	1.5 (0.8-3.0)	0.989
Baseline ALT level (IU/L)	42 (22-88)	70 (42-163)	33 (20-68)	<0.001	61 (39-109)	60 (28-144)	0.110
Baseline ALT level (x ULN)	1.1 (0.7-2.4)	1.9 (1.2-4.3)	0.9 (0.6-1.8)	<0.001	1.7 (1.0-3.3)	1.6 (0.8-3.7)	0.086
Baseline GGTP level (IU/L)	28 (16-59)	39 (24-72)	24 (14-52)	<0.001	34 (23-64)	34 (18-68)	0.088
Baseline total bilirubin level (mg/dL)	0.7 (0.5-0.9)	0.7 (0.5-1.0)	0.6 (0.5-0.9)	<0.001	0.7 (0.5-1.0)	0.7 (0.5-0.9)	0.210
Baseline serum albumin level (g/L)	4.2 (3.9-4.5)	3.9 (3.6-4.1)	4.4 (4.1-4.6)	<0.001	3.9 (3.7-4.2)	4.0 (3.8-4.3)	0.084
†Platelet count (10^5/mm^3) (SD)	19.1 (6.3)	16.9 (5.6)	20.0 (6.4)	<0.001	17.5 (5.2)	17.2 (6.0)	0.349
Follow-up duration (yrs)	5.4 (3.1-13.2)	3.2 (2.1-4.3)	9.5 (4.4-16.1)	<0.001	3.3 (2.3-4.3)	7.6 (3.4-13.7)	<0.001
Person-years of follow-up	13,986	1561	12381	–	1064	2978	–
No. of HCC cases	156	12	144	–	6	72	–
Incidence rates per 1000 person-years	11.15	7.69	11.63	–	5.63	24.1	–
Progression of cirrhosis within 5 year	21 (1.3)	0	21 (1.8)	0.001	0	10 (3.2)	0.001
HBV DNA <400 copies/mL at 1 yearr	–	421 (89)	NA	–	288 (90)	NA	–
Emergence of drug-resistant mutants during ETV treatment	–	4 (0.8)	NA	–	2 (0.6)	NA	–

HBeAg, hepatitis B e antigen; HBV, hepatitis B virus; AST, aspartate aminotransferase; GGTP, gamma glutamyltransferase (ULN=33 IU/L); ALT, alanine aminotransferase (ULN=42 IU/L for men and 27 IU/L for women); HCC, hepatocellular carcinoma; ETV, entecavir.

*P <0.05.

**P <0.001, comparison of entecavir-treated group and control group.

†Data displayed as mean ± standard deviation. ‡All other values are expressed as median (25th to 75th percentile) or number (percentage of total, %).

matched control group were 4.0% at year 2, 7.2% at year 3, 10.0% at year 4, and 13.7% at year 5. Log-rank test revealed a statistically significant difference between the incidence of HCC in the ETV group and the control group over time ($P < 0.001$) (Fig. 2). We then used Cox proportional regression analysis to estimate the effects of ETV treatment on HCC risk. Factors that were associated with HCC at year 5 in the propensity score matched cohort were age, gender, alcohol consumption (>200 kg), the presence of cirrhosis, HBeAg positivity, baseline viral load, ALT, γ-GTP, total bilirubin, serum albumin, and platelet counts (Table 2). For ETV treatment effect, we estimated the hazard ratio of HCC development, adjusting for multiple baseline variables (age, gender, alcohol consumption, smoking, preexisting cirrhosis, HBeAg, HBV DNA, ALT, albumin, γ-GTP, total bilirubin, and platelet count) in the propensity matched cohort. Progression of cirrhosis within 5 years was used as a time-dependent covariate in the proportional hazard regression but it did not show a statistically significant hazard to HCC development.

