polymorphic in the human genome, presumably in order to be able to respond to all potential foreign antigens [9].

Recently, many genome-wide association studies (GWAS) have been performed to seek associations between human genetic variation and the outcome of HBV infection [10–15]. Studies in the Japanese population showed that 11 single nucleotide polymorphisms (SNPs) located within or around the *HLA-DPA1* and *HLA-DPB1* loci are significantly associated with the occurrence of CHB. Of these 11 SNPs, the most strongly associated with the outcome of HBV infection were rs9277535 and rs3128917 in *HLA-DPB1* and rs3077 in *HLA-DPA1* [10].

Thereafter, GWAS studies in the Korean population confirmed the presence of these host factors related to HBV outcome and reported two new SNPs significantly associated with CHB within the *HLA* region, namely rs1419881 and rs652888 in transcription factor 19 (*TCF19*) and euchromatic histone-lysine methyltransferase 2 (*EHMT2*), respectively [16]. *TCF19* (or transcription factor SC1) is a *trans*-activating factor that mainly influences the transcription of genes required for late growth regulation at the G1-S checkpoint and during S phase [17]. *EHMT2* is a histone methyltransferase responsible for mono- and di-methylation of H3K9 (lysine at 9th residue of histone subunit 3) in euchromatin [18], which modifies the conformation of chromatin from its tightly packed form, heterochromatin, and thus influences gene repression or transcriptional silencing [19].

In the present study, we determined associations between the SNPs of *HLA-DPA1* (rs3077), *HLA-DPB1* (rs9277378 and rs3128917), *TCF19* (rs1419881) and *EHMT2* (rs652888) in HBV infected patients compared to those with resolved infections and those who had never been infected.

Materials and Methods

Ethics Statement

This study was approved by the Institutional Review Board of the Faculty of Medicine, University (Bangkok, Thailand) code IRB.455/54. Written informed consent was obtained from each patient and all samples were anonymized.

Sample Collection

All blood samples were negative for hepatitis C virus and human immunodeficiency virus. Subjects were defined into 4 groups: 230 hepatitis B surface antigen (HBsAg)-positive HCC, and 219 CHB who had been HBsAg-positive for at least 6 months were recruited at the King Chulalongkorn Memorial Hospital, whereas patients with resolved HBV and uninfected subjects were from the Thai Red Cross Society and from the north-eastern part of Thailand (age>40 years) which had been screened by Immunoassay (Architect i2000SR, Abbott, USA.) for HBsAg, antibody to hepatitis B surface antigen (anti-HBs) and antibody to hepatitis B core protein (anti-HBc). Of these subjects, 113 were negative for HBsAg but positive for anti-HBc and/or positive for anti-HBs after resolution of infection, while 123 uninfected subjects were all negative for HBsAg, anti-HBc and anti-HBs. All samples in this study were collected from subjects who have lived at the same area in Thailand, suggesting that the genetic background would be balanced between a case and control.

Genotyping assays

DNA was extracted from peripheral blood mononuclear cell using phenol-chloroform DNA extraction. The concentration of DNA was determined by NanoDrop 2000c spectrophotometer (Thermo Scientific, Wilmington, DE). We determined SNPs of *HLA-DPA1* (rs3077), *HLA-DPB1* (rs9277378 and rs3128917), and the genes *TCF19* (rs1419881) and *EHMT2* (rs652888) by commercial TaqMan PCR assays (Applied Biosystems, USA). In this study we investigated *HLA-DPB1* (rs9277378) because this SNP had a high level of linkage disequilibrium with rs9277535 ($D' = 1.00$, $R^2 = 0.954$) [20] and was clearly detectable by the TaqMan assay rather than rs9277535.

Statistical analyses

In this study, Hardy-Weinberg equilibrium was performed on each SNP. The Chi-square test of independence and Odds Ratio (OR) from two-by-two tables for comparisons between case and control groups was performed using Microsoft Excel. Statistical significance was defined by $P<0.05$. The calculated of possibility level was established using Chi-square contingency table analysis.

Results

Subjects were defined into 4 groups: group 1) HCC (age = 58.2±12 years, 190/230 (82.6%) male); group 2) CHB (age = 46.6±10 years, 144/219 (65.7%) male); group 3) those with resolved HBV (age = 48.2±6 years, 83/113 (73.5%) male); and group 4) HBV uninfected subjects (age = 46.7±6 years, 73/123 (59.3%) male). The details are given in Table 1. To find the genetic factor associated with chronicity of HBV infection, however, the two groups (group 1 and 2) were combined (designated "HBV carriers"). Indeed, according to the frequencies of minor alleles of the SNPs in the *HLA-DP*, *TCF19* and *EHMT2* genes listed in Table 2, the frequencies of minor alleles of these 5 SNPs in HCC and CHB were similar (data shown in Table S1). The composite HBV carriers group had a minor allele frequency for rs3077 and rs9277378 lower than in groups 3 and 4 (OR = 0.57, 95% CI = 0.42–0.78, $p<0.001$ and OR = 0.63, 95% CI = 0.47–0.85, $p = 0.008$ for rs3077, OR = 0.59, 95% CI = 0.44–0.81, $p = 0.001$ and OR = 0.56, 95% CI = 0.42–0.75, $p<0.001$ for rs9277378, respectively). In contrast, the minor allele frequency for rs1419881 in HBV carriers was similar to group 3 (OR = 0.80, 95% CI = 0.60–1.08, $p = 0.142$) but lower than in group 4 (OR = 0.64, 95% CI = 0.48–0.85, $p = 0.002$). Moreover, minor allele frequency for rs3128917 and rs652888 in HBV carriers was comparable to groups 3 and 4 (OR = 1.14, 95% CI = 0.85–1.53, $p = 0.371$ and OR = 1.06, 95% CI = 0.80–1.41, $p = 0.673$ for rs3128917; OR = 1.14, 95% CI = 0.84–1.55, $p = 0.400$ and OR = 1.12, 95% CI = 0.83–1.50, $p = 0.471$ for rs652888, respectively).

The results of Hardy-Weinberg equilibrium analysis of each SNPs were shown in Table 3. All data were over 0.01 ($p>0.01$), indicating that the frequencies did not deviate from Hardy-Weinberg equilibrium. The genotype distribution in HBV carriers compared to subjects with HBV resolution showed that both rs3077 and rs9277378 were significantly associated with protective effects against CHB in minor dominant model (OR = 0.45, 95% CI = 0.30–0.69, $p<0.001$ for rs3077 and OR = 0.47, 95% CI = 0.31–0.72, $p<0.001$ for rs9277378, are described in Table 3), suggesting that major homozygous genotypes were risk factors with the chronicity of HBV. The other SNPs rs3128917, rs1419881 and rs652888 were not associated against HBV carrier status (OR = 1.22, 95% CI = 0.76–1.97, $p = 0.413$ for rs3128917, OR = 0.67, 95% CI = 0.42–1.06, $p = 0.084$ for rs1419881 and OR = 1.31, 95% CI = 0.87–2.00, $p = 0.198$ for rs652888, respectively).

The genotype frequencies for 5 SNPs are shown in Table 3. Comparing HBV carriers with uninfected subjects showed that rs3077, rs9277378 and rs1419881 were all protectively associated with chronic HBV infection (OR = 0.63, 95% CI = 0.42–0.95,

Table 1. Characteristics of participants in HCC, CHB, resolved HBV and HBV uninfected subjects in Thailand.

	HCC (n = 230)	CHB[a] (n = 219)	Resolved[b] (n = 113)	Uninfected[c] (n = 123)
Age (years)	58.2±12	46.6±10	48.2±6	46.7±6
Male	190 (82.6%)	144 (65.7%)	83 (73.5%)	73 (59.3%)
HBsAg positive	230 (100%)	219 (100%)	0	0
ALT>40 (IU/L)	43 (18.7%)	61 (27.8%)	-	-
Alb (g/dl)	3.7 (2.5–5.6)	4.5 (3–5.2)	-	-
TB (mg/dl)	1.2 (0.17–14.8)	0.56 (0.2–2.67)	-	-

Abbreviation: HCC, hepatocellular carcinoma; CHB, chronic hepatitis B; HBsAg, hepatitis B surface antigen; ALT, Alanine transaminase; Alb, Albumin; TB, Total bilirubin.
[a]Defined as chronic hepatitis B includes chronic HBV infection but not cirrhosis and HCC.
[b]Defined as HBsAg negative but anti-HBc or/and anti-HBs positive.
[c]Defined as any HBV serological markers negative.
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$p = 0.025$ for rs3077 and OR = 0.55, 95% CI = 0.36–0.82, $p = 0.003$ for rs9277378 and OR = 0.57, 95% CI = 0.36–0.90, $p = 0.015$ for rs1419881, respectively). Comparing HBV carriers and uninfected subjects rather than those with resolved infection regarding rs1419881 was significantly protective association against CHB, but rs3128917 and rs652888 were not associated against CHB (OR = 1.58, 95% CI = 1.02–2.46, $p = 0.042$ for rs3128917 and OR = 1.09, 95% CI = 0.65–1.82, $p = 0.080$ for rs652888). When we consider the Bonferroni corrections (5 SNPs), however, the P value for rs1419881 did not reach the level of significant difference (0.015>0.05/5) between HBV carriers and HBV uninfected subjects. These data suggested that other SNPs, rs1419881, rs3128917 and rs652888 were not associated with HBV carriers in this study.

