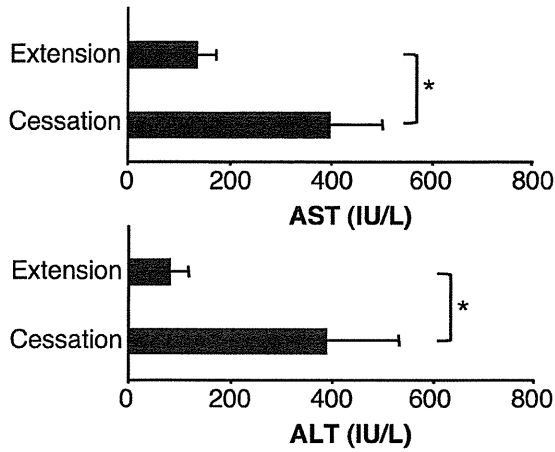
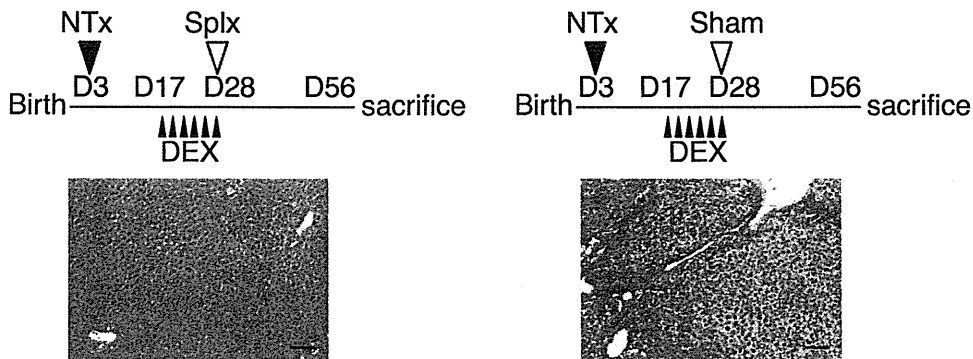


