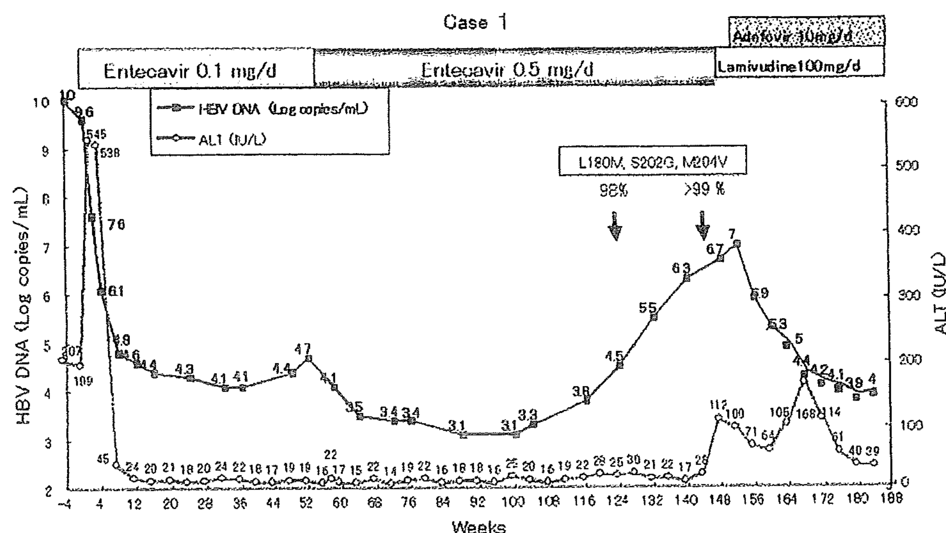

Fig. 1 Clinical course of case 1, a 44-year-old man with nucleoside-naive CHB. ETV treatment reduced ALT levels to below the upper normal limit at week 12 and reduced HBV DNA load to a nadir of 3.1 $\log_{10}$ copies/ml at week 88. However, HBV DNA re-elevated to 4.5 $\log_{10}$ copies/ml at week 124 (virologic breakthrough) and 6.3 $\log_{10}$ copies/ml at week 140, as well as ALT level re-elevated at week 148 (biochemical breakthrough). Sequence analysis of the HBV DNA polymerase gene using serum sample obtained at weeks 124 and 144 revealed the emergence of L180M, M204V (related to LVD resistance), and S202G (related to ETVr) substitutions. SNP-PCR assay revealed that LVDr M204V and ETVr S202G substitutions were detected first at week 124 (98%) and increased at week 148 (>99%). Switching from ETV to LVD/ADV combination treatment at week 148 was successful in reducing HBV DNA load and ALT again

Table 2 Population sequence analysis of isolates from case 1 on ETV therapy

Week	Reverse transcriptase position				
	180	202	204	223	238
0	L	S	M	S/A	N
24	L	S	M	S/A	N
100	L	S	M/I	S/A	N/H
124	M	G	V	S	N/H
144	M	G	V	S	N/H

polymorphic residue S223, which was mixed as S/A at baseline, was found to be only S at weeks 124 and 144.

In addition, preserved serum samples from this patient at baseline and at every 24 weeks were analyzed by an ultrasensitive, single-nucleotide-polymorphism (SNP)-PCR assay, using a method similar to Punia et al. [19] for identification of resistance substitutions, as well as analyzing the sequence of individual clones to determine the genetic linkage of substitutions. SNP-PCR analysis was performed for the two LVD-resistance (LVDr) substitutions, M204V (codon GTG) and M204I (codons ATA and ATT), and the ETVr substitution S202G. Both wild-type and positive control plasmids containing the correct sequence were used at various concentrations to establish the background level as well as the level of detection for each substitution. For clonal analysis, the amplified RT gene from the patient's HBV was cloned into plasmids, as well as 22 to 24 individual clones were selected and sequenced, to determine the genetic linkage of the different substitutions observed.

SNP-PCR analysis for ultrasensitive detection of the resistance substitutions revealed that the LVDr M204V(GTG) and ETVr S202G(GGT) substitutions were not detected (<0.1%) at baseline, week 24, or week 100. The M204I substitution (codon ATA) was detected at low levels at week 24 (0.4%), increased levels at week 100 (6.6%), and was present but at reduced levels at weeks 124 and 148 (0.4% at both time points). The LVDr M204V and ETVr S202G substitutions were detected first at week 124 (98%) and increased levels at week 148 (>99%). The levels of M204I(ATA) were lower at weeks 124 and 144, likely as a result of the dominant M204V/S202G virus (Table 3). Samples at weeks 48 and 76 could not be analyzed conclusively because of low yields of HBV DNA from serum samples.

Clonal analysis revealed that position 223 was a mixture of S and A residues at baseline, the LVDr substitutions L180M and M204V, as well as the ETVr substitution S202G, all emerged simultaneously and were linked in the same virus isolate clones at week 124, isolates that also contained S at position 223. These substitutions did not appear to arise from the LVDr isolates with M204I because the M204I substitution emerged in an isolate with substitution S223A.

Table 3 SNP-PCR analysis of case 1 isolates

Week	M204V		S202G		M204I (ATA)		M204I (ATT)	
	Mut/WT	Ave (%)	Mut/WT	Ave (%)	Mut/WT	Ave (%)	Mut/WT	Ave (%)
0	1/5,424	0.018	1/15,453	0.0065	1/4,199	0.024	1/37,940	0.0026
24	1/5,655	0.018	1/19,000	0.0052	**1/243**	**0.410**	1/46,518	0.0021
100	1/3,846	0.026	1/16,038	0.0062	**1/14**	**6.569**	1/50,456	0.0020
124	**48/1**	**97.973**	**59/1**	**98.327**	**1/265**	**0.377**	1/12,879	0.0078
144	**706/1**	**99.859**	**1,250/1**	**99.920**	**1/237**	**0.421**	1/10,573	0.0095

Cells with bold and underlined font are considered positive (>1/1000 or >0.1% mutant/wild-type)

Mut/WT, mutant/wild type, mean ($N = 3$)

Ave %, average % in total HBV DNA

Case 2

A 47-year-old Japanese male CHB patient was positive for HBsAg, HBeAg, serum HBV DNA, and had HBV genotype C, had elevated ALT levels, and had no history of nucleoside analogue treatment. At age 33, he was diagnosed for the first time as an asymptomatic HBV carrier in the immune-tolerant phase because of positive HBsAg and normal liver enzymes. At age 44, he was found to have ALT elevation, referred to our hospital, and diagnosed with CHB. Histologic diagnosis by percutaneous liver biopsy revealed chronic hepatitis with moderate fibrosis and moderate activity (CH F2/A2 according to the New Inuyama Classification). He was treated with ursodeoxycholic acid at a daily dose of 600 mg orally and glycyrrhizin preparation (stronger Neo-Minophagen C™) 40 ml i.v. thrice per week for 3 months. However, liver enzymes did not normalize. Interferon-α2b administration, three mega units i.m. thrice per week, was started at age 45 and continued for 24 weeks. Although HBV DNA level was reduced transiently to below 3.7 $\log_{10}$ copies/ml at the end of therapy, it rose 9 months after cessation of interferon therapy to 8.2 $\log_{10}$ copies/ml and ALT level increased to 483 IU/l. At age 47, the patient was started on ETV treatment as the subject enrolled in the ETV clinical trial (ETV-053) in Japan at a daily oral dose of 0.5 mg and continued for 188 weeks. A liver biopsy performed 1 month before starting the ETV treatment showed chronic hepatitis with moderate fibrosis and moderate activity (CH F2/A2, according to the New Inuyama Classification). The baseline serum HBV DNA level was 8.2 $\log_{10}$ copies/ml, ALT level was 74 IU/l, and other baseline characteristics were as shown in Table 1. The serum HBV DNA level declined to 3.2 $\log_{10}$ copies/ml and ALT level decreased to below the upper limit of normal at week 32. Liver histology improved to mild-to-moderate fibrosis and mild activity (CH F1-2/A1) at week 48 and chronic hepatitis with mild-to-moderate fibrosis and mild activity (CH F1/A1) at week 148. HBV DNA level was suppressed to a nadir of 2.9 $\log_{10}$ (794) copies/ml at week 124 and rose again to 4.7 $\log_{10}$ copies/ml at week 148, 5.4 $\log_{10}$ copies/ml at week 152, and 6.4 $\log_{10}$ copies/ml at week 160 and 7.0 $\log_{10}$ copies/ml at week 164. ALT level rose to 79 IU/l at week 172 and remained between 40 and 50 IU/l thereafter. ETV at 0.5 mg/day was continued until this time (Fig. 2).

HBV DNA sequence analysis revealed no resistance substitutions in the patient's baseline virus. However, the LVDr-related substitutions L180M and M204V, as well as ETVr-related substitution S202G, were detected at week 124, as a mixed population with wild type, and at week 148, as a pure population (Table 4). In addition, the patient displayed evidence of several polymorphic substitutions at baseline, indicating a mixed quasi-species, which became enriched for those with the resistant virus over time.

SNP-PCR analysis was used to determine the first appearance of the resistance substitutions, using the same method as for case 1. There was no antiviral resistance detected at baseline (<0.1%). The M204V (0.65%) and S202G substitutions were detected first at week 24 but not again until week 124. At weeks 124 and 148, the resistant isolate had become enriched to 43% (M204V) and 98% (M204V), respectively (Table 5).

Clonal analysis was performed to determine the genetic linkage of the various substitutions observed, using the same method as for case 1. The amplified RT gene from the patient's virus was cloned into plasmids, and 24 to 27 individual clones were selected and sequenced. From the clonal analysis, it can be seen that there are three positions that contain mixtures at baseline; position 55 is a mixture of H and R residues, position 221 is a mixture of Y and F residues, and position 269 is a mixture of I and L residues. The substitutions L180M and M204V, as well as the ETVr-related substitution S202G, all emerge simultaneously and in an isolate with H at position 55, Y at position 221, and I at position 269.