Results of meta-analysis for 3 SNPs (rs3077, rs9277378 and rs3128917) in the *HLA* gene were shown in Table S2 and S3; HBV carriers were compared to HBV resolved or HBV uninfected subjects, respectively. While the other 2 SNPs were published only from Korean population, thus the meta-analysis appeared only between HBV carriers and HBV uninfected subjects. All SNPs analyzed by the meta-analysis were significantly associated with HBV carriers.

The associations between these 5 SNPs and HBV status are depicted graphically in Figure S1. Each histogram compares HBV carriers with subjects that have resolved HBV infection or were never infected. The results showed that the minor dominant model of rs3077 and rs9277378 was highly protective associated against chronic HBV, while no significant associations were observed with rs3128917 and rs652888. Furthermore, comparing the frequency of rs1419881 between HBV carriers and uninfected subjects also revealed its association against chronic HBV infection but the association with resolved HBV did not achieve statistical significance.

Discussion

Genetic variations of rs3077 and rs9277378, but not rs3128917, rs1419881 and rs652888, were significantly associated with HBV carriers relative to resolved HBV in Thai population. In the human genome, single nucleotide polymorphisms are found in every 300–570 nucleotides. Many SNPs have no effect on the function of the encoded proteins, but some variants do appear in regulatory or coding part of the gene and affect gene expression level or protein function which can give rise to disease [21] such as the 3 SNPs including rs3077, rs9277378 and rs3128917 in *HLA-*

Table 2. Minor allele frequencies in HBV carriers, resolved HBV and uninfected subjects in Thailand.

						HBV carriers vs. Resolved		HBV carriers vs. Uninfected	
SNPs	Gene	Minor alleles[a]	HBV carriers[b] (2n = 898)	Resolved (2n = 226)	Uninfected (2n = 246)	OR (95% CI)	*P* values	OR (95% CI)	*P* values
rs3077	*HLA-DPA1*	T	227 (25.3%)	84 (37.2%)	86 (35.0%)	0.57 (0.42–0.78)	<0.001	0.63 (0.47–0.85)	0.008
rs9277378	*HLA-DPB1*	A	237 (26.4%)	85 (37.6%)	96 (39.0%)	0.59 (0.44–0.81)	0.001	0.56 (0.42–0.75)	<0.001
rs3128917	*HLA-DPB1*	G	459 (51.1%)	108 (47.8%)	122 (49.6%)	1.14 (0.85–1.53)	0.372	1.06 (0.80–1.41)	0.673
rs1419881	*TCF19*	C	361 (40.2%)	103 (45.6%)	126 (51.2%)	0.80 (0.60–1.08)	0.142	0.64 (0.48–0.85)	0.002
rs652888	*EHMT2*	C	329 (36.6%)	76 (33.6%)	84 (34.1%)	1.14 (0.84–1.55)	0.400	1.11 (0.83–1.50)	0.478

Abbreviation: CI, confidence interval; OR, odds ratio.
[a]Defined by using data from public database (NCBI).
[b]Defined as the combination between HCC and CHB.
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Table 3. Genotype frequencies in HBV carriers, resolved HBV and uninfected subjects in Thailand.

					HBV carriers vs. Resolved		HBV carriers vs. Uninfected	
SNP	**Genotype**	**HBV carriers[a] (n = 449)**	**Resolved (n = 113)**	**Uninfected (n = 123)**	**OR (95% CI)**	***P* values**	**OR (95% CI)**	***P* values**
rs3077	CC	259 (57.7%)	43 (38.1%)	57 (46.3%)	1.00	-	1.00	-
HLA-DPA1	CT	153 (34.1%)	56 (49.6%)	46 (37.4%)	0.45 (0.29–0.71)	<0.001	0.73 (0.47–1.13)	0.161
	TT	37 (8.2%)	14 (12.4%)	20 (16.3%)	0.44 (0.22–0.88)	0.018	0.41 (0.22–0.75)	0.003
	Dominant[b]				**0.45 (0.30–0.69)**	**<0.001**	**0.63 (0.42–0.95)**	**0.025**
	HWEp	0.038	0.516	0.049				
rs9277378	GG	242 (53.9%)	40 (35.4%)	48 (39.0%)	1.00	-	1.00	-
HLA-DPB1	AG	177 (39.4%)	61 (54.0%)	54 (43.9%)	0.48 (0.31–0.75)	0.001	0.65 (0.42–1.00)	0.051
	AA	30 (6.7%)	12 (10.6%)	21 (17.1%)	0.41 (0.20–0.87)	0.018	0.28 (0.15–0.54)	<0.001
	Dominant				**0.47 (0.31–0.72)**	**<0.001**	**0.55 (0.36–0.82)**	**0.003**
	HWEp	0.757	0.110	0.390				
rs3128917	TT	99 (22.0%)	29 (25.7%)	38 (30.9%)	1.00	-	1.00	-
HLA-DPB1	TG	241 (53.7%)	60 (53.1%)	48 (39.0%)	1.18 (0.71–1.94)	0.525	1.93 (1.19–3.13)	0.008
	GG	109 (24.3%)	24 (21.2%)	37 (30.1%)	1.33 (0.73–2.44)	0.355	1.13 (0.67–1.92)	0.648
	Dominant				**1.22 (0.76–1.97)**	**0.413**	**1.58 (1.02–2.46)**	**0.042**
	HWEp	0.117	0.496	0.015				
rs1419881	TT	162 (36.1%)	31 (27.4%)	30 (24.4%)	1.00	-	1.00	-
TCF19	TC	213 (47.4%)	61 (54.0%)	60 (48.8%)	0.67 (0.41–1.08)	0.097	0.66 (0.41–1.07)	0.088
	CC	74 (16.5%)	21 (18.6%)	33 (26.8%)	0.67 (0.36–1.25)	0.210	0.42 (0.24–0.73)	0.002
	Dominant				**0.67 (0.42–1.06)**	**0.084**	**0.57 (0.36–0.90)**	**0.015**
	HWEp	0.778	0.349	0.792				
rs652888	TT	169 (37.6%)	50 (44.2%)	57 (46.3%)	1.00	-	1.00	-
EHMT2	TC	231 (51.4%)	50 (44.2%)	48 (39.0%)	1.37 (0.88–2.12)	0.162	1.62 (1.05–2.50)	0.027
	CC	49 (10.9%)	13 (11.5%)	18 (14.6%)	1.12 (0.56–2.22)	0.756	0.92 (0.49–1.70)	<0.001
	Dominant				**1.31 (0.87–2.00)**	**0.198**	**1.09 (0.65–1.82)**	**0.080**
	HWEp	0.022	0.926	0.142				

Abbreviation: CI, confidence interval; OR, odds ratio ; HWEp, Hardy-Weinberg equilibrium analysis.
[a]Defined as the combination between HCC and CHB.
[b]Defined as a minor dominant according to the comparison between heterozygous+minor homozygous genotype and major homozygous genotype (*eg.* rs3077; CT+TT vs. CC).
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DP region of MHC class II. The function of HLA-DP is to present bound peptide antigens, e.g. from HBV, at the surface of antigen-presenting cells. CD4+ T cells recognize these antigens and initiate the adaptive immune response. They assist the MHC class I-restricted CD8+ T cells which are the primary cellular effectors mediating HBV clearance from the liver during acute viral infection [22]. HBV infection will either be cleared by these means, or establish itself as a chronic infection. The reason for the latter is unclear but may be related to variation of *HLA-DP* alleles. Thus, the position of *HLA-DP* SNPs might be associated with possibility of clearance or chronicity. The rs3077 and rs9277535 SNPs are located within the 3′ untranslated region (UTR) of *HLA-DPA1* and *HLA-DPB1*, respectively while rs3128917 is located downstream of *HLA-DPB1*.