Subanalyses Showing HCC Suppression Effect Between ETV and LAM. PS matching of the LAM-treated patients without rescue therapy (n = 492) with ETV-treated patients resulted in a matched cohort of 182 patients (Supporting Table 3). The rate of non-rescued LAM-treated group having undetectable HBV DNA at 1 year after treatment was lower when compared with the ETV-treated group. The LAM-treated group also had a higher drug-resistant mutation rate. Comparisons of HCC incidence among the ETV-treated group, nonrescued LAM-treated group, and control showed that the HCC suppression effect was greater in ETV-treated ($P < 0.001$) than nonrescued LAM-treated ($P = 0.019$) when compared with the

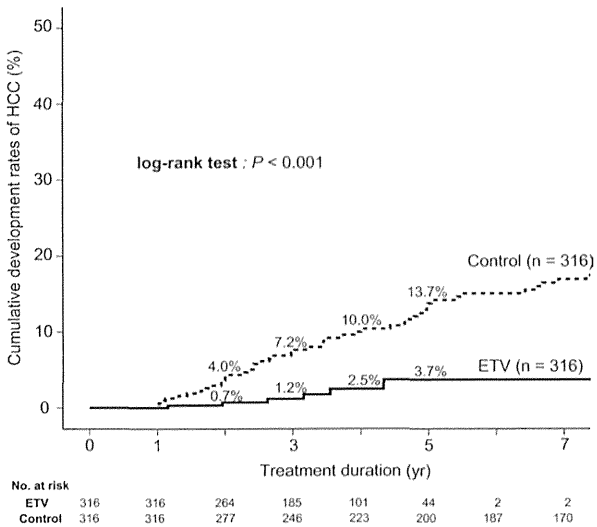

Fig. 2. Comparison of HCC cumulative incidence rates between the entecavir-treated group and the nontreated control group after propensity score matching. The log-rank test revealed a statistically significant difference between the ETV and the control group in the incidence of HCC at 5 years time (log-rank test: $P < 0.001$).

control group (Fig. 3). The difference of effect between ETV and LAM was also significant ($P = 0.043$). The treatment effect was seen in cirrhosis patients but not in noncirrhosis patients. The result showed ETV's superiority to LAM in suppressing HCC.

Effect of ETV on the Reduction of HCC Development by Preexisting Cirrhosis and Risk Scores. To further examine the ETV treatment effect, we compared the ETV and the control groups by preexisting cirrhosis and published risk scores. Viral response rates (HBV DNA < 400 copies/mL) of 1-year post-ETV treatment was 87% in the noncirrhosis patients and 91% in the cirrhosis patients (LC). ALT normalization was 94% and 90% in the chronic hepatitis and cirrhosis patients, respectively. The treatment effect was not inferior by cirrhosis status. Among those who developed HCC, 97 out of 144 patients in the control group and 9 out of 12 patients in the ETV group had cirrhosis. Interactions between preexisting cirrhosis and ETV treatment were not observed ($P = 0.177$).

Cumulative HCC incidence rates by risk scores are compared between the two cohorts in Fig. 4A-G. Figure 4A,B shows the risk scores developed by Yang et al.[10] Figure 4C,D shows the risk scores developed by Yuen et al.[11] Figure 4E-G shows the risk scores developed by Wong et al.[12] All three risk score scales showed that ETV significantly reduced HCC incidence in patients with a higher risk (risk score ≥ 12, $P = 0.006$; risk score ≥ 82, $P = 0.002$; medium risk, $P = 0.062$; high risk, $P < 0.001$). Interactions between risk scores and ETV treatment were not observed (Yang et al.: $P = 0.713$, Yuen et al.: $P = 0.267$, Wong et al.: $P = 0.265$).

Discussion

Our study suggests that long-term ETV therapy would significantly suppress the development of HCC in HBV-infected patients when compared with HBV-infected patients in the control group. The treatment effect was more prominent among patients at high risk of HCC than those at low risk.