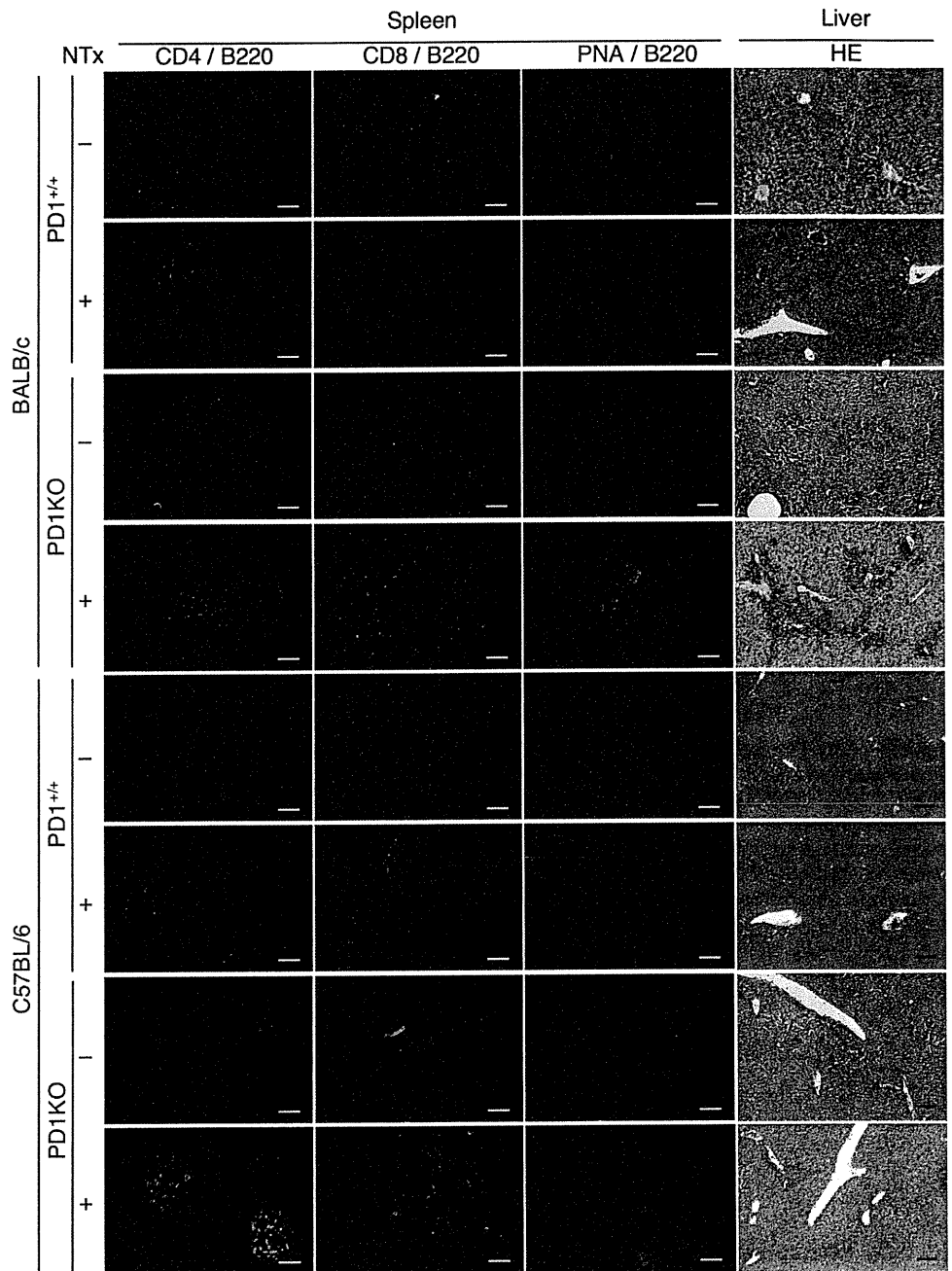
DEX-Tx



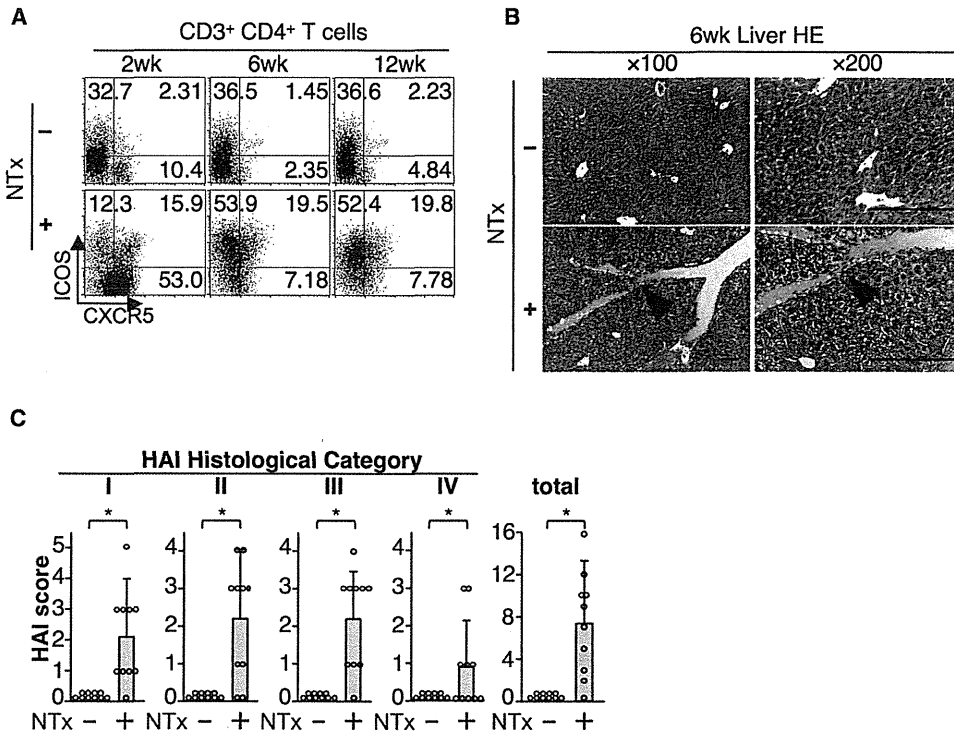
Supplementary Figure 3. Cessation of DEX therapy induces relapse of hepatitis in BALB/c-NTx-PD-1^{-/-} mice. Starting 14 days after thymectomy, BALB/c-NTx-PD-1^{-/-} mice were intraperitoneally injected every other day with 1.0 mg/kg DEX. Mice with DEX injections extended until 40 days of age (Extension, n = 7) and mice with cessation of DEX injections at 4 weeks of age (Cessation, n = 10) were killed at 40 days of age, and sera were harvested. Serum levels of the liver transaminases AST and ALT are shown. The bars indicate the mean of each group, and the error bars indicate SD. *P < .05.



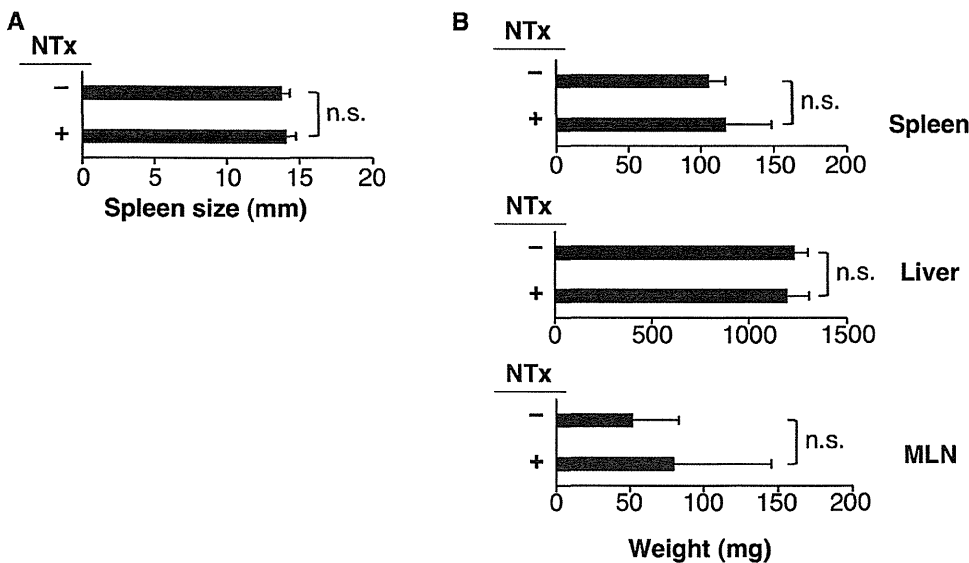
Supplementary Figure 4. Splenectomy overcomes therapeutic insufficiency of corticosteroids and induces prolonged remission of AIH in BALB/c-NTx-PD-1^{-/-} mice. Starting 14 days after thymectomy, BALB/c-NTx-PD-1^{-/-} mice were intraperitoneally injected every other day with 1.0 mg/kg DEX. After 6 injections, mice stopped receiving DEX injections and underwent splenectomy (Splx, n = 3) or sham operation (n = 3) at 28 days of age. Mice at 56 days of age were killed, and the livers were harvested. Histological analysis of the liver from each group is shown, with staining of the liver with H&E. All scale bars = 100 μm.



