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_{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 1.7×10^{-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 mete-analysis of all our study. The result did not show any association between rs17401966 and HBV-derived HCC (P_{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				OR		
	(cases/controls)	GG	AG	AA	GG	AG	AA	HWE p	(95 % CI)	P [a]	P_{het} [b]
replication 1	179/769	13	61	105	45	261	463	0.599	1.09	0.578	
(Japan 1)		(7.2)	(34.1)	(58.7)	(5.9)	(33.9)	(60.2)		(0.82-1.43)		
replication 2	142/251	5	46	91	14	91	146	1	0.79	0.212	
(Japan 2)		(3.5)	(32.4)	(64.1)	(5.6)	(36.2)	(58.2)		(0.54-1.15)		
replication 3	164/144	17	59	88	15	55	74	0.616	0.95	0.790	
(Korea)		(10.4)	(36.0)	(53.6)	(10.4)	(38.2)	(51.4)		(0.66-1.36)		
replication 4	94/187	10	39	44	13	80	94	0.767	1.17	0.432	
(Hong Kong)		(10.6)	(41.5)	(46.8)	(6.9)	(42.8)	(50.3)		(0.79-1.75)		
Meta-analysis [c]									0.996	0.965	0.423
									(0.84-1.18)		

[a]P value of fisher's exact test for allele model.
[b]Result of Breslow-Day test.
[c]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: $P_{het} = 0.0066$; Chan's study versus Zhang's study: $P_{het} = 0.035$). 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 hepatisis 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, Nationa 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-μl reaction mixture containing 1 μl of genomic DNA, 2.5 μl 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.

Acknowledgements

This study was supported by a grant-in-aid from the Ministry of Health, Labour, and Welfare of Japan (H23-kanen-005), and Japan Science and Technology Agency (09038024). We thank Dr. Minae Kawashima to giving us technical advices.

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Received: 2 March 2012 Accepted: 19 June 2012
Published: 19 June 2012

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doi:10.1186/1471-2350-13-47
Cite this article as: Sawai *et al.*: No association for Chinese HBV-related hepatocellular carcinoma susceptibility SNP in other East Asian populations. *BMC Medical Genetics* 2012 **13**:47.

Hepatology Research

Hepatology Research 2012 doi: 10.1111/j.1872-034X.2012.01038.x

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

INTRODUCTION

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

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Received 29 January 2012; revision 11 April 2012; accepted 15 April 2012.

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

Lamivudine (LAM) treatment has been used to prevent the progression of CHB and the development of HCC.[5] 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.[6–8] 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.[9,10] 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 (≥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, γ-glutamyl transpeptidase (γ-GTP), platelet (PLT) counts, and total bilirubin (T-Bil), albumin (Alb), prothrombin time (PT) and α-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 $n = 99$	HBeAg negative $n = 59$
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 ($\times 10^4/mm^3$)	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

IN 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 population; 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 ($P < 0.001$, $P = 0.002$,