Table 2. Factors Associated with HCC Development as Determined by Cox Proportional Hazard Regression Analysis at 5-Year (Propensity Score Matched Cohort)

Variable	Univariate HR (95% CI)	P	Multivariate Adjusted HR (95% CI)	P
Age (per year)	1.05 (1.02-1.07)	<0.001	1.06 (1.03-1.09)	<0.001
Gender (M)	2.81 (1.25-6.32)	0.012		
Alcohol consumption (>200kg)	2.71 (1.49-4.92)	0.001	2.21 (1.18-4.16)	0.013
Cigarette smoking	1.53 (0.84-2.80)	0.164		
Preexisting cirrhosis	12.0 (5.57-25.9)	<0.001	4.28 (1.88-9.73)	0.001
HBV genotype (C)	2.73 (0.98-7.65)	0.056		
HBeAg (positive)	2.64 (1.41-4.94)	0.002	2.26 (1.18-4.34)	0.014
HBV DNA (≥5.0 log copies/mL)	4.66 (1.44-15.1)	0.010		
ALT (≥45 IU/L)	2.29 (1.10-4.77)	0.027		
GGTP (≥50 IU/L)	3.79 (2.02-7.09)	<0.001		
Total bilirubin (≥1.5 mg/dL)	5.51 (2.87-10.6)	<0.001		
Serum albumin (<3.8 g/L)	4.44 (2.42-8.14)	<0.001		
Platelet count ($<1.5\times10^5/mm^3$)	14.8 (5.84-37.7)	<0.001	5.64 (2.13-15.0)	0.001
*Progression of cirrhosis within 5 years	1.80 (0.25-13.2)	0.562		
ETV treatment	0.23 (0.09-0.55)	0.001	0.37 (0.15-0.91)	0.030

Asterisks (*) indicate time-dependent covariates.

†Adjusted for age, gender, alcohol, cigarette, cirrhosis, genotype, HBeAg, HBV DNA, ALT, albumin, GGTP, total bilirubin, and platelet counts

Abbreviations: ETV, entecavir; HR, hazard ratio; CI, confidence interval; HBV, hepatitis B virus; HBeAg, hepatitis B e antigen; ALT, alanine aminotransferase; GGTP, gamma glutamyltransferase.

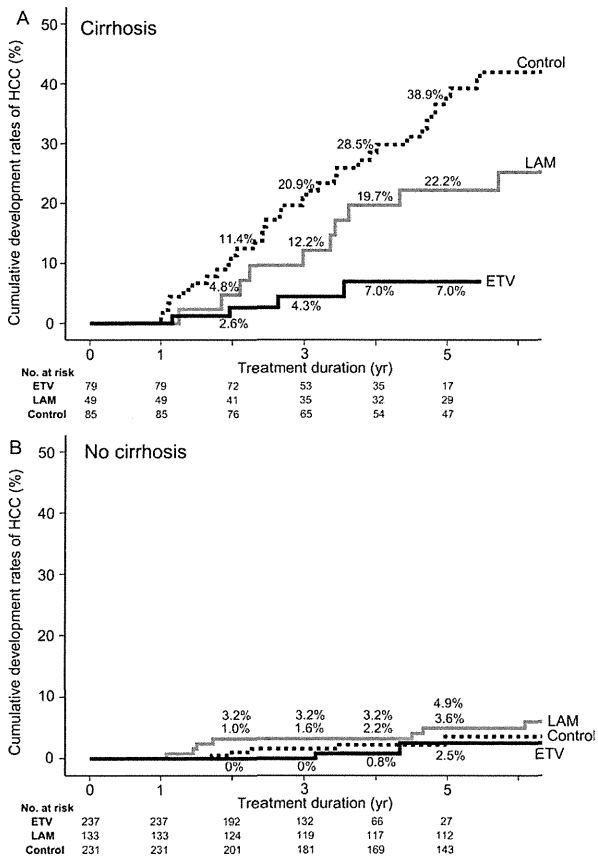

Fig. 3. Comparison of HCC cumulative incidence rates between the entecavir (ETV)-treated group, lamivudine (LAM)-treated, and the nontreated control group after PS matching stratified by cirrhosis. The log-rank test revealed a statistically significant difference in the incidence of HCC at 5 years time in cirrhosis patients: ETV versus control group ($P < 0.001$); LAM versus control ($P = 0.019$); ETV versus LAM ($P = 0.043$). The differences were not seen in the noncirrhosis patients: ETV versus control ($P = 0.440$); LAM versus control ($P = 0.879$); ETV versus LAM ($P = 0.126$).