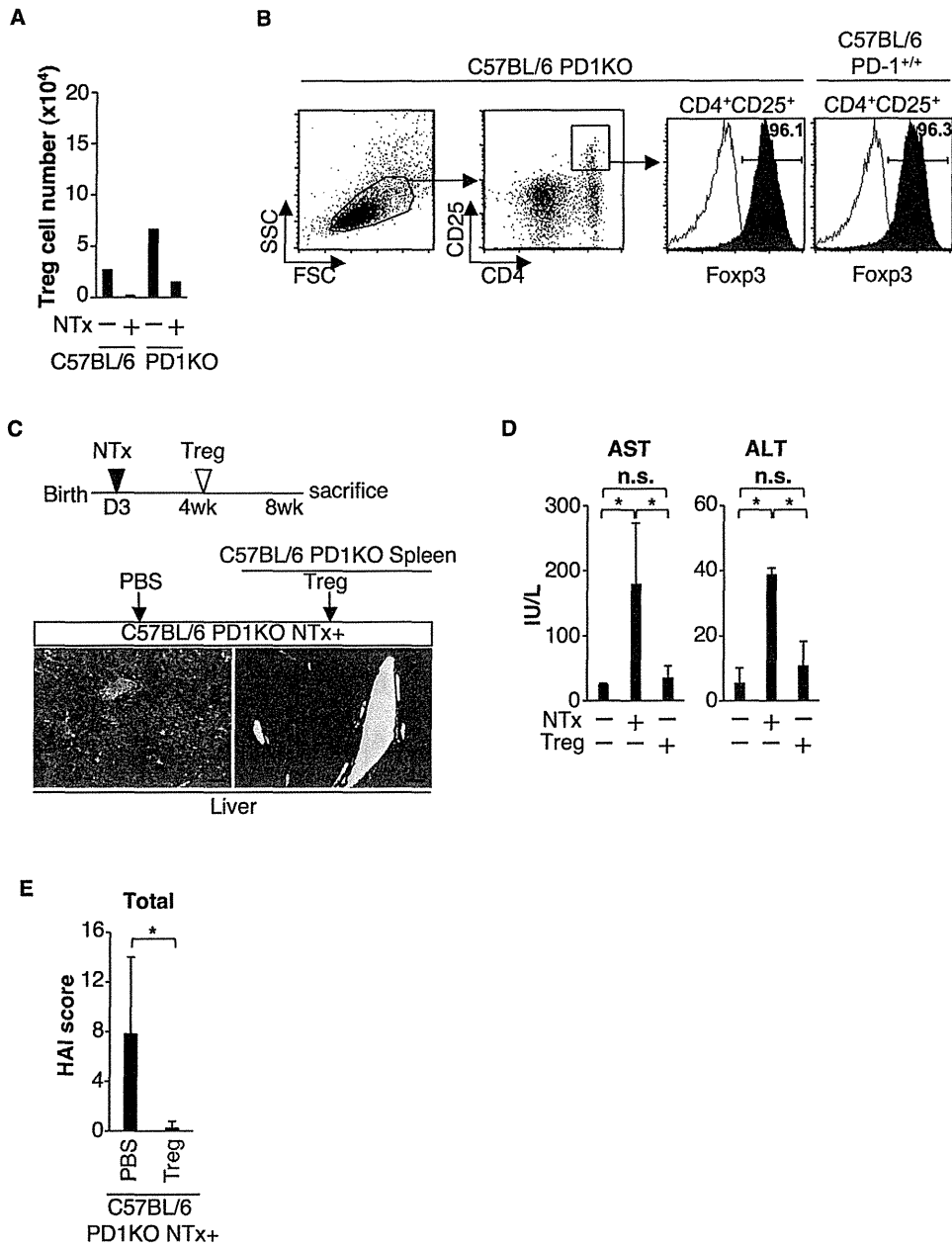
Supplementary Figure 5. Splenic CD4⁺ T cells are preferentially localized within B220⁺ B-cell follicles in NTx-*PD-1*^{-/-} mice at 2 weeks of age, whereas PNA⁺ germinal centers exist in B220⁺ B-cell follicles in the spleen of AIH-bearing NTx-*PD-1*^{-/-} mice on the BALB/c background but in not those on the C57BL/6 background. Immunohistological staining of the spleen (*left panels*) and H&E (HE) staining of the liver (*right panels*) is shown. The spleens and livers were from 2-week-old *PD-1*^{+/+} mice or *PD-1*^{-/-} mice with or without NTx on the BALB/c or C57BL/6 background. The spleens were stained with FITC-conjugated anti-CD4, anti-CD8, or PNA (*green*) and biotin-labeled anti-B220 followed by Texas Red-conjugated avidin (*red*). All scale bars = 100 μ m.



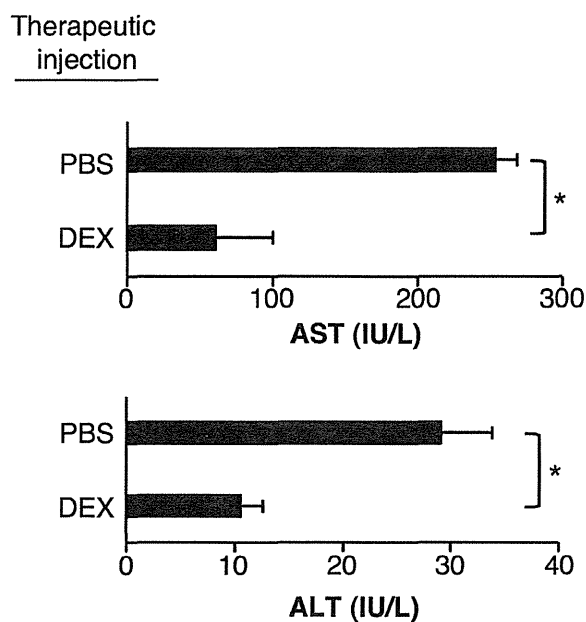
Supplementary Figure 6. Adult C57BL/6-NTx-PD-1^{-/-} mice with increased numbers of splenic T_{FH} cells develop chronic hepatitis. (A) Flow cytometric analysis of splenic CD4⁺ T cells in C57BL/6-PD-1^{-/-} mice with or without NTx at indicated ages. The cells were stained with FITC/anti-CXCR5, PE/anti-ICOS, and APC-Cy7/anti-CD4. The numbers in plots indicate the percentage of cells in each gate in the CD4⁺ T-cell population. (B) Histological findings from the livers of 6-week-old C57BL/6-PD-1^{-/-} mice with or without NTx. In contrast to mice without NTx, those with NTx showed dense packing of inflammatory cells marked in portal branches (black arrowhead). Scale bars = 100 μm. (C) Knodell's HAI score for livers in 8-week-old C57BL/6-PD-1^{-/-} mice with (n = 10) or without NTx (n = 10). HAI score is graded in 4 categories: I, periportal and/or bridging necrosis; II, intralobular degeneration and focal hepatocellular necrosis; III, portal inflammation; and IV, fibrosis. All are described in Supplementary Materials and Methods.



