

**Table 2.** Results of replication study for protective effects against CHB.

dbSNP rsID		Position		MAF <sup>a</sup>	Allele	Stage	HBV carriers			Healthy controls			OR <sup>b</sup>	95% CI	P-value <sup>c</sup>	P <sub>het</sub> <sup>d</sup>
rsID	Chr	Buld	36.3 Nearest Gene	(allele)	(1/2)	(population)	11	12	22	11	12	22	HWEp			
rs3077	6	33141000	HLA-DPA1	0.44	T/C	GWAS	13	51	117	28	88	67	0.919	0.42	1.14×10 <sup>-7</sup>	
						(Japanese)	(7.2)	(28.2)	(64.6)	(15.3)	(48.1)	(36.6)		(0.30–0.58)		
						Replication-1	26	95	134	46	125	65	0.309	0.48	2.70×10 <sup>-8</sup>	
						(Japanese)	(10.2)	(37.3)	(52.5)	(19.5)	(53.0)	(27.5)		(0.37–0.62)		
						Replication-2	23	81	111	31	74	40	0.767	0.47	2.08×10 <sup>-6</sup>	
						(Korean)	(10.7)	(37.7)	(51.6)	(21.4)	(51.0)	(27.6)		(0.35–0.65)		
						Meta-analysis <sup>e</sup>							0.46	4.40×10 <sup>-19</sup>	0.80	
														(0.39–0.54)		
rs9277542	6	33163225	HLA-DPB1	0.45	T/C	GWAS	18	53	110	29	102	52	0.073	0.42	5.32×10 <sup>-8</sup>	
						(Japanese)	(9.9)	(29.3)	(60.8)	(15.8)	(55.7)	(28.4)		(0.31–0.58)		
						Replication-1	30	106	118	54	114	67	0.681	0.54	3.33×10 <sup>-6</sup>	
						(Japanese)	(11.8)	(41.7)	(46.5)	(23.0)	(48.5)	(28.5)		(0.42–0.70)		
						Replication-2	30	87	94	35	72	36	0.933	0.54	8.29×10 <sup>-5</sup>	
						(Korean)	(14.2)	(41.2)	(44.5)	(24.5)	(50.3)	(25.2)		(0.40–0.74)		
						Meta-analysis <sup>e</sup>							0.50	1.28×10 <sup>-15</sup>	0.40	
														(0.43–0.60)		

<sup>a</sup>Minor allele frequency and minor allele in 198 healthy Japanese (ref#19).

<sup>b</sup>Odds ratio of minor allele from two-by-two allele frequency table.

<sup>c</sup>P value of Pearson's chi-square test for allelic model.

<sup>d</sup>Heterogeneity was tested using general variance-based method.

<sup>e</sup>Meta-analysis was tested using the random effects model.

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comparison of HBV patients with HBV resolved individuals (OR = 0.56 for rs3077, 0.66 for rs9277535 and 0.55 for rs9277542) than in the one with healthy controls (OR = 0.55 for rs3077, 0.61 for rs9277535 and 0.51 for rs9277542), which was the opposite result as we expected (Table S2 and Table S4). These results may suggest that other unknown immune system(s) exist to eliminate the HBV in the HBV resolved individuals.

Among the HLA class II loci (*HLA-DPA1*, *HLA-DPB1* and *HLA-DQB2*), which were associated with CHB and HBV clearance, a weak linkage disequilibrium ( $r^2 < 0.1$ ) was observed between *HLA-DQB2* locus and *HLA-DPA1*/*-DPB1* loci in Japanese and Korean populations (Figure S2). We also found that similar linkage disequilibrium blocks ( $r^2$ ) were observed among three subgroups (HBV carriers, HBV resolved individuals and Healthy controls). Moreover, logistic regression analysis of *HLA-DP* (rs3077 and rs9277542) with use of *HLA-DQ* (rs9276431 and rs768538) as covariates showed that the same level of significant associations of *HLA-DP* with CHB and HBV clearance as shown in the single-point association analysis, while no associations of *HLA-DQ* with  $P_{log} > 0.05$  were detected both in Japanese and in Korean (Table S5). These results show that *HLA-DP* is the main genetic factor for susceptibility to CHB and HBV clearance, and the associations of *HLA-DQB2* would result from linkage disequilibrium of *HLA-DPA1*/*-DPB1*.

In this study, we confirmed the significant associations between *HLA-DPA1* and *HLA-DPB1*, and protective effects against CHB and HBV clearance in Japanese and Korean individuals. These results suggest that the associations between the *HLA-DP* locus, CHB and HBV clearance are widely replicated in East Asian populations, including Chinese, Thai, Japanese and Korean individuals; however, there have been no similar GWAS performed in Caucasian and African populations. Moreover,

there were no significant SNPs associated with HCC development in this study, thus suggesting that it is necessary to increase the sample size. To clarify the pathogenesis of CHB or the mechanisms of HBV clearance, further studies are necessary, including a functional study of the *HLA-DP* molecule, identification of novel host genetic factors other than *HLA-DP*, and variation analysis of HBV.

## Materials and Methods

### Ethics Statement

All study protocols conform to the relevant ethical guidelines, as reflected in the *a priori* approval by the ethics committees of all participating universities and hospitals. The written informed consent was obtained from each patient who participated in this study and all samples were anonymized.

### Genomic DNA Samples and Clinical Data

All of the 1,793 Japanese and Korean samples, including individuals with CHB, healthy controls and HBV-resolved individuals (HBsAg-negative and anti-HBc-positive), were collected at 20 multi-center hospitals (liver units with hepatologists) throughout Japan and Korea. The 19 hospitals in Japan were grouped into the following 8 areas: Hokkaido area (Hokkaido University Hospital, Teine Keijinkai Hospital), Tohoku area (Iwate Medical University Hospital), Kanto area (Musashino Red Cross Hospital, Saitama Medical University, Kitasato University Hospital, University of Tokyo), Koshin area (Shinshu University Hospital, Kanazawa University Hospital), Tokai area (Nagoya City University Hospital, Nagoya Daini Red Cross Hospital), Kinki area (Kyoto Prefectural University of Medicine Hospital, National Hospital Organization Osaka National Hospital, Osaka

**Table 3.** Results of replication study for clearance of hepatitis B virus.

dbSNP rsID	Position			MAF <sup>a</sup> (allele)	Allele (1/2)	Stage (population)	HBV carriers			Resolved individuals			OR <sup>b</sup> 95% CI	P-value <sup>c</sup>	P <sub>het</sub> <sup>d</sup>	
	Chr	Buld	36.3 Nearest Gene				11	12	22	11	12	22				
rs3077	6	33141000	HLA-DPA1	0.44 (T)	T/C	GWAS (Japanese)	13	51	117	29	82	74	0.44	9.24 × 10 <sup>-7</sup>		
							(7.2)	(28.2)	(64.6)	(15.7)	(44.3)	(40.0)	(0.32–0.61)			
							Replication-1	26	95	134	20	64	60	0.72	3.32 × 10 <sup>-2</sup>	
							(Japanese)	(10.2)	(37.3)	(52.5)	(13.9)	(44.4)	(41.7)	(0.53–0.97)		
							Replication-2	23	81	111	29	48	28	0.41	2.35 × 10 <sup>-7</sup>	
							(Korean)	(10.7)	(37.7)	(51.6)	(27.6)	(45.7)	(26.7)	(0.29–0.58)		
Meta-analysis <sup>e</sup>									0.51	1.56 × 10 <sup>-4</sup>	0.03					
										(0.36–0.72)						
						Meta-analysis <sup>e</sup> (GWAS+replication-2)						0.43	1.89 × 10 <sup>-12</sup>	0.75		
												(0.34–0.54)				
rs9277542	6	33163225	HLA-DPB1	0.45 (T)	T/C	GWAS (Japanese)	18	53	110	28	88	69	0.51	3.15 × 10 <sup>-5</sup>		
							(9.9)	(29.3)	(60.8)	(15.1)	(47.6)	(37.3)	(0.37–0.70)			
							Replication-1	30	106	118	28	62	52	0.68	1.25 × 10 <sup>-2</sup>	
							(Japanese)	(11.8)	(41.7)	(46.5)	(19.7)	(43.7)	(36.6)	(0.51–0.92)		
							Replication-2	30	87	94	30	53	22	0.46	4.97 × 10 <sup>-6</sup>	
							(Korean)	(14.2)	(41.2)	(44.5)	(28.6)	(50.5)	(21.0)	(0.33–0.64)		
Meta-analysis <sup>e</sup>									0.55	5.91 × 10 <sup>-7</sup>	0.19					
										(0.43–0.69)						
						Meta-analysis <sup>e</sup> (GWAS+replication-2)						0.49	9.69 × 10 <sup>-10</sup>	0.65		
												(0.39–0.61)				

<sup>a</sup>Minor allele frequency and minor allele in 198 healthy Japanese (ref#19).

<sup>b</sup>Odds ratio of minor allele from two-by-two allele frequency table.

<sup>c</sup>P value of Pearson's chi-square test for allelic model.

<sup>d</sup>Heterogeneity was tested using general variance-based method.

<sup>e</sup>Meta-analysis was tested using the random effects model.

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City University), Chugoku/Shikoku area (Tottori University Hospital, Ehime University Hospital, Yamaguchi University Hospital, Kawasaki Medical College Hospital) and Kyushu area (Kurume University Hospital). Korean samples were collected at Yonsei University College of Medicine.

