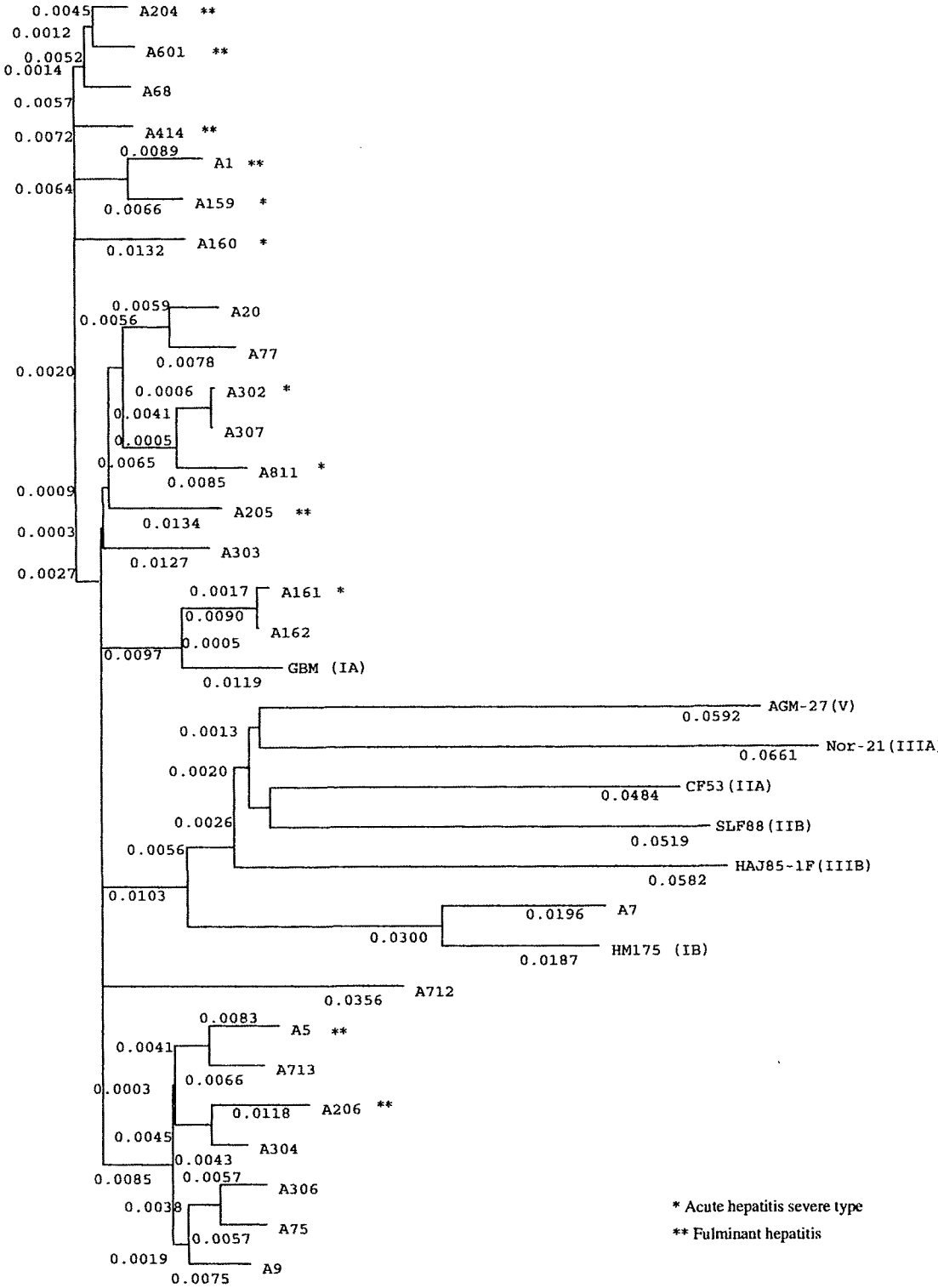

Fig. 2. Genetic relatedness between individual hepatitis A virus (HAV) of entire 2B and 2C recovered from 25 patients and HAV reference strains GBM (subgenotype IA), HM175 (subgenotype IB), CF53 (subgenotype IIA), SLF88 (subgenotype IIB), Nor-21 (subgenotype IIIA), HAJ85-1F (subgenotype IIIB) and AGM27 (genotype V). Numbers beside the phylogenetic roots are the results of bootstrap analyses.