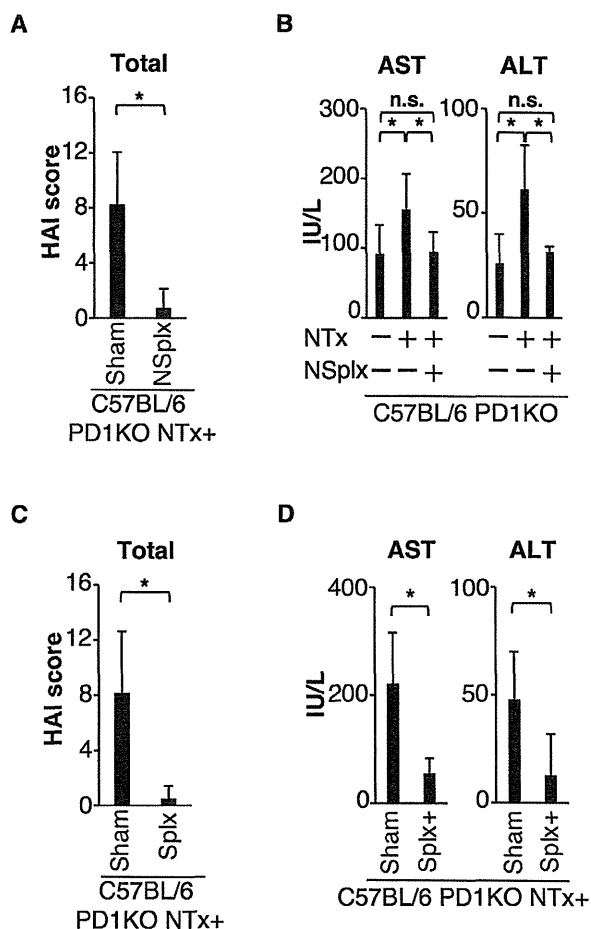
Supplementary Figure 7. Adult C57BL/6-NTx-PD-1^{-/-} mice do not show obvious splenomegaly. (A) Splenic sizes and (B) weights of the spleen, liver, and mesenteric lymph node (MLN) in 12-week-old C57BL/6-PD-1^{-/-} mice with or without NTx. Bars indicate the mean of each group, and the error bars indicate SD. n.s., not significant.



Supplementary Figure 8. Transfer of Tregs has therapeutic efficacy for chronic AIH in C57BL/6-NTx-PD-1^{-/-} mice. (A) Flow cytometric analysis of Tregs in the liver from the indicated mice at 6 weeks of age. The cells were stained with FITC/anti-CD4, PE/anti-CD3, and APC/anti-CD25. The numbers of CD3⁺CD4⁺CD25⁺ Tregs were calculated as follows: (Percentage of Cells in the Cell Types) × (Number of Viable Cells). Data shown are from one of 3 separate experiments. (B) Flow cytometric analysis of CD4⁺CD25⁺ T cells in the spleen from the 8-week-old C57BL/6-PD-1^{-/-} or PD-1^{+/+} mice. The cells were isolated from the spleen and stained with FITC/anti-CD4, APC/anti-CD25, and PE/anti-Foxp3. Filled histograms represent staining of CD4⁺CD25⁺ T cells with Foxp3, and open histograms represent the isotype control. Data represent one of 5 experiments. The numbers in histograms indicate percentages of Foxp3⁺ cells in viable CD4⁺CD25⁺ T cells. (C-E) For transfer of Tregs, 1 × 10⁶ of Tregs were prepared from 8-week-old C57BL/6-PD-1^{-/-} mice. Four-week-old C57BL/6-NTx-PD-1^{-/-} mice were intravenously injected with 1 × 10⁶ Tregs (n = 5) or PBS (n = 5). Recipient mice were analyzed at 8 weeks of age. (C) Histological findings of the liver. Scale bars = 100 μm. (D) Serum levels of the liver transaminases AST and ALT. (E) Knodell's HAI score. Bars indicate the mean of each group, and the error bars indicate SD. *P < .05. n.s., not significant.



Supplementary Figure 9. Therapeutic injections of DEX suppress chronic AIH in C57BL/6-NTx-PD-1^{-/-} mice. Intraperitoneal injections of DEX (n = 5) or PBS (n = 5) were started at 4 weeks of age in C57BL/6-NTx-PD-1^{-/-} mice. After 14 injections every other day at 8 weeks of age, mice were killed and examined. Serum levels of the liver transaminases AST and ALT are shown. Bars indicate the mean of each group, and the error bars indicate SD. *P < .05.



Supplementary Figure 10. The spleen is the induction site of chronic AIH in C57BL/6-NTx-PD-1^{-/-} mice, and splenectomy suppresses chronic AIH. (A and B) C57BL/6-NTx-PD-1^{-/-} mice underwent a splenectomy (NSplx, n = 5) or a sham operation (n = 5) at 1 day after NTx and were analyzed at 8 weeks of age. (C and D) Four-week-old C57BL/6-NTx-PD-1^{-/-} mice underwent a splenectomy (Spix, n = 5) or a sham operation (n = 5) and were analyzed at 8 weeks of age. (A and C) Total HAI scores for livers were as described in Supplementary Materials and Methods. (B and D) Serum levels of the liver transaminases AST and ALT are shown. Bars indicate the mean of each group, and horizontal short bars indicate SD. *P < .05. n.s., not significant. Scale bars = 100 μ m.