Recent investigations have identified 11 risk alleles for CHB related to mRNA expression of *HLA-DPA1* and *HLA-DPB1* [23]. The results showed that only these two alleles, rs3077 and rs9277535 were strongly associated with the risk of CHB and decreased expression of *HLA-DPA1* and *HLA-DPB1*, respectively. In contrast, while rs3128917 was associated with CHB, it was not associated with the level of HLA-DPB1 expression [23]. Variation at 5′ and 3′ UTRs can alter the binding sites of regulatory proteins which protect and stabilize newly synthesized RNA, either increasing or decreasing binding [24,25]. Nevertheless, the present study showed that rs3128917 was not associated with HBV carrier status in Thailand. Because rs3128917 is located downstream of the direction of transcription of the gene, this suggests that it does not affect regulation or coding of the gene and would have no effect on HLA protein expression.

The results from the present study not only establish the importance of variation at the *HLA-DP* gene but also explore two new SNPs, rs1419881 located in *TCF19* and rs652888 in the *EHMT2* gene [16]. *TCF19* (or transcription factor SC1) is a late growth regulatory gene like histone, thymidine kinase etc, maximally expressed at the onset of DNA synthesis at the G1-S boundary and S phase of cell cycle. This protein is also involved in regulations of growth and transcription factors controlling the number and development of peripheral-blood monocytes and erythrocytes [26]. The *EHMT2* gene is a histone methyltransferase [18] mainly responsible for mono- and di-methylation of H3K9 in euchromatin. This changes the conformation of chromatin from euchromatin to heterochromatin and then affects gene repression

[19]. Histone methylation has a critical role in gene transcription and epigenetic events [27–30].

According to recently published GWAS data [11], two SNPs associated with the risk for CHB in the Korea population were identified. These were the top signals in the genome-wide significance level analysis and were independently associated with *HLA-DP* and *HLA-DQ*, respectively. The authors then confirmed the results in a replication sample, showing that the frequency of their two SNPs strongly associated with CHB; OR = 0.76, 95% CI = 0.68–0.86, $p = 4.51E\text{-}11$ for rs1419881 and OR = 1.26, 95% CI = 1.07–1.47, $p = 2.78E\text{-}06$ for rs652888 [16]. Furthermore, another GWAS study focused on HLA, of hepatitis B vaccinated people in Indonesia, showed that rs652888 was also associated with risk of CHB ($p \leq 0.0001$) in that population [31].

In the present study, however, we found that rs1419881 tended to be associated with chronic HBV infection, based on the results of a comparison between HBV carriers and uninfected subjects. Nonetheless, it did not reach the significance by the Bonferroni corrections, as well as when HBV carriers were compared with patients who had their HBV infection resolved, no association with rs1419881 was observed. The second SNP, rs652888, was not associated with chronic HBV infection in the Thai population. Although our study had sampling error due to small samples, it might be another effect that the result between rs652888 in *EHMT2* gene and chronic hepatitis B in Thai population was not associated. The reason for these negative findings for the two SNPs might be due to the affected gene functions that were not involved with the immune system or processes of persistent infection. Data supporting this notion are to be found in the GWAS data for the Korean population, where pathway analysis of genes involved in the regulation of immune function showed that *TCF19* and *EHMT2* genes are not significantly involved in human immunity [16].

Mapping the position of the two new SNPs showed that rs1419881 located at the 3′ UTR of exon 4, with a tendency towards association with CHB and rs652888 which is not associated with CHB located on an intron. The position of each SNP might affect the phenotype of gene expression and susceptibility to disease, explaining why some are associated with chronic HBV infection, and others not. According to previous publications, the 3′ UTR of the *HLA-DP* region is strongly involved with regulating HLA-DP expression and influences the outcome of HBV infection [32]. In addition, another study showed that variation of the 3′ UTR of HLA-C was strongly associated with HLA-C expression levels and with control of human immunodeficiency virus [33]. This illustrated the general principle that the position of SNPs affects association with diseases.

The prevalence of HBV in Eastern countries, i.e. Asia, sub-Saharan Africa and the Pacific is much higher than in Western Europe and America. Most people in Eastern countries are infected with HBV during childhood and 8–10% of these develop CHB. In contract, the frequency of chronic carriers in Western Europe and North America is ≤1%. Furthermore, previous GWAS and meta-analysis reported that A alleles at rs3077 and rs9277353 have protective effects against CHB. Asian and African populations, especially Chinese, have lower frequencies of A alleles than European and American populations [10,34,35]. Moreover, the previous study showed no associations of rs3077 and rs9277535 with progressive CHB infection; however rs3077 was highly significant associated with HBV infection but not associated with rs9277353 in Caucasian populations [36].

While the frequency of alleles at rs3128917 and rs1419881 in Asian and African populations are quite similar, Northern and Western European populations have high frequencies of the protective T allele at rs3128917 but have low T allele frequencies (a risk allele for CHB) at rs1419881. The allele frequencies of populations in the worldwide for conspicuous details came from dbSNP Short Genetic Variations available at http://www.ncbi.nlm.nih.gov/projects/SNP/snp_ref.cgi. Lastly, both ethnic Eastern and Western populations have similar allele frequencies at rs652888, carrying a risk for CHB, with T allele frequencies very much higher than C allele frequencies, which has a protective effect. In addition, evolution of genomic characteristics, the migratory history of different populations, as well as HBV genotypes [37], HBV carrier rate [38] and pathological procession of liver disease [39] in each country may affect the distribution of *HLA* alleles. This was illustrated by a recent report in two Han Chinese populations (southern and northern) having different distributions of *HLA-DP* genes [39]. Thus, the genetics of the host is one of the factors influencing and predicting disease outcome [40].

According to less number of samples, it might influence statistical power in this study. Thus, we made another statistic meta-analysis of data obtained from previous reports and this study in Table S3. We compared HBV carriers with HBV uninfected subjects, because most previous studies also compared CHB with HBV clearance and/or healthy (negative for any HBV serological markers). Interestingly, all SNPs analyzed by the meta-analysis were significantly associated with HBV carriers. These results could support our data in Thailand. Additionally, no heterogeneity was observed between HBV carriers and HBV-resolved subjects ($P_{het} = 0.10$ for rs3077, 0.79 for rs9277378, and 0.07 for rs3128917), as well as between HBV carriers and HBV uninfected subjects ($P_{het} = 0.10$ for rs3077, 0.02 for rs9277378, 0.91 for rs1419881, and 0.04 for rs652888) except for rs9277378 ($P_{het} = 0.000$), for the minor allele frequency (MAF) of only rs9277378 was different between HapMap-CHB (MAF = 46.3% of G allele) and HapMap-JPT (MAF = 44.8% of T allele).

In the present study, we determined associations of variations at the *HLA-DP* gene with outcome in HBV infected Thai patients and the major homozygous genotypes of rs3077 and rs9277378, but not rs3128917, were significantly associated with HBV carrier status. Although genetic variation of two new SNPs, rs1419881 in the *TCF19* gene and rs652888 in the *EHMT2* gene, were not associated with the outcome of HBV infection in the Thai population, a large-scale study should be required.

Supporting Information

Figure S1 Association of 5 SNPs with HBV carriers, resolved HBV and uninfected subjects in Thailand. The results were compared between percentages of combination of heterozygous genotypes and minor homozygous genotypes (White square) with percentages of major homozygous genotypes (Grey square). Five SNPs applied in this study were rs3077, rs9277378 and rs3128917 in *HLA-DP* gene, rs1419881 in *TCF19* gene and rs652888 in *EHMT2* gene. OR, odds ratio; (lower-upper), 95% confidence interval.
(PPTX)

Table S1 Minor allele frequencies in HCC, CHB, resolved HBV and uninfected subjects in Thailand.
(DOC)

Table S2 The meta-analysis of minor allele frequencies in HBV carriers and resolved HBV.
(DOC)

Table S3 The meta-analysis of minor allele frequencies in HBV carriers and uninfected subject.
(DOC)

Author Contributions

Conceived and designed the experiments: SP TW YP YT. Performed the experiments: NP. Analyzed the data: NP SP SI KM NS. Contributed reagents/materials/analysis tools: PT SO SM. Wrote the paper: NP.

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Impact of alpha-fetoprotein on hepatocellular carcinoma development during entecavir treatment of chronic hepatitis B virus infection

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Abstract

Background Entecavir (ETV) is one of the first-line nucleoside analogs for treating patients with chronic hepatitis B virus (HBV) infection. However, the hepatocellular carcinoma (HCC) risk for ETV-treated patients remains unclear.