Table 2. Clinical, biochemical and viral characteristics of six patients with fulminant and severe hepatitis located in the near parts of the phylogenetic tree

Patient	Diagnosis	Age/sex	Origin	Onset	Outcome	ALT (IU/L)	T-Bil (mg/dl)	PT (%)	IgM-HA (cut-off index)	Viral load (log copies/ml)	Days from onset
A204	FH	39/F	Tohoku	February 1990	Death	4470	5.3	10	3.8	ND	3
A601	FH	64/F	Shinetsu	January 1997	Death	12 500	7.0	13	2.9	3.7	9
A414	FH	49/M	Shinetsu	January 1989	Recovery	5276	26.3	13	+	5.0	7
A160	AHs	39/M	Kanto	June 1998	Recovery	9164	1.6	38	3.1	5.1	4
A1	FH	29/M	Kanto	March 1992	Recovery	1175	7.3	17	5.1	3.3	6
A159	AHs	50/M	Kanto	May 1998	Recovery	5655	2.5	20	4.6	4.6	5

Patient	Genotype	5′ NTR homology (%)	2B nt homology (%)	2C nt homology (%)
A204	IA	99.0	93.8	90.0
A601	IA	99.3	94.3	89.3
A414	IA	98.7	95.0	88.6
A160	IA	97.7	95.2	88.3
A1	IA	98.7	96.0	88.5
A159	IA	98.7	96.9	88.8

Homology, sequences were compared with wild-type HAV genotype IA strain GBM.
AH, acute hepatitis; AHs, acute hepatitis severe type; ALT, alanine aminotransferase; FH, fulminant hepatitis; 5′NTR, 5′-nontranslated region; ND, not done; nt, nucleotide; Ti-Bil, total bilirubin.

patients. Rezende *et al.* (29) reported that HAV-related liver failure is because of an excessive host response associated with a marked reduction in viral load, and there is a discrepancy between their data and ours. But they did not show the time points of serum sampling that represent critical data about viraemia in AH, and so we cannot discuss the discrepancy.

Thus, genetic variations not in one specific region but in 5′NTR, 2B and 2C might cooperatively influence replication of the virus and thereby affect virulence. Our findings are in accordance with the basic reports that the pathogenicity of HAV could be related to cooperative mutations within 5′NTR and P2 in cultured cells and simians, and the clinical finding that there has been only one report about a cluster of fulminant hepatitis A, unlike the many reports of clusters of fulminant hepatitis B.

Our current study suggests that both viral and host factors should be considered and examined when discussing the mechanisms responsible for the severity of hepatitis A. Further, we should examine several portions of the HAV genome including 5′NTR, 2B and 2C rather than focus on one specific region when analysing viral factors. Our study also suggests that vaccination should be considered all the more if HAV itself is involved in the pathogenicity of hepatitis A, because safe and extremely effective inactivated HAV vaccines are available.

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Hepatol Int (2009) 3:403–410
DOI 10.1007/s12072-008-9108-8

CASE REPORT

Two cases of development of entecavir resistance during entecavir treatment for nucleoside-naive chronic hepatitis B

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Received: 27 June 2008 / Accepted: 2 October 2008 / Published online: 9 December 2008

Abstract
Background Entecavir (ETV) is a potent nucleoside analogue against hepatitis B virus (HBV), and emergence of drug resistance is rare in nucleoside-naive patients because development of ETV resistance (ETVr) requires at least three amino acid substitutions in HBV reverse transcriptase. We observed two cases of genotypic ETVr with viral rebound and biochemical breakthrough during

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ETV treatment of nucleoside-naive patients with chronic hepatitis B (CHB).

Results *Case 1*: A 44-year-old HBeAg-positive man received ETV 0.1 mg/day for 52 weeks and 0.5 mg/day for 96 weeks consecutively. HBV DNA was 10.0 $\log_{10}$ copies/ml at baseline, declined to a nadir of 3.1 at week 100, and rebounded to 4.5 at week 124 and 6.7 at week 148. Alanine aminotransferase (ALT) level increased to 112 IU/l at week 148. Switching to a lamivudine (LVD)/adefovir-dipivoxil combination was effective in decreasing HBV DNA. *Case 2*: A 47-year-old HBeAg-positive man received ETV 0.5 mg/day for 188 weeks. HBV DNA was 8.2 $\log_{10}$ copies/ml at baseline, declined to a nadir of 2.9 at week 124, and then rebounded to 4.7 at week 148 and 6.4 at week 160. ALT level increased to 72 IU/l at week 172. The ETVr-related substitution (S202G), along with LVD-resistance-related substitutions (L180M and M204V), was detected by sequence analysis at week 124 in both case 1 and case 2.