Supplementary Table 1. Incidence of Autoimmunity in Various Organs of C57BL/6 PD1KO Mice With or Without NTx

Mice Age (Month) (Total number)	C57BL/6 PD1KO NTx+				C57BL/6 PD1KO NTx-			
	~1M (n=14)	1~2M (n=19)	2~3M (n=15)	3M~ (n=12)	~1M (n=12)	1~2M (n=15)	2~3M (n=16)	3M~ (n=16)
Hepatitis	6 (42.9%)	16 (84.2%)	15 (100%)	12 (100%)	0 (0%)	0 (0%)	0 (0%)	2 (12.5%)
Sialoadenitis	0 (0%)	9 (47.4%)	5 (33.3%)	4 (33.3%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
Pancreatitis	0 (0%)	7 (36.8%)	4 (26.7%)	2 (16.7%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
Myocarditis	0 (0%)	3 (15.8%)	2 (13.3%)	2 (16.7%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
Nephritis	0 (0%)	2 (10.5%)	2 (13.3%)	2 (16.7%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
Gastritis	0 (0%)	0 (0%)	0 (0%)	1 (8.3%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
Enteritis	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
Pneumonitis	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)

The tissues of various organs in C57BL/6 PD1KO mice with or without NTx at the indicated ages (n = 12~19 in each group) were evaluated histologically. Incidence was determined by inflammation characterized by slight infiltration of mononuclear cells in the organs by lymphocytes.

Supplementary Table 2. Survival Rate With or Without Splenectomy

Mice Splenectomy Total number	C57BL/6 PD1 ^{-/-} NTx+ Splx 6 week	
	+	-
	n = 8	n = 16
Survival rate at 20 weeks of age	8 (100%)	16 (100%)

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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Received: 27 March 2012 / Accepted: 12 September 2012
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Abstract

Background Clearance of hepatitis B surface antigen (HBsAg) is considered the ultimate goal in chronic hepatitis B treatment. One treatment option is long-term nucleot(s)ide analog (NA) therapy. We followed a group of long-term NA therapy patients to evaluate the efficacy of this treatment in promoting clearance and longitudinal declines of HBsAg.

Method The study included 791 NA therapy patients who received lamivudine as their first drug. At the baseline, 442 patients were hepatitis B e antigen (HBeAg)+ and 349 were HBeAg-. All analyses were performed after separating the HBeAg+ and HBeAg- cohorts. Cox proportional hazards models were used to determine which factors were associated with HBsAg clearance.

Results HBsAg clearance was observed in 18 (4.1 %) of the HBeAg+ patients and 20 (5.7 %) of the HBeAg- patients at baseline, giving seroclearance rates of 6.4 and 6.9 %, respectively, over the nine-year study period. HBsAg clearance was influenced by several independent factors that varied according to HBeAg cohort. For HBeAg+ patients, these included previous interferon therapy, infection with hepatitis B virus (HBV) genotype A, a ≥ 0.5 log IU/mL decline in HBsAg level within six months, and clearance of HBeAg at six months. For

HBeAg- patients, these included infection with HBV genotype A, decline in HBsAg at six months, and a baseline HBsAg level of < 730 IU/mL.

Conclusion This study suggests that both direct antiviral potential and host immune response are needed to achieve HBsAg clearance by NA therapy. Viral genotype strongly influenced HBsAg clearance during NA therapy.

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

Introduction

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

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

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Antiviral therapy and drug resistance

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

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

Clinical data collection and follow-ups

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

At least every 1–3 months, liver function and virological markers of HBV infection were measured in all patients. All serum HBsAg titers were measured from frozen serum samples collected at six months, one year, three years, five years, and once annually for 6–10 years, and then stored at -80 °C. The day of HBsAg clearance

was defined by the measurement in consecutive available serum samples before it was undetected in subsequent samples. A genotypic analysis of drug resistance was performed in cases of insufficient virological response or VBT, defined as an increase in serum HBV DNA levels ≥ 1 log above the nadir measured after the initial virological response. Cirrhosis was diagnosed by laparoscopy, liver biopsy, or clinical data such as imaging modalities and portal hypertension. The primary outcome for this study was HBsAg clearance. The endpoint of the follow-up was HBsAg clearance or last visit before January 2011.

Markers of HBV infection

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

Statistical analyses

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

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

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

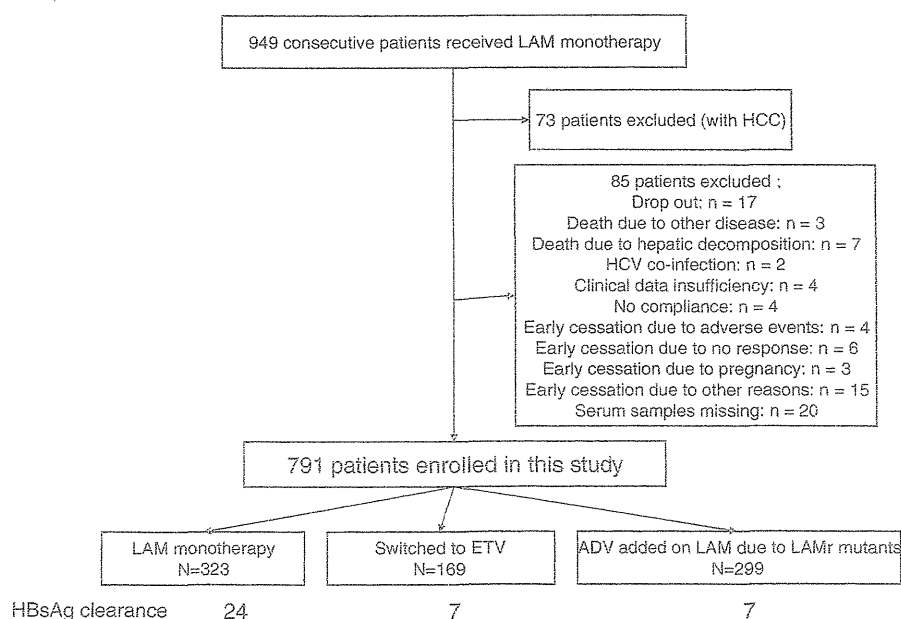
In order to evaluate this possibility empirically, we conducted a ten-year-long study in which we followed patients who received NA therapy initiated by the administration of LAM. We evaluated the resulting clearance and longitudinal declines of HBsAg using highly sensitive assays. Our aim was to determine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