HBV status was measured based on serological results for HBsAg and anti-HBc with a fully automated chemiluminescent enzyme immunoassay system (Abbott ARCHITECT; Abbott Japan, Tokyo, Japan, or LUMIPULSE f or G1200; Fujirebio, Inc., Tokyo, Japan). For clinical staging, inactive carrier (IC) state was defined by the presence of HBsAg with normal ALT levels over 1 year (examined at least four times at 3-month intervals) and without evidence of portal hypertension. Chronic hepatitis (CH) was defined by elevated ALT levels (>1.5 times the upper limit of normal [35 IU/L]) persisting over 6 months (at least by 3 bimonthly tests). Liver cirrhosis (LC) was diagnosed principally by ultrasonography (coarse liver architecture, nodular liver surface, blunt liver edges and hypersplenism), platelet counts <100,000/cm<sup>3</sup>, or a combination thereof. Histological confirmation by fine-needle biopsy of the liver was performed as required. Hepatocellular carcinoma (HCC) was diagnosed by ultrasonography, computerized tomography, magnetic resonance imaging, angiography, tumor biopsy or a combination thereof.

The Japanese control samples from HBV-resolved subjects (HBsAg-negative and anti-HBc-positive) at Nagoya City University-affiliated healthcare center were used by comprehensive agree-

ment (anonymization in an unlinkable manner) in this study. Some of the unrelated Japanese healthy controls were obtained from the Japan Health Science Research Resources Bank (Osaka, Japan). One microgram of purified genomic DNA was dissolved in 100 µl of TE buffer (pH 8.0) (Wako, Osaka, Japan), followed by storage at -20°C until use.

### SNP Genotyping and Data Cleaning

For GWAS, we genotyped a total of 550 individuals, including 181 Japanese HBV carriers, 184 Japanese healthy controls and 185 spontaneously HBV-resolved Japanese individuals (HBsAg-negative and anti-HBc-positive), using the Affymetrix Genome-Wide Human SNP Array 6.0 (Affymetrix, Inc., Santa Clara, CA), in accordance with the manufacturer's instructions. The average QC call rate for 550 samples reached 98.47% (95.00–99.92%), which had an average sample call rate of 98.91% (93.55–99.74%) by determining the genotype calls of over 900 K SNPs using the Genotyping Console v4.1 software (with Birdseed v1 algorithm) provided by the manufacturer [19]. We then applied the following thresholds for SNP quality control in data cleaning: SNP call rate ≥95% and MAF ≥1% for three groups (HBV carriers, healthy controls and HBV-resolved individuals), and HWE P-value ≥0.001 for healthy controls [20]. Here, SNP call rate is defined for each SNP as the number of successfully genotyped samples divided by the number of total samples genotyped. A total of 597,789 SNPs and 590,278 SNPs on autosomal chromosomes

passed the quality control filters in the genome-wide association analysis using HBV carriers and healthy controls, and using HBV carriers and HBV-resolved individuals, respectively (Figure 1). All cluster plots for the SNPs showing  $P < 0.0001$  on association analyses in the allele frequency model were confirmed by visual inspection, and SNPs with ambiguous cluster plots were excluded.

In the following replication stage, we selected a set of 32 SNPs with  $P < 0.0001$  in the GWAS using HBV carriers and HBV-resolved individuals. SNP genotyping in two independent sets of 256 Japanese HBV carriers, 236 Japanese healthy controls and 150 Japanese HBV-resolved individuals (Table 1, replication-1), and 344 Korean HBV carriers, 151 Korean healthy controls and 106 Korean HBV-resolved individuals (Table 1, replication-2) was completed for the selected 32 SNPs using the DigITag2 assay [21,22] and custom TaqMan SNP Genotyping Assays (Applied Biosystems, Foster City, CA) on the LightCycler 480 Real-Time PCR System (Roche, Mannheim, Germany).

### Statistical Analysis

The observed associations between SNPs and the protective effects on chronic hepatitis B or clearance of hepatitis virus B were assessed by chi-squared test with a two-by-two contingency table in allele frequency model. SNPs on chromosome X were removed because gender was not matched among HBV carriers, healthy controls and HBV-resolved individuals. A total of 597,789 SNPs and 590,278 SNPs passed the quality control filters in the GWAS stage; therefore, significance levels after Bonferroni correction for multiple testing were  $P = 8.36 \times 10^{-8}$  (0.05/597,789) and  $P = 8.47 \times 10^{-8}$  (0.05/590,278), respectively. For the replication study, 29 of 32 SNPs were successfully genotyped; therefore, we applied  $P = 0.0017$  (0.05/29) as a significance level, and none of the 29 markers genotyped in the replication stage showed deviations from the Hardy-Weinberg equilibrium in healthy controls ( $P > 0.01$ ).

The genetic inflation factor  $\lambda$  was estimated by applying the Cochran-Armitage test on all SNPs and was found to be 1.056 and 1.030 in the GWAS using HBV carriers and healthy controls, and using HBV carriers and HBV-resolved individuals, respectively (Figure S3). These results suggest that the population substructure should not have any substantial effect on statistical analysis. In addition, the principal component analysis in a total of 550 individuals in the GWAS stage together with the HapMap samples also revealed that the effect of population stratification was negligible (Figure S4).

Based on the genotype data of a total of 1,793 samples including 1,192 Japanese samples and 601 Korean samples in both GWAS and replication stages, haplotype blocks were estimated using the Gabriel's algorithm using the Haploview software (v4.2) (Figure S2). In the logistic regression analysis, two SNPs (rs9276431 and rs7768538) within the HLA-DQ locus were individually involved as a covariate (Table S5). Statistical analyses were performed using the SNP & Variation Suite 7 software (Golden Helix, MT, USA).

### Supporting Information

**Figure S1 GWAS using samples from HBV carriers with LC or HCC, and HBV carriers without LC and HCC.**  $P$  values were calculated using chi-squared test for allele frequencies. (PPTX)

**Figure S2 Estimation of linkage disequilibrium blocks in HBV patients, HBV resolved individuals and healthy controls in Japanese and Korean.** The LD blocks ( $r^2$ ) were analyzed using the Gabriel's algorithm. (PPTX)

**Figure S3 Quantile-quantile plot for test statistics (allele-based chi-squared tests) for GWAS results.** Dots represent  $P$  values of each SNP that passed the quality control filters. Inflation factor  $\lambda$  was estimated to be: a) 1.056 in the analysis with HBV carriers and healthy controls; and b) 1.030 with HBV carriers and HBV-resolved individuals. (PPTX)

**Figure S4 Principal component analysis on a total of 550 individuals in GWAS, together with HapMap samples (CEU, YRI and JPT).** (PPTX)

**Table S1 Results for 29 SNPs selected in replication study using samples of HBV carriers and healthy controls.** <sup>a</sup> $P$  values by chi-squared test for allelic model. <sup>b</sup>Odds ratio of minor allele from two-by-two allele frequency table. <sup>c</sup>Meta-analysis was tested using additive, two-tailed CMH fixed-effects model. (XLSX)

**Table S2 Results of meta-analysis for protective effects against persistent HB infection across 6 independent studies, including this study.** <sup>a</sup>Minor allele frequency and minor allele in 198 healthy Japanese (ref#19). <sup>b</sup>Odds ratio of minor allele from two-by-two allele frequency table. <sup>c</sup> $P$  value of Pearson's chi-squared test for allele model. <sup>d</sup>Heterogeneity was tested using general variance-based method. <sup>e</sup>Meta-analysis was tested using the random effects model. (XLSX)

**Table S3 Results for 29 SNPs selected in replication study using samples from HBV carriers and HBV-resolved individuals.** <sup>a</sup> $P$  values by chi-squared test for allelic model. <sup>b</sup>Odds ratio of minor allele from two-by-two allele frequency table. <sup>c</sup>Meta-analysis was tested using additive, two-tailed CMH fixed-effects model. (XLSX)

**Table S4 Results of meta-analysis for clearance of HBV across 6 independent studies, including this study.** <sup>a</sup>Minor allele frequency and minor allele in 198 healthy Japanese (ref#19). <sup>b</sup>Odds ratio of minor allele from two-by-two allele frequency table. <sup>c</sup> $P$  value of Pearson's chi-squared test for allele model. <sup>d</sup>Heterogeneity was tested using general variance-based method. <sup>e</sup>Meta-analysis was tested using the random effects model. (XLSX)

**Table S5 Logistic regression analysis of HLA-DP (rs3077 and rs9277542) and HLA-DQ (rs9276431 and rs7768538) with susceptibility to CHB and HBV clearance using the HLA-DQ genotypes individually as a covariate.** (XLSX)

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### Author Contributions

Conceived and designed the experiments: NN HS YT. Performed the experiments: HS Y. Mawatari M. Sageshima YO. Analyzed the data: NN MK AK. Contributed reagents/materials/analysis tools: KM M. Sugiyama SHA JYP SH JHK KS M. Kurosaki YA SM MW ET MH SK EO YI EM AT Y. Murawaki YH IS M. Korenaga KH TI NI KHH YT MM. Wrote the paper: NN M. Kawashima YT KT MM.

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RESEARCH ARTICLE

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# No association for Chinese HBV-related hepatocellular carcinoma susceptibility SNP in other East Asian populations

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## Abstract

**Background:** A recent genome-wide association study (GWAS) using chronic HBV (hepatitis B virus) carriers with and without hepatocellular carcinoma (HCC) in five independent Chinese populations found that one SNP (rs17401966) in *KIF1B* was associated with susceptibility to HCC. In the present study, a total of 580 HBV-derived HCC cases and 1351 individuals with chronic hepatitis B (CHB) or asymptomatic carrier (ASC) were used for replication studies in order to evaluate the reported association with HBV-derived HCC in other East Asian populations.