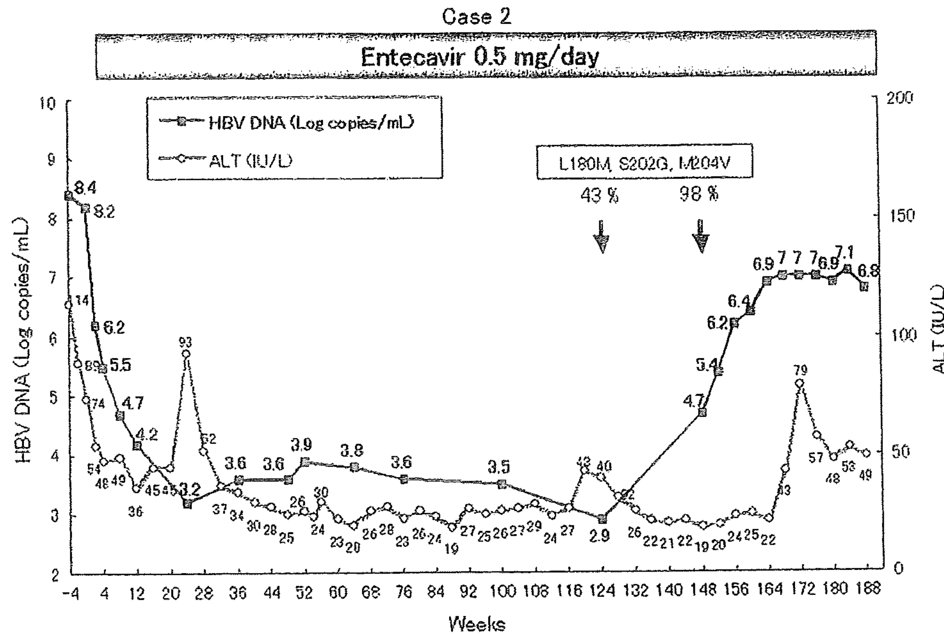

Fig. 2 Clinical course of case 2, a 47-year-old man with nucleoside-naive CHB. ETV treatment reduced ALT level to below the upper normal limit at week 30 and reduced serum HBV DNA level to a nadir of 2.9 $\log_{10}$ copies/ml at week 124. However, HBV DNA level re-elevated to 4.7 $\log_{10}$ copies/ml (virologic breakthrough) at week 148 and 7.0 $\log_{10}$ copies/ml at week 168, as well as ALT level re-elevated to 79 IU/l at week 172. Sequence analysis of the HBV DNA polymerase gene using serum sample obtained at weeks 124 and 148 revealed the emergence of L180M, M204V (related to LVD resistance), and S202G (related to ETVr) substitutions. SNP-PCR assay revealed that the resistant isolate was enriched to 43 (M204V) and 98% (M204V), respectively

Table 4 Population sequence analysis of isolates from case 2 on ETV therapy

Week	RT position								
	55	76	180	191	195	202	204	221	269
0	H/R	S	L	V	F	S	M	Y/F	I/L
24	H/R	S	L	V	F	S	M	Y/F	I/L
52	H/R	S	L	V	F	S	M	Y/F	I/L
100	H	S	L	V	F	S	M	Y/F	I/L
124	H	S/T	L/M	V/I	F/S	S/G	M/V	Y	I/L
148	H	S	M	V	F	G	V	Y	I

Table 5 SNP-PCR analysis of case 2 isolates

Week	S202G[a]	M204V (GTG, %)	M204I (ATA, %)	M204I (ATT, %)
0	Negative	0.016	0.020	0.0065
24	Positive	0.65	0.029	0.018
52	Negative	0.021	0.020	0.018
100	Negative	0.020	0.021	0.010
124	Positive	43	0.33	0.010
148	Positive	98	2.9	0.016

[a] S202G PCR was non-quantitative. A positive indicates 4-fold, 5085-fold, and 10475-fold the wild-type background for weeks 24, 124, and 148, respectively. The baseline isolate gave 1.1-fold the wild-type background

Discussion

The most important limitation of long-term nucleoside analogue treatment for CHB is the emergence of drug-resistant mutant HBV followed by viral breakthrough and hepatitis flare [12]. The most common mutation associated with LVDr involves substitution of methionine in the tyrosine-methionine-aspartate-aspartate (YMDD) motif of the HBV DNA polymerase gene RT domain with valine or isoleucine (M204V/I), with or without a leucine-to-methionine substitution in an upstream region (rtL180M) [20]. It was reported that LVDr was detected at a rate of 14 to 32% after 1 year and 60 to 70% after 5 years of LVD treatment [12]. The substitutions conferring resistance to ADV are asparagine to threonine (N236T) and alanine to valine or threonine (A181V/T) [21], and the cumulative probability of ADV resistance with elevation of HBV DNA level has been reported to be 20% at 5 years in HBeAg-negative patients [22] and as high as 42% in HBeAg-positive patients [23].

In the case of ETV, it has been reported that resistance to the drug requires at least one of three substitutions in HBV RT, that is, rtT184, rtS202, and rtM250, as well as LVDr-related substitutions rtL180M and M204V [24]. Phenotypic analyses of samples associated with virologic breakthrough confirmed that ETV susceptibility correlates

with the spectrum of these additional substitutions conferring genotypic resistance and the increased level of circulating HBV DNA [25].

There is a high genetic barrier to resistance to ETV in nucleoside-naive patients and <1% experience virologic breakthrough with ETVr through 4 years of therapy [15]. However, in LVD-refractory patients, the barrier to resistance is lower because the suppression of HBV replication is not as great and these patients mostly harbor virus with two of the three substitutions required for high-level ETVr [26]. This results in virologic breakthrough with ETVr in LVD-refractory patients at 1% in the first year but increasing to 39.5% after 4 years of therapy [15].

In this article, we report two cases with confirmed genotypic resistance to ETV, virologic rebound, and biochemical breakthrough during long-term ETV treatment for nucleoside-naive CHB patients. In the first case, the patient received a lower dose of ETV (0.1 mg daily for 52 weeks) than is currently recommended in product labeling. It was shown that LVD–ADV combination therapy was apparently effective for the ETV-resistant strain, presumably because there is no cross-resistance between ETV and ADV [26, 27].

SNP-PCR analysis for resistance substitutions revealed that the LVDr M204V(GTG) and the ETVr S202G(GGT) substitutions were negative at baseline and emerged simultaneously at week 124 in both patients. The three resistance substitutions L180M, M204V, and S202G appeared to be genetically linked and did not arise in a stepwise manner in nucleoside-naive patients, as has been described previously.

ETV displays several properties for consideration as the first-line nucleoside analogue because of its potent antiviral activity and a lower frequency of drug resistance than LVD, ADV, or telbivudine [13]. Although ETV is effective in LVD-refractory patients, the potency is reduced somewhat and the barrier to resistance is diminished by the presence of rtM204I/V and rtL180M substitutions. The fact that ETVr may develop in nucleoside-naive patients, even if the chance is small, is noteworthy. In case 1, the patient received a lower dose of ETV (0.1 mg daily), which may be a possible contributing factor to resistance. The common features of our two cases were: HBeAg-positivity, male, high viral load, slow decrease of HBV DNA, and persistently detectable HBV DNA by PCR (>2.6 $\log_{10}$ copies/ml) during the treatment course; however, these characteristics were also present in some other patients who did not develop ETVr. Patient compliance with prescribed therapy also should be assessed in such situations. It is believed that some subpopulations of HBV that proliferate very actively and are not completely suppressed by ETV may have a chance of being selected for the resistance substitutions required for ETV virologic failure. Accordingly, such cases with persistent HBV DNA after extended ETV treatment should be evaluated for emergence of drug-resistance substitutions with close monitoring of HBV DNA level, even in nucleoside-naive patients.

The rate at which resistant mutants are selected is related to pretreatment serum HBV DNA level, rapidity of viral suppression, duration of treatment, and prior exposure to nucleoside analogue therapies [12]. For the management of the emergence of drug resistance in nucleoside analogue treatment of CHB and cirrhosis, prediction and early detection of drug-resistant HBV by close monitoring of serum viral load and genotypic resistance are necessary. Keeffe et al. [28] reported the "road-map concept," that is, on-treatment monitoring strategy, for selection of nucleoside analogues by early prediction of efficacy and resistance using assessment of viral responses at weeks 12 and 24. Although this "concept" seems imperfect because the probability of emergence of resistance to particular nucleoside analogue is not taken into account, a similar strategy for management of drug resistance by close monitoring of viral load and confirming genotypic resistance with consideration of the property of each nucleoside analogue should be established for antiviral treatment of CHB using nucleoside analogues.

Conclusions

We reported two cases of emergence of genotypic resistance to ETV accompanied by virologic breakthrough in nucleoside-naive CHB patients. One patient was treated with a lower than recommended dose of ETV. Although development of ETVr-related gene mutations is rare in nucleoside-naive patients, the patients with a slow decline of HBV DNA or persistent HBV DNA (>2.6 $\log_{10}$ copies/ml) after ETV administration should be evaluated carefully for the potential emergence of ETVr.