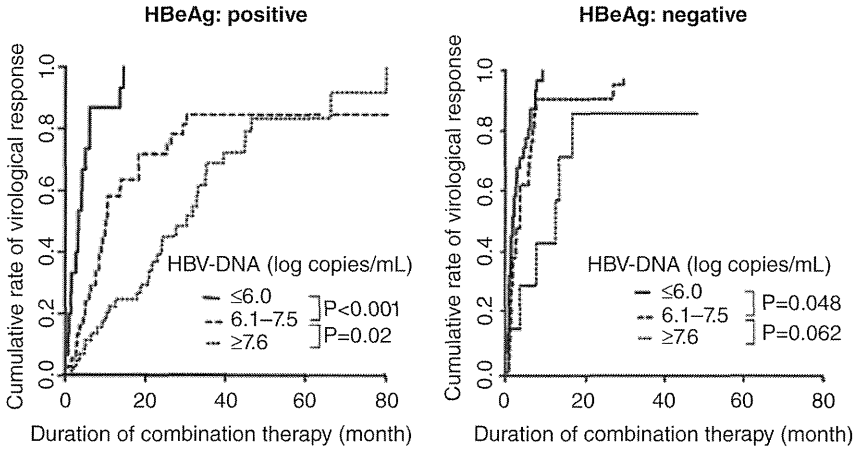

Figure 1 Cumulative rate of virological response on treatment with lamivudine plus adefovir dipivoxil depending on hepatitis B virus (HBV) DNA load in HBeAg positive and negative patients. hepatitis B e antigen (HBeAg) negativity and low HBV replication had a higher probability of virological response 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 $n = 99$				HBeAg negative $n = 59$	
	Univariate		Multivariate		Univariate	
	HR	*P*-value	HR	*P*-value	HR	*P*-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 ($\times 10^4/mm^3$) (<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, $n = 99$				ALT normalization			
	Univariate		Multivariate		Univariate		Multivariate	
	Odds ratio	*P*-value	Odds ratio	*P*-value	Odds ratio	*P*-value	Odds ratio	*P*-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 ($\times 10^4/mm^3$) (<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.[11–17] 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 treatment. 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.[10,18] 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.[19–21] 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[22] as well as nucleos(t)ide analog therapy.[23,24]

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,[25] 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.

ACKNOWLEDGMENTS

THIS STUDY WAS supported in part by a Grant-in-Aid from the NHO (no. H21-NHO(Kan)-01). We express our deepest appreciation to the members of the Liver Disease Network Group of the NHO and to their institutions. For complete details, please see Appendix I.

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SUPPORTING INFORMATION

ADDITIONAL 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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J Gastroenterol (2012) 47:577–585
DOI 10.1007/s00535-011-0522-7

ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

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

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Received: 30 August 2011 / Accepted: 29 November 2011 / Published online: 11 January 2012

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.

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 ± 27.4 months.

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

Electronic supplementary material The online version of this article (doi:10.1007/s00535-011-0522-7) contains supplementary material, which is available to authorized users.

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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 ≥50 years, platelet count $<14.0 \times 10^4/mm^3$, 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 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°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 ± 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–Meiercurve 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 ± 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 ± 151 IU/l, the ALT level was 203 ± 252 IU/l, the total bilirubin level was 1.2 ± 1.6 mg/dl, the albumin (Alb) level was 3.8 ± 0.5 g/dl, and the platelet count was 13.7 ± 5.4 × 10^4/mm^3. The mean follow-up period for all patients was 67.6 ± 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	*p* value
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
HBe Ag (positive	163 (56%)	121 (59%)	42 (48%)	0.068
HBV DNA (log copies/ml)[a]	7.0 (3.0 to 8.5<)	6.8±1.1	6.6 ± 1.1	0.162
AST (IU/l)	131 ± 151	143 ± 162	104 ± 120	0.045*
ALT (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**
Alb (g/dl)	3.8 ± 0.5	3.9 ± 0.4	3.5 ± 0.6	<0.001**
Platelets ($\times 10^4$/mm^3)	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

HBe Ag Hepatitis B e antigen, *HBV* hepatitis B virus, *AST* aspartate aminotransferase, *ALT* alanine aminotransferase, *Alb* albumin

[a] Values are expressed as medians

* $p < 0.05$, ** $p < 0.001$, comparing patients with chronic hepatitis and those with liver cirrhosis using the Mann–Whitney *U*-test for quantitative variables and Fisher's exact test for categorical variables