**Results:** We did not detect any associations between rs17401966 and HCC in the Japanese cohorts (replication 1: OR = 1.09, 95 % CI = 0.82-1.43; replication 2: OR = 0.79, 95 % CI = 0.54-1.15), in the Korean cohort (replication 3: OR = 0.95, 95 % CI = 0.66-1.36), or in the Hong Kong Chinese cohort (replication 4: OR = 1.17, 95 % CI = 0.79-1.75). Meta-analysis using these cohorts also did not show any associations with  $P = 0.97$ .

**Conclusions:** None of the replication cohorts showed associations between rs17401966 and HBV-derived HCC. This may be due to differences in the genetic diversity among the Japanese, Korean and Chinese populations. Other reasons could be the high complexity of multivariate interactions between the genomic information and the phenotype that is manifesting. A much wider range of investigations is needed in order to elucidate the differences in HCC susceptibility among these Asian populations.

**Keywords:** Hepatitis B, hepatocellular carcinoma, candidate SNP, replication study, genome-wide association study

## Background

Hepatitis B (HB) is a potentially life-threatening liver infection caused by the hepatitis B virus (HBV), and approximately 360 million people worldwide are thought to be chronically infected with HBV. The clinical course of HBV infection is variable, including acute self-limiting infection, fulminant hepatic failure, inactive carrier state and chronic hepatitis with progression to cirrhosis and

hepatocellular carcinoma (HCC). Although some HBV carriers spontaneously eliminate the virus, 2-10 % of individuals with chronic HB (CHB) develop liver cirrhosis every year, and a subset of these individuals suffer from liver failure or HCC. Around 600,000 new HCC cases are diagnosed annually worldwide, with HCC being relatively common in Asia-Pacific countries and sub-Saharan Africa; more than 70 % of HCC patients are diagnosed in Asia (with 55 % in China) [1]. However, HCC is relatively uncommon in the USA, Europe and Australia [1,2]. The majority of HCC develops in patients with cirrhosis, which is most often attributable

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to chronic HBV infection followed by chronic HCV in the Asia-Pacific region [3].

A recent genome-wide association study (GWAS) using Japanese CHB cases and controls confirmed that 11 SNPs in a region including *HLA-DPA1* and *-DPB1* were associated with CHB [4]. Moreover, a GWAS using chronic HBV carriers with and without HCC in five independent Chinese populations reported that one SNP (rs17401966) in *KIF1B* was associated with HCC susceptibility [5]. In the present study, we performed replication studies using Japanese, Korean and Hong Kong Chinese cases and controls in order to evaluate the reported association with HBV-derived HCC in other East Asian populations.

### Results

We performed SNP genotyping of rs17401966 located in the *KIF1B* gene for the purpose of replication analysis of the previous GWAS report [5]. Four distinct cohorts were used for these replication analyses (Table 1). We first examined two independent Japanese case-control samples including 179 cases and 769 controls from Biobank Japan (replication 1), and 142 cases and 251 controls from various hospitals (replication 2). We did not detect any associations between rs17401966 and HCC in the Japanese cohorts (replication 1: OR = 1.09; 95 % CI = 0.82-1.43, replication 2: OR = 0.79; 95 % CI = 0.54-1.15). We further examined Korean case-control samples comprising 164 cases and 144 controls (replication 3) and Hongkongese 94 HCC cases and 187 CHB controls (replication 4), but again did not detect any association (replication 3: OR = 0.95; 95 % CI = 0.66-1.36, replication 4: OR = 1.17; 95 % CI = 0.79-1.75). Logistic regression analysis adjusted for age and gender also did not show any association ( $P_{\log} = 0.65, 0.27, 0.11, 0.56$  for each replication

panel). Moreover, we conducted meta-analysis to combine these studies, also not detect any association ( $P_{\text{meta}} = 0.97$ ).

### Discussion and conclusions

Zhang et al. [5] reported that SNP rs17401966 was significantly associated with HBV-related HCC (joint OR = 0.61). They conducted a GWAS using 348 cases and 359 controls in a population in Guangxi in southern China, and selected 45 SNPs for the replication study based on the results ( $P < 10^{-4}$ ). In the first replication study, they used 276 cases and 266 controls from Beijing in northern China, and 5 SNPs showed the same direction of association as in the GWAS ( $P < 0.05$ ). They performed a further replication study (of 507 cases and 215 controls) in Jiangsu in eastern China and only one SNP showed the same trend ( $P = 3.9 \times 10^{-5}$ ). Guangdong and Shanghai samples from southern and eastern China were used for further replication studies. The association yielded a p-value of  $1.7 \times 10^{-18}$  on meta-analysis.

We performed four replication analyses using Japanese, Korean and Hong Kong Chinese samples (Table 1). Although sample size of each cohort is smaller than that of the previous GWAS, we conducted meta-analysis of all our study. The result did not show any association between rs17401966 and HBV-derived HCC ( $P_{\text{meta}} = 0.97$ ).

This may be due to differences in genetic diversity among Japanese, Korean and Chinese populations. A maximum-likelihood tree of 126 populations based on 19,934 SNPs showed that Japanese and Korean populations form a monophyletic clade with a 100 % bootstrap value [6]. However, Chinese populations form a paraphyletic clade with two other populations. This indicates that Japanese and Korean populations are genetically closer to one another than the Chinese population.

**Table 1 Association between rs17401966 and HBV-derived HCC**

cohort	sample size (cases/controls)	cases			controls			HWE p	OR (95 % CI)	$P^a$	$P_{\text{het}}^b$
		GG	AG	AA	GG	AG	AA				
replication 1 (Japan 1)	179/769	13 (7.2)	61 (34.1)	105 (58.7)	45 (5.9)	261 (33.9)	463 (60.2)	0.599	1.09 (0.82-1.43)	0.578	
replication 2 (Japan 2)	142/251	5 (3.5)	46 (32.4)	91 (64.1)	14 (5.6)	91 (36.2)	146 (58.2)	1	0.79 (0.54-1.15)	0.212	
replication 3 (Korea)	164/144	17 (10.4)	59 (36.0)	88 (53.6)	15 (10.4)	55 (38.2)	74 (51.4)	0.616	0.95 (0.66-1.36)	0.790	
replication 4 (Hong Kong)	94/187	10 (10.6)	39 (41.5)	44 (46.8)	13 (6.9)	80 (42.8)	94 (50.3)	0.767	1.17 (0.79-1.75)	0.432	
Meta-analysis <sup>c</sup>									0.996 (0.84-1.18)	0.965	0.423

<sup>a</sup>P value of fisher's exact test for allele model.

<sup>b</sup>Result of Breslow-Day test.

<sup>c</sup>Results of meta-analysis were calculated by the Mantel-Haenzel method.

We did not find any association with Hong Kong Chinese cohort ( $P = 0.43$ ). Moreover, a study using 357 HCC cases and 354 HBV-positive non-HCC controls in Hong Kong Chinese did not show any significant difference ( $P = 0.91$ ) [7]. Previous population studies have revealed that various Han Chinese populations show varying degrees of admixture between a northern Altaic cluster and a southern cluster of Sino-Tibetan/Tai-Kadai populations in southern China and northern Thailand [6]. Although Hong Kong is located closed to the Guangdong (cohort 3 of Zhang et al study), there is great heterogeneity for rs17401966 between Hong Kong cohorts (our study and Chan's study [7]) and Guangdong cohort (our study versus Zhang's study:  $P_{\text{het}} = 0.0066$ ; Chan's study versus Zhang's study:  $P_{\text{het}} = 0.035$ ). This result suggests the existence of other confounding factors, which can differentiate the previous study in China and this study.

One of the possible reasons could be the high complexity of multivariate interactions between the genomic information and the phenotype that is manifesting. HCC development is a multiple process which links to causative factors such as age, gender, environmental toxins, alcohol and drug abuse, higher HBV DNA levels, and HBV genotype variations [8]. The eight HBV genotypes display distinct geographical and ethnic distributions. Genotypes B and C are prevalent in Asia. Specific variations in HBV have been associated with cirrhosis and HCC. These variations include in particular mutations in pre-core region (Pre-C), in basal core promoter (BCP) and in ORF encoding Pre-S1/Pre-S2/S and Pre-C/C. Because there is an overlap between Pre-C or BCP mutations and genotypes, these mutations appear to be more common in genotype C as compared to other genotypes [9].

Aflatoxins are a group of 20 related metabolites and Aflatoxin B1 is the most potent naturally occurring chemical liver carcinogen known. Aflatoxin exposures multiplicatively increase the risk of HCC in people chronically infected with HBV, which illustrates the deleterious impact that even low toxin levels in the diet can have on human health [10–12]. Liu and Wu estimated population risk for aflatoxin-induced HCC around the world [13]. Most cases occur in sub-Saharan Africa, Southeast Asia and China, where populations suffer from both high HBV prevalence and largely uncontrolled exposure to aflatoxin in food. But we could not obtain the information of these confounding factors from both of the previous GWAS study and this study. A much wider range of investigations is thus needed in order to elucidate the differences in HCC susceptibility among these Asian populations.