HBV has been previously shown to influence HCC development. Ikeda et al.[20] reported that the cumulative HCC incidence rates among Japanese HBV patients were 2.1% at 5 years, 4.9% at 10 years, and 18.8% at 15 years among NA-naïve patients. Other studies, both from Japan and other countries, have reported a 5-year cumulative HCC incidence rate of 3.3% among chronic HBV, and 21.2% to 59% among cirrhosis patients.[21,22] The incidence of HCC varies significantly by country and ethnic group,[4] which seems to be attributable to diverse exposure to HCC risk factors.

Carcinogenicity related to HBV infection is somewhat complex and multifactorial when compared with carcinogenicity related to HCV infection. Known HCC risk factors among HBV-infected patients include older age, male gender, cirrhotic status, diabetes mellitus, family history, alcohol consumption, AST, HBsAg, HBeAg, and genotype C.[20,23,25] Chen et al.[5] found a dose-response relationship between pretreatment serum HBV DNA levels and the development of HCC. Baseline ALT is another risk factor for HCC, as elevated ALT levels indicate an active immune response against HBV, resulting in repetitive hepatocyte injury.[5] Our study corroborates these findings on these factors influence on HCC development.

The potential ability of ETV to reduce the risk of HCC is an additional example of a long-term NA treatment effect. Some studies have shown that ETV has low incidence of HCC but these studies did not have a control arm.[9] A meta-analysis and a systematic review showed that NAs can reduce liver complications, including HCC.[26,27] Other studies have begun to show that control of sustained viral loads through drugs such as NAs is important in preventing long-term complications. Chen et al.[28] showed that greater decreases in serum HBV DNA levels ($<10^4$ copies/mL) during follow-up were associated with a lower risk of HCC.

Our comparison among the PS-matched ETV-treated group, nonrescued LAM-treated patients, and the control showed that ETV is superior to LAM in HCC suppression. Kurokawa et al.[29] showed that treatment with lamivudine for an average of 5 years reduced the incidence of HCC in HBV-infected cirrhosis patients, who showed sustained viral response at a median HBV DNA of <4.0 log copies/mL. Unfortunately, only 48% of the patients in this study achieved sustained viral response, while 51% developed lamivudine-resistant tyrosine-methionine-aspartate-aspartate mutation (YMDD mutation) during follow-up.[29] Patients with drug resistance were reported to have a 2.6 times greater chance of developing long-term complications.[26] A systematic review of 21 studies showed that HCC occurred more (2.3% versus 7.5%, $P < 0.001$) in nonresponding patients or in patients with viral breakthrough compared with those who experienced remission.[28] On-treatment drug resistance could subject patients to a variable viral status. Suppression of HCC by NAs requires NAs that do not lead to drug resistance. Compared with other NAs, ETV shows minimal drug resistance. Our results showed that ~90% of the ETV-treated patients had sustained viral suppression at year 1, and that drug resistance was minimal (0.8%) during the median follow-up period of 3.2 years.

We found that the effect of ETV treatment in reducing the risk of HCC was more prominent among high-risk patients. This phenomenon was observed by examining the combination of parameters associated with the recently developed risk scores (Fig. 4). The published risk scores were developed mainly to create