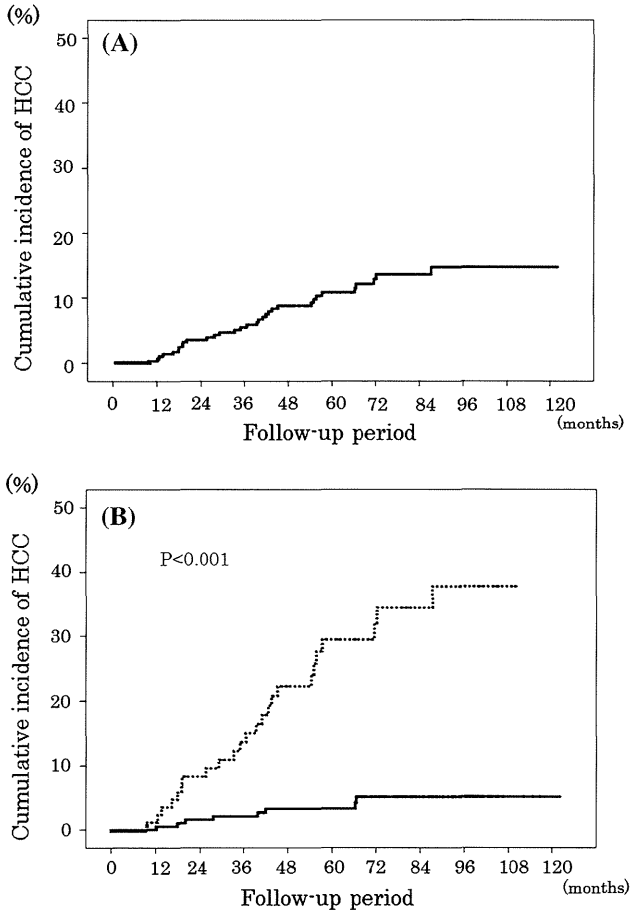

Fig. 1 Cumulative incidence of development of hepatocellular carcinoma (*HCC*) in patients with hepatitis B virus infection treated with lamivudine (LAM). **a** All cases; **b** chronic hepatitis or cirrhosis. *Solid line* Chronic hepatitis, *dotted line* cirrhosis

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 (≥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 × 10^4/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 ≥50 years [hazard ratio (HR) 3.20, 95% confidence interval [CI] 1.08–9.53, $p = 0.036$], platelet count <14.0 × 10^4/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 (≥50 years) ($p = 0.002$), HBe Ag negativity ($p = 0.005$), and low platelet count (<14 × 10^4/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 ≥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 ($\times 10^4$/mm^3) (<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 ($\times 10^4$/mm^8) (<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 compared with non-MVR in all HBV-infected patients ($p = 0.035$).

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

	95% CI	*p* value
Chronic hepatitis		
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 ($\times 10^4$/mm^3) (<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
Cirrhosis		
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 ($\times 10^4$/mm^3) (<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

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).