## Methods

### Samples

Case and control samples used in this study were collected from Japan, Korea and Hong Kong listed in supplementary

Additional file 1: Table S1. A total of 179 cases and 769 control subjects were analyzed in the first replication study. DNA samples from both CHB controls and HBV-related HCC cases used in this study were obtained from the BioBank Japan at the Institute of Medical Science, the University of Tokyo [14]. Among the BioBank Japan samples, we selected HBsAg-seropositive CHB patients with elevated serum aminotransferase levels for more than six months, according to the guidelines for diagnosis and treatment of chronic hepatitis from The Japan Society of Hepatology (<http://www.jsh.or.jp/medical/gudelines/index.html>). The mean (and standard deviation; SD) age was 62.0 (9.4) years for the cases and 54.7 (13.5) years for the controls. The second Japanese replication sample sets for the cases ( $n = 142$ ) and controls ( $n = 251$ ) study were obtained from 16 hospitals. The case samples for the second replication included 142 HCC patients and the controls included 135 CHB patients and 116 asymptomatic carriers (ASC). The mean (SD) age was 61.3 (10.2) years for the cases and 56.2 (10.9) years for the controls. The Korean replication samples were collected from Yonsei University College of Medicine. The third replication set was composed of 165 HCC patients and 144 CHB patients. The mean (SD) age was 52.2 (8.9) and 37.3 (11.3) years for the cases and controls, respectively. The samples in Hong Kong were collected from the University of Hong Kong, Queen Mary Hospital. The fourth replication set was composed of 94 HCC patients and 187 CHB patients. The mean (SD) age was 58.0 (10.5) and 56.9 (8.3) years for the cases and controls, respectively. All participants provided written informed consent. This research project was approved by the Research Ethics Committees at the Institute of Medical Science and the Graduate School of Medicine, the University of Tokyo, Yonsei University College of Medicine, the University of Hong Kong, National Center for Global Health and Medicine, Hokkaido University Graduate School of Medicine, Teine Keijinkai Hospital, Iwate Medical University, Saitama Medical University, Kitasato University School of Medicine, Musashino Red Cross Hospital, Kanazawa University Graduate School of Medicine, Shinshu University School of Medicine, Nagoya City University Graduate School of Medical Sciences, Kyoto Prefectural University of Medicine, National Hospital Organization Osaka National Hospital, Kawasaki Medical College, Tottori University, Ehime University Graduate School of Medicine, and Kurume University School of Medicine.

### SNP Genotyping

For the first replication samples, we genotyped rs17401966 using PCR-based Invader assay (Third Wave Technologies, Madison, WI) [15], and for the second, third and fourth replication samples, we used TaqMan genotyping assay (Applied Biosystems, Carlsbad, CA). In the TaqMan SNP

genotyping assay, PCR amplification was performed in a 5- $\mu$ l reaction mixture containing 1  $\mu$ l of genomic DNA, 2.5  $\mu$ l of KAPA PROBE FAST qPCR Master Mix (Kapa Biosystems, Woburn, MA), and 40 x TaqMan SNP Genotyping Assay probe (ABI) for this SNP. QPCR thermal cycling was performed as follows: 95°C for 3 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. The SNP call rate of each replication panel was 100 %, 100 %, 99.7 % and 99.6 %.

### Statistical analysis

We performed Hardy-Weinberg equilibrium test for the case and control samples in each replication study. Fisher's exact test was applied to two-by-two contingency tables for three different genetic models; allele frequency, dominant and recessive model. Odds ratios and confidence intervals were calculated using the major alleles as references. Meta-analysis was conducted using the Mantel-Haenszel method. Heterogeneity among studies was examined by using the Breslow-Day test. Genotype-phenotype association for the SNP rs17401966 was assessed using logistic regression analysis adjusted for age and gender in plink 1.07 (<http://pngu.mgh.harvard.edu/~purcell/plink/index.shtml>).

### Additional file

**Additional file 1: Table S1.** Samples used in this study.

### Abbreviations

HB: Hepatitis b; HBV: Hepatitis b virus; HCC: Hepatocellular carcinoma; CHB: Chronic hepatitis b; HCV: Hepatitis c virus; GWAS: Genome-wide association study; ASC: Asymptomatic carrier.

### Competing interests

The authors declare that they have no competing interests.

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**Original Article**

# Long-term outcomes of add-on adefovir dipivoxil therapy to ongoing lamivudine in patients with lamivudine-resistant chronic hepatitis B

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**Aim:** Add-on adefovir dipivoxil (ADV) therapy has been a standard rescue treatment for patients with lamivudine (LAM)-resistant chronic hepatitis B, but the overall benefits of long-term add-on ADV therapy are still limited. The aim of this study was to evaluate the long-term efficiency of add-on ADV treatment and to explore predictive factors associated with it.

**Methods:** A total of 158 patients with LAM-resistant chronic hepatitis B were included in this retrospective, multicenter, nationwide study in Japan. After confirming LAM resistance, ADV was added to LAM treatment. Three types of events were considered as outcomes: virological response, hepatitis B e antigen (HBeAg) clearance and alanine aminotransferase (ALT) normalization. Virological response was defined as serum hepatitis B virus (HBV) DNA levels of less than 3 log copies/mL. Baseline factors contributing to these outcomes were examined by univariate and multivariate analyses.

**Results:** The median total duration of ADV treatment was 41 months (range, 6–84). The rate of virological response was

90.8% at 4 years of treatment; HBeAg clearance and ALT normalization were achieved by 34.0% and 82.7%, respectively, at the end of follow up. Each outcome had different predictive factors: baseline HBV DNA and albumin level were predictive factors for virological response, history of interferon therapy and ALT level for HBeAg clearance, and sex and baseline albumin level for ALT normalization.

**Conclusion:** Long-term add-on ADV treatment was highly effective in LAM-resistant chronic hepatitis B patients in terms of virological and biochemical responses. Lower HBV replication and lower albumin level at baseline led to better outcomes.

**Key words:** adefovir dipivoxil, alanine aminotransferase normalization, chronic hepatitis B, hepatitis B e antigen clearance, lamivudine resistance, virological response

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## INTRODUCTION

CHRONIC HEPATITIS B (CHB) is an important cause of morbidity and mortality worldwide.<sup>1–3</sup> The main goals of therapy in CHB patients are to prevent the development of liver failure, due to subsequent liver

cirrhosis, and the emergence of hepatocellular carcinoma (HCC). All of these are likely to be achieved by suppressing hepatitis B virus (HBV) replication, which thereby leads to remission of liver disease.<sup>4</sup>

Lamivudine (LAM) treatment has been used to prevent the progression of CHB and the development of HCC.<sup>5</sup> LAM is an effective and well-tolerated treatment for patients with CHB, but it has the major limitation of drug-resistant mutants arising at a rate of 16–32% during the first year of treatment and increasing by 15% with each additional year of treatment.<sup>6–8</sup> The widespread use of LAM monotherapy in CHB patients before introduction of entecavir, which is more potent, has progressively increased the numbers of patients with LAM-resistant HBV mutant strains.

Adefovir dipivoxil (ADV) has been reported to be effective in suppressing HBV replication and approved as a standard therapy in LAM-resistant patients.<sup>9,10</sup> However, data concerning the long-term efficacy of ADV treatment in LAM-resistant CHB patients are still limited. The aims of this study were to evaluate the long-term efficiency of ADV add-on treatment based on virological response (VR), hepatitis B e antigen (HBeAg) clearance and alanine aminotransferase (ALT) normalization, and to explore the predictive factors associated with ADV add-on treatment.

## METHODS

### Patients

A TOTAL OF 158 patients (109 males and 49 females) were included in this retrospective study from 21 medical centers of the National Hospital Organization (NHO) in Japan. Both HBeAg positive and negative CHB patients were considered eligible if they had documented LAM resistance confirmed by detection of mutations in the YMDD motif of the reverse transcriptase gene of the virus (genotypic resistance), elevated serum HBV DNA levels ( $\geq 4$  log copies/mL and/or  $>1$  log copies/mL elevation from the LAM on-treatment nadir) and/or elevated serum ALT levels ( $>40$  IU/L). Patients were excluded if they had decompensated liver cirrhosis, HCC at the initiation of ADV, or if they had co-infections (human immunodeficiency virus, hepatitis C virus) or other concomitant liver diseases such as autoimmune liver disease. Patients with no available clinical, biochemical, serological or virological data at baseline as well as every 6 months during treatment were also excluded.

Patient records were extracted from each institutional database. All data were labeled with their respective

institution and pooled. In total, 20 variables were examined to evaluate the long-term responses. The following variables were used as baseline factors: sex, HBeAg status, liver disease, age, body mass index, duration of LAM monotherapy, history of interferon (IFN) therapy, serum HBV DNA level, aspartate aminotransferase (AST), ALT,  $\gamma$ -glutamyl transpeptidase ( $\gamma$ -GTP), platelet (PLT) counts, and total bilirubin (T-Bil), albumin (Alb), prothrombin time (PT) and  $\alpha$ -fetoprotein (AFP) levels. All were measured at the initiation of ADV therapy. For each variable, it was not used in the stepwise analysis if missing data accounted for more than 10% of the cases.

The study conformed to the ethical guidelines of the 1975 Declaration of Helsinki. Written informed consent was obtained from all patients and approval of this study was obtained from the NHO.

### Statistical analysis

Three types of events were considered as outcomes: (i) VR; (ii) HBeAg clearance; and (iii) ALT normalization. VR was defined as serum HBV DNA levels of less than 3 log copies/mL by a quantitative real-time polymerase chain reaction assay, and ALT normalization was defined as a decrease in ALT levels to less than 31 IU/L during the on-treatment follow-up period. Baseline factors that could have an impact in the prediction of VR, HBeAg clearance as well as ALT normalization were investigated. The predictive value of several baseline parameters for VR was evaluated using time-to-event methods, because of the varying length of follow up. Time-to-event analysis was carried out using Kaplan–Meier estimates to draw cumulative incidence curves, compared by log–rank tests, as well as using univariate and multivariate Cox's proportional hazards models in combination with stepwise regression analysis. Factors contributing to HBeAg clearance and ALT normalization during ADV add-on therapy were estimated using multivariate multiple logistic regression analysis in combination with stepwise regression analysis. A stepwise variable selection procedure was used for variables that were at least marginally associated with the outcomes.