Methods

Study population

Over a period of 12 years (September 1995 to September 2007), 949 consecutive patients who were chronically monoinfected with HBV (confirmed HBsAg positivity for at least six months), were treated with LAM monotherapy at the Department of Hepatology, Toranomon Hospital, Metropolitan Tokyo. The indication for antiviral therapy was abnormal ALT levels accompanying the increase in HBV DNA (over 4 log copies/mL) as a rule. However, in cases where ALT levels were normal, patients with advanced fibrosis were administered LAM. We did not treat patients without fibrosis who had low HBV DNA and normal ALT levels as a rule. We selected 791 patients for the final study after we had excluded all those who had been treated with LAM for <6 months, were co-infected with hepatitis C virus, had not provided sufficient serum samples, and/or had insufficient clinical records (Fig. 1). No patient was co-infected with human immunodeficiency virus in this cohort. Seven hundred ninety-one patients were enrolled in this cohort study. Of these 791 patients, 442 were HBeAg+ and 349 were HBeAg– at baseline. All analyses were performed after separating the HBeAg+ and HBeAg– cohorts. Written informed consent was obtained from each patient. The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki and was approved a priori by the institution's human research committee. This study has been registered in the University Hospital Medical Information Network Clinical Trials Registry (UMIN CTR) as the number UMIN000007993.

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



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

Results

Patient characteristics

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

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

Characteristics	All patients (<i>n</i> = 791)	HBeAg+ at baseline (<i>n</i> = 442)			HBeAg– at baseline (<i>n</i> = 349)		
		Persistently HBeAg+ (<i>n</i> = 424)	HBsAg seroclearance (<i>n</i> = 18)	<i>P</i>	Persistently HBeAg+ (<i>n</i> = 329)	HBsAg seroclearance (<i>n</i> = 20)	<i>P</i>
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 HBeAg+ (n = 424)	HBsAg seroclearance (n = 18)	P	Persistently HBeAg+ (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)	<i>P</i>	HBsAg clearance rate ratio (95 % CI)	<i>P</i>
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 ($\geq 4.5 \times$ ULN)	4.53 (1.68–12.2)	0.003		
ALT ($\geq 7.2 \times$ 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 ($< 1.2 \times 10^5$ /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 \rightarrow 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* values. Variables 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 $4.5 \times$ ULN for AST and $7.2 \times$ 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)	<i>P</i>	HBsAg clearance rate ratio (95 % CI)	<i>P</i>
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

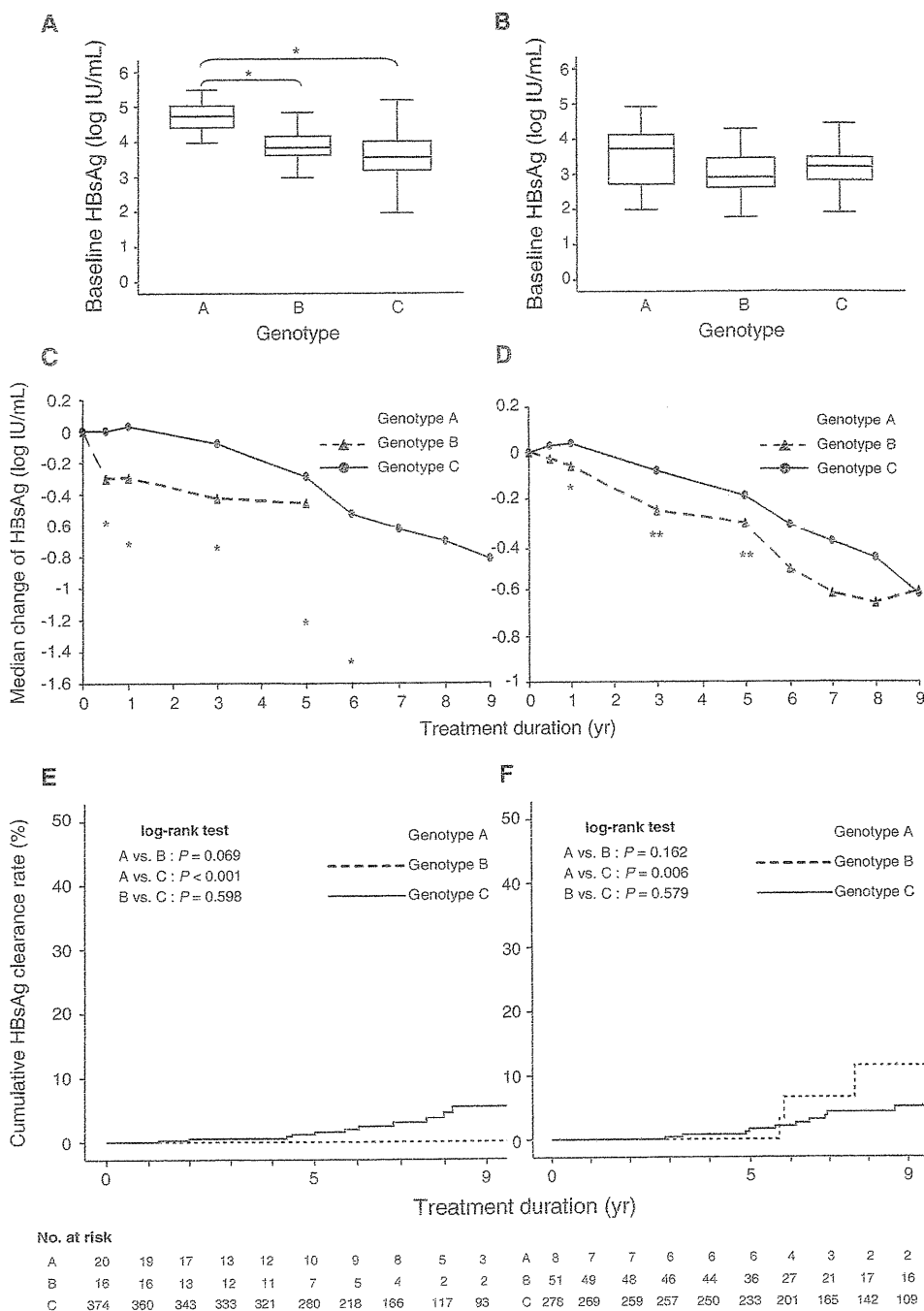
Variables analyzed in multivariate analysis: HBV genotype, baseline HBsAg, decline of HBsAg levels

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).