Methods A total of 496 Japanese patients with chronic HBV infection undergoing ETV treatment were enrolled in this study. The baseline characteristics were as follows: age 52.6 ± 12.0 years, males 58 %, positive for hepatitis B e antigen 45 %, cirrhosis 19 %, and median HBV DNA level 6.9 log copies (LC) per milliliter. The mean treatment duration was 49.9 ± 17.5 months.

Results The proportions of HBV DNA negativity (below 2.6 LC/mL) were 68 % at 24 weeks and 86 % at 1 year, and the rates of alanine aminotransferase (ALT) level normalization were 62 and 72 %, respectively. The mean serum alpha-fetoprotein (AFP) levels decreased significantly at 24 weeks after ETV treatment initiation (from 29.0 ± 137.1 to 5.7 ± 27.9 ng/mL, $p < 0.001$). The cumulative incidence of HCC at 3, 5, and 7 years was 6.0, 9.6, and 17.2 %, respectively, among all enrolled patients. In a multivariate analysis, advanced age [55 years or older, hazard ratio (HR) 2.84; $p = 0.018$], cirrhosis (HR 5.59, $p < 0.001$), and a higher AFP level (10 ng/mL or greater) at 24 weeks (HR 2.38, $p = 0.034$) were independent risk

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factors for HCC incidence. HCC incidence was not affected by HBV DNA negativity or by ALT level normalization at 24 weeks.

Conclusions The AFP level at 24 weeks after ETV treatment initiation can be the on-treatment predictive factor for HCC incidence among patients with chronic HBV infection.

Keywords Hepatitis B virus · Entecavir · Risk factors for hepatocellular carcinoma incidence · Alpha-fetoprotein

Abbreviations

AFP	Alpha-fetoprotein
ALT	Alanine aminotransferase
cccDNA	Covalently closed circular DNA
ETV	Entecavir
HBV	Hepatitis B virus
HCV	Hepatitis C virus
HCC	Hepatocellular carcinoma
IFN	Interferon
NA	Nucleos(t)ide analog
ROC	Receiver operating characteristic

Introduction

More than 350 million people worldwide have hepatitis B virus (HBV) infection, and persistent hepatic damage following HBV infection is associated with liver disease progression [1–3]. Chronic HBV infection accounts for approximately 52.3 % of hepatocellular carcinoma (HCC) cases worldwide [4], and antiviral treatment such as interferon (IFN) or nucleos(t)ide analogs (NAs) that aims to improve the prognosis of patients with chronic HBV infection has been developed [5]. Entecavir (ETV), one of the first-choice NAs, is a more potent antiviral agent with a higher genetic barrier to resistance than lamivudine; ETV administration over the long term has been reported to enable most patients to maintain a state of viral suppression [6–9]. With regard to the suppressive effect of NAs on HCC, in a randomized controlled trial of patients who were treated with lamivudine or placebo, the lamivudine-treatment group showed a significantly lower HCC rate than the placebo group during the observation period of 32.4 months (3.9 % vs 7.4 %, $p = 0.047$) [10]. In other cohort studies of patients who were treated with lamivudine, HCC incidence has been reported to be significantly lower in those who maintained low HBV DNA levels [less than 4 or 5 log copies (LC) per milliliter], especially in those with cirrhosis [11–13]. In contrast, the suppressive effect of ETV on HCC incidence remains unclear because a randomized controlled study of patients treated with ETV or placebo has not been performed.

To date, many studies have assessed the relationship between clinical factors and HCC incidence, such as male gender, advanced age, presence of cirrhosis, and high HBV DNA levels, during the natural course of chronic HBV infection [14, 15]. Among patients who were treated with IFN, it has been reported that hepatitis B e antigen seroconversion achieved with IFN treatment was associated with lower HCC incidence rates compared with nonseroconversion [16]. However, neither the pretreatment factors nor the on-treatment factors that are associated with HCC incidence among patients receiving ETV have been fully examined. ETV treatment for patients with chronic HBV infection reduces serum HBV DNA levels and may also have anti-inflammatory and antineoplastic effects. That is, among patients receiving ETV, various factors, such as HBV DNA, alanine aminotransferase (ALT), total bilirubin, albumin, and alpha-fetoprotein (AFP) levels, have the possibility to change and be associated with HCC suppression.

In this study, we evaluated the risk factors for HCC, especially the on-treatment factors in patients with chronic HBV infection who were undergoing ETV treatment.

Patients and methods

Study population

This study was a retrospective, multicenter study conducted by Osaka University Hospital and other institutions

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that participate in the Osaka Liver Forum. A total of 840 NA-naïve patients chronically infected with HBV started treatment with 0.5 mg of ETV per day between July 2004 and July 2012. Of these patients, we excluded 51 patients with HBV DNA levels under 3 LC/mL at the baseline, 13 patients who were co-infected with hepatitis C virus (HCV) or with human immunodeficiency virus, one patient who had undergone liver transplantation, and 140 patients with a history of HCC at the baseline. In addition, we excluded 51 patients who had been treated with ETV for less than 1 year and 88 patients who developed HCC within 1 year after the initiation of ETV treatment. As a result, 496 patients were enrolled in this cohort study. This study was conducted according to the ethical guidelines of the Declaration of Helsinki, amended in 2002, and was approved by the Institutional Review Board of Osaka University Hospital (approval number 12380-2).

HCC surveillance and data collection

The patients were followed up once every 3–6 months, and clinical symptoms, HBV DNA and other virological markers, complete blood count, liver biochemistry, and AFP levels were assessed. AFP levels measured between 20 and 28 weeks from the initiation of ETV treatment were regarded as valid AFP levels at 24 weeks. Ultrasonography of the abdomen, computed tomography, and/or magnetic resonance imaging was performed every 3–6 months for HCC surveillance. HCC was diagnosed by the presence of typical hypervascular characteristics evident on the computed tomography and/or magnetic resonance imaging scans. If no typical signs of HCC were observed, either hepatic angiography or fine-needle aspiration biopsy was performed with the patient's consent, or the patient was carefully followed until a diagnosis was possible on the basis of a definite observation. Liver cirrhosis was defined by a shrunken, small liver with a nodular surface as noted on liver imaging and by clinical features of portal hypertension.

Definition of treatment response

The surveillance start date was defined as the time of ETV treatment initiation. HBV DNA was measured by the COBAS Amplicor HBV Monitor Test (Roche Diagnostics, Tokyo, Japan) with a linear range of detection from 2.6 to 7.6 LC/mL or by the COBAS Taqman HBV Test v2.0 (Roche Diagnostics) with a linear range of detection from 2.1 to 9.0 LC/mL. The achievement of a virological response by ETV treatment was defined by serum HBV DNA levels that were continuously under 2.6 LC/mL. ALT level normalization was defined by serum ALT levels that were 30 IU/L or less.

Statistical analyses

Statistical analyses were performed using SPSS version 19.0 (IBM, Armonk, NY, USA) and SAS for Windows version 9.3 (SAS Institute, Cary, NC, USA). The continuous variables were expressed as the mean ± standard deviation or standard error of the mean or as the median (range), as appropriate, whereas the categorical variables were expressed as frequencies. The Wilcoxon signed-rank sum test was used to analyze differences between continuous variables before and after treatment. The cutoff value of AFP levels at 24 weeks from the initiation of ETV treatment for prediction of HCC incidence was assessed by the time-dependent receiver operating characteristic (ROC) curve, and the 95 % confidence interval for the area under the ROC curve was constructed using the bootstrap method. The Kaplan–Meier method was used to assess the cumulative HCC incidence, and the groups were compared using the log-rank test. The Cox proportional-hazards model was used to identify the independent factors associated with HCC incidence. The factors that were selected as significant by simple Cox regression analysis were evaluated by multiple Cox regression analysis. The risks were expressed as hazard ratios and 95 % confidence intervals. We considered $p < 0.05$ as significant.

Results

The characteristics of the 496 patients at the baseline and at 24 weeks after ETV treatment initiation are summarized in Table 1. The average age of the patients was 52.6 ± 12.0 years at the baseline, and there were 288 males (58 %) and 92 patients with cirrhosis (19 %). The patients were followed up for an average of 49.9 ± 17.5 months.