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nature genetics

A genome-wide association study identifies variants in the *HLA-DP* locus associated with chronic hepatitis B in Asians

Yoichiro Kamatani[1,2], Sukanya Wattanapokayakit[3], Hidenori Ochi[4,5], Takahisa Kawaguchi[4], Atsushi Takahashi[4], Naoya Hosono[4], Michiaki Kubo[4], Tatsuhiko Tsunoda[4], Naoyuki Kamatani[4], Hiromitsu Kumada[6], Aekkachai Puseenam[7], Thanyachai Sura[7], Yataro Daigo[1,2], Kazuaki Chayama[4,5], Wasun Chantratita[8], Yusuke Nakamura[1,4] & Koichi Matsuda[1]

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Chronic hepatitis B is a serious infectious liver disease that often progresses to liver cirrhosis and hepatocellular carcinoma; however, clinical outcomes after viral exposure vary enormously among individuals[1]. Through a two-stage genome-wide association study using 786 Japanese chronic hepatitis B cases and 2,201 controls, we identified a significant association of chronic hepatitis B with 11 SNPs in a region including *HLA-DPA1* and *HLA-DPB1*. We validated these associations by genotyping two SNPs from the region in three additional Japanese and Thai cohorts consisting of 1,300 cases and 2,100 controls (combined $P = 6.34 \times 10^{-39}$ and 2.31×10^{-38}, OR = 0.57 and 0.56, respectively). Subsequent analyses revealed risk haplotypes (*HLA-DPA10202-*DPB1**0501 and *HLA-DPA1**0202-*DPB1**0301, OR = 1.45 and 2.31, respectively) and protective haplotypes (*HLA-DPA1**0103-*DPB1**0402 and *HLA-DPA1**0103-*DPB1**0401, OR = 0.52 and 0.57, respectively). Our findings show that genetic variants in the *HLA-DP* locus are strongly associated with risk of persistent infection with hepatitis B virus.**

Chronic hepatitis B is one of the most common infectious liver diseases caused by hepatitis B virus (HBV). HBV infection shows a marked regional diversity and is very prevalent in the Asia-Pacific region; HBsAg seropositivity rates are as high as 5–12% in Thai and China, but as low as 0.2–0.5% in North America and Europe[2]. It is estimated that, at present, more than 400 million people worldwide are chronically infected with HBV, and nearly 60% of liver cancers are considered to be related to chronic hepatitis B and subsequent liver cirrhosis[3]. Most HBV carriers are considered to have been infected through maternal transmission in the neonatal period or infancy, particularly in Japan[4]. Although some HBV carriers spontaneously eliminate the virus, 2–10% of individuals with chronic hepatitis B are estimated to develop liver cirrhosis every year, and a subset of these individuals suffer from liver failure or hepatocellular carcinoma[1]. Because clinical outcomes after exposure to HBV are highly variable, identification of genetic and environmental factors that are related to progression of HBV-induced liver diseases is critical.

Several epidemiological factors such as age at infection, sex, chronic alcohol abuse[5] and co-infection with other hepatitis viruses[6] were suspected to affect viral persistence. In addition, a twin study in Taiwan indicated that host genetic background influences infection outcome[7]. Although genetic variants in *IFNG*, *TNF*, *VDR*, *ESR1* and several *HLA* loci were shown to associate with chronic hepatitis B[8–12], none of the associations has been proven to be conclusive. To identify disease-predisposing variants, we carried out a two-stage association study for chronic hepatitis B using genome-wide SNPs as genetic markers.

Characteristics of each cohort group are shown in **Supplementary Table 1** online. We carried out a two-stage genome-wide association approach as described in the Methods. In the first stage, we genotyped 179 Japanese individuals with chronic hepatitis B and 934 control individuals using Illumina HumanHap550 BeadChip (**Fig. 1a**). For the second stage, we selected the top 12,000 SNPs that had the smallest P values on the basis of minimum P value considering three genetic models: allelic, dominant or recessive. Analysis of an independent set of 607 cases and 1,267 controls using these sub-selected SNPs showed 11 SNPs to be significantly associated ($P = 3.62 \times 10^{-8} \sim 1.16 \times 10^{-13}$) with chronic hepatitis B after Bonferroni correction (**Fig. 1b** and **Supplementary Table 2** online). Application of the Cochrane-Armitage

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test to all the tested SNPs indicated that the genetic inflation factor lambda was 1.02 for the second stage (**Supplementary Fig. 1a** online), implying a low possibility of false positive associations due to population stratification. All 11 SNPs are located within or around the *HLA-DPA1* and *HLA-DPB1* locus (**Fig. 2**). We also conducted age- and sex-adjusted analysis using a logistic regression model, and confirmed similar association after adjustment (data not shown).

To validate the result of the discovery-phase analysis, we carried out replication analyses using three independent cohorts. We selected the most or second-most strongly associated SNPs from each *HLA-DP* locus (rs9277535 on *HLA-DPB1* and rs3077 on *HLA-DPA1*, respectively), as we failed to design a Taqman or Invader probe for rs2395309 on *HLA-DPA1*. We first examined two independent sets of Japanese case–control samples comprising 274 cases and 274 controls (age-, sex- and alcohol consumption–matched cohort from BioBank Japan) as well as 718 cases and 1,280 controls. We found significant associations at two SNP loci in both studies ($P = 1.06 \times 10^{-16} \sim 1.96 \times 10^{-6}$; **Table 1**). We also genotyped 308 individuals with chronic hepatitis B and 546 healthy controls in Thailand, and further confirmed the association at the two loci, rs3077 ($P = 6.53 \times 10^{-6}$) and rs9277535 ($P = 6.52 \times 10^{-8}$).

To combine these studies, we conducted a meta-analysis with a fixed-effects model using the Mantel-Haenszel method. As shown in **Table 1** and **Supplementary Figure 1b**, the odds ratios (OR) were quite similar across the four studies (the second stage of GWAS and three replication studies) and no heterogeneity was observed. Mantel-Haenszel *P* values for independence were 2.31×10^{-38} for rs3077 (OR = 0.56, 95% confidence interval (CI) = 0.51–0.61), and 6.34×10^{-39} for rs9277535 (OR = 0.57, 95% CI = 0.52–0.62).

The 11 SNPs showing significant associations are located within a 50-kb region including *HLA-DPA1* and *HLA-DPB1* (**Fig. 2**). Although the *HLA* region is known to show extensive linkage disequilibrium (LD) spanning over 7 Mb, the LD block including these 11 SNPs (surrounded by a bold line in **Fig. 2a**) was not in strong LD with the other *HLA* loci. In accordance with the extent of LD, only SNPs around the *HLA-DPA1* and *HLA-DPB1* genes showed very strong associations with chronic HBV (surrounded by a bold line in **Fig. 2b**), and SNPs outside of this particular LD block did not have significant association.

HLA-DPA1 and *HLA-DPB1* encode the HLA-DP α and β chains, respectively. HLA-DPs belong to the HLA class II molecules that form heterodimers on the cell surface and present antigens to CD4-positive T lymphocytes. HLA-DPs are highly polymorphic, especially in exon 2, which encodes antigen-binding sites. We thus considered that the association of these SNPs with chronic HBV might reflect variations in antigen-binding sites that might affect the immune response to HBV. We genotyped *HLA-DPA1* and *HLA-DPB1* alleles by direct sequencing of exon 2 (cases at second stage and controls at first stage) and found significant association of chronic hepatitis B with *HLA-DPA1**0103, *DPA1**0202, *DPB1**0402 and *DPB1**0501 ($P = 2.93 \times 10^{-11}$, 4.45×10^{-8}, 2.27×10^{-7} and 6.98×10^{-7}, respectively; **Supplementary Table 3** online). Because sequence variants in exon 2 of *HLA-DPA1* and *HLA-DPB1* could be linked to individual nucleotide variants, we inferred haplotypes using the 11 SNPs and variants in exon 2, and found very strong LD among them (**Supplementary Fig. 2**

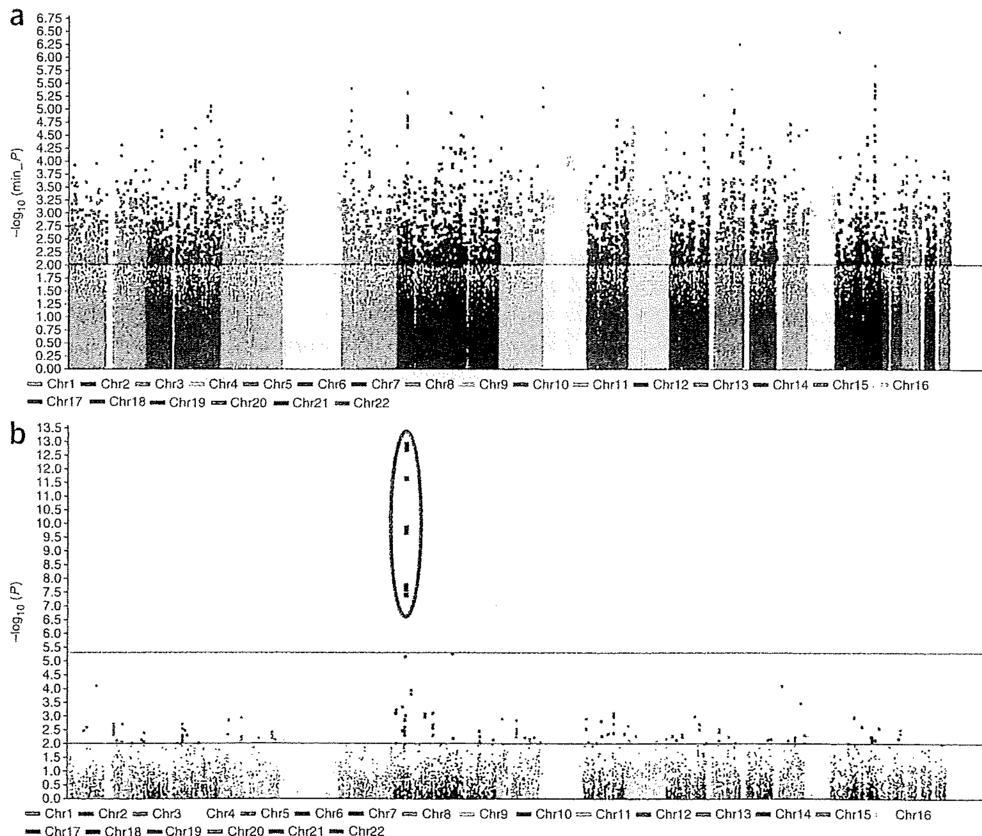

Figure 1 Results from a two-stage genome-wide association study. (**a**) $-\log_{10}$ *P* value plot at the first stage. Each *P* value is the minimum of Fisher's exact tests for three models: dominant, recessive and allele frequency model. (**b**) $-\log_{10}$ *P* value plot at the second stage. *P* values were calculated by 1-d.f. Cochrane-Armitage trend test. The large dots circled by red on the chromosome 6 showed significant associations ($P < 5.06 \times 10^{-6}$) with chronic hepatitis B.