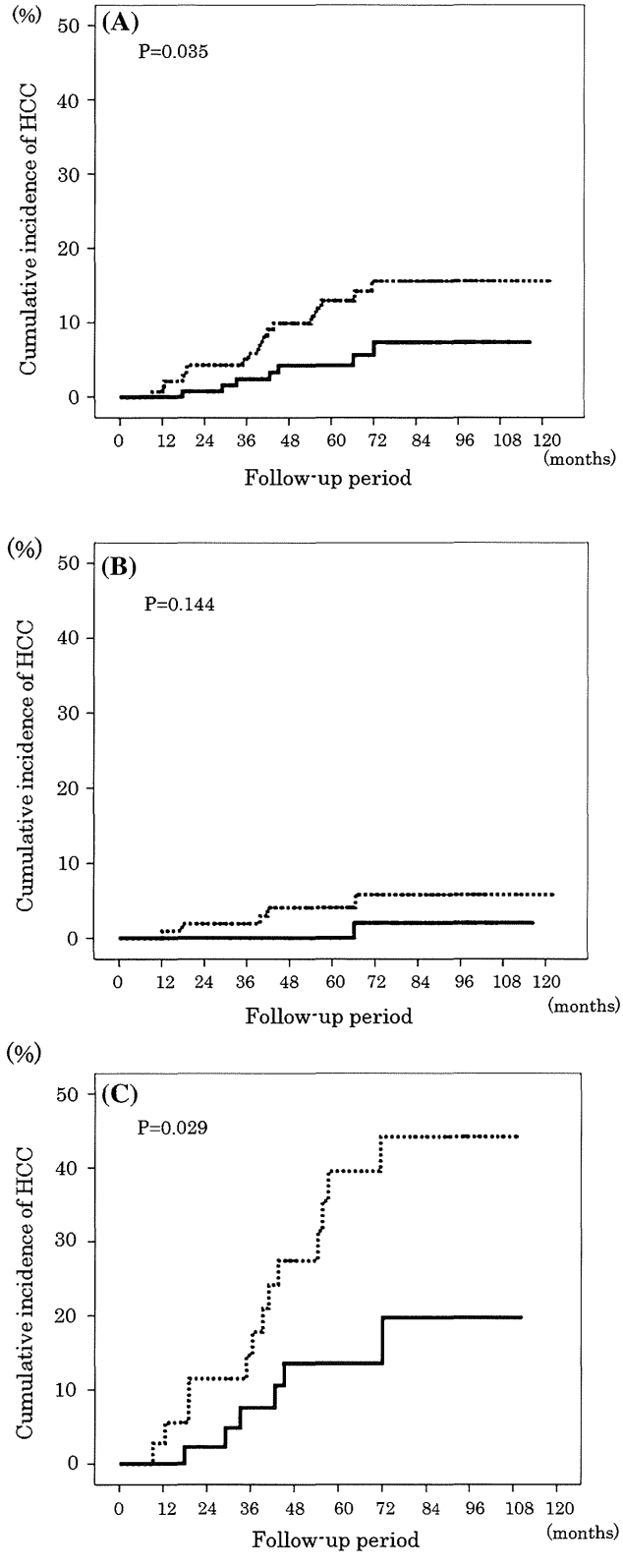

Fig. 2 Cumulative incidence of development of HCC according to the effectiveness of treatment (MVR vs. non-MVR). **a** All cases; **b** chronic hepatitis; **c** cirrhosis. *Solid lines* MVR, *dotted lines* non-MVR. *MVR* Maintained viral response

The cumulative carcinogenesis rate for MVR-positive patients was 8% at 3 years, 14% at 5 years, and 14% at 7 years. On the other hand, the cumulative carcinogenesis rate for MVR-negative patients was 18% at 3 years, 40% at 5 years, and 44% at 7 years. MVR during LAM significantly suppressed the cumulative HCC incidence compared with non-MVR in the cirrhosis patients ($p = 0.029$).

Relationship between IVR and MVR

Maintained viral response (MVR) was achieved by 142 (48%) of the 293 patients enrolled in this study. IVR was achieved by 204 (79%) of the 259 patients who were examined for IVR. The relationship between IVR and MVR is shown in Fig. 3; 60% (122/204) of the IVR-positive patients achieved an MVR, while only 16% (9/55) of the IVR-negative patients achieved an MVR ($p < 0.001$). The LAM-resistant YMDD mutant virus was found in 149 (51%) of all patients during follow-up, and in 52% (107/204) of the IVR-positive patients, a finding which was nearly equal to that for the IVR-negative patients (51%, 28/55). Among the IVR-positive patients, the MVR rate was lower in patients with the YMDD mutation, compared with that in those without the YMDD mutation (44%, 47/107 vs. 77%, 75/97, $p = 0.016$), while the MVR rates were low in the IVR-negative patients, irrespective of their YMDD mutation status (with and without the mutation, 15 vs. 18%, respectively). ADV was added to LAM treatment for 73 (68%) of the 107 IVR-positive patients with the YMDD mutation and 20 (36%) of the 55 IVR-negative patients with the YMDD mutation. However, MVR was only achieved at the low rates of 33% (24/73) for the former patients and 20% (4/20) for the latter.

Discussion

Lamivudine treatment has been shown to improve the liver histological findings in patients with HBV-infected liver disease by reducing the HBV load and stabilizing inflammatory activity [16–18]. One report has shown that LAM effectively reduced the incidence of HCC in patients with chronic hepatitis B, but the study only compared LAM-treated patients with non-treated patients in a matched case-controlled study [19]. However, there have been few detailed reports about the relationship between virological response and HCC development in HBV-infected patients during LAM treatment. In the present study, we retrospectively examined the incidence of HCC to clarify the indicators of LAM therapy, including median HBV-DNA levels, for reducing the risk of HCC in HBV-infected patients.