The cumulative incidence of virological response (HBV DNA level less than 2.6 LC/mL) at 24 weeks, 1 year, and 3 years after the initiation of ETV treatment was 68, 86, and 95 %, respectively. The median levels of HBV DNA were significantly decreased among noncirrhotic (6.9 LC/mL to less than 2.6 LC/mL, $p < 0.001$) and cirrhotic (6.9 LC/mL to less than 2.6 LC/mL, $p < 0.001$) patients from the baseline to 24 weeks after ETV treatment initiation (Table 1). ALT level normalization (30 IU/L or lower) was achieved in 62 % of patients at 24 weeks and in 72 % of patients at 1 year. The median ALT levels were significantly decreased among noncirrhotic (72.0–25.0 IU/L, $p < 0.001$) and cirrhotic (51.0–29.0 IU/L, $p < 0.001$) patients from the baseline to 24 weeks after ETV treatment initiation. The following parameters were also significantly increased from the baseline to 24 weeks after ETV treatment initiation: platelet counts and serum albumin levels

Table 1 Characteristics of patients at the baseline and 24 weeks after initiation of entecavir (ETV) treatment

	All patients, $n = 496$		Noncirrhotic patients, $n = 404$		Cirrhotic patients, $n = 92$	
	Baseline	24 weeks	Baseline	24 weeks	Baseline	24 weeks
Age (years)	52.6 ± 12.0 (15–82)		51.3 ± 12.1 (15–82)		58.2 ± 9.8 (32–81)	
Gender: male/female	288/208 (58 %)		233/171 (58 %)		55/37 (60 %)	
HBeAg[a]: positive/negative	220/270 (45 %)		181/219 (45 %)		39/51 (43 %)	
Histology[b], activity: A0/1/2/3	3/82/74/14		3/75/63/12		0/7/11/2	
Histology[b], fibrosis: F0/1/2/3/4	8/63/51/32/20		8/63/52/32/0		0/0/0/0/20	
History of IFN therapy: presence	50 (11 %)		44 (11 %)		6 (7 %)	
Platelet count ($\times 10^4/\mu L$)	16.0 ± 5.8	16.5 ± 6.4*	17.3 ± 5.2	17.7 ± 5.3*	10.3 ± 5.8	11.5 ± 7.9
Total bilirubin (mg/dL)	1.01 ± 1.48	0.83 ± 0.45*	0.91 ± 0.95	0.78 ± 0.42*	1.45 ± 2.78	1.09 ± 0.48
Albumin (g/dL)	3.94 ± 0.52	4.11 ± 0.44*	4.03 ± 0.44	4.18 ± 0.39*	3.56 ± 0.64	3.79 ± 0.50*
PT (%)	83.8 ± 16.3		86.7 ± 15.7		72.4±16.3	
ALT (IU/L)	143.7 ± 199.3 (9–1,885)	29.6 ± 16.5* (6–166)	156.1 ± 210.8 (9–1,885)	29.2 ± 16.9* (6–166)	89.2 ± 124.7 (12–763)	31.5 ± 14.0* (10–84)
ALT ≤ 30 (IU/L)	11 %	62 %	10 %	64 %	13 %	53 %
30 < ALT ≤ 60 (IU/L)	31 %	33 %	28 %	31 %	48 %	43 %
60 < ALT (IU/L)	58 %	5 %	62 %	5 %	39 %	4 %
HBV DNA (LC/mL) (median)	6.9	<2.6*	6.9	<2.6*	6.9	<2.6*
HBV DNA < 2.6 (LC/mL)	–	68 %	–	68 %	–	70 %
2.6 ≤ HBV DNA < 4.0 (LC/mL)	4 %	24 %	4 %	21 %	3 %	30 %
4.0 ≤ HBV DNA (LC/mL)	96 %	8 %	96 %	11 %	97 %	0 %
AFP (ng/mL)[c]	29.0 ± 137.1 (1–2,225)	5.7 ± 7.9* (1–126)	29.5 ± 152.7 (1–2,225)	4.9 ± 4.6* (1–126)	27.4 ± 48.0 (1–318)	9.3 ± 14.6* (1–52)
Observation periods (months)	49.9 ± 17.5 (14–109)		49.2 ± 17.6 (14–109)		52.8 ± 16.6 (18–82)	

Data are expressed as the mean ± standard deviation except for hepatitis B virus (*HBV*) DNA (median)

AFP alpha-fetoprotein, *ALT* alanine aminotransferase, *HBeAg* hepatitis B e antigen, *IFN* interferon, *LC* log copies, *PT* prothrombin time

* $p < 0.05$ (Wilcoxon signed-rank sum test)

[a] HBeAg measurement at the baseline was missing in six patients

[b] Liver biopsy was performed in 174 patients

[c] AFP data were missing in 78 noncirrhotic patients and five cirrhotic patients with cirrhosis

among noncirrhotic patients ($p = 0.008$ and $p < 0.001$, respectively) and serum albumin levels in cirrhotic patients ($p < 0.001$).

Mean serum AFP levels decreased significantly from 29.0 ± 137.1 ng/mL at the baseline to 5.7 ± 7.9 ng/mL at 24 weeks after the initiation of ETV treatment ($p < 0.001$). Mean AFP levels were assessed according to the severity of liver disease and decreased significantly from the baseline to 24 weeks in both the noncirrhotic group and the cirrhotic group (noncirrhotic group 29.5 ± 152.7 to 4.9 ± 4.6 ng/mL, $p < 0.001$; cirrhotic group 27.4 ± 48.0 to 9.3 ± 14.6 ng/mL, $p < 0.001$; Table 1). The proportion of patients with AFP levels below 10 ng/mL increased from 73 % at the baseline to 95 % at 24 weeks among noncirrhotic patients and from 48 % at the baseline to 76 % at 24 weeks among cirrhotic patients (Fig. 1).

A total of 42 patients developed HCC during the observation period (16 noncirrhotic patients, 26 cirrhotic patients). The cumulative incidence of HCC at 3, 5, and 7 years was 6.0, 9.6, and 17.2 %, respectively. The mean time point of HCC development was 34.0 ± 18.4 months from the initiation of ETV treatment. AFP levels among patients who developed HCC decreased from 24 weeks (13.1 ± 3.9 ng/mL) (mean ± standard error of the mean) to 48 weeks (10.2 ± 3.0 ng/mL) after the initiation of ETV treatment and increased again from 24 weeks before HCC incidence (7.6 ± 1.6 ng/mL) to the time of HCC incidence (35.4 ± 12.8 ng/mL) (Fig. S1). The cutoff value of AFP levels at 24 weeks from the initiation of ETV treatment for prediction of HCC incidence was set as 10 ng/mL on the basis of the calculated cutoff value (12.1 ng/mL) assessed using the time-dependent ROC curve (Table S1).

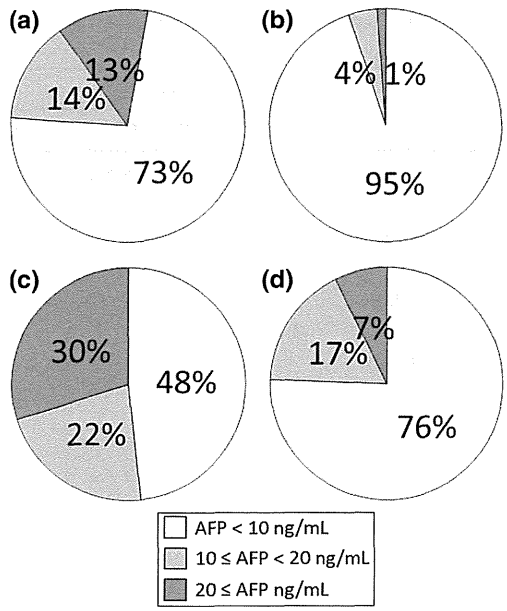

Fig. 1 Distribution of alpha-fetoprotein (*AFP*) levels at the baseline and at 24 weeks after the initiation of entecavir (ETV) treatment according to the severity of liver disease: **a** patients without cirrhosis at the baseline ($n = 326$); **b** patients without cirrhosis at 24 weeks after ETV treatment initiation ($n = 326$); **c** patients with cirrhosis at the baseline ($n = 87$); **d** patients with cirrhosis at 24 weeks after ETV treatment initiation ($n = 87$)

Factors associated with HCC incidence at the baseline

In a univariate analysis, factors at the baseline such as advanced age, cirrhosis, lower platelet counts, and higher total bilirubin, lower albumin, and higher AFP levels were significant, and a multivariate analysis demonstrated that advanced age (55 years or older) and cirrhosis were significant independent risk factors for HCC incidence (Table 2). After a stratified analysis of HCC incidence according to those risk factors at the baseline, the cumulative incidence of HCC at 5 years was 2.5 % in younger patients (younger than 55 years) and 18.6 % in older patients (55 years or older, $p < 0.001$; Fig. 2a). The cumulative incidence of HCC at 5 years was 5.3 % in noncirrhotic patients and was 30.0 % in cirrhotic patients ($p < 0.001$; Fig. 2b).