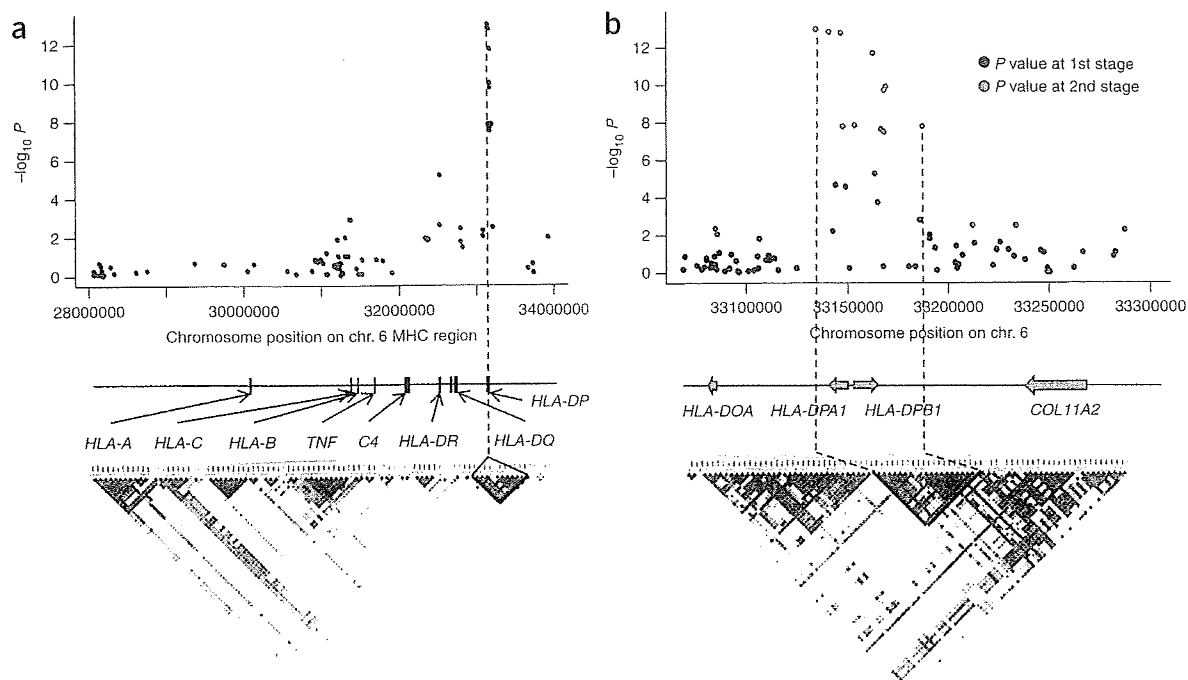

Figure 2 Case–control association results and linkage disequilibrium map of the MHC region. (**a**) *P*-value plot, genomic structure and LD map of the second stage within the extended MHC region of chromosome 6. The LD map based on *D′* was drawn using the genotype data of the cases and the controls in the second stage. (**b**) *P*-value plot, genomic structure and LD map around the *HLA-DPA1* and *HLA-DPB1* region. Black dots and red dots represent *P* values in the first and the second stage, respectively. The LD map based on *D′* was drawn using the genotype data of the cases and the controls in the first stage.

online). Case–control analyses revealed four associated haplotypes: *DPA1**0103-*DPB1**0402 and *DPA1**0103-*DPB1**0401 showed protective effects ($P = 6.00 \times 10^{-8}$, OR = 0.52, 95% CI = 0.35–0.75 and $P = 0.002$, OR = 0.57, 95% CI = 0.33–0.96, respectively), whereas *DPA1**0202-*DPB1**0501 and *DPA1**0202-*DPB1**0301 were associated with susceptibility to chronic hepatitis B ($P = 5.79 \times 10^{-6}$, OR = 1.45, 95% CI = 1.16–1.81 and $P = 0.002$, OR = 2.31, 95% CI = 1.39–3.84, respectively; **Table 2**). We also found various sets of SNPs (tagging SNPs) that could predict *HLA-DP* alleles (**Supplementary Table 4** online). Taken together, our findings strongly implicate an association of genetic variants in the *HLA-DPA1* and *HLA-DPB1* genes with chronic hepatitis B.

HLA-DR13 was reported to have a protective effect against persistent HBV infection in different populations[9,13,14]. Comparison of genotypes of *HLA-DRB1**1301 and *1302 alleles (both corresponding to HLA-DR13) and Illumina HumanHap550 SNPs in 333 of the first-stage control samples revealed that the A allele of rs11752643 was in strong LD with HLA-DR13 ($r^2 = 0.83$, $D' = 1$). However, the association between rs11752643 and chronic hepatitis B was not significant in our second stage GWAS, with an uncorrected *P* value of 1.04×10^{-4} (**Supplementary Table 5** online). In addition, the association of chronic hepatitis B with rs3077 and rs9277535 remained highly significant ($P = 2.11 \times 10^{-10}$ and 1.73×10^{-9}, respectively) after adjustment for rs11752643 using a logistic

Table 1 Results of replication studies and meta-analysis

SNP	Nearest gene	Allele (1/2)	Stage	Cases 11	Cases 12	Cases 22	Controls 11	Controls 12	Controls 22	OR (95%CI)[a]	P^b	P_{het}^c
rs3077	*HLA-DPA1*	A/G	GWAS second stage	42	240	324	197	598	472	0.57 (0.49–0.66)	1.26E–13	
			First replication	25	95	152	50	122	102	0.53 (0.41–0.69)	1.73E–06	
			Second replication	64	237	410	197	596	485	0.55 (0.47–0.63)	1.06E–16	
			Third replication	28	109	163	85	250	210	0.61 (0.49–0.75)	6.53E–06	
			Meta-analysis[d]							0.56 (0.51–0.61)	2.31E–38	0.84
rs9277535	*HLA-DPB1*	A/G	GWAS second stage	58	254	294	230	619	418	0.59 (0.51–0.69)	1.78E–12	
			First replication	26	102	144	49	132	91	0.54 (0.42–0.69)	1.96E–06	
			Second replication	68	264	376	227	604	445	0.56 (0.48–0.64)	1.81E–16	
			Third replication	29	136	139	107	273	155	0.56 (0.46–0.69)	6.52E–08	
			Meta-analysis[d]							0.57 (0.52–0.62)	6.34E–39	0.85

Odds ratio and *P* values for independence test were calculated by the Mantel-Haenszel method.
[a]Odds ratio of minor allele from two-by-two allele frequency table. [b]*P* values of Pearson's χ^2 test for allele model. [c]Result of Breslow-Day test. [d]Meta-analysis of all four studies.

Table 2 Haplotype analysis

No.	Haplotype[a]	Frequency (cases)	Frequency (controls)	P^b	OR[b] (95% CI)
1	GG-*DPA1*0202*-TCG-*DPB1*0501*-GAGATT	0.428	0.347	5.79E−06	1.45 (1.16–1.81)
2	AA-*DPA1*0103*-CCA-*DPB1*0201*-AGTGCC	0.165	0.192	0.052	Reference
3	GG-*DPA1*0201*-TCG-*DPB1*0901*-GGGGTC	0.129	0.124	0.642	1.21 (0.91–1.61)
4	AA-*DPA1*0103*-CTA-*DPB1*0402*-AGTGCC	0.042	0.096	6.00E−08	0.52 (0.35–0.75)
5	AA-*DPA1*0103*-CCA-*DPB1*0401*-AGTGCC	0.018	0.038	0.002	0.57 (0.33–0.96)
6	GG-*DPA1*0202*-TCG-*DPB1*0301*-GGGGTC	0.036	0.018	0.002	2.31 (1.39–3.84)
7	GG-*DPA1*0202*-TCG-*DPB1*0202*-AGTGCC	0.020	0.027	0.257	0.88 (0.51–1.52)
8	GG-*DPA1*0202*-TCG-*DPB1*0201*-AGTGCC	0.022	0.024	0.662	0.97 (0.57–1.65)
9	GG-*DPA1*0201*-TCG-*DPB1*0501*-GAGATT	0.029	0.018	0.057	1.81 (1.06–3.08)
10	GG-*DPA1*0201*-TCA-*DPB1*1301*-GGTGCC	0.022	0.016	0.172	1.69 (0.95–3.03)
11	AA-*DPA1*0103*-CTG-*DPB1*0301*-GGGGTC	0.011	0.016	0.246	0.74 (0.36–1.53)
12	GG-*DPA1*0201*-TCG-*DPB1*1401*-GGGGTC	0.012	0.012	0.877	1.25 (0.61–2.53)

Controls of the first stage and cases of the second stage were analyzed.
[a]Haplotypes consisting of rs2595309, rs3077, *HLA-DPA1*, rs2301220, rs9277341, rs3135021, *HLA-DPB1*, rs9277535, rs10484569, rs3128917, rs2281388, rs3117222 and rs9380343 are shown. [b]*P* values, odds ratios and its 95% confidence intervals of each haplotype were calculated as described in the Methods.

regression model. Thus, our findings clearly indicate that hepatitis B is associated with variants in the *HLA-DP* loci.

A number of reports have described association of several *HLA* and non-*HLA* genes with persistent HBV infection[12,15], but their results were not consistent among the studies, and none of them indicated a possible involvement of the *HLA-DP* locus. This study is the first GWAS to investigate host genetic factors associated with chronic hepatitis B. One genome-wide linkage analysis using 318 microsatellite markers in the Gambian population suggested that the chromosome 21q22 region contains a susceptibility locus for persistent HBV infection[16]. However, our GWAS analysis failed to support this result, possibly owing to ancestry differences or different modes of viral transmission (the vertical transmission in Japan versus the horizontal transmission in Gambia).

To investigate the correlation between the incidence of hepatitis B infection and these polymorphisms, we evaluated the frequencies of rs3077 and rs9277535 in 11 different HapMap3 populations (**Supplementary Table 6** online). Our association analysis indicated that A alleles at both rs3077 and rs9277535 were associated with protective effects for chronic hepatitis B. Notably, the frequencies of these two alleles were lower in Asian and African populations, especially in the Chinese population, compared with European and Central American populations. Although disease prevalence is not determined solely by genetic factors, the findings presented in our manuscript suggest that genetic factors might exert substantial influence on the prevalence of infectious disease.