Covariates included in these analyses were binomial or continuous variables. Quartile analysis was initially performed separately for each continuous variable to make the decision regarding cut-off points. At first, we divided each continuous data into quarters to convert numerical values into four categorical values. Then, we estimated whether there was a regular trend among these four ordinal categorical data with outcome and selected a cut-off point among the 25th, 50th and 75th percentiles so that these variables could be appropriately

**Table 1** Baseline characteristics at the initiation of add-on ADV therapy in LAM-resistant CHB patients based on HBeAg status

Baseline characteristics	HBeAg positive <i>n</i> = 99	HBeAg negative <i>n</i> = 59
Age (years)	51.6 (25.5–80.4)	59.3 (33.3–76.9)
Sex (male/female)	73/26	36/23
Liver disease (CH/cirrhosis)	79/20	38/21
Duration of LAM therapy (months)	29.8 (6.0–82.4)	39.3 (8.4–91.2)
History of IFN therapy (months)	39	15
HBV DNA (log copies/mL)	7.5 (2.1–7.6)	5.9 (2.1–7.6)
≤6	15	31
6–7.5	38	21
>7.5	46	7
Total bilirubin (mg/dL)	0.8 (0.3–5.2)	0.9 (0.41–3.7)
AST (IU/L)	60 (18–959)	60 (17–464)
ALT (IU/L)	80 (11–697)	86 (17–724)
γ-GTP (IU/L)	38 (12–325)	53 (10–740)
Albumin (g/dL)	4.3 (2.6–5.4)	4.3 (2.7–5.2)
Platelet count (×10 <sup>4</sup> /mm <sup>3</sup> )	15.5 (3.7–50.0)	12.3 (1.7–33.2)

Continuous variables are expressed in median (range) and categorized variables in number.

ALT, alanine aminotransferase; AST, aspartate aminotransferase; CH, chronic hepatitis; γ-GTP, γ-glutamyl transpeptidase; HBeAg, hepatitis B e antigen; HBV, hepatitis B virus; LAM, lamivudine.

dichotomized. The hazards ratio (HR) and the odds ratio (OR) are presented with 95% confidence intervals (CI) and *P*-values, with less than 0.05 being considered statistically significant. All data analyses were processed using the R statistical software ver. 2.13.

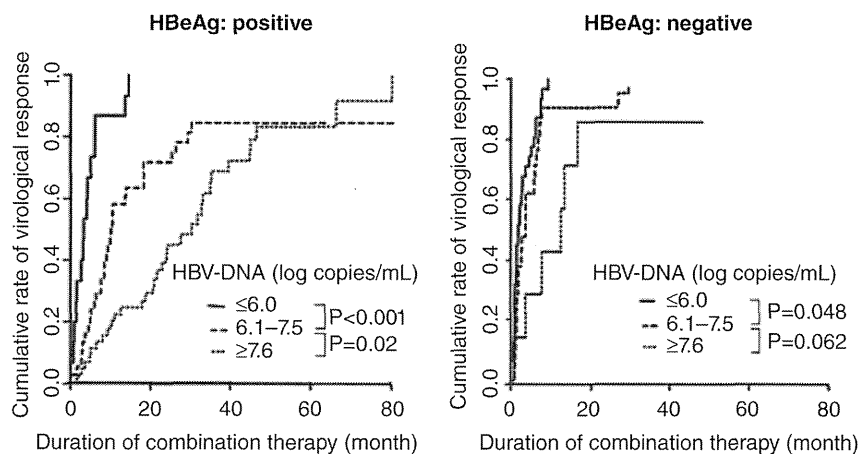
## RESULTS

**I**N THIS RETROSPECTIVE nationwide analysis of add-on ADV therapy in Japan, a total of 158 patients were enrolled from 2003–2010, consisting of 99 HBeAg positive and 59 HBeAg negative patients. Table 1 summarizes the baseline characteristics of the study popula-

tion; most were HBV genotype C. At the time of this analysis, the median total duration of ADV treatment was 41 months (range, 6–84), and the median time of LAM monotherapy, prior to initiation of ADV, was 34 months (range, 6–91).

## VR

Figure 1 shows a Kaplan–Meier curve displaying the cumulative probability of VR based on HBV DNA levels among HBeAg positive and negative patients. Patients with a lower HBV DNA level displayed earlier VR than those with a higher HBV DNA level among both HBeAg positive and negative patients (*P* < 0.001, *P* = 0.002,



**Figure 1** Cumulative rate of virological response on treatment with lamivudine plus adefovir dipivoxil depending on hepatitis B virus (HBV) DNA load in HBeAg positive and negative patients. hepatitis B e antigen (HBeAg) negativity and low HBV replication had a higher probability of virological response compared with HBeAg positivity or higher HBV replication. —, ≤6.0; ---, 6.1–7.5; ···, ≥7.6.

**Table 2** Univariate and multivariate Cox's regression analysis of predictors of virological response

Variable	HBeAg positive <i>n</i> = 99				HBeAg negative <i>n</i> = 59	
	Univariate		Multivariate		Univariate	
	HR	<i>P</i> -value	HR	<i>P</i> -value	HR	<i>P</i> -value
Age (years) (<45/45≤)	0.91	0.69			0.66	0.34
Sex (male/female)	1.07	0.86			0.71	0.21
Liver disease (CH/cirrhosis)	0.61	0.069			1	0.99
Duration of LAM therapy (months) (<34/34≤)	0.92	0.76			1.72	0.076
History of IFN therapy (-/+)	0.83	0.43			0.89	0.73
HBV DNA (log copies/mL) (<7.0/7.0≤)	0.28	<0.001	<0.001	<0.001	0.44	0.012
Total bilirubin (mg/dL) (<1.0/1.0≤)	1.66	0.067	1.73	0.06	1.54	0.13
AST (IU/L) (<100/100≤)	1.57	0.061			1.11	0.71
ALT (IU/L) (<130/130≤)	1.51	0.085			1.05	0.87
γ-GTP (IU/L) (<70/70≤)	1.53	0.113			1.33	0.3
Albumin (g/dL) (<4.1/4.1≤)	0.51	0.011	0.48	0.0065	1.41	0.32
Platelet count (×10 <sup>4</sup> /mm <sup>3</sup> ) (<15/15≤)	0.93	0.77			1.1	0.74

ALT, alanine aminotransferase; AST, aspartate aminotransferase; CH, chronic hepatitis; HBeAg, hepatitis B e antigen; HBV, hepatitis B virus; HR, hazard ratio; IFN, interferon; γ-GTP, γ-glutamyl transpeptidase; LAM, lamivudine.

respectively; log-rank test). HBeAg negative patients displayed higher VR rates than HBeAg positive patients at month 12 (89.9% vs 45.5%), month 24 (95.0% vs 61.5%), month 36 (98.4% vs 79.6%) and month 48 (98.4% vs 86.4%) of treatment. Even at a higher HBV DNA level (HBV DNA ≥7.0 log copies/mL), HBeAg negative patients displayed more rapid VR than HBeAg positive patients ( $P < 0.001$ ). Seven patients did not achieve VR during the 4-year treatment, and one HBeAg positive patient developed ADV-resistant mutations without VR at month 44 of treatment. According to the results of the univariate Cox regression model, HBV DNA level and Alb level were associated with VR in HBeAg positive patients, while only the HBV DNA level was in HBeAg negative patients (HR = 0.44, 95% CI = 0.24–0.84,  $P = 0.012$ ). In multivariate analysis, both lower HBV DNA level and lower Alb level were independent predictive factors associated with VR in HBeAg positive patients (HR = 0.26, 0.48, 95% CI = 0.15–0.44, 0.28–0.81,  $P < 0.001$ ,  $P = 0.0065$ , respectively) (Table 2), while only the HBV DNA level was selected by a stepwise analysis for HBeAg negative patients.

### HBeAg clearance or HBeAg seroconversion

Among 99 HBeAg positive patients, HBeAg clearance and seroconversion were achieved by 17.1% and 11.0% at month 24, by 24.3% and 14.3% at month 36 of treatment, and by 34.0% and 16.0% by the end of follow up, respectively. Except for a history of IFN

therapy (OR = 2.46, 95% CI = 0.94–6.6,  $P = 0.047$ ), none of the other baseline variables were significantly associated with HBeAg clearance, according to the results of the univariate logistic regression analysis. In multivariate analysis, serum ALT level and history of IFN therapy were independent predictive factors for HBeAg clearance (Table 3). No patient experienced a reappearance of HBeAg or reverse seroconversion to HBeAg positive status during this treatment.

### Normalization of ALT levels

The mean ALT level declined from 138.2 to 24.7 IU/L by add-on ADV therapy. Furthermore, addition of ADV to LAM-resistant CHB led to normalization of ALT levels in 75.2%, 79.5% and 82.7% of the patients at months 24 and 36, and at the final follow up, respectively. We next estimated the predictive factors for ALT normalization. Univariate logistic regression analysis revealed that only the baseline Alb level was significantly related to the ALT normalization. In the multivariate model, female patients (OR = 0.19,  $P = 0.037$ ) and lower Alb level (OR = 0.19,  $P = 0.0017$ ) were found to be independent predictors of ALT normalization.