Factors associated with HCC incidence at 24 weeks after the initiation of ETV treatment

The association between HCC incidence and posttreatment factors at 24 weeks after the initiation of ETV treatment was estimated. In a univariate analysis, advanced age, cirrhosis, lower platelet counts,and lower albumin, higher total bilirubin, and higher AFP levels at 24 weeks were significant, and a multivariate analysis showed that a higher AFP level (10 ng/mL or greater) at 24 weeks was the only additional factor independently associated with HCC incidence other than advanced age and cirrhosis, which were found to be significant risk factors at the baseline (Table 3). The cumulative incidence of HCC at 5 years was 8.2 % among patients with an AFP level below 10 ng/mL at 24 weeks and was 34.2 % among patients with an AFP level of 10 ng/mL or higher at 24 weeks (Fig. 3a). Although the American Association for the Study of Liver Disease practical guidelines for chronic hepatitis B indicate that the aims of treatment for patients infected with HBV are to achieve a reduction in the serum HBV DNA levels and a normalization of serum ALT levels [17], in this study, neither virological response nor biochemical response (ALT level of 30 IU/L or lower) at 24 weeks by ETV treatment affected HCC incidence (Table 3). The cumulative incidence of HCC was almost equivalent between patients with and without virological response at 24 weeks in the analysis among all enrolled patients ($p = 0.685$; Fig. 3b). Additionally, there was no significant difference in the cumulative incidence of HCC between patients with or without normalization of ALT levels at 24 weeks ($p = 0.076$; Fig. 3c). The cumulative incidence of HCC significantly increased with higher AFP levels (10 ng/mL or greater) at 24 weeks even among patients who achieved virological response ($p = 0.023$) or normalization of ALT levels at 24 weeks ($p = 0.002$). The AFP levels at 24 weeks were closely related to HCC incidence irrespective of the virological response or biochemical response at 24 weeks in patients with HBV infection who were undergoing treatment with ETV.

The impact of AFP at 24 weeks on HCC incidence according to baseline factors

Because AFP levels at 24 weeks were found to be a significant factor related to HCC incidence among multiple factors that varied during treatment, the impact of AFP at 24 weeks on HCC incidence was assessed in the subgroups stratified by HCC-related factors at the baseline: age and the severity of liver disease. In the subgroup analysis stratified by age, AFP levels at 24 weeks were significantly related to HCC incidence, and the cumulative incidence of HCC at 5 years was significantly higher in patients with AFP levels of 10 ng/mL or higher at 24 weeks than those with AFP levels below 10 ng/mL, irrespective of age (younger than 55 years, 16.1 % vs 2.2 %, $p = 0.009$; 55 years or older, 45.4 % vs 14.9 %, $p < 0.001$; Fig. 4a, b). In the subgroup analysis that was stratified according to the severity of liver disease, the AFP level at 24 weeks was a significant factor in the cirrhotic group ($p = 0.029$) but not in the noncirrhotic group ($p = 0.377$); the cumulative incidence of HCC at 5 years in the cirrhotic group was

Table 2 Risk factors at the baseline for hepatocellular carcinoma (HCC) incidence in chronic hepatitis B patients receiving ETV treatment (Cox proportional-hazards model)

Factors	Category	Univariate analysis		*p*	Multivariate analysis		
		HR	95 % CI		HR	95 % CI	*p*
Age (years)	0:<55	1	2.601–13.243	<0.001	1	1.592–8.560	0.002
	1:≥55	5.869			3.691		
Gender	0:male	1	0.365–1.319	0.265			
	1:female	0.694					
Severity of liver disease	0:no cirrhosis	1	4.050–14.085	<0.001	1	2.415–9.404	<0.001
	1:cirrhosis	7.553			4.765		
HBeAg	0:negative	1	0.412–1.436	0.410			
	1:positive	0.770					
Histology: activity	0:A0-1	1	0.352–3.800	0.810			
	1:A2-3	1.157					
Histology: fibrosis	0:F0-2	1	0.865–5.910	0.096			
	1:F3-4	2.262					
History of IFN therapy	0:none	1	0.032–1.718	0.154			
	1:presence	0.236					
Platelet count ($\times 10^4/\mu L$)	0:<15	1	0.103–0.449	<0.001			
	1:≥15	0.215					
Total bilirubin (mg/dL)	0:<1.0	1	1.235–4.141	0.008			
	1:≥1.0	2.261					
Albumin (g/dL)	0:<4.0	1	0.201–0.725	0.003			
	1:≥4.0	0.381					
PT (%)	0:<80	1	0.301–1.056	0.074			
	1:≥80	0.564					
ALT (IU/L)	0:<80	1	0.345–1.246	0.197			
	1:≥80	0.656					
HBV DNA(LC/mL)	0:<6.5	1	0.748–2.701	0.283			
	1:≥6.5	1.422					
AFP (ng/mL)	0:<10	1	1.040–3.721	0.038			
	1:≥10	1.967					

CI confidence interval, *HR* hazard ratio

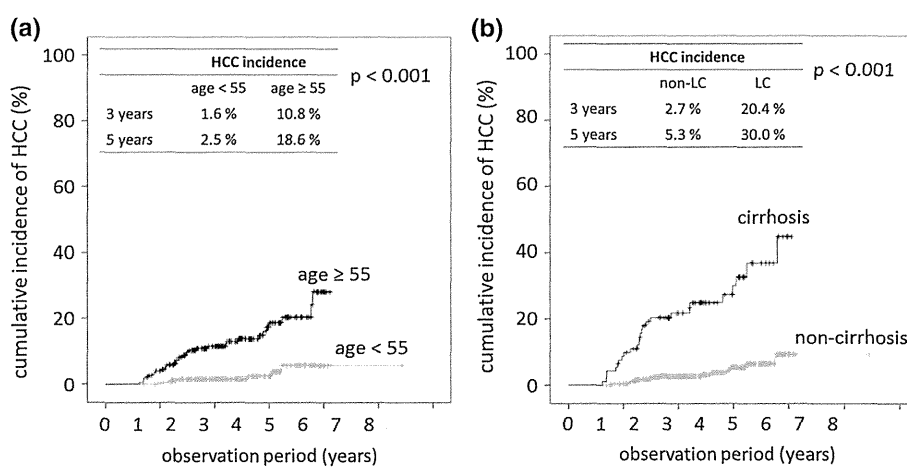

Fig. 2 Cumulative hepatocellular carcinoma (*HCC*) incidence among patients with hepatitis B virus (HBV) infection according to factors at the baseline (log-rank test). **a** Cumulative HCC incidence according to the age at the baseline (*black line* 55 years or older, *gray line* younger than 55 years). **b** Cumulative HCC incidence according to the severity of liver disease (*black line* cirrhosis, *gray line* no cirrhosis)

Table 3 Risk factors at 24 weeks after initiation of ETV treatment for HCC incidence in chronic hepatitis B patients receiving ETV treatment (Cox proportional-hazards model)

Factors	Category	Univariate analysis			Multivariate analysis		
		HR	95 % CI	*p*	HR	95 % CI	*p*
Age (years)	0:<55	1	2.601–13.243	<0.001	1	1.198–6.748	0.018
	1:≥55	5.869			2.843		
Gender	0:male	1	0.365–1.319	0.265			
	1:female	0.694					
Severity of liver disease	0:no cirrhosis	1	4.050–14.085	<0.001	1	2.518–12.411	<0.001
	1:cirrhosis	7.553			5.590		
Platelet count ($\times 10^4/\mu L$) at 24 weeks	0:<15	1	0.114–0.473	<0.001			
	1:≥15	0.233					
Total bilirubin (mg/dL) at 24 weeks	0:<1.0	1	1.360–4.569	0.003			
	1:≥1.0	2.493					
Albumin (g/dL) at 24 weeks	0:<4.0	1	0.201–0.725	0.003			
	1:≥4.0	0.381					
ALT (IU/L) at 24 weeks	0:≤30	1	0.938–3.157	0.080			
	1:>30	1.720					
VR[a] at 24 weeks	0:none	1	0.461–1.664	0.685			
	1:presence	0.875					
AFP (ng/mL) at 24 weeks	0:<10	1	2.589–11.496	<0.001	1	1.066–5.316	0.034
	1:≥10	5.456			2.381		

VR virological response

[a] VR is defined as HBV DNA of less than 2.6 LC/mL

higher in patients with AFP levels of 10 ng/mL or greater at 24 weeks than in those with AFP levels below 10 ng/mL (50.0 % vs 24.7 %; Fig. 4c, d).