Antigen presentations on HLA class II molecules to CD4-positive helper T cells and on class-I molecules to CD8-positive cytotoxic T cells are considered to be critical for the immune response against exposure to HBV. Although cytotoxic T cells are suspected to have major roles in viral clearance, helper T cells are also essential in the immune response to acute infections[17]. HLA-DPs have a structure similar to other classical HLA class II molecules, but their roles in the immune response have not been well characterized, except the association with berylliosis[18]. The 11 SNPs we found showing strong association with chronic HBV infection were in very strong LD with *HLA-DP* alleles. Because the subsequent haplotype analyses identified significant association of chronic hepatitis B with haplotypes containing the *HLA-DPA1* and *HLA-DPB1* genes, we suspected that variations in HLA-DP molecules would affect the ability for antigen presentation of HLA class II molecules on immune cells and result in weak (or no) immune response and persistent HBV infection. A previous report that implicated *HLA-DPA1**0103 and *DPB1**0402 to be candidate predictive factors for antibody production after HBV vaccination[19] supports this hypothesis. It should be noted that the lack of information regarding exposure to HBV for each control might underestimate the effect size obtained in this study but does not inflate the type 1 error rate.

In summary, we have demonstrated that genetic variants in the *HLA-DP* genes are strongly associated with chronic hepatitis B in the Asian population. Considering the function of HLA-DP molecules, our findings suggest that antigen presentation on HLA-DP molecules might be critical for virus elimination and have an important role in the pathogenesis of chronic hepatitis B. An understanding of the molecular mechanism by which *HLA-DP* variants confer risk of chronic hepatitis B should shed light on its pathogenesis and facilitate development of new therapies for treatment of the disease and prevention of disease progression.

METHODS

Samples. Characteristics of each cohort group are shown in **Supplementary Table 1**. Case and control samples used in this study for the Japanese population were obtained from the BioBank Japan at the Institute of Medical Science, the University of Tokyo[20], except case samples of the second replication and control samples of the first stage of the GWAS. From the registered samples in BioBank Japan, we selected individuals that were clinically diagnosed as having chronic hepatitis B. The diagnosis of chronic hepatitis B was conducted based on HBsAg-seropositivity and elevated serum aminotransferase levels for more than six months according to the guideline for diagnosis and treatment of chronic hepatitis (see URLs section below). The control groups consisted of 2,821 individuals that were registered in BioBank Japan as subjects with diseases other than chronic hepatitis B. Subjects who were positive for HBsAg were excluded from the controls. We obtained 934 Japanese control DNAs in the first stage from volunteers in the Osaka-Midosuji Rotary Club, Osaka, Japan. Case samples for the second replication cohort ($n = 718$, RIKEN) were collected at Toranomon Hospital as well as at hospitals participating in the Hiroshima Liver Study Group (for a list of doctors participating in this study group, see URLs section below). Cases and controls for the Thai replication study ($n = 308$ and 546, respectively) were collected at Ramathibodi Hospital, Mahidol University, Thailand. The diagnosis of chronic hepatitis B was based on HBsAg-seropositivity and elevated serum aminotransferase levels. All participants provided written informed consent. This research project was approved by the ethical committees at the Institute of Medical Science, the University of Tokyo, the Center for Genomic Medicine (formerly SNP Research Center), RIKEN and Ramathibodi Hospital, Mahidol University.

SNP genotyping. We applied the two-stage approach as described previously[21]. For the first stage, we genotyped 188 individuals with chronic hepatitis B and 934 controls using the Illumina HumanHap550v3 Genotyping BeadChip. After excluding nine cases with call rate of <0.98, we applied SNP quality control (call rate of ≥ 0.99 in both cases and controls and P value of Hardy-Weinberg equilibrium test of $\geq 1.0 \times 10^{-6}$ in controls): 499,544 SNPs on autosomal chromosomes passed the quality control filters and were further analyzed. Among the SNPs analyzed in the first stage, we selected the top 12,000 SNPs showing the smallest P values for the second stage. SNPs with minor allele frequency (MAF) of ≤ 0.1 in both case and control samples were excluded from the further analysis. In the second stage, we genotyped an additional panel of 616 cases using an

Affymetrix GeneChip Custom 10K array. After excluding nine cases with call rate of <0.95, all cluster plots were checked by visual inspection by trained staff, and SNPs with ambiguous calls were excluded. Ninety-four randomly selected case samples in the first stage were re-genotyped in the second stage, and SNPs with concordance rates of $<98\%$ between two assays (Illumina and Affymetrix) were excluded from the further analysis. We used genome-wide screening data of other diseases (uterine cervical cancer, esophageal cancer, hematological cancer, pulmonary tuberculosis, ovarian cancer, uterine body cancer and keroid) as controls for the second stage. All the samples were genotyped using the Illumina HumanHap550v3 Genotyping BeadChip, and the same quality-control filters as the first screening were applied. As a result, we analyzed 9,875 SNPs in 607 cases and 1,267 controls in the second stage and found 11 SNPs ($P < 5.06 \times 10^{-6}$) to be significantly associated with chronic hepatitis B after Bonferroni correction. These first and second stages are defined as the discovery phase of the research, and the following replication studies are defined as the replication phase. In the replication analyses, we used TaqMan genotyping system (Applied Biosystems) or the multiplex PCR-based Invader assay (Third Wave Technologies).

***HLA-DPA1* and *HLA-DPB1* genotyping.** We analyzed *HLA-DP* genotypes using 607 cases (in the second stage of GWAS) and 934 controls (in the first stage of GWAS). Exon 2 of the *HLA-DPA1* and *HLA-DPB1* genes were amplified and directly sequenced according to the protocol of International Histocompatibility Workshop Group[22]. *HLA-DPA1* and *DPB1* alleles were determined based on the alignment database of dbMHC.

Statistical analysis. In the first stage of the GWAS, Fisher's exact test was applied to a two-by-two contingency table in three genetic models: an allele frequency model, a dominant-effect model and a recessive-effect model. At the second stage of GWAS and replication analyses, statistical significance of the association with each SNP was assessed using a 1-degree-of-freedom Cochrane-Armitage trend test. Significance levels after Bonferroni correction for multiple testing were $P = 5.06 \times 10^{-6}$ (0.05/9,875) in the second stage and $P = 0.025$ (0.05/2) in replication analyses. Age- and sex-adjusted odds ratios were obtained by logistic regression analysis. Odds ratios and confidence intervals were calculated using the major allele as a reference. The meta-analysis was conducted using the Mantel-Haenszel method. Heterogeneity among studies was examined by using the Breslow-Day test. To assess the association of each *HLA* allele, we used Fisher's exact tests on two-by-two contingency tables with or without each *HLA* allele. To analyze the association of haplotypes, we used R package haplo.stats. *P* values for each haplotype were given by the results of a score test, and odds ratios and 95% confidence intervals were calculated from coefficients of GLM model. Odds ratios of each haplotype were calculated relative to the second major haplotype in **Table 2**, because the most common haplotype was the disease-associated haplotype. All of these statistical values were calculated by function haplo.cc. We used Haploview software to analyze linkage disequilibrium values between *HLA-DR13* and SNPs.

Software. For general statistical analysis, we used R statistical environment version 2.6.1 or PLINK1.03 (ref. 23). To draw the LD map, we used Haploview software[24]. Estimation of haplotype frequencies and analysis of haplotype association were performed by R package haplo.stats[25]. Sequence variants in exon2 of *HLA-DPA1* and *HLA-DPB1* were analyzed by Polyphred.

URLs. The Japan Society of Hepatology, http://www.jsh.or.jp/medical/gudelines/index.html; Hiroshima Liver Study Group, http://home.hiroshima-u.ac.jp/naika1/hepatology/english/study.html; PLINK1.03, http://pngu.mgh.harvard.edu/~purcell/plink/; R package haplo.stats, http://mayoresearch.mayo.edu/mayo/research/schaid_lab/software.cfm; Polyphred, http://droog.gs.washington.edu/polyphred/.

Note: Supplementary information is available on the Nature Genetics website.

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AUTHOR CONTRIBUTIONS

Y.N. conceived the study; Y.N., Y.K., Y.D., M.K. and K.M. designed the study; Y.K., S.W., H.O. and N.H. performed genotyping; Y.K., T.T., M.K., N.K., Y.N. and K.M. wrote the manuscript; T.K., A.T., T.T. and N.K. performed data analysis at the genome-wide phase; Y.N., K.M. and M.K. managed DNA samples belong to BioBankJapan; K.C. and H.K. managed second replication samples; W.C., A.P. and T.S. managed third replication samples in Thailand; Y.K. summarized the whole results; Y.N. obtained funding for the study.

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ORIGINAL ARTICLE

Antiviral activity, dose–response relationship, and safety of entecavir following 24-week oral dosing in nucleoside-naive Japanese adult patients with chronic hepatitis B: a randomized, double-blind, phase II clinical trial

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Abstract
Purpose A randomized, double-blind, multicenter study (ETV-047) was conducted to evaluate the dose–response relationship of entecavir and compare its antiviral activity and safety with lamivudine in Japanese patients with chronic hepatitis B (CHB).
Methods One hundred thirty-seven nucleoside-naive adult patients with CHB were randomized to once-daily oral doses of entecavir 0.01, 0.1, or 0.5 mg or lamivudine 100 mg for 24 weeks. The primary efficacy end point used to evaluate the dose–response relationship was mean change from baseline in serum hepatitis B virus (HBV) DNA level at week 22, as determined by polymerase chain reaction assay.
Results Entecavir demonstrated a clear dose–response relationship, with mean change from baseline in serum

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HBV DNA level of −3.11, −4.77, and −5.16 $\log_{10}$ copies/ml with entecavir 0.01, 0.1, and 0.5 mg, respectively. Entecavir 0.5 mg was superior to lamivudine 100 mg for the mean change in HBV DNA level (−5.16 vs. −4.29 $\log_{10}$ copies/ml; $P = 0.007$). The overall incidence of adverse events was comparable between treatment groups. Two patients discontinued treatment because of adverse events (one with liver cirrhosis [entecavir 0.5 mg] and one with grade 4 serum alanine aminotransferase (ALT) elevation, nausea, and malaise [lamivudine 100 mg]). Serum ALT flares were observed in four patients; flares were associated with 2 $\log_{10}$ reductions or more in HBV DNA level and resolved without dose interruption.