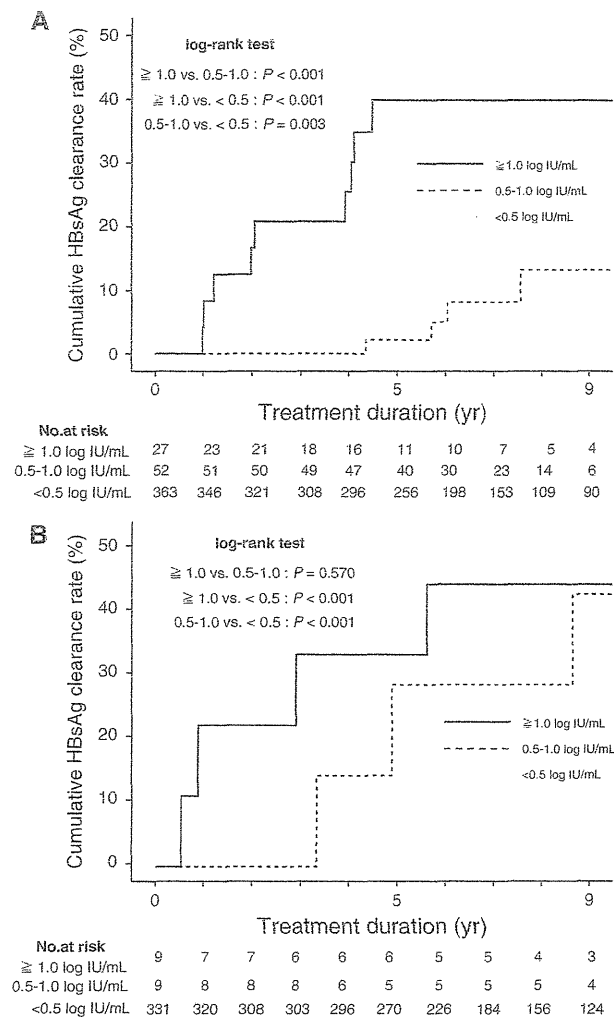


Fig. 3 a Kaplan–Meier life table showing cumulative HBsAg clearance rates in patients with varying rates of HBsAg decline within the first six months (HBeAg+ cohort). Clearance rates were highest in the rapid decline group, followed by the intermediate decline group and the slow or steady group (log-rank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

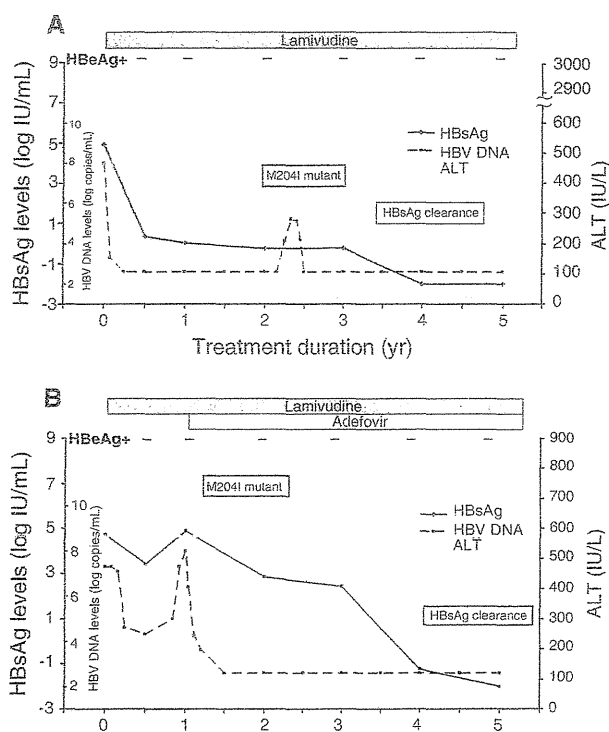


Fig. 4 Case presentation of the typical clinical and virological courses of two representative patients who achieved HBsAg clearance after VBT occurred. **a** Patient 1, a 45-year-old man who was HBeAg+ at baseline and had genotype A. **b** Patient 2, a 38-year-old man who was HBeAg+ at baseline and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

Acknowledgments This research was partly supported by grants from the Ministry of Health, Labour and Welfare of Japan.

Conflict of interest Dr. Kumada reports having received investigator, lecture, and consulting fees from Bristol-Myers Squibb, Dai-
nippon 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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Association of genes involved in bile acid synthesis with the progression of primary biliary cirrhosis in Japanese patients

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Received: 31 August 2012 / Accepted: 26 November 2012
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Abstract

Background Patients with primary biliary cirrhosis (PBC) exhibit a variety of clinical manifestations and patterns of disease progression. The aim of this study was to identify genetic determinants of PBC progression.

Methods A total of 52 tag single nucleotide polymorphisms (SNPs) of 11 candidate genes involved in regulating bile acid synthesis were analyzed by polymerase chain reaction (PCR)-restriction fragment length polymorphism, -high resolution melting curve analysis, or -direct DNA sequencing in 315 Japanese patients with PBC.

Results In this study, four tag SNPs of *CYP7A1* (rs1457043, rs8192870, rs3808607, and rs3824260), two

tag SNPs of *HNF4A* (rs6017340 and 6031587), and one SNP of *PPARGC1A* (rs8192678) showed a significant association with PBC progression. In addition, a dual luciferase assay revealed that the polymorphism of rs3808607 in *CYP7A1* altered the expression of *CYP7A1* in HepG2. Specifically, the *CYP7A1* promoter carrying the risk G allele for PBC progression induced higher expression of *CYP7A1* under both the normal and cholestatic conditions in vitro as compared to another promoter carrying the non-risk T allele.

Conclusion These results suggested that the genetic variants of *CYP7A1* and its transcriptional activators (*HNF4A* and *PPARGC1A*) may activate bile acid synthesis, resulting in the accumulation of bile acids in hepatocytes and eventually leading to the predisposition to PBC progression. Thus, the regulation of *CYP7A1* expression may represent an attractive therapeutic target for cholestatic liver diseases including PBC.