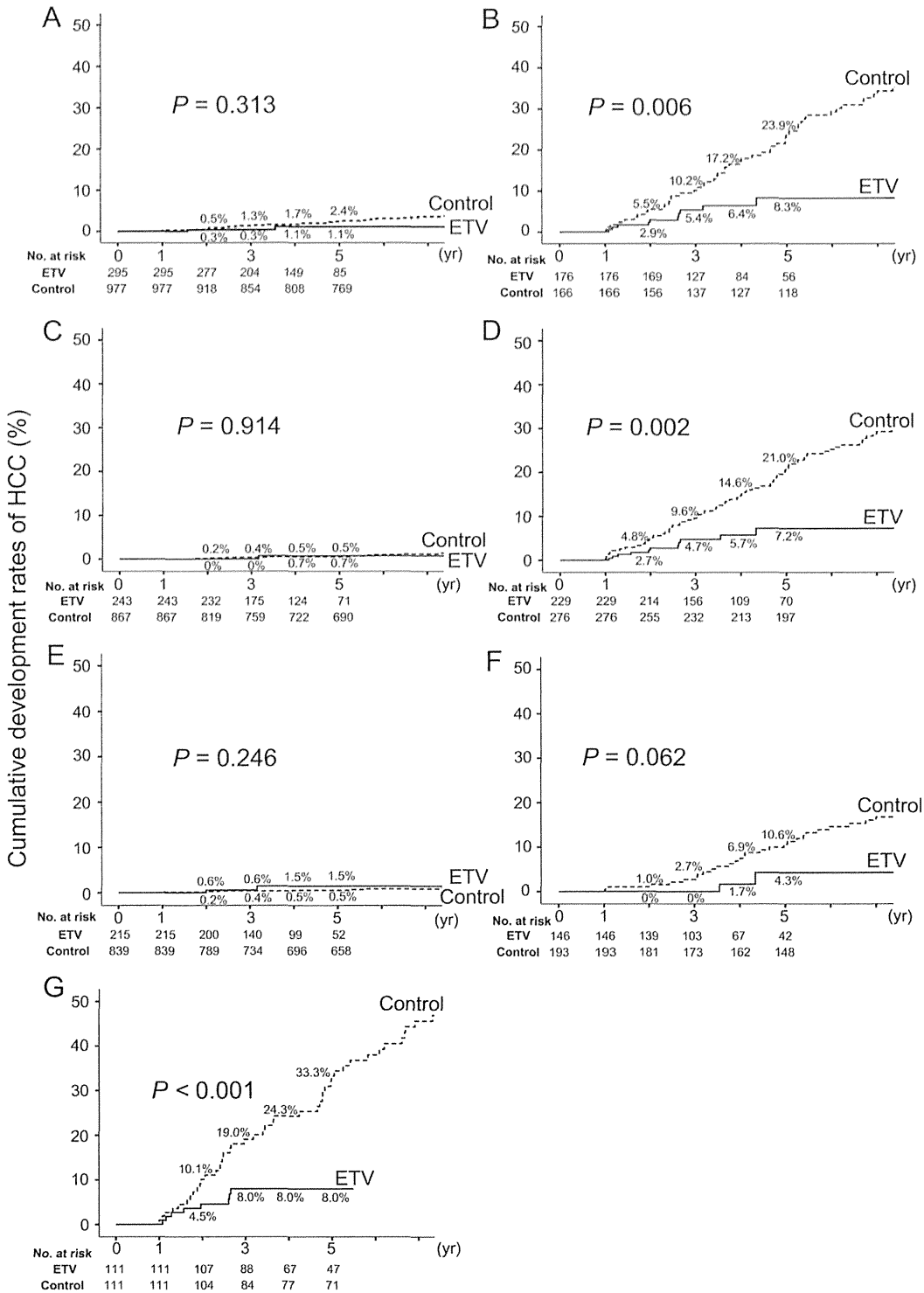

Fig. 4. Cumulative incidence of HCC by risk score scales: comparison between entecavir-treated and nontreated control patients: Risk score cutoff points were based on those presented in articles by the following: A,B (Yang et al.[10]): low-risk score cutoff point < 12; high-risk score cutoff point ≥ 12. C,D (Yuen et al.[11]): low-risk score cutoff point < 82; high-risk score cutoff point ≥ 82. E-G (Wong et al.[12]): low-risk score cutoff point < 4; medium-risk cutoff point 4-19; high-risk score cutoff point ≥ 20. A statistically significant difference in HCC incidence was seen between the ETV group and the control group in the higher-risk groups when observed the incidence of HCC over time (log-rank test $P = 0.006$ for risk score ≥ 12; $P = 0.002$ for risk score ≥ 82; $P = 0.062$ for patients with medium risk; $P < 0.001$ for patients at high risk for HCC).