Conclusion Entecavir 0.01–0.5 mg is well tolerated and produces a dose-dependent reduction in viral load in nucleoside-naive Japanese patients with CHB. Compared with lamivudine 100 mg, entecavir 0.1 mg demonstrated noninferiority and entecavir 0.5 mg was superior in this population.

Keywords Chronic hepatitis B · Entecavir · Lamivudine · HBV DNA · ALT flare

Introduction

It is reported that more than 2 billion individuals worldwide have been infected with hepatitis B virus (HBV) and approximately 350 million people are long-term HBV carriers [1]. Chronic hepatitis B (CHB) is induced by chronic replication of HBV in the liver and has a poor prognosis, with 20–40% of infected individuals developing liver cirrhosis, noncompensated liver disorder, or hepatocellular carcinoma [2]. Treatment of CHB is aimed at sustained inhibition of HBV replication and remission of liver disease [3], ultimately preventing progression to liver cirrhosis or hepatocellular carcinoma [4].

Prior to the advent of the nucleoside analog lamivudine, interferon-α formed the mainstay of treatment, but this immunoregulatory cytokine requires parenteral administration and is poorly tolerated [5]. Lamivudine is well tolerated on oral administration and has been proven to be highly effective in the treatment of CHB, but the emergence of resistance mutations (including the YMDD motif) in the reverse-transcriptase domain of HBV polymerase frequently results in overt viral rebound and disease progression [6–9]. The novel nucleoside analog adefovir is effective against wild-type HBV and lamivudine-resistant strains and is well tolerated on long-term administration, but its clinical use is restricted by the need for renal monitoring in patients with impaired renal function [10].

Entecavir, a cyclopentylguanine-derived nucleoside analog and selective inhibitor of HBV replication, was approved by the U.S. Food and Drug Administration in 2005 for the treatment of CHB. Entecavir displays potent antiviral activity in the woodchuck and duck models of HBV infection [11, 12] and is reported to be 100- to 2,200-fold more potent than lamivudine and adefovir in inhibiting HBV replication in vitro [13, 14]. Phase II clinical trials of entecavir conducted in non-Japanese patients with CHB have demonstrated entecavir to be well tolerated and more effective than lamivudine [15, 16].

A global dose-finding study (ETV-005) conducted in lamivudine-naive patients with CHB compared three doses of entecavir (0.01, 0.1, and 0.5 mg once daily) with lamivudine 100 mg once daily over a 22-week treatment period. Entecavir showed a clear dose–response relationship and was well tolerated at all three dose levels; in addition, 0.1 and 0.5 mg of entecavir showed superior antiviral activity compared with 100 mg of lamivudine [15].

Phase I studies of single-dose (0.05–2.5 mg) and multiple-dose (0.1–1.0 mg daily) entecavir conducted in Japan have confirmed the drug's safety in healthy men. As in Caucasian populations, entecavir displayed linear plasma pharmacokinetics over a wide range of doses, including putative therapeutic doses (0.5 and 1.0 mg), in Japanese subjects; there was no evidence of significant ethnic differences in its pharmacokinetics and pharmacodynamics. Similar findings to those obtained in the global phase II clinical trials of entecavir might therefore be expected from corresponding studies conducted in Japanese patients.

To evaluate the dose–response relationship, the antiviral activity and safety of entecavir in Japanese CHB patients, we conducted a 24-week phase II study comparing entecavir (0.01, 0.1, and 0.5 mg daily) to lamivudine (100 mg daily).

Materials and methods

Study design

This randomized, double-blind, double-dummy study was conducted at 38 institutions in Japan from August 2003 to March 2005. Eligible patients comprised 20- to 75-year-old men and women with CHB who fulfilled the following criteria: (i) HBsAg-positive for 24 weeks or more or IgM HBcAb-negative with biopsy-confirmed CHB; (ii) HBeAg-positive or HBeAg-negative for 12 weeks or more; (iii) serum HBV DNA level 40 MEq/ml or more (143 pg/ml) by Quantiplex™ branched DNA hybridization method (bDNA assay) (≥7.6 $\log_{10}$ genome equivalent by the transcription-mediated amplification method or $\geq 10^{7.6}$ copies/ml by Roche Amplicor™ polymerase chain reaction method [PCR assay]) measured 2 weeks or more before screening and serum HBV DNA level 40 MEq/ml or more (by bDNA assay) at screening; (iv) serum alanine

aminotransferase (ALT) level 1.25–10 times the upper limit of normal (ULN); and (v) well-compensated liver disease with prothrombin time prolongation 3 s or less or international normalized ratio 1.5 or less, serum albumin level 3.0 g/dl or more, and total bilirubin 2.5 mg/dl or less (42.75 μmol/l). After a 6-week screening period, eligible patients were stratified according to HBeAg status and study site and randomized (1:1:1:1) to oral treatment with entecavir (0.01, 0.1, or 0.5 mg plus matching placebo capsule) or lamivudine (100 mg plus matching placebo tablet) once daily for 24 weeks. All doses were administered at fixed times of the day, avoiding the 2 h before and after meals. Pregnant women were excluded from the study, as were patients with liver cirrhosis, patients with a history or evidence of variceal bleeding, patients with hepatic encephalopathy or ascites requiring diuretics, or patients with paracentesis. Patients with other liver disease (e.g., autoimmune hepatitis) were excluded from the study. In addition, patients were excluded if they had a serum creatinine level more than 1.5 × ULN, hemoglobin level less than 10.0 g/dl, platelet count less than 70,000/mm^3, granulocyte count less than <1,500/mm^3 or plasma α-fetoprotein level more than 100 ng/ml, a history of allergy induced by nucleoside analog or exposure to nucleoside analogs, a recent history (previous 24 weeks) of treatment with immunosuppressives or interferon-α/β, or current treatment of CHB.

Treatment efficacy was assessed after 22 weeks, and all eligible patients who completed 24 weeks of blinded therapy were given the option of enrolling in a separate entecavir trial. Patients who discontinued therapy prematurely were followed up for 24 weeks postdosing. Patients began anti-HBV therapy as recommended by their physician during the postdosing follow-up period.

Informed consent was obtained from all patients in writing prior to their inclusion in the study. The study was conducted in accordance with the principles of the Declaration of Helsinki and Good Clinical Practice guidelines and notifications were issued by the Ministry of Health and Labor.

Efficacy and safety assessment

The primary efficacy end point for the evaluation of the dose–response relationship of entecavir was the change from baseline in mean serum HBV DNA level at week 22, as determined by PCR assay. Secondary efficacy end points for the assessment of the noninferiority of entecavir at each dose to lamivudine included the change from baseline in mean serum HBV DNA level at week 22, as determined by PCR assay, the percentage of patients with a reduction in serum HBV DNA level 2 $\log_{10}$ copies/ml or more or a serum HBV DNA level below the limit of detection (400 copies/ml by PCR assay; 2.5 pg/ml or 0.7 MEq/ml by bDNA assay) at week 22, the percentage of patients with HBeAg loss, the percentage of patients with HBeAg seroconversion (HBeAg loss and appearance of HBe-antibody), the percentage of patients achieving ALT normalization (World Health Organization grade 0: <1.25 × ULN), and the percentage of patients achieving a protocol-defined response (HBV DNA level <0.7 MEq/ml by bDNA assay, HBeAg negativity and serum ALT level <1.25 × ULN for HBeAg-positive patients; HBV DNA level <0.7 MEq/ml by bDNA assay and serum ALT level <1.25 ULN for HBeAg-negative patients) at week 22. The incidence of genotypic drug resistance was also assessed in patients who had a 1 $\log_{10}$ copies/ml or more increase in HBV DNA by PCR from nadir while on study drug.

Based on the results of the global dose–response study of entecavir conducted in nucleoside-naive patients (ETV-005 study) [15], noninferiority of entecavir 0.1 or 0.5 mg compared with lamivudine (100 mg) was confirmed if the upper 95% confidence interval (CI) for the difference in mean HBV DNA levels at week 22 was 0.8 $\log_{10}$ copies/ml or less.

Assay methods

Serum HBV DNA level was determined by Roche Amplicor™ PCR assay (Roche Diagnostics K.K., Tokyo, Japan) and Quantiplex™ (Chiron) bDNA assay. Clinical laboratory tests, serum HBV DNA assays, and HBV serology were performed at the central clinical laboratory designated by the trial sponsor. Genotypic analysis of HBV isolates was performed using samples collected from patients on the first day of treatment. Genotypic analysis of HBV DNA polymerase was performed at SRL Inc. (Tokyo, Japan).

Statistical analysis

Numerical data were expressed by descriptive statistics. Serum HBV DNA level, a continuous variable, was analyzed after logarithmic transformation. For treatment group, comparisons of continuous variables, analysis of variance models, incorporating baseline HBV DNA level and HBeAg status as covariates were employed. For intertreatment comparisons of binary data, Cochran–Mantel–Haenszel tests were employed using baseline HBeAg status as a stratification factor. For analysis of dose–response relationships, Student's t test was applied to linear regression plots of serum HBV DNA level against log dose. A two-sided $P < 0.05$ was taken to indicate statistical significance. For analysis of dose–response relationships using efficacy data, a two-sided $P < 0.05/3$ was taken to

indicate statistical significance following Bonferroni adjustment.

Results

Study population and demographic characteristics

A total of 137 patients, including 20- to 73-year-old men and women, met the study eligibility criteria and were randomized to the following treatment groups: entecavir 0.01 mg ($n = 35$), entecavir 0.1 mg ($n = 34$), entecavir 0.5 mg ($n = 34$), and lamivudine 100 mg ($n = 34$). Three patients (two in the entecavir 0.5 mg group and one in the lamivudine 100 mg group) discontinued the study prematurely; the reasons for discontinuation were noncompliance (one patient in the entecavir 0.5 mg group) and adverse events (liver cirrhosis in one patient [entecavir 0.5 mg group] and grade 4 serum ALT elevation with nausea and malaise in one patient [lamivudine 100 mg group]). Accordingly, a total of 134 patients (entecavir 0.01 mg group, 35 patients; entecavir 0.1 mg group, 34 patients; entecavir 0.5 mg group, 32 patients; and lamivudine 100 mg group, 33 patients) completed 24 weeks of treatment and were included in the efficacy assessment.