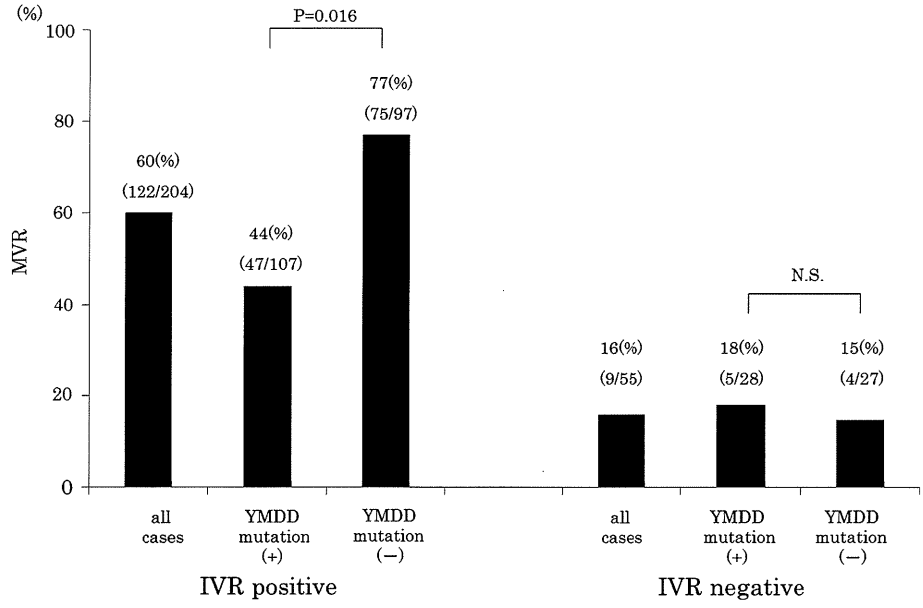

Fig. 3 Relationship between IVR and MVR. *IVR* Initial viral response, *MVR* maintained viral response, *N.S.* not significant

Many investigators have reported that serum HBV DNA levels higher than 4.0–4.5 log copies/ml before HBV treatment serve as a strong risk predictor of HCC [23–25]. Di Marco et al. [26] have reported that the incidence of HCC was higher in patients with serum HBV levels of more than 5.0 log copies/ml, at least once, during LAM therapy than in those in whom serum HBV levels were maintained at 5.0 log copies/ml or less. However, the add-on ADV therapy had not been adopted when the study of Di Marco et al. was reported. When the use of ADV is possible, an evaluation method is needed to measure the antiviral effects of nucleoside/nucleotide analogues against HBV-related liver disease. In the present study, we set the cut-off value for HBV-DNA at 4.0 log copies/ml. The basis of this cut-off value is that a serum HBV DNA level higher than 4.0 log copies/ml before HBV treatment was reported to serve as a strong risk predictor of HCC [23]. MVR, defined as a median HBV-DNA level of less than 4.0 log copies/ml measured every 6 months during therapy, was adopted as an indicator of viral replication, and non-MVR (median HBV-DNA >4.0 log copies/ml) during LAM therapy was shown to be significantly associated with the development of HCC in HBV-infected patients. We also found that a median HBV-DNA level of >4.0 log copies/ml during LAM therapy was a risk factor for HCC development. On the other hand, IVR, defined as HBV-DNA of <4.0 log copies/ml in the first 6 months of the follow-up period after the initiation of therapy, was not associated with the development of HCC in HBV patients in this study. As shown in Fig. 3, 84% of the IVR-negative patients could not achieve an MVR, suggesting that it is crucial to achieve an IVR in order to achieve an MVR. The reason why IVR was not a significant factor for MVR seemed to be the appearance of the YMDD mutation, which reduced the antiviral effect of LAM for HBV in IVR-positive patients. The LAM-resistant YMDD mutant virus was found in 52% of the IVR-positive patients. Although ADV was added to LAM treatment for 73 patients, only 33% of these patients could achieve an MVR. We speculate that the antiviral effect of ADV is not very strong [27] and it takes time to reduce the YMDD mutant virus, which may explain the low MVR rate (33%) in patients with the add-on ADV therapy. The immediate administration of ADV when the LAM-resistant YMDD mutant virus appears can be important [28]. A switch to ETV, which induces resistant virus less frequently, could also raise MVR rates among IVR-positive patients without the YMDD mutant virus.

As the duration of the add-on ADV therapy was included in this study, we compared the cumulative incidence of HCC in patients receiving LAM monotherapy with that in patients who also received the add-on ADV therapy. Sixteen (10%) of the 164 patients who received the LAM monotherapy developed HCC and the cumulative carcinogenesis rate was 6% at 3 years, 10% at 5 years, and 15% at 7 years. On the other hand, 16 (12%) of the 129 patients who received LAM plus ADV developed HCC and the cumulative carcinogenesis rate was 6% at 3 years, 12% at 5 years, and 14% at 7 years. No significant difference was found between these two groups ($p = 0.986$). In addition, we examined the cumulative incidence of HCC development according to the effectiveness of treatment (MVR vs. non-MVR) in patients for whom the observation period was terminated when ADV was added, and the same results were obtained (data not shown).