easy-to-use nomograms based on clinical characteristics to predict the risk of HCC in patients with HBV. These scales have been validated, and can accurately estimate the risk of HCC up to 10 years. The cutoff scores used in these studies were based on their sensitivity to detect HCC derived and validated with non-treated HBV cohorts. The importance of our study using these risk scales in our cohorts was to see the change in risk with the initiation of therapy. We found that the ETV treatment effect to reduce the risk of HCC was more prominent among cirrhosis and high-risk patients despite the lack of interactions between ETV treatment and preexisting cirrhosis or risk factors. The lower treatment effect among lower-risk patients was somewhat not surprising. HCC development among low-risk patients is generally rare, and therefore, the treatment effect may not have occurred in large enough numbers during the treatment period allotted in our study to be able to detect a difference. In addition, HCC development differs greatly by cirrhotic status and risk factors in the control group. The treatment effect of ETV to reduce HCC is probably more likely reflected among cirrhosis or high-risk patients. A study with a longer observation period and higher patient numbers might be necessary to examine this ETV treatment effect among low-risk patients. The development of a scoring system to predict treatment effect of HBV patients with different risk levels will be useful in determining the most appropriate timing of treatment initiation in clinical settings.

Study Limitations. There were several limitations to our study. First, because our patients were recruited from one hospital, they might not have been representative of the general Japanese HBV population. Second, our control group included historically observed patients who entered the cohort long before the ETV group, resulting in treatment differences during the time gap. However, we used PS matching and a similar follow-up period between the two cohorts to minimize this bias. Third, our study was an observational study with patients having large demographic differences. Although we used a PS to match ETV-treated and control groups, our sample size did not take into account other unobserved confounding factors such as HCC family history, stage of cirrhosis, and comorbidities when determining associating factors for carcinogenesis in HBV. Finally, the observation period of the ETV group was relatively short, and patients in the ETV-treated cohort at 5 years consisted of only less than ~25% of the initial recruited patients. Because of this limitation, we censored patients who were followed for more than 5 years. The observed treatment effect would require confirmation over a longer period and a more complete follow-up.

Conducting a long-term study to examine the effect of antiviral therapy with HCC as the endpoint would be time-consuming and challenging. Such a study would require a large sample size and would, therefore, be costly. In addition, the increases in choices of therapy over time would make it difficult to conduct a long-term study using a single therapy. Owing to ethical issues, it would be difficult to recruit or follow a naïve, untreated cohort over an extended period of time. Because of these challenges, most studies have examined the relationship between antiviral treatment and the risks of HCC involved older drugs, lacked a control group, or were of relatively short duration. Consequently, the association between antiviral treatment and carcinogenesis is inferential and requires additional confirmatory studies.

In conclusion, in our study we observed the effect of HCC risk among HBV-infected patients treated by ETV by comparing them with a group of NA-naïve patients. We followed these Japanese patients for a relatively long period of time and compared them with a large pool of untreated control patients. In this long-term study among Japanese patients, ETV significantly reduced the incidence of HCC among chronic HBV-infected patients, and was more prominent among patients at higher risk for HCC.

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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 $\geq$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

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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

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.