Risk analysis for HCC incidence among patients who achieved virological response by ETV treatment

Among patients with HBV infection who achieved virological response by ETV treatment, the risk analysis for HCC incidence was performed in a Cox proportional-hazards model according to the number of the following three risk factors: AFP levels at 24 weeks, age, and the presence of cirrhosis (Fig. S2). When the AFP level remained high (10 ng/mL or higher) at 24 weeks, the cumulative incidence of HCC at 5 years was 6.7 % with no other risk factors (Fig. S2a), 14.8 % with the factor of age of 55 years or older, 27.9 % with the factor of cirrhosis, and 57.7 % with the factors of age of 55 years or older and cirrhosis (Fig. S2b).

Discussion

ETV treatment has been reported to reduce serum HBV DNA levels and ALT levels in patients with chronic HBV infection and to improve hepatitis [18]. On the basis of a study that showed that a higher HBV DNA level at the baseline is associated with a higher HCC incidence in the natural history cohort (the REVEAL study) [15], a reduction of HBV DNA levels by ETV treatment has been considered to have the possibility to suppress HCC incidence among patients with chronic HBV infection. However, it was still unknown whether a lower or an undetectable level of serum HBV DNA, which was achieved by ETV treatment, has a suppressive effect on HCC incidence as shown in the natural course. In the present study, factors associated with HCC incidence during ETV treatment among patients with chronic HBV infection were investigated.

In a previous study that used a historical control group, a significant suppressive effect of ETV on HCC incidence was shown in cirrhotic but not noncirrhotic patients [19]. Furthermore, Wong et al. [20] reported that HCC incidence was significantly lower among patients with cirrhosis who had undetectable levels of HBV DNA compared with those with detectable levels of HBV DNA. In the present study, reduced serum HBV DNA levels were associated with a decrease in the cumulative incidence of HCC only in patients with cirrhosis, and not in those without cirrhosis (Fig. S3). Originally, HBV covalently closed circular DNA (cccDNA) levels in the hepatocyte nuclei were nearly parallel to the serum HBV DNA levels in the natural

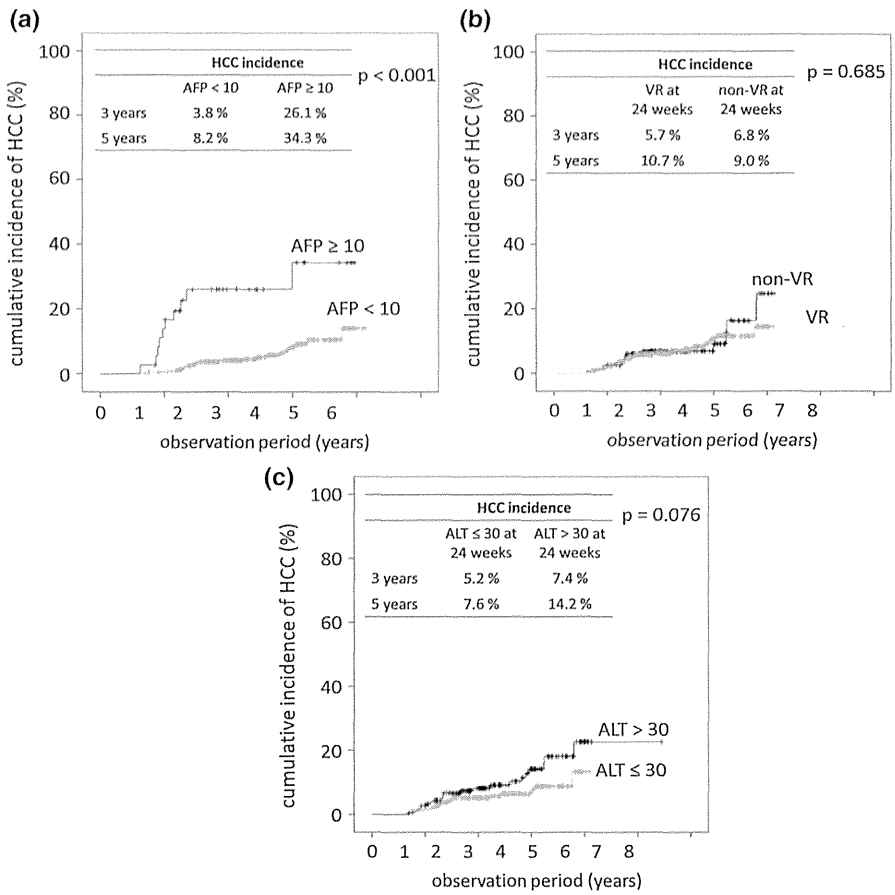

Fig. 3 Cumulative HCC incidence among patients with HBV infection according to factors at 24 weeks after ETV treatment initiation (log-rank test). Virological response (*VR*) is defined as HBV DNA of less than 2.6 log copies per milliliter. **a** Cumulative HCC incidence according to AFP levels at 24 weeks (*back line* AFP level of 10 ng/mL or greater at 24 weeks, *gray line* AFP level below 10 ng/mL at 24 weeks). **b** Cumulative HCC incidence according to virological response at 24 weeks (*black line* no VR at 24 weeks, *gray line* VR at 24 weeks). **c** Cumulative HCC incidence according to biochemical response at 24 weeks [*black line* alanine aminotransferase (*ALT*) level above 30 IU/L at 24 weeks, *gray line* ALT level of 30 IU/L or lower at 24 weeks]

course. However, low levels of serum HBV DNA achieved by ETV treatment do not always indicate low intracellular HBV cccDNA levels [21, 22]. Therefore, it is possible that an insufficient decrease of intracellular HBV DNA levels cannot bring the apparent HCC suppression in noncirrhotic liver with low malignant potential. A longer observation period is required to clarify the suppressive effect on HCC incidence among noncirrhotic patients. The relationship between HBV cccDNA levels in the liver and HCC incidence should also be examined.

In this study, in the analysis of the relationship between on-treatment factors and HCC incidence, only higher AFP levels (10 ng/mL or higher) at 24 weeks after the initiation of ETV treatment were found to be associated with HCC incidence. This is the first study to investigate the significance of AFP levels as a representative marker for the potential of HCC development among patients with chronic HBV infection undergoing ETV treatment. Originally, AFP was known as a tumor-associated antigen in HCC and as a target for immunotherapy. AFP has been used in the surveillance of HCC and in the evaluation of treatment response in HCC patients. The use of AFP as a marker to identify HCC among patients with HBV infection has previously been shown in patients with a natural course of the disease [23]. In recent reports that have focused on AFP levels for HCC diagnosis in patients undergoing ETV treatment, elevated AFP levels at 6 months before or at the time of HCC incidence were shown to be useful in detecting existing HCC [24, 25]; that is, elevated AFP levels implied the existence of cancer cells. However, the present study clarified that a high AFP level at 24 weeks did not suggest the existence of cancer cells, but indicates a potential for HCC incidence before the initiation of carcinogenesis. A possible reason is as follows. The AFP levels among patients who developed HCC decreased from 24 to 48 weeks after the initiation of ETV treatment and increased again from 24 weeks before HCC incidence to the time of HCC incidence. Furthermore, it took a considerably long time before HCC incidence, on average 32.6 months of the observation period (Fig. S1). With regard to the relationship between serum AFP levels and HCC incidence among HCV-infected patients, AFP levels at 24 weeks after the end of IFN treatment have been associated with HCC [26, 27]. AFP levels after the