Conclusions ETVr emerged in two Japanese nucleoside-naive CHB patients after prolonged therapy and incomplete suppression and in one patient after <0.5 mg of dosing. ETV patients with detectable HBV DNA or breakthrough after extended therapy should be evaluated for compliance to therapy and potential emergence of resistance.

Keywords Entecavir · HBV · Chronic hepatitis B · Drug resistance · Nucleoside-naive

Introduction

Hepatitis B virus (HBV) infection is a serious health problem because of its high prevalence, estimated to be infecting more than 350 million people worldwide, and its potential for inducing chronic hepatitis, cirrhosis, hepatic decompensation, and hepatocellular carcinoma (HCC) [1, 2]. It has been demonstrated that the most potent risk factor for development of cirrhosis or HCC is serum HBV DNA level [3, 4], and it seems that suppressing serum HBV load is essential for improving the prognosis of HBV carriers. Treatment of chronic hepatitis B (CHB) has evolved markedly with the introduction of nucleoside-analogue antivirals, that is, lamivudine (LVD), adefovir-dipivoxil (ADV), entecavir (ETV), and telbivudine, to clinical practice. LVD, the first approved nucleoside analogue against HBV, was shown to be effective in suppressing HBV DNA replication, improving transaminase levels, improving liver histology, inducing hepatitis B e antigen (HBeAg) seroconversion, and suppressing hepatic insufficiency and hepatocarcinogenesis in CHB and compensated cirrhosis [5, 6]. However, the effectiveness of LVD is limited because of frequent development of drug resistance followed by a hepatitis flare and, occasionally, hepatic failure [7, 8].

ETV, a novel anti-HBV nucleoside analogue, has more than 1,500 times greater potency than LVD in vitro [9]. In clinical trials, ETV administration demonstrated potent anti-HBV activity with a marked decline in serum HBV DNA level and a significant improvement in liver histology than LVD in nucleoside-naive HBeAg-positive and -negative patients [10, 11]. In addition, emergence of ETV resistance (ETVr) or viral rebound was shown in these studies to be rare. From these results, recent treatment guidelines have recommended ETV as the first-line nucleoside analogue for nucleoside-naive CHB patients, including those with cirrhosis [12, 13].

It has been reported that the development of ETVr in nucleoside-naive patients is very rare, even after 4 years of therapy. Recently, however, rare cases of ETVr, which developed in nucleoside-naive patients in clinical studies, have been reported [14–16]. We also observed two patients who developed ETVr-associated HBV reverse transcriptase (RT) substitutions, followed by *virologic rebound*, defined as an elevation in serum HBV DNA of more than 1 $\log_{10}$ copy/ml from nadir, and biochemical breakthrough in long-term ETV treatment of nucleoside-naive CHB patients. In this article, we report these two cases in detail.

Case report

Case 1

A 44-year-old Japanese male CHB patient was positive for hepatitis B surface antigen (HBsAg), HBeAg, serum HBV DNA, and had HBV genotype C, had elevated alanine aminotransferase (ALT) levels, and had no history of nucleoside analogue treatment. The patient had a history of acute appendicitis at age 30, ureteral stone at age 35, and hyperlipidemia at age 43. He had a habit of drinking alcohol (700 ml) daily but did not smoke. At age 27, he was diagnosed for the first time by health screening as an asymptomatic HBV carrier in the immune-tolerant phase, defined by HBsAg positivity and normal liver enzymes, and he was followed up regularly elsewhere with blood tests for liver enzymes. He was found to have ALT elevation. He was referred to our hospital at age 44 and was diagnosed with CHB. Serum HBV DNA level determined by Roche Amplicor™ Monitor PCR assay (lower limit of detection is 2.6 $\log_{10}$ copies/ml = 400 copies/ml; Roche Diagnostics K.K., Tokyo, Japan) [17] was 10.0 $\log_{10}$ copies/ml and serum ALT level was 199 IU/l. Histologic diagnosis by percutaneous liver biopsy at baseline revealed chronic hepatitis with mild fibrosis and mild activity (CH F1/A1, according to the New Inuyama Classification) [18].