## DISCUSSION

ADD-ON ADV therapy has been a standard rescue treatment for patients with LAM-resistant HBV, but the overall benefits of long-term add-on ADV therapy

**Table 3** Univariate and multivariate logistic regression analysis of predictors of HBeAg clearance and ALT normalization

Variable	HBeAg loss, <i>n</i> = 99				ALT normalization			
	Univariate		Multivariate		Univariate		Multivariate	
	Odds ratio	<i>P</i> -value	Odds ratio	<i>P</i> -value	Odds ratio	<i>P</i> -value	Odds ratio	<i>P</i> -value
Age (years) (<45/45≤)	0.42	0.065			0.94	0.85		
Sex (male/female)	3.02	0.075	2.99	0.081	0.4	0.34	0.19	0.037
Liver disease (CH/cirrhosis)	0.76	0.59			0.54	0.73		
Duration of LAM therapy (months) (<34/34≤)	1.1	0.97			0.59	0.39		
History of IFN therapy (-/+)	2.46	0.047	2.67	0.041	1.2	0.78		
HBV DNA (log copies/mL) (<7.0/7.0≤)	0.49	0.15			0.32	0.21		
Total bilirubin (mg/dL) (<1.0/1.0≤)	1.03	0.83			1.83	0.72		
AST (IU/L) (<100/100≤)	1.52	0.47			3.99	0.075		
ALT (IU/L) (<130/130≤)	2.44	0.061	2.74	0.043	3.71	0.13		
γ-GTP (IU/L) (<70/70≤)	2.16	0.17			1.29	0.98		
Albumin (g/dL) (<4.4/4.4≤)	0.9	0.99			0.17	0.0047	0.19	0.0017
Platelet count (×10 <sup>4</sup> /mm <sup>3</sup> ) (<15/15≤)	1.21	0.82			0.52	0.39		

ALT, alanine aminotransferase; AST, aspartate aminotransferase; CH, chronic hepatitis; HBeAg, hepatitis B e antigen; HBV, hepatitis B virus; HR, hazard ratio; IFN, interferon; γ-GTP, γ-glutamyl transpeptidase; LAM, lamivudine.

have not yet been fully assessed. In this multicenter study of 158 patients from 21 hospitals over a mean follow-up period of 43.5 months, we tried to evaluate the long-term efficacy of add-on ADV therapy to LAM-resistant patients, and also to investigate which baseline factors were associated with VR, HBeAg clearance and ALT normalization. We found long-term add-on ADV treatment produced long-term virological and biochemical improvement. In addition, each outcome had different predictive factors; baseline HBV DNA and Alb level were predictive factors for VR in HBeAg positive patients, history of IFN therapy and ALT level for HBeAg clearance, and sex and Alb level for ALT normalization.

The rate of VR was 90.8% at 4 years of treatment. The strongest predictive factor for VR in both HBeAg positive and negative patients were confirmed by previous observations showing that add-on ADV therapy achieves more rapid and higher rates of VR when ADV is initiated in LAM-resistant patients with low viral replication levels.<sup>11-17</sup> We also found that lower Alb level was an independent predictive factor for VR in HBeAg positive patients. In fact, baseline Alb correlated with PLT counts ( $r = 0.51$ ,  $P < 0.001$ ) and T-Bil ( $r = -0.38$ ,  $P < 0.001$ ), indicating that a lower Alb level reflected progression of liver disease. Little attention has been given to the relation of Alb level with VR – further studies will be needed to confirm our findings and understand its underlying mechanisms – but progression of chronic hepatitis might be predictive of VR under the add-on ADV treat-

ment. This is the first report to show the significance of baseline Alb levels as we used a time-to-event method for large populations, which is a more powerful and informative method to assess the association of factors to time-to-event outcomes.

The rate of HBeAg clearance was 34% at the end of follow up, which was compatible with previous observations.<sup>10,18</sup> According to the results of multivariate analysis, IFN history was the strongest predictor of HBeAg clearance. Of the 37 patients, 17 (46%) who had previously received IFN therapy achieved HBeAg loss, suggesting that previous IFN therapy might have some immune modulatory effect on the ongoing combination therapy. IFN-induced HBeAg loss has been reported to be durable after a follow-up period of 4–8 years.<sup>19-21</sup> In addition, baseline ALT levels were also significantly associated with HBeAg clearance in this study. Our results agree with those of many clinical studies that have shown baseline ALT levels to be the strongest predictor of HBeAg seroconversion in response to IFN therapy<sup>22</sup> as well as nucleos(t)ide analog therapy.<sup>23,24</sup>

Alanine aminotransferase normalization was achieved in 82.7% of the patients. ALT normalization and VR were independent of each other. Actually, among 24 patients who did not achieve ALT normalization, only seven had not achieved VR, suggesting that ALT elevation after sustained suppression of HBV replication might be associated with some conditions other than CHB. In addition, lower baseline Alb was revealed

to be an independent and positive predictive factor for ALT normalization. Considering that patients who did achieve ALT normalization had lower Alb levels than patients with elevated ALT at the final follow up (4.4 vs 4.6 g/dL,  $P < 0.01$ ), and Alb levels are significantly higher in non-alcoholic fatty liver disease,<sup>25</sup> we speculate that fatty liver disease is related to the abnormal ALT. To clarify this, further studies by liver biopsy and/or ultrasonography will be needed.

In conclusion, long-term ADV treatment was highly effective in LAM-resistant CHB patients in terms of virological and biochemical response. In addition, the emergence of resistance to the add-on ADV therapy appears to be delayed and infrequent, in contrast to LAM. Furthermore, lower HBV DNA level and lower Alb level were significant predictive factors for better outcomes. Even though add-on ADV therapy in LAM-resistant CHB patients was highly effective in the long term, CHB patients with LAM or entecavir monotherapy need to be carefully followed-up and the optimal timing of ADV intervention should be determined on the basis of HBV DNA level and progression of liver disease.

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## SUPPORTING INFORMATION

**A**DDITIONAL SUPPORTING INFORMATION may be found in the online version of this article:

**Appendix S1** Relationship of liver cirrhosis with virological response on the basis of fibrosis, using 60 out of 158 patients liver biopsy had been performed. Fibrosis was related with platelet counts but neither with albumin levels nor with the virological response.

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## APPENDIX I

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## Long-term effect of lamivudine treatment on the incidence of hepatocellular carcinoma in patients with hepatitis B virus infection

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### Abstract

**Background** Nucleotide analogues have recently been approved for the treatment of patients with hepatitis B virus (HBV) infection. However, it is still controversial whether the decrease of HBV-DNA amount induced by treatment with nucleotide analogues can reduce the risk of hepatocellular carcinoma (HCC) development in HBV patients.

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**Methods** A total of 293 HBV patients without HCC who were treated with lamivudine (LAM) were enrolled in a multicenter trial. The incidence of HCC was examined after the start of LAM therapy, and the risk factors for liver carcinogenesis were analyzed. The mean follow-up period was  $67.6 \pm 27.4$  months.

**Results** On multivariate analysis for HCC development in all patients, age  $\geq 50$  years, platelet count  $< 14.0 \times 10^4/\text{mm}^3$ , cirrhosis, and median HBV-DNA levels of  $\geq 4.0$  log copies/ml during LAM treatment were significant risk factors. The cumulative carcinogenesis rate at 5 years was

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3% in patients with chronic hepatitis and 30% in those with cirrhosis. For the chronic hepatitis patients, the log-rank test showed the significant risk factors related to HCC development to be age  $\geq 50$  years, platelet count  $< 14.0 \times 10^4/\text{mm}^3$ , and hepatitis B e antigen negativity, but median HBV-DNA levels of  $< 4.0$  log copies/ml (maintained viral response, MVR) did not significantly suppress the development of HCC. In cirrhosis patients, however, the attainment of MVR during LAM treatment was revealed to reduce the risk of HCC development.

**Conclusions** These results suggest that the incidence of HCC in HBV patients with cirrhosis can be reduced in those with an MVR induced by consecutive LAM treatment.

**Keywords** Lamivudine · Chronic hepatitis B · Cirrhosis · Hepatocellular carcinoma · HBV-DNA level

### Abbreviations

HBV	Hepatitis B virus
HCC	Hepatocellular carcinoma
LAM	Lamivudine
ADV	Adefovir
ETV	Entecavir
Hbs Ag	Hepatitis B surface antigen
PCR	Polymerase chain reaction
TMA	Transcription-mediated amplification
IVR	Initial viral response
MVR	Maintained viral response
HBe Ag	Hepatitis B e antigen
CT	Computed tomography
MRI	Magnetic resonance imaging
ALT	Alanine aminotransferase

### Introduction

More than 350 million people worldwide suffer from chronic infection with hepatitis B virus (HBV) [1–3]. Chronic HBV infection eventually leads to the development of cirrhosis and hepatocellular carcinoma (HCC), and raises the risk of hepatic disease-related death [4–6]. In Japan, up to 15% of HCC patients are diagnosed with HBV-related liver disease [7].

HCC is one of the most common malignancies in Japan and its incidence has been increasing over the past 30 years. Recently, various treatments such as transcatheter arterial embolization/chemoembolization, radio-frequency ablation, and hepatic resection have been reported to yield significant improvements in overall patient survival [8–11]. However, HCC relapse has thus far been observed in a majority of treated patients due to its highly malignant potential. In this regard, successful treatment of chronic

HBV infection should prevent the patient's liver from progressing to cirrhosis and reduce the risk of HCC development. In recent years, the treatment of chronic hepatitis has changed greatly with the development of various antiviral therapies with nucleoside/nucleotide analogues such as lamivudine (LAM), adefovir (ADV), and entecavir (ETV) [12–15]. LAM has long been used against chronic hepatitis, and many reports have demonstrated that LAM is effective in stabilizing inflammatory activity, suppressing HBV-DNA replication, and improving liver histological findings in chronic hepatitis patients [16, 17] and in HBV-related cirrhosis patients [18]. Furthermore, LAM has been reported to reduce the incidence of HCC in patients with chronic hepatitis B [19]. However, it is still controversial whether or not treatment using nucleotide analogues can reduce the risk of HCC development in HBV-infected patients [20, 21], and the relationship between the effect of HBV suppression and HCC development during LAM treatment has not yet been discussed in detail. Also, the risk factors for HCC development in HBV-infected patients who have been treated with LAM have not been sufficiently evaluated. In this study, we aimed to clarify whether the decrease of HBV-DNA amount induced by LAM therapy could reduce the incidence of HCC in HBV-infected patients.