The four treatment groups were matched with respect to gender, age, body weight, and proportion of HBeAg-positive patients (Table 1). Serum HBV DNA levels by PCR assay (mean ± SD) at baseline were 7.94 ± 0.87, 8.09 ± 1.05, 8.39 ± 0.73, and 7.94 ± 0.83 $\log_{10}$ copies/ml for the entecavir 0.01, 0.1, and 0.5 mg and lamivudine 100 mg groups, respectively. With regard to HBV genotype, 124 patients were genotype C, 6 patients were genotype A, 5 patients were genotype B, and 2 patients were genotype F. All patients were nucleos(t)ide-naive and none had been pretreated with interferon therapy.

Virologic response

Mean changes (from baseline) in serum HBV DNA level at week 22 were −3.11, −4.77, and −5.16 $\log_{10}$ copies/ml with entecavir 0.01, 0.1, and 0.5 mg, respectively (Fig 1; Table 2). Estimated differences in serum HBV DNA levels between the 0.1 and 0.5 mg entecavir groups and the low-dose entecavir group (0.01 mg) were determined after adjustment for baseline level and HBeAg status. Estimated intertreatment group differences (adjusted 95% CI) were −1.61 (−2.20 to −1.02) $\log_{10}$ copies/ml between the entecavir 0.01 and 0.1 mg groups and −1.95 (−2.53 to −1.37) $\log_{10}$ copies/ml between the entecavir 0.5 and 0.01 mg groups; both of these differences were statistically significant ($P < 0.0001$). In contrast, the difference in serum HBV DNA levels between the high-dose (0.5 mg) and medium-dose (0.1 mg) entecavir groups was not statistically significant (estimated difference [adjusted 95% CI] −0.23 [−0.69 to 0.23] $\log_{10}$ copies/ml). Taken together, these results demonstrate the superiority of high- and medium-dose entecavir (0.1 and 0.5 mg) compared with low-dose entecavir (0.01 mg) in terms of viral load reduction (Table 3). Linear regression analyses indicated a

Table 1 Baseline demographics and clinical characteristics of treated subjects

	ETV 0.01 mg ($n = 35$)	ETV 0.1 mg ($n = 34$)	ETV 0.5 mg ($n = 34$)	LVD 100 mg ($n = 34$)
Male, n (%)	25 (71.4)	23 (67.6)	23 (67.6)	28 (82.4)
Female, n (%)	10 (28.6)	11 (32.4)	11 (32.4)	6 (17.6)
Age (years), mean ± SD	42.0 ± 12.5	40.1 ± 9.8	39.8 ± 10.4	42.3 ± 12.6
Weight (kg), mean ± SD	66.2 ± 12.5	64.6 ± 11.9	65.3 ± 11.1	64.4 ± 9.0
Ethnicity Japanese, n (%)	35 (100)	34 (100)	34 (100)	34 (100)
HBV DNA ($\log_{10}$ copies/ml by PCR), mean ± SD	7.94 ± 0.87	8.09 ± 1.05	8.39 ± 0.73	7.94 ± 0.83
HBeAg positive, n (%)	30 (85.7)	30 (88.2)	30 (88.2)	31 (91.2)
ALT (IU/l), mean ± SD	150.1 ± 111.8	162.0 ± 127.1	142.4 ± 82.2	185.0 ± 130.8
AST (IU/l), mean ± SD	83.2 ± 40.0	114.3 ± 109.4	81.0 ± 43.0	121.6 ± 85.4
Total bilirubin (mg/dl), mean ± SD	0.65 ± 0.25	0.56 ± 0.15	0.66 ± 0.25	0.71 ± 0.28
HBV genotype (%)				
C	32 (91.4)	30 (88.2)	32 (94.1)	30 (88.2)
A	1 (2.86)	2 (5.88)	1 (2.94)	2 (5.88)
B	1 (2.86)	1 (2.94)	1 (2.94)	2 (5.88)
F	1 (2.86)	1 (2.94)	0	0

ETV entecavir; *LVD* lamivudine

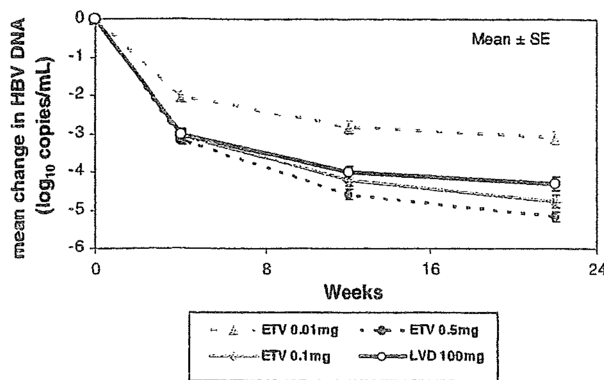

Fig. 1 Mean change from baseline in serum HBV DNA level by PCR assay through 22 weeks in patients treated with entecavir (ETV) 0.01, 0.1, and 0.5 mg and lamivudine 100 mg. Mean change in serum HBV DNA level was plotted as a function of time after the initiation of the protocol therapy (weeks). Data expressed as mean ± SE

significant dose–response relationship between $\log_{10}$ entecavir dose and reduction in $\log_{10}$ serum HBV DNA level ($P < 0.0001$).

Mean change (from baseline) in serum HBV DNA level at week 22 for the lamivudine 100 mg group was −4.29 $\log_{10}$ copies/ml (Fig. 1; Table 2). Estimated mean differences (95% CI) in serum HBV DNA level (after adjustment for baseline level and HBeAg status) were −0.39 (−0.83 to 0.05) $\log_{10}$ copies/ml between the entecavir 0.1 mg and lamivudine 100 mg groups and −0.62 (−1.06 to −0.18) $\log_{10}$ copies/ml between the entecavir 0.5 mg and lamivudine 100 mg groups, indicating the noninferiority of the entecavir 0.1 and 0.5 mg groups to the lamivudine 100 mg group and the superiority of the entecavir 0.5 mg group to the lamivudine 100 mg group ($P = 0.007$) (Table 2). In contrast, the entecavir 0.01 mg group was significantly inferior to the lamivudine 100 mg group (estimated mean difference = 1.20 [0.69–1.71]; $P < 0.0001$) (Table 2).

The secondary efficacy end point of a reduction in serum HBV DNA level 2 $\log_{10}$ copies/ml or more or HBV DNA level less than 400 copies/ml by PCR assay was achieved by 88.6% of patients in the entecavir 0.01 mg group and by 100% of patients in the entecavir 0.1 and 0.5 mg groups at week 22. Ninety-seven percent of patients in the lamivudine 100 mg group achieved this end point at week 22. HBV DNA level less than 0.7 MEq/ml by bDNA assay was achieved by 65.7%, 94.1%, and 100% of patients in the 0.01, 0.1, and 0.5 mg entecavir groups, respectively, and by 93.9% of patients in the lamivudine 100 mg treatment group.

Serologic response

Among HBeAg-positive patients, there was no significant difference between seroconversion rates at week 22 for the entecavir 0.01, 0.1, and 0.5 mg treatment groups (10.0%, 13.3%, and 3.6%, respectively) versus the lamivudine 100 mg treatment group (3.3%; Table 2). All patients who lost HBeAg also experienced HBeAg seroconversion.

Biochemical response

At baseline, elevated serum ALT levels (>1.25 × ULN) were present in more than 90% of patients in all four treatment groups. At week 22, normal serum ALT levels (World Health Organization grade 0, <1.25 × ULN) were recorded in similar proportions of patients in the entecavir 0.01, 0.1, and 0.5 mg treatment groups (75.0%, 85.3%, and 80.0% of patients, respectively) and the lamivudine treatment group (78.1% of patients), with no significant intergroup difference (Table 2).

Response

Response (HBV DNA level <0.7 MEq/ml by bDNA assay, HBeAg loss, and serum ALT level <1.25 × ULN for HBeAg-positive patients and HBV DNA level <0.7 MEq/ml by bDNA assay and serum ALT <1.25 × ULN for HBeAg-negative patients) was achieved by 14.3%, 20.6%, and 15.6% of patients in the entecavir 0.01, 0.1, and 0.5 mg

Table 2 Differences in HBV DNA levels between entecavir dose groups by PCR at week 22 in evaluable subjects

	0.1 mg ETV–0.01 mg ETV (n = 34, n = 35)	0.5 mg ETV–0.01 mg ETV (n = 32, n = 35)	0.5 mg ETV–0.1 mg ETV (n = 32, n = 34)
Estimated difference[a] ($\log_{10}$ copies/ml)	−1.61	−1.95	−0.23
Standard error	0.24	0.24	0.19
95% Confidence interval[b]	−2.20, −1.02	−2.53, −1.37	−0.69, 0.23
P-value	<0.0001	<0.0001	0.227

[a] Estimated differences are regression-adjusted for baseline serum HBV DNA and HBeAg status

[b] 95% Confidence interval is adjusted by modified Bonferroni procedures

ETV entecavir

Table 3 Virology and biochemical responses at week 22 and comparison of entecavir treatment groups with lamivudine in evaluable subjects

Response	ETV 0.01 mg (n = 35)	ETV 0.1 mg (n = 34)	ETV 0.5 mg (n = 32)	LVD 100 mg (n = 33)
HBV DNA by PCR assay				
Reduction from baseline at week 22 ($\log_{10}$ copies/ml), mean ± S.E.	−3.11 ± 0.18	−4.77 ± 0.17	−5.16 ± 0.13	−4.29 ± 0.18
HBV DNA estimated difference[a] (vs. LVD) ($\log_{10}$ copies/ml)	1.20	−0.39	−0.62	–
Standard error	0.26	0.22	0.22	–
95% Confidence interval	0.69, 1.71	−0.83, 0.05	−1.06, -0.18	–
P-value	<0.0001[b]	0.081	0.007[c]	–
HBV DNA by Roche Amplicor™ PCR assay				
Change in $\log_{10}$ HBV DNA reduction >2 or HBV DNA <400 copies/ml at week 22, n (%)	31 (88.6)	34 (100)	32 (100)	32 (97.0)
P-value (vs. LVD)	0.206	NR[d]	NR[d]	–
HBV DNA by Quantiplex assay				
HBV DNA <0.7 MEq/ml (2.5 pg/ml) at week 22, n (%)	23 (65.7)	32 (94.1)	32 (100)	31 (93.9)
P-value (vs. LVD)	0.002	1.000	NR[d]	–
Normalization of ALT levels[e]				
At week 22, n/n with abnormal baseline (%)	24/32 (75.0)	29/34 (85.3)	24/30 (80.0)	25/32 (78.1)
P-value (vs. LVD)	0.842	0.439	0.880	–
Loss of HBeAg and seroconversion at week 48[f]				
HBeAg loss, n/n HBeAg positive at baseline (%)	3/30 (10.0)	4/30 (13.3)	1/28 (3.6)	1/30 (3.3)
HBeAg seroconversion	3/30 (10.0)	4/30 (13.3)	1/28 (3.6)	1/30 (3.3)
P-value (vs. LVD)	0.605	0.350	1.000	–
Response[g] at week 22, n (%)	5 (14.3)	7 (20.6)	5 (15.6)	3 (9.1)
P-value (vs. LVD)	0.735	0.190	0.480	–