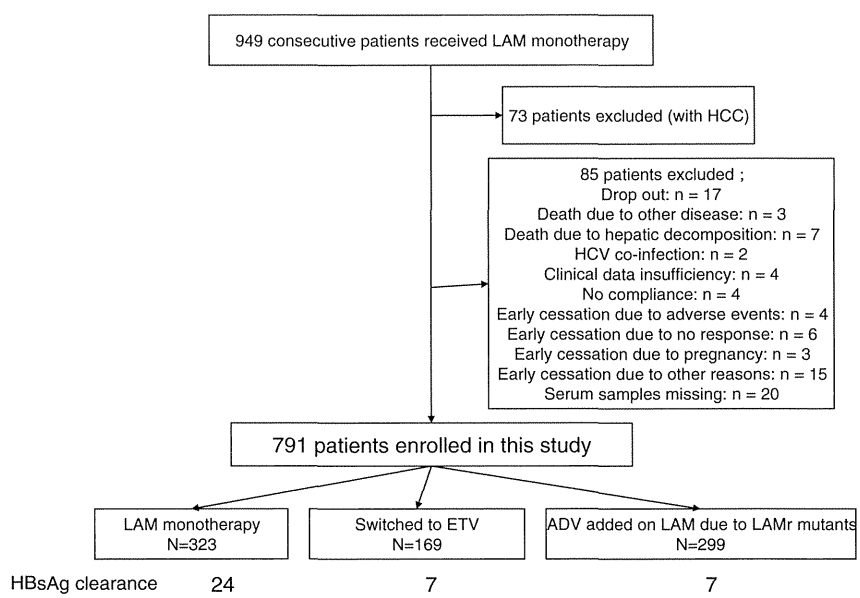

Fig. 1 Schematic of study protocol. *LAM* lamivudine, *HCC* hepatocellular carcinoma, *HCV* hepatitis C virus, *ETV* entecavir, *ADV* adefovir dipivoxil, *HBsAg* hepatitis B surface antigen