### Patients and methods

#### Patient selection and study design

This study was conducted at Osaka University Hospital and other institutions participating in the Osaka Liver Forum in Japan. The subjects were 293 consecutive patients with HBV infection who underwent continuous LAM therapy for more than 24 weeks from September 2000 to September 2006. All patients tested positive for hepatitis B surface antigen (HBs Ag) or had detectable levels of HBV DNA in their sera according to findings from a polymerase chain reaction (PCR)-based method or a transcription-mediated amplification (TMA) method. Exclusion criteria were patients with anti-hepatitis C antibody, anti-human immunodeficiency virus antibody, and other liver diseases (alcoholic liver disease, drug-induced liver disease, and autoimmune hepatitis). Also excluded were patients with a history of HCC and those who developed HCC within the first 24 weeks of the follow-up period after the initiation of LAM therapy (because of the possibility that microscopic HCC had been present before the initiation of treatment).

All patients were treated with 100 mg of LAM daily. Of the 293 patients, 129 underwent ADV (10 mg/day) therapy in addition to receiving ongoing LAM treatment. For 43 patients who started ETV administration in lieu of LAM, the observation period was terminated when they started

ETV. LAM resistance was confirmed by virological breakthrough and was defined as an increase in serum HBV-DNA by  $>1 \log_{10}$  greater than the nadir [22]. If virological breakthrough developed and alanine aminotransferase (ALT) was elevated over the upper normal limit, the patients received add-on ADV at 10 mg/day.

In this study, all patients were examined for serum HBV-DNA level just before therapy initiation and every 6 months during treatment. The initial viral response (IVR) was defined as HBV-DNA  $<4.0 \log$  copies/ml in the first 24 weeks of the follow-up period after the initiation of LAM therapy, and the maintained viral response (MVR) was defined as median HBV-DNA levels of less than 4.0 log copies/ml measured every 6 months during therapy.

This study protocol followed the ethical guidelines of the Declaration of Helsinki amended in 2008, and informed consent was obtained from each patient.

### HBV testing

HBs Ag, hepatitis B e antigen (HBe Ag) and anti-hepatitis B e antibody (anti-HBe) levels were examined by chemiluminescence immunoassay or enzyme immunoassay. HBV DNA was measured by a PCR-based method (Amplicor HBV monitor; Roche Diagnostics, Tokyo, Japan) or a TMA method (TMA-HPA; Fujirebio, Tokyo, Japan), which have lower detection limits of 2.6 and 3.7 log copies/ml, respectively. The LAM-resistant YMDD mutant virus was examined by a PCR-ELMA method. Serum samples were stored frozen at  $-80^{\circ}\text{C}$ .

### Diagnosis of HCC and cirrhosis

Ultrasonography was carried out before LAM therapy and every 3–6 months during the follow-up period. New space-occupying lesions detected or suspected at the time of ultrasonography were further examined by computed tomography (CT), magnetic resonance imaging (MRI), or hepatic angiography. HCC was diagnosed by the presence of typical hypervascular characteristics on angiography, in addition to the findings from CT or MRI. If no typical image of HCC was observed, fine-needle aspiration biopsy was carried out with the patient's consent or the patient was carefully followed until a diagnosis was possible with definite observation by CT, MRI, or hepatic angiography. Cirrhosis was diagnosed by liver biopsy or laparoscopy, and for patients without this information, by clinical data, imaging modalities, and portal hypertension.

### Statistical analysis

Quantitative variables were expressed as means  $\pm$  SD. Quantitative variables at the baseline were compared

among two groups, the chronic hepatitis and cirrhosis groups, using the Mann–Whitney *U*-test. Categorical data, such as gender and status of HBe Ag, were compared using Fisher's exact test. The cumulative incidence of HCC was evaluated with a Kaplan–Meier curve and the differences between groups were analyzed by the log-rank test. For multivariate analysis to investigate factors affecting the cumulative incidence of HCC, Cox's regression analysis was carried out. A value of  $p < 0.05$  (two-tailed) was considered to be statistically significant. All calculations were performed with SPSS version 15.0J (SPSS, Chicago, IL, USA).

## Results

### Baseline characteristics of patients

The baseline clinical features of the enrolled patients before LAM administration are shown in Table 1. The mean age of the patients was  $48.0 \pm 10.7$  years, 214 (73%) of the entire group were male, and 163 (56%) tested positive for HBe Ag. Of the 293 patients, 205 (70%) were diagnosed as having chronic hepatitis and 88 (30%) as having cirrhosis. The median HBV-DNA level was 7.0 (range 3.0 to 8.5) log copies/ml. At baseline, the aspartate aminotransferase (AST) level was  $131 \pm 151$  IU/l, the ALT level was  $203 \pm 252$  IU/l, the total bilirubin level was  $1.2 \pm 1.6$  mg/dl, the albumin (Alb) level was  $3.8 \pm 0.5$  g/dl, and the platelet count was  $13.7 \pm 5.4 \times 10^4/\text{mm}^3$ . The mean follow-up period for all patients was  $67.6 \pm 27.4$  months, with a range of 12–110 months from the start of LAM treatment. There were significant differences between patients with chronic hepatitis and those with liver cirrhosis in age, AST, ALT, total bilirubin, Alb, and platelet counts.

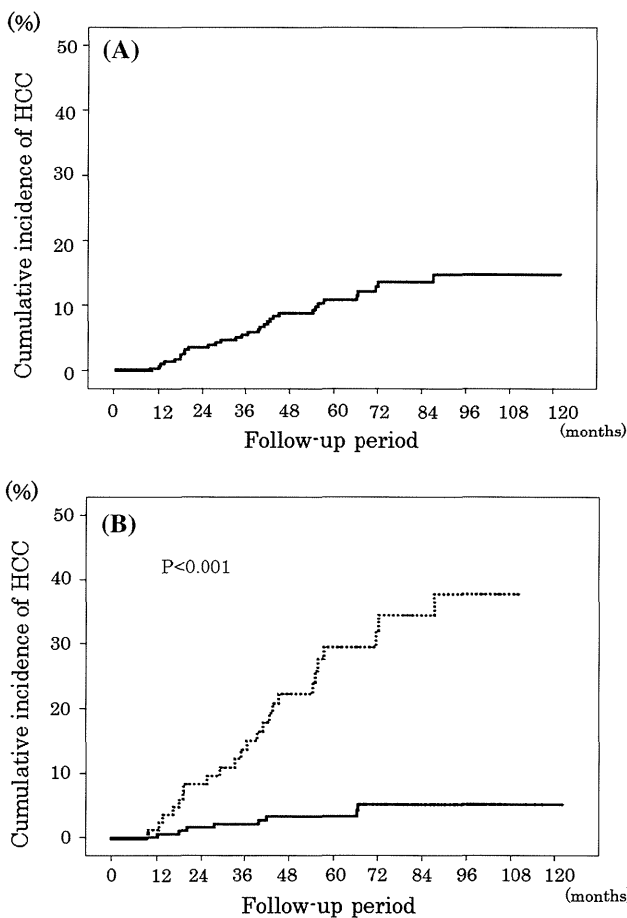
### Cumulative incidence of development of HCC

Figure 1a shows the Kaplan–Meier curve of the cumulative HCC incidence for all HBV patients treated with LAM or LAM plus ADV. Of the 293 patients with HBV infection, 32 (10.9%) developed HCC and the cumulative carcinogenesis rate was 6% at 3 years, 12% at 5 years, and 15% at 7 years.

Figure 1b shows the Kaplan–Meier curve of the cumulative HCC incidence according to initial diagnosis (chronic hepatitis vs. cirrhosis). Eight (4%) of the 205 enrolled chronic hepatitis patients developed HCC and the cumulative carcinogenesis rate was 2% at 3 years, 3% at 5 years, and 5% at 7 years. On the other hand, 24 (27%) of the 88 enrolled cirrhosis patients developed HCC and the cumulative carcinogenesis rate was 15% at 3 years, 30% at 5 years, and 35% at 7 years.