[a] Estimated differences are regression-adjusted for baseline HBV DNA and HBeAg status

[b] Two-sided test indicates inferiority of the entecavir 0.01 mg dose

[c] Two-sided test indicates superiority of the entecavir dose

[d] Not reported because expected counts <5

[e] WHO grade 0, ALT <1.25 × upper limit of normal

[f] Seroconversion was defined as disappearance of HBe-antigen and appearance of HBe-antibody

[g] Response was defined as HBV DNA levels <0.7 MEq/ml, HBeAg negativity and ALT <1.25 × ULN for HBeAg-positive patients and HBV DNA levels <0.7 MEq/ml and ALT <1.25 × ULN for HBeAg-negative patients

ETV entecavir

LVD lamivudine

treatment groups, respectively, and by 9.1% of patients in the lamivudine treatment group at week 22, and there were no significant differences in the rates of response between the four treatment groups (Table 2).

Resistance analysis

During the treatment period, serum HBV DNA level increased by 1 $\log_{10}$ copies/ml or more from its nadir in one patient in the entecavir 0.01 mg group and one patient in the lamivudine 100 mg group. Nucleotide sequence analysis of the DNA polymerase coding region, using viral samples collected from these two patients at day 1 and at week 22, revealed no lamivudine-resistance substitutions (rt180 and rt204 amino acid residues) [17, 18] or entecavir-resistance substitutions (rt184, rt202, and rt250 amino acid residues) [19].

Safety

During the study, adverse events were experienced by similar proportions of patients in the entecavir 0.01, 0.1, and 0.5 mg groups and the lamivudine 100 mg treatment group (97.1%, 97.1%, 91.2%, and 100.0%, respectively). Most adverse events were of mild or moderate intensity (grade 1/2) and transient. The most frequently reported adverse events (affecting ≥ 10% of patients in any one treatment group) included nasopharyngitis, headache, and

Table 4 Summary of adverse events and laboratory abnormalities during the 24-week blinded treatment phase

	ETV 0.01 mg (n = 35)	ETV 0.1 mg (n = 34)	ETV 0.5 mg (n = 34)	LVD 100 mg (n = 34)
Any adverse events	34 (97)	33 (97)	31 (91)	34 (100)
Most frequent clinical adverse events,[a] n (%)				
Nasopharyngitis	9 (25.7)	10 (29.4)	11 (32.4)	10 (29.4)
Headache	6 (17.1)	7 (20.6)	2 (5.9)	7 (20.6)
Diarrhea	1 (2.9)	1 (2.9)	4 (11.8)	4 (11.8)
Grade 3/4 clinical adverse events, n (%)	0	0	1 (2.9)	1 (2.9)
Grade 3/4 laboratory adverse events, n (%)	2 (5.7)	4 (11.8)	2 (5.9)	4 (11.8)
Any serious adverse events, n (%)	0	1 (2.9)	2 (5.9)	1 (2.9)
Discontinuations due to adverse events,[b] n (%)	0	0	1 (2.9)	1 (2.9)
ALT flares,[c] n (%)	0	1 (2.9)	1 (2.9)	2 (5.9)
Death, n (%)	0	0	0	0

[a] Occurring in at least 10% of patients

[b] One patient treated with ETV 0.5 mg discontinued the study drug due to hepatic cirrhosis. One patient treated with lamivudine discontinued due to increased ALT

[c] ALT flare defined ALT >2 × baseline and 10 × ULN

ETV entecavir

LVD lamivudine

diarrhea (Table 4). Grade 3/4 clinical adverse events occurred in one patient in the entecavir 0.5 mg group (colon carcinoma) and one patient in the lamivudine group (anal ulcer); neither of these events was considered to be related to the study drug. Serious adverse events were limited to the above-mentioned case of colon carcinoma, serum ALT elevation (entecavir 0.1 mg group [n = 1], entecavir 0.5 mg group [n = 1]), and serum aspartate aminotransferase (AST)/ALT elevation (lamivudine 100 mg group [n = 1]), but these were not considered to be causally related to the study drug and did not necessitate treatment discontinuation. Transient ALT flares (serum ALT >2 × baseline level and >10 × ULN) occurred in four patients (entecavir 0.1 mg group [n = 1], entecavir 0.5 mg group [n = 1], and lamivudine 100 mg group [n = 2]) and were associated with HBV DNA level decreases of 2 $\log_{10}$ copies/ml or more. None of the ALT flares were associated with hepatic decompensation and serum ALT and AST levels recovered to less than 1.25 × baseline level on continuation of the study treatment.

Discussion

The global ETV-005 study reported that entecavir was superior to lamivudine at reducing viral load in nucleoside-naive patients with CHB infection [15]. We conducted the present study, using an identical design to the ETV-005 study, to determine whether the findings from this earlier study are applicable to Japanese patients. In keeping with the previous findings, our results indicate that entecavir produces a dose-related reduction in serum HBV DNA level ($0.01 < 0.1 \leq 0.5$ mg) in nucleoside-naive Japanese patients with CHB; the log dose–response curves for the reduction in serum HBV DNA level with entecavir in the two studies were similar, with estimated regression curve slopes of −1.24 (Japanese study) and −1.32 (global study). In addition, both studies demonstrated the noninferiority of the entecavir 0.1 mg group compared with the lamivudine 100 mg group and the superiority of the entecavir 0.5 mg group compared with the lamivudine 100 mg group. The demonstration of a dose–response relationship for entecavir and the superiority of the entecavir 0.5 mg dose over lamivudine confirm that the antiviral activity of entecavir in Japanese patients is similar to that observed in study ETV-005. In a previous study, Ono et al. [14] demonstrated that the in vitro potency of entecavir was up to 2,200 times greater than that of lamivudine. The results presented here substantiate these earlier in vitro data and confirm the greater potency of entecavir over lamivudine in patients with CHB.

Serum ALT normalization rates with entecavir 0.5 mg and lamivudine 100 mg (~80%) were higher in the present study than those reported in the ETV-005 study (entecavir 0.5 mg, 69.0%; lamivudine 100 mg, 59.1%) [15]. In keeping with previous findings [20, 21], the incidence of entecavir-associated serum ALT flares in Japanese patients was low. The serum ALT flares occurred against a background of 2 $\log_{10}$ copies/ml or more reductions in serum

HBV DNA level, and serum ALT levels subsequently normalized without discontinuation of entecavir. Therefore, the serum ALT flare noted here may indicate recovery of the host's immune response arising from the reduction in HBV viral titer [22, 23]. ALT flares have been reported after the discontinuation of entecavir therapy [15, 16], thus necessitating long-term follow-up to identify possible posttreatment viral rebound.

In conclusion, the results of this dose-ranging study demonstrate a clear dose–response relationship for entecavir in terms of mean HBV DNA level reduction at week 22. Entecavir 0.5 mg was significantly more effective than lamivudine 100 mg in reducing HBV DNA levels in nucleoside-naive Japanese adult patients with CHB. At this dose level, entecavir treatment resulted in serum HBV DNA levels of less than 400 copies/ml in 100% of patients and normalization of serum ALT levels in 80% of patients after 22 weeks. Moreover, entecavir 0.5 mg once daily was well tolerated and showed a comparable safety profile to lamivudine.

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Association of Amino Acid Substitution Pattern in Core Protein of Hepatitis C Virus Genotype 2a High Viral Load and Virological Response to Interferon-Ribavirin Combination Therapy

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Key Words

Hepatitis C virus · Genotype 2a · Core region · Interferon · Ribavirin · Rapid response

Abstract

Background: Substitution of amino acids (aa) 70 and 91 in the core region of HCV genotype 1b is a useful pretreatment predictor of poor response to interferon + ribavirin combination therapy, but the impacts of aa substitutions in the core region of HCV genotype 2a are still not clear. ***Methods:*** 154 consecutive Japanese adults with a high viral load (≥100 kIU/ml) of genotype 2a who could complete combination therapy for 24 weeks were evaluated. To examine the differences in virological characteristics between non-sustained virological response (non-SVR) and rapid responder (SVR patients who could achieve a HCV-RNA-negative status within 8 weeks), 86 patients could be analyzed by pretreatment substitution patterns of the core region. ***Results:*** SVR was achieved in 127 of 154 patients (83%), and rapid response in 113 of 127 (90%). In all 154 patients, multivariate analysis identified younger age, lower level of viremia, and higher level of albumin as significant determinants of SVR. As significant determinants of rapid response in 86 patients, multivariate analysis identified substitution of aa 4 (non-asparagine) in addition to the significant determinants of SVR. ***Conclusions:*** Our results suggest that the aa substitution pattern of the core region in patients with a high titer of genotype 2a may partly affect the virological response to combination therapy.

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Introduction

The response to interferon (IFN)-related therapy varies according to hepatitis C virus (HCV) genotype [1, 2]. In Japan, about 70% of patients with chronic hepatitis C are infected with HCV genotype 1b, and about 25% with genotype 2a [3]. Sustained virological response (SVR) to 48-week IFN + ribavirin combination therapy is about 50% in genotype 1b infection, and SVR to 24-week combination therapy is more than 80% in genotype 2 infection [4–9].

IFN + ribavirin combination therapy carries potential serious side effects and is costly, especially when used

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