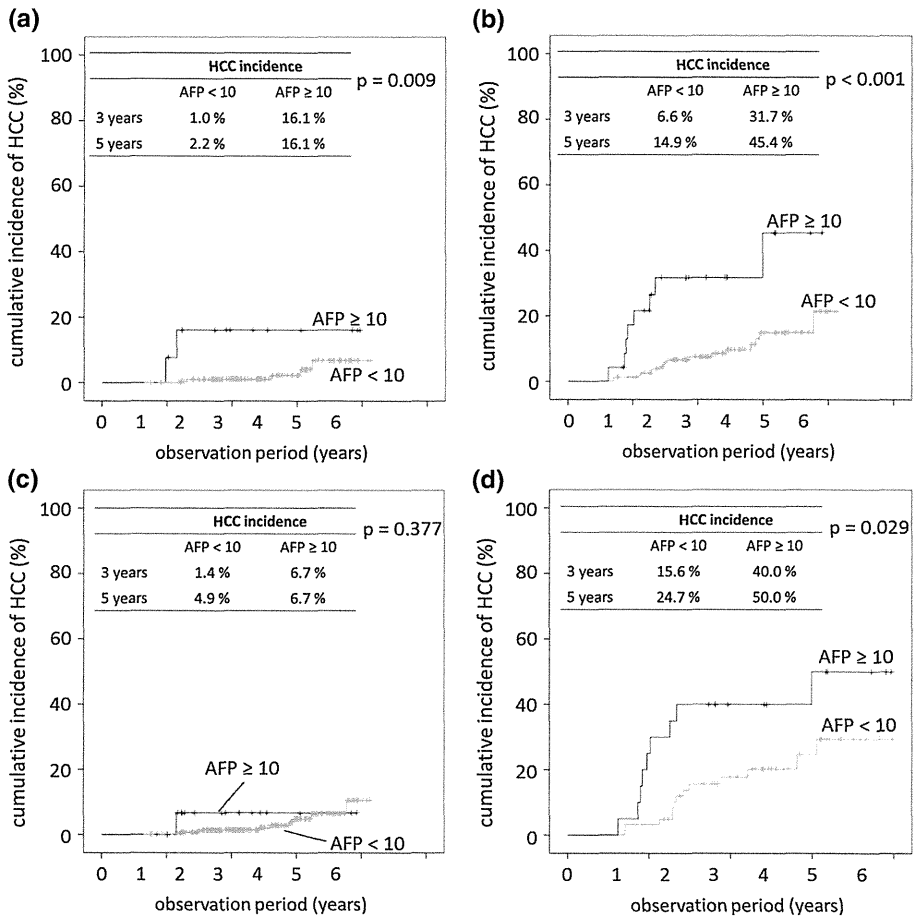

Fig. 4 Cumulative HCC incidence among patients with HBV infection according to AFP levels at 24 weeks after ETV treatment initiation, stratified with baseline factors (log-rank test). **a** Patients younger than 55 years. **b** Patients 55 years or older. **c** Patients without cirrhosis. **d** Patients with cirrhosis. *Black line* AFP level of 10 ng/mL or higher at 24 weeks, *gray line* AFP level below 10 ng/mL at 24 weeks

initiation of treatment of both HBV infection and HCV infection appear to have important implications for HCC incidence.

What the AFP levels at 24 weeks actually represent in patients undergoing ETV treatment is uncertain. The AFP level is a surrogate marker that appears to predict a disease condition from various pathological factors including inflammation, fibrosis, and liver regeneration, which involve carcinogenesis. Moreover, a previous study reported that the activation of natural killer cells by dendritic cells was inhibited when they were co-cultured with AFP; this result suggests an association between HCC development and the maintenance of high AFP levels [28]. Therefore, AFP is thought to be an important biomarker that can reflect various aspects of liver disease.

American Association for the Study of Liver Disease practice guidelines for the management of HBV have defined the goal of NA treatment as to decrease serum HBV DNA levels to undetectable levels to suppress HCC development. In this study, the HBV DNA levels and ALT levels were rapidly lowered in most patients. However, this study shows that the virological and biochemical treatment responses had no association with HCC development, whereas advanced age, liver cirrhosis, and a higher AFP level at 24 weeks after the initiation of ETV treatment were independent risk factors that were significantly associated with HCC development. It is considered that decreasing serum HBV DNA levels to undetectable levels is the necessary, but not sufficient condition to suppress HCC development. In fact, the HCC incidence rate even in patients undergoing ETV treatment who achieved virological response at 24 weeks with the three factors of age of 55 years or older, liver cirrhosis, and AFP level of 10 ng/mL or higher increased to as high as approximately 60 % at 5 years (Fig. S2). Accordingly, the undetectable HBV DNA level in patients with chronic HBV infection undergoing ETV treatment is in itself of little consequence and does not mean a riskless environment.

The limitation of this study is that analysis including other HCC-related factors, such as hepatitis B surface antigen levels, precore and core promotor mutations, and family history of HCC or alcohol consumption, was not

performed. Especially, further investigation is needed to clarify the relationship between the change in hepatitis B surface antigen levels during treatment and HCC incidence in patients with HBV infection.

In conclusion, in the consecutive surveillance for HCC after the initiation of ETV treatment, monitoring the change in AFP levels at 24 weeks is essential, especially among patients of advanced age or with cirrhosis.

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ORIGINAL ARTICLE—LIVER, PANCREAS, and BILIARY TRACT

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

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

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

Abbreviations

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

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VBT Viral breakthrough
AST Aspartate aminotransferase
CI Confidence interval
Pt Patient

Introduction

Hepatitis B virus (HBV) infection is a common disease that can induce a chronic carrier state, and is associated with the risk of developing progressive disease and hepatocellular carcinoma [1]. Interferon (IFN) and several nucleoside/nucleotide analogues (NA) such as lamivudine (LAM), adefovir dipivoxil (ADV), entecavir (ETV), and tenofovir disoproxil fumarate (TDF) are currently approved for treatment of chronic hepatitis B (CHB) in most countries [2–8]. Successful treatment of CHB with clearance of hepatitis B e antigen (HBeAg), reduction in serum HBV DNA levels, and normalization of alanine aminotransferase (ALT) levels are associated with favorable long-term outcomes, independent of the antiviral drug used [9–11].

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

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

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

Patients and methods

Patients

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

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

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

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

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

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

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

Blood tests and serum viral markers

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

Determination of nucleotide sequences of HBV DNA

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

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

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

HBV genotype

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

Statistical analysis

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

Results

Study population

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

Response to ADV plus LAM combination therapy

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

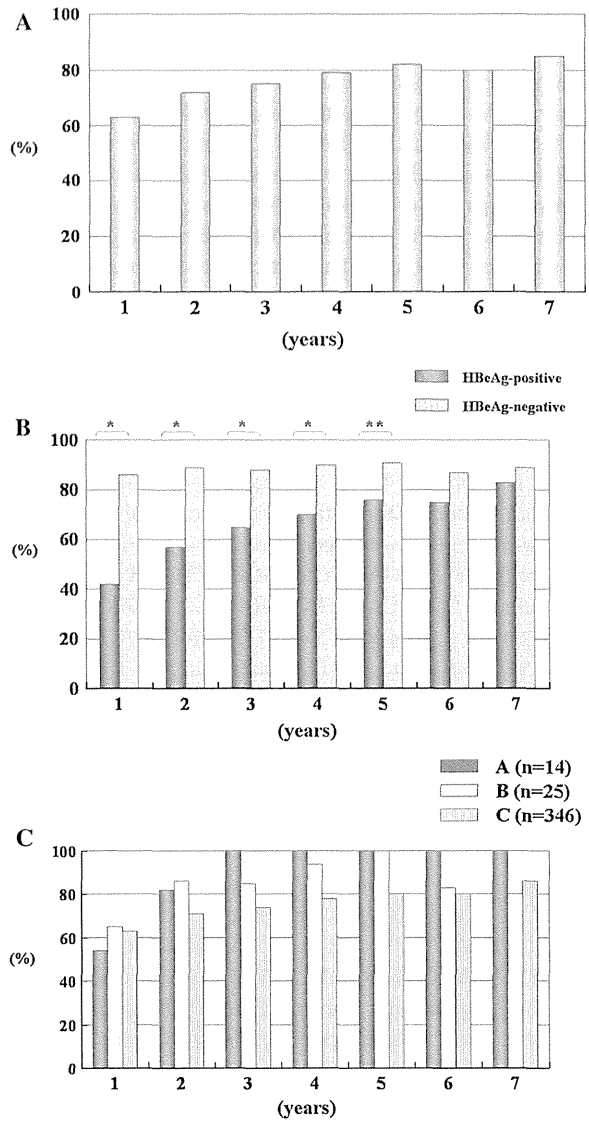

Fig. 1 Undetectable serum HBV DNA levels (<2.6 log copies/mL) in years 1 through 7, respectively. **a** All patients. **b** HBeAg status. A *single asterisk* indicates a statistical significance of $P < 0.0001$ and a *double asterisk* indicates $P = 0.0044$, as determined at the χ^2 test. **c** Genotypes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

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

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

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

HBeAg clearance

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

HBsAg clearance

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

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

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

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

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

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