Table 1 Baseline characteristics

	Normal range	Unit	Case 1	Case 2
Age	–	–	44 years	47 years
Gender	–	–	Male	Male
T. Bil	0.2–1.0	mg/dl	0.8	0.5
AST	10–40	IU/l	113	48
ALT	5–40	IU/l	199	74
ALP	115–359	IU/l	268	216
BUN	6–20	mg/dl	9.5	15.9
CREA	0.61–1.04	mg/dl	0.95	0.83
ALB	4.0–5.0	g/dl	4.2	4.3
WBC	3500–8500	/μl	6,800	5,650
Hb	13.5–17.0	g/dl	15.7	14.8
PLT	13.1–36.2	10^4/μl	18.9	14.5
Prothrombin time	10–13	second	10.8	11.2
INR		–	1.0	0.9
HBsAg (CLIA)	0–0.05	IU/ml	>100 (positive)	>100 (positive)
anti-HBs (CLIA)	0–10	IU/ml	0 (negative)	0 (negative)
HBeAg (CLIA)	0–1		120 (positive)	190 (positive)
anti-HBe (CLIA)	0–50	%	<35 (negative)	0 (negative)
HBV DNA (PCR)	<2.6	$\log_{10}$ copies/ml	10.0	8.2
HBV genotype			Genotype C	Genotype C
YMDD (sequencing)			YMDD+	YMDD+
			YVDD–	YVDD–
			YIDD–	YIDD–
Liver histology[a]			CH F1/A1	CH F2/A2

[a] Diagnosed according to New Inuyama classification. T. Bil: total bilirubin, AST: aspartate aminotransferase, ALT: alanine aminotransferase, ALP: alkalinephosphatase, BUN: blood urea nitrogen, CREA: serum creatinine, ALB: serum albumin, WBC: white blood cell count, Hb: hemoglobin, PLT: platelet count, INR: international normalized ratio, HBsAg: hepatitis B surface antigen, CLIA: chemiluminescent immunoassay, anti-HBs: antibody to hepatitis B surface antigen, HBeAg: hepatitis B e antigen, anti-HBe: antibody to hepatitis B e antigen, HBV: hepatitis B virus, PCR: polymerase chain reaction, YMDD: tyrosine-methionine-aspartate-aspartate motif, YVDD: tyrosine-valine-aspartate-aspartate motif, YIDD: tyrosine-isoleucine-aspartate-aspartate motif, CH F1/A1: chronic hepatitis with mild fibrosis and mild activity, CH F2/A2: chronic hepatitis with moderate fibrosis and moderate activity

Other baseline characteristics are shown in Table 1. He was enrolled in a phase II clinical trial of ETV and was randomized into 0.1- and 0.5-mg dosage groups. The trial was conducted in Japan in compliance with the ethical principles of the Declaration of Helsinki, Good Clinical Practice guidelines, and Articles/Notifications of the Ministry of Health, Labor and Welfare (H. Kobashi et al., J Gastroenterol Hepatol, in press). He was assigned into the 0.1-mg dosage group and administered ETV at daily dose of 0.1 mg for an initial 52 weeks. Subsequently, he was administered ETV continuously at a daily dose of 0.5 mg for the following 96 weeks. The serum HBV DNA level, which was 10.0 $\log_{10}$ copies/ml at baseline, declined to a nadir of 3.1 $\log_{10}$ copies/ml at week 88 of ETV treatment. Thereafter, HBV DNA level increased from 4.5 $\log_{10}$ copies/ml at week 124 to 6.3 $\log_{10}$ copies/ml at week 140 and 6.7 $\log_{10}$ copies/ml at week 148. ALT levels increased from 28 IU/l at week 144 to 112 IU/l at week 148. The patient discontinued ETV therapy at week 148, and then received a combination therapy of 100 mg of LVD and 10 mg of ADV per day. Afterwards, HBV DNA level dropped to below 2.6 $\log_{10}$ copies/ml and ALT level was normalized after 28 weeks of LVD/ADV dosing (Fig. 1).