**Table 1** Patient characteristics

Factor	All	Chronic hepatitis	Cirrhosis	<i>p</i> value
<i>HBe Ag</i> Hepatitis B e antigen,				
<i>HBV</i> hepatitis B virus,				
<i>AST</i> aspartate aminotransferase,				
<i>ALT</i> alanine aminotransferase,				
<i>Alb</i> albumin				
<sup>a</sup> Values are expressed as medians				
* <i>p</i> < 0.05, ** <i>p</i> < 0.001, comparing patients with chronic hepatitis and those with liver cirrhosis using the Mann–Whitney <i>U</i> -test for quantitative variables and Fisher’s exact test for categorical variables				
Number of patients	293	205	88	
Age (years)	48.0 ± 10.7	46.3 ± 10.7	51.9 ± 9.8	<0.001**
Sex (male/female)	214/79	147/58	67/21	0.475
<i>HBe Ag</i> (positive)	163 (56%)	121 (59%)	42 (48%)	0.068
<i>HBV</i> DNA (log copies/ml) <sup>a</sup>	7.0 (3.0 to 8.5<)	6.8±1.1	6.6 ± 1.1	0.162
<i>AST</i> (IU/l)	131 ± 151	143 ± 162	104 ± 120	0.045*
<i>ALT</i> (IU/l)	203 ± 252	235 ± 269	129 ± 189	<0.001**
Total bilirubin (mg/dl)	1.2 ± 1.6	0.9 ± 0.6	1.8 ± 2.7	<0.001**
<i>Alb</i> (g/dl)	3.8 ± 0.5	3.9 ± 0.4	3.5 ± 0.6	<0.001**
Platelets (×10 <sup>4</sup> /mm <sup>3</sup> )	13.7 ± 5.4	15.6 ± 9.3	9.3 ± 3.8	<0.001**
Follow-up period (months)	67.6 ± 27.4	68.5 ± 26.5	65.5 ± 29.5	0.393



**Fig. 1** Cumulative incidence of development of hepatocellular carcinoma (*HCC*) in patients with hepatitis B virus infection treated with lamivudine (LAM). **a** All cases; **b** chronic hepatitis or cirrhosis. Solid line Chronic hepatitis, dotted line cirrhosis

**Risk factors for cumulative incidence of HCC development in all HBV-infected patients**

Univariate analysis with the log-rank test was performed for all HBV-infected patients treated with LAM, with the

results shown in Table 2. Univariate analysis with the log-rank test showed that the following were significant risk factors for the development of HCC: older age ( $\geq 50$  years) ( $p < 0.001$ ), cirrhosis ( $p < 0.001$ ), high total bilirubin level ( $>1.2$  g/dl) ( $p = 0.004$ ), low *Alb* level ( $<3.8$  g/dl) ( $p = 0.019$ ), low platelet count ( $<14 \times 10^4/\text{mm}^3$ ) ( $p < 0.001$ ), and non-MVR ( $p = 0.035$ ).

Stepwise multivariate analyses of four of these variables were performed by Cox’s regression analysis for all patients treated with LAM with the results shown in Table 3. The analysis indicated the following factors as independent significant risk factors related to the development of HCC: age  $\geq 50$  years [hazard ratio (HR) 3.20, 95% confidence interval [CI] 1.08–9.53,  $p = 0.036$ ], platelet count  $<14.0 \times 10^4/\text{mm}^3$  (HR 4.76, 95% CI 0.05–0.96,  $p = 0.045$ ), cirrhosis (HR 4.64, 95% CI 1.75–12.4,  $p = 0.002$ ), and non-MVR (HR 2.70, 95% CI 1.09–6.56,  $p = 0.032$ ).

**Cumulative incidence of and risk factors for HCC development in patients with chronic hepatitis and cirrhosis**

The results of univariate analysis with the log-rank test for the development of HCC in chronic hepatitis patients treated with LAM are shown in Table 4, and the following were significant risk factors: older age ( $\geq 50$  years) ( $p = 0.002$ ), *HBe Ag* negativity ( $p = 0.005$ ), and low platelet count ( $<14 \times 10^4/\text{mm}^3$ ) ( $p = 0.004$ ). Suppression of median *HBV*-DNA levels to  $<4.0$  log copies/ml by LAM treatment was not associated with the development of HCC in the chronic hepatitis patients. Only non-MVR (median *HBV*-DNA amount  $\geq 4.0$  log copies/ml) was shown to be a significant risk factor for the development of HCC in the cirrhosis patients ( $p = 0.029$ ), while the factors of age, *HBe Ag* status, and platelet count were not significant in these patients (Table 4).

**Table 2** Risk factors for HCC development in all HBV-infected patients by univariate analysis

Factor	95% CI	p value
Age (years) (<50/≥50)	2.15–14.5	<0.001
Sex (male/female)	0.33–1.76	0.520
Initial diagnosis (chronic hepatitis/cirrhosis)	3.75–1.176	<0.001
HBe Ag (positive/negative)	0.31–1.29	0.209
HBV DNA (log copies/ml) (<7.0/≥7.0)	0.33–1.35	0.262
AST (IU/l) (<40/≥40)	0.33–2.22	0.742
ALT (IU/l) (<40/≥40)	0.17–1.16	0.188
Total bilirubin (mg/dl) (<1.2/≥1.2)	1.43–6.72	0.004
Alb (g/dl) (<3.8/≥3.8)	0.19–0.86	0.019
Platelets (×10 <sup>4</sup> /mm <sup>3</sup> ) (<14/≥14)	0.02–0.31	<0.001
Emergence of LAM-resistant viruses (positive/negative)	0.51–2.03	0.968
IVR (positive/negative)	0.52–3.25	0.575
MVR (positive/negative)	1.04–5.95	0.035

HCC Hepatocellular carcinoma, HBV hepatitis B virus, CI confidence interval, HBe Ag hepatitis B e antigen, HBV hepatitis B virus, AST aspartate aminotransferase, ALT alanine aminotransferase, Alb albumin, IVR initial viral response, MVR maintained viral response, LAM lamivudine

**Table 3** Risk factors for HCC development in all HBV-infected patients by multivariate analysis

Factor	Category	Risk ratio	95% CI	p value
Age (years)	<50	1	1.08–9.53	0.036
	≥50	3.20		
Initial diagnosis	Chronic hepatitis	1	1.75–12.4	0.002
	Cirrhosis	4.64		
Platelets (×10 <sup>4</sup> /mm <sup>3</sup> ) (<14/≥14)	≥14	1	0.05–0.96	0.045
	<14	4.76		
MVR	Negative	1	1.09–6.56	0.032
	Positive	0.37		

HCC Hepatocellular carcinoma, HBV hepatitis B virus, CI confidence interval, MVR maintained viral response

Cumulative incidence of HCC development according to effectiveness of treatment (MVR vs. non-MVR)

Figure 2a shows the Kaplan–Meier curve of cumulative HCC incidence in all HBV-infected patients treated with LAM according to the effectiveness of treatment (MVR vs. non-MVR). The cumulative carcinogenesis rate for MVR-positive patients was 2% at 3 years, 4% at 5 years, and 6% at 7 years. On the other hand, the cumulative carcinogenesis rate for MVR-negative patients was 5% at 3 years, 13% at 5 years, and 16% at 7 years. MVR during LAM significantly suppressed the cumulative HCC incidence

**Table 4** Risk factors for HCC development by univariate analysis (chronic hepatitis/cirrhosis)

	95% CI	p value
<b>Chronic hepatitis</b>		
Age (years) (<50/≥50)	0.26–8.38	0.002
Sex (male/female)	0.37–6.42	0.556
HBe Ag (positive/negative)	0.01–0.74	0.005
HBV DNA (log copies/ml) (<7.0/≥7.0)	0.11–1.99	0.296
AST (IU/l) (<40/≥40)	0.11–2.64	0.482
ALT (IU/l) (<40/≥40)	0.06–1.41	0.101
Total bilirubin (mg/dl) (<1.2/≥1.2)	0.67–6.67	0.574
Alb (g/dl) (<3.8/≥3.8)	0.13–8.58	0.960
Platelets (×10 <sup>4</sup> /mm <sup>3</sup> ) (<14/≥14)	0.01–0.72	0.004
Emergence of LAM-resistant viruses (positive/negative)	0.27–4.28	0.927
IVR (positive/negative)	0.29–8.67	0.590
MVR (positive/negative)	0.51–37.10	0.144
<b>Cirrhosis</b>		
Age (years) (<50/≥50)	0.86–6.17	0.089
Sex (male/female)	0.21–1.82	0.380
HBe Ag (positive/negative)	0.80–4.17	0.149
HBV DNA (log copies/ml) (<7.0/≥7.0)	0.40–2.01	0.795
AST (IU/l) (<40/≥40)	0.27–3.07	0.873
ALT (IU/l) (<40/≥40)	0.13–1.47	0.167
Total bilirubin (mg/dl) (<1.2/≥1.2)	0.82–4.80	0.126
Alb (g/dl) (<3.8/≥3.8)	0.28–1.58	0.354
Platelets (×10 <sup>4</sup> /mm <sup>3</sup> ) (<14/≥14)	0.03–1.51	0.084
Emergence of LAM-resistant viruses (positive/negative)	0.44–2.18	0.948
IVR (positive/negative)	0.90–8.32	0.063
MVR (positive/negative)	1.07–0.029	

HCC Hepatocellular carcinoma, HBV hepatitis B virus, CI confidence interval, HBe Ag hepatitis B e antigen, HBV hepatitis B virus, AST aspartate aminotransferase, ALT alanine aminotransferase, Alb albumin, IVR initial viral response, MVR maintained viral response

compared with non-MVR in all HBV-infected patients ( $p = 0.035$ ).

Figure 2b shows the Kaplan–Meier curve of the cumulative HCC incidence in chronic hepatitis patients according to the effectiveness of treatment (MVR vs. non-MVR). The cumulative carcinogenesis rate for MVR-positive patients was 0% at 3 years, 0% at 5 years, and 2% at 7 years. On the other hand, the cumulative carcinogenesis rate for MVR-negative patients was 2% at 3 years, 4% at 5 years, and 6% at 7 years. MVR during LAM did not significantly suppress the cumulative HCC incidence compared with non-MVR in the chronic hepatitis patients ( $p = 0.144$ ).

Figure 2c shows the Kaplan–Meier curve of the cumulative HCC incidence in cirrhosis patients according to the effectiveness of treatment (MVR vs. non-MVR).