Older age (≥50 years), cirrhosis, and low platelet count ($<14 \times 10^4/mm^3$) were shown to be significantly associated with the development of HCC in patients with HBV infection. These results were consistent with those of

previous reports [29–31], suggesting that patients of older age with advanced fibrosis should be followed up carefully for longer periods in order to detect early stages of HCC even if LAM therapy does effectively suppress HBV. Of note, in the present study we estimated the cumulative HCC incidence according to the initial diagnosis of chronic hepatitis or cirrhosis. In the chronic hepatitis patients, older age (≥50 years), HBe Ag negativity, and low platelet count ($<14 \times 10^4/mm^3$) were significant risk factors for the development of HCC, but this was not the case in the cirrhosis patients. Because liver biopsies had not been performed, the liver fibrosis stage could not be evaluated with respect to the risk factors for HCC in this study. Instead, the factors of age, HBe Ag status, and platelet count may reflect the degree of liver fibrosis in chronic hepatitis patients. In fact, cirrhotic patients, in comparison with chronic hepatitis patients, were of older age (chronic hepatitis vs. cirrhosis: 46.3 ± 10.7 vs. 51.9 ± 9.8 years, $p < 0.001$), had higher rates of HBe Ag negativity (chronic hepatitis vs. cirrhosis: 39 vs. 51%, $p = 0.065$), and had lower platelet counts (chronic hepatitis vs. cirrhosis: 15.6 ± 4.9 vs. $9.3 \pm 3.8 \times 10^4/mm^3$, $p < 0.001$). This seems to explain why none of these factors were significant risk factors for HCC in cirrhotic patients. On the other hand, in the chronic hepatitis patients, MVR was not a significant factor for HCC development, while MVR was a significant factor for HCC development in the cirrhotic patients. We speculate that HBV suppression induced by LAM therapy could reduce the incidence of HCC in patients infected with HBV, especially those with cirrhosis, who displayed higher malignant potential. Investigation over a longer period is needed to clarify the effect of HBV suppression on the development of HCC in chronic hepatitis patients.

In conclusion, the present study shows that the attainment of an MVR induced by LAM therapy has a significant beneficial effect on the clinical course of HBV-infected patients by decreasing the incidence of HCC. The newer nucleotide analogues, such as ETV and tenofovir, should be able to further reduce the incidence of HCC, given their greater potency.

Acknowledgments This work was supported by Grants-in-Aid for Research on Hepatitis and BSE from the Ministry of Health Labour and Welfare of Japan and for Scientific Research from the Ministry of Education, Science, and Culture of Japan.

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

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J Gastroenterol (2012) 47:334–342
DOI 10.1007/s00535-011-0498-3

ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

Reducing Peg-IFN doses causes later virologic response or no response in HCV genotype 1 patients treated with Peg-IFN alfa-2b plus ribavirin

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Received: 15 August 2011 / Accepted: 21 September 2011 / Published online: 23 November 2011

Abstract
Background The timing to the first undetectable hepatitis C virus (HCV) RNA level is strongly associated with sustained virologic response in pegylated interferon (Peg-IFN) plus ribavirin combination therapy for patients with chronic hepatitis C (CH-C) with genotype 1. This study was conducted to clarify the impact of drug exposure to Peg-IFN on the timing of HCV RNA negativity in Peg-IFN plus ribavirin combination therapy for CH-C patients with genotype 1.

Methods A total of 1409 patients treated with Peg-IFN alfa-2b plus ribavirin were enrolled and classified into four categories according to the Peg-IFN dosage. Furthermore, 100 patients were extracted from each Peg-IFN dosage category to adjust for characteristic factors, using the propensity score method.

Results Peg-IFN exposure was dose-dependently associated with the timing of HCV RNA negativity ($p \leq 0.001$). The HCV RNA negative rate at week 4 decreased from 12% with a Peg-IFN dose of >1.5 μg/kg/week to 1–3% with a dose of <1.5 μg/kg/week ($p \leq 0.001$), and at week 12 the rate had decreased from 44% with a dose of

T. Oze and N. Hiramatsu contributed equally to this work.

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