Electronic supplementary material The online version of this article (doi:10.1007/s00535-012-0730-9) contains supplementary material, which is available to authorized users.

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Keywords PBC progression · Bile acid synthesis ·
CYP7A1 · PGC-1 α · *HNF4 α*

Introduction

Primary biliary cirrhosis (PBC) is a chronic and slowly progressive liver disease characterized by immune-mediated destruction of the intrahepatic small bile ducts that leads to cholestasis, fibrosis, cirrhosis, and eventually liver failure. PBC is considered to be an organ-specific autoimmune disease because autoantibodies against mitochondrial and/or nuclear proteins are closely associated with its pathogenesis [1, 2]. Development of PBC is attributed to genetic predispositions and environmental triggers [2, 3]. Previous studies have shown that almost all of the

disease-susceptibility genes are immune-related, e.g., encoding human leukocyte antigens [4–6], cytotoxic T-lymphocyte antigen 4 (CTLA-4) [7–9], interleukin-12 α [5, 10], interleukin-12 receptor β 2 [5, 10], and interferon regulatory factor 5-transportin 3 locus [10, 11].

Although the etiology of PBC is due to the dysregulation of immune systems, a majority of PBC patients are treated with ursodeoxycholic acid (UDCA), a secondary bile acid comprising less than 5 % of endogenous bile acids. Clinical manifestations of PBC vary with respect to symptoms, course of progression, and response to treatment [3, 12]. Patients who respond to UDCA have a normal life expectancy, whereas those who do not are at risk for severe disease progression that could lead to cirrhosis and liver failure. Several genes related to PBC progression have been reported, including tumor necrosis factor α [7], solute carrier 4, anion exchanger 2 [7], and *CTLA-4* [13]. By contrast, there is scant information on the genes associated with PBC subphenotypes. To understand the genetic mechanisms underlying these subphenotypes and how they relate to disease progression is an unresolved problem for the clinical management of PBC.

UDCA acts on cholestatic liver diseases through multiple pharmacological actions, including: (1) decreasing the proportion of hydrophobic bile acids, which are toxic to cellular membranes, to the total amount of biliary bile acids; (2) preventing apoptosis of hepatic cells; and (3) positively modulating ductular bile flow by partially altering the expression of genes involved in bile acid homeostasis [14]. The accumulation of endogenous bile acids, such as chenodeoxycholic acid (CDCA), reduces the intrahepatocellular bile acids by repressing both their synthesis and their influx of circulating bile acids from the portal vein and hepatic vessels into hepatocytes (referred to as negative feedback regulation) [15]. At the same time, the accumulation of endogenous bile acids increases their detoxification and efflux to the bile ducts and systemic circulation [15].

From an etiological perspective of bile acid synthesis in hepatocytes, the expression of cholesterol 7 α -hydroxylase (*CYP7A1*), a rate-limiting bile acid synthetic enzyme that plays an important role in determining the size of the bile acid pool, is intricately controlled via multiple mechanisms [16]. Under normal conditions, hepatocyte nuclear factor 4 α (HNF4 α), a transcription factor, activates the expression of *CYP7A1* by interaction with peroxisome proliferator-activated receptor γ coactivator 1 α (PGC-1 α) [17, 18]. However, in the case of cholestasis, this transactivation is inhibited by bile acid-activated nuclear receptors and their downstream signals. Indeed, *CYP7A1* messenger RNA (mRNA) levels are decreased in the liver of patients with advanced PBC [19, 20]. Furthermore, UDCA also represses *Cyp7a1* expression in a rodent model [21]. Therefore, the

dysregulation of bile acid synthesis may contribute to severe PBC progression.

In order to dissect the mechanisms contributing to individual differences in PBC progression, we investigated whether polymorphisms of candidate target genes involved in bile acid synthesis and its regulatory pathways are associated with PBC progression in Japanese patients. In this study, we focused on the rate-limiting enzyme *CYP7A1* in the bile acid synthesis and its regulation pathways, which play an important role in bile acid homeostasis. Then, 11 candidate genes were selected as follows: (1) the bile acid synthetic enzyme, *CYP7A1* (encoded by *CYP7A1*); (2) activators of *CYP7A1* expression, e.g., HNF4 α (encoded by *HNF4A*) and PGC-1 α (encoded by *PPARGC1A*); and (3) repressors of *CYP7A1* expression, e.g., farnesoid X receptor (FXR; encoded by *NR1H4*), short heterodimer partner (SHP; encoded by *NROB2*), G protein pathway suppressor 2 (GPS2; encoded by *GPS2*), pregnane X receptor (PXR; encoded by *NR1I2*), fibroblast growth factor 19 (FGF19; encoded by *FGF19*), fibroblast growth factor receptor 4 (FGFR4; encoded by *FGFR4*), Klotho β (encoded by *KLB*), and forkhead box O1 (FOXO1; encoded by *FOXO1*).

Methods

Subjects

The cohort study consisted of 315 unrelated Japanese patients with PBC. The patients were registered in the PBC cohort study of the National Hospital Organization Study Group for Liver Disease in Japan (NHOSLJ) from August 1982 to September 2008. The time of entry was defined as the date of the initial PBC diagnosis. The study protocol was approved by the Ethics Committee dealing with the Human Genome and Gene Analysis at Nagasaki University and National Hospital Organization Nagasaki Medical Center, and written informed consent was obtained from each patient.

The patients were diagnosed with PBC if they met at least two of the following internationally accepted criteria [22]: biochemical evidence of cholestasis based upon alkaline phosphatase elevation, the presence of serum antimitochondrial antibodies, and histological evidence of nonsuppurative destructive cholangitis and destruction of the interlobular bile ducts. A liver biopsy was performed in 233 out of 315 patients at the initial diagnosis. Patients with acute or autoimmune hepatitis (alanine aminotransferase >200 IU/L, aspartate aminotransferase >200 IU/L), a maintenance dose of prednisolone >5 mg/body weight for concomitant autoimmune hepatitis, persistent hepatitis