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

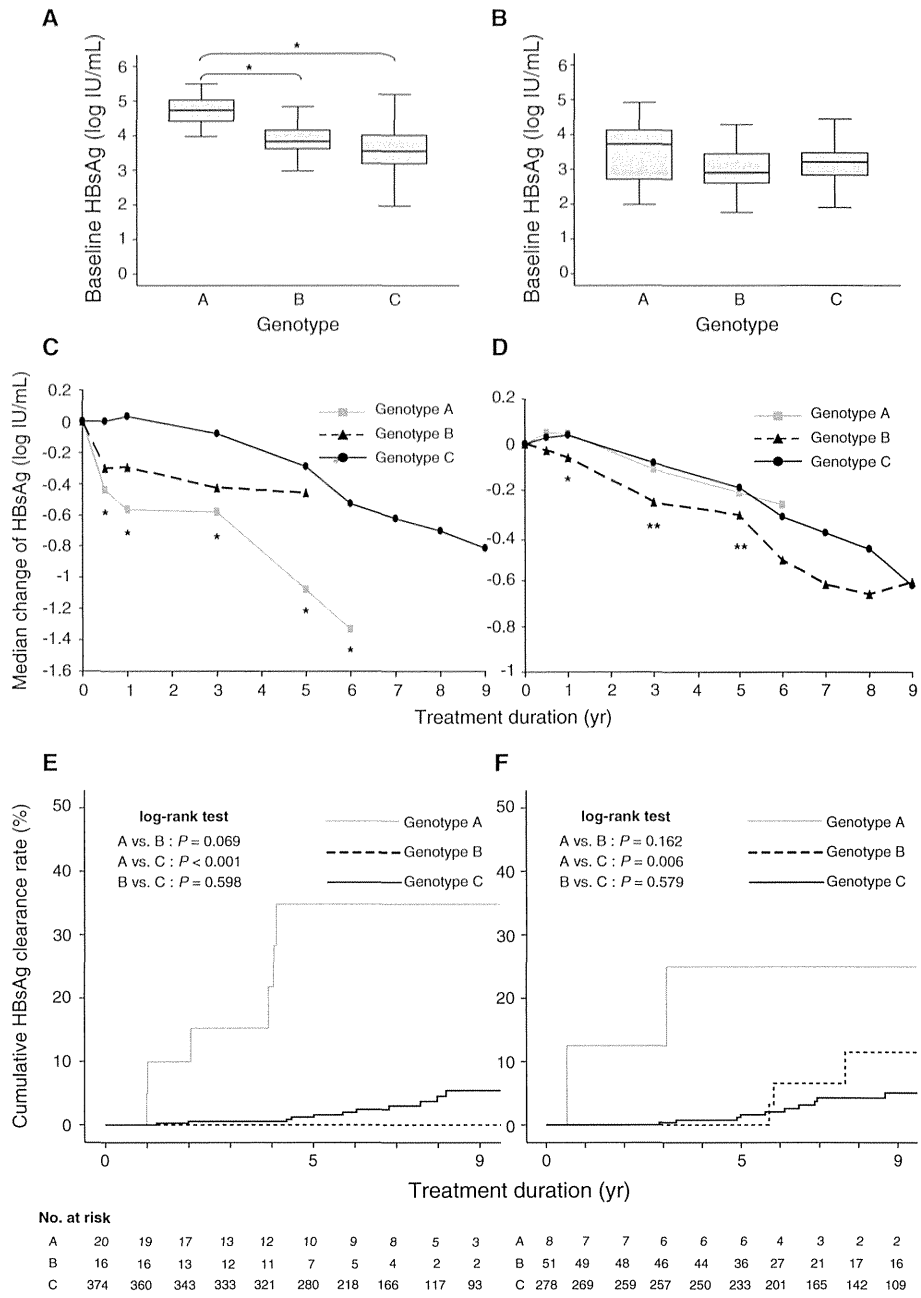

Fig. 2 **a** Box plot of baseline HBsAg levels in patients with different HBV genotypes (HBeAg+ cohort). The *asterisk* (*) indicates a statistical significance of $P < 0.001$, as determined by the Mann–Whitney U test and Bonferroni correction. **b** Box plot of baseline HBsAg levels in patients with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

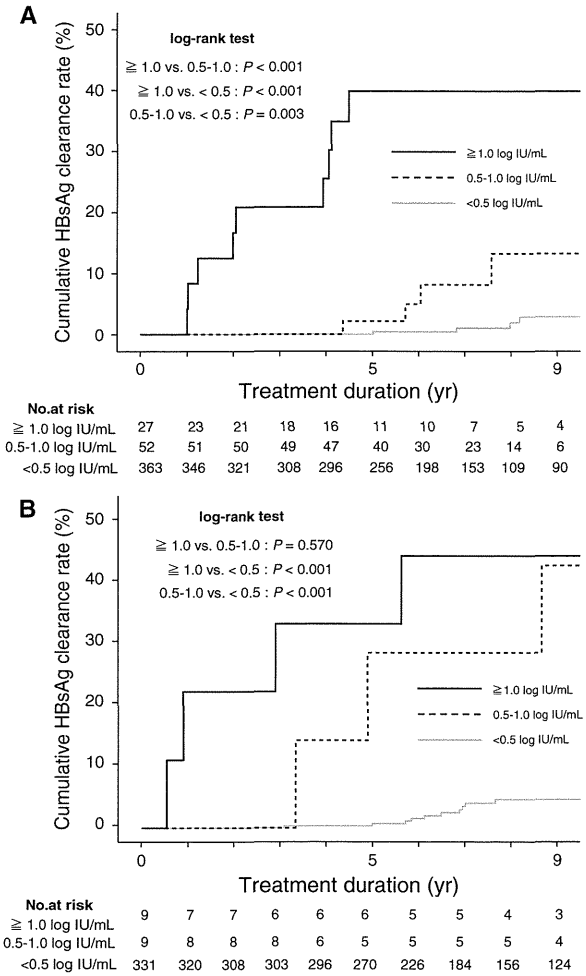

Fig. 3 **a** Kaplan–Meier life table showing cumulative HBsAg clearance rates in patients with varying rates of HBsAg decline within the first six months (HBeAg+ cohort). Clearance rates were highest in the rapid decline group, followed by the intermediate decline group and the slow or steady group (log-rank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