HBV DNA sequence analysis was performed using PCR-amplified HBV DNA from preserved serum samples at baseline and at every 24 weeks via HBV DNA polymerase sequence assay (developed at SRL, Inc., Tokyo, Japan). Although sequence analysis of the baseline isolate revealed no substitution in the RT domain of the HBV DNA polymerase gene, analysis of the isolates collected over time revealed the M204I substitution at week 100 and the L180M, S202G, and M204V substitutions at weeks 124 and 144, respectively (Table 2). In addition, a polymorphic residue N238 was found as mixed N238 N/H at week 100 and thereafter. The

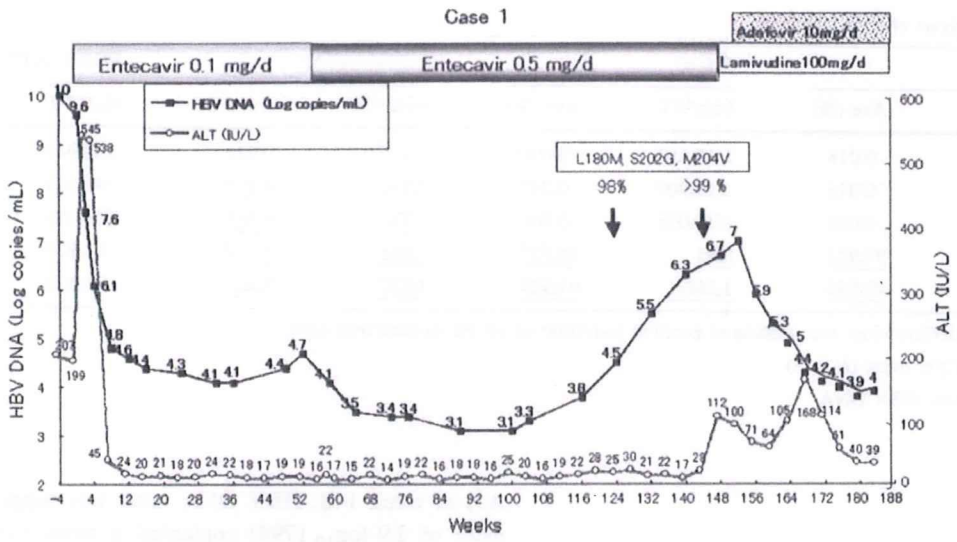

Fig. 1 Clinical course of case 1, a 44-year-old man with nucleoside-naive CHB. ETV treatment reduced ALT levels to below the upper normal limit at week 12 and reduced HBV DNA load to a nadir of 3.1 $\log_{10}$ copies/ml at week 88. However, HBV DNA re-elevated to 4.5 $\log_{10}$ copies/ml at week 124 (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.

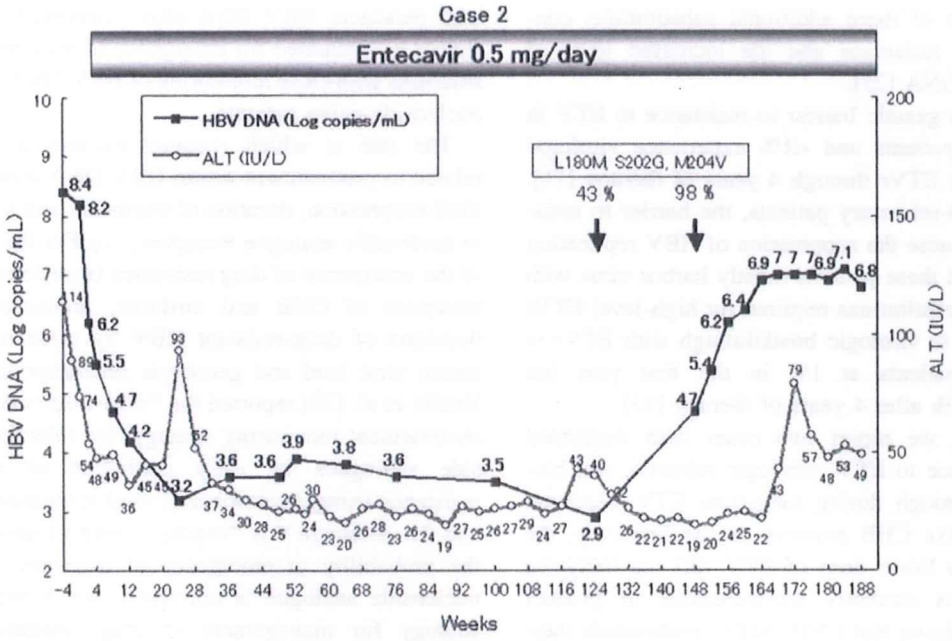

Fig. 2 Clinical course of case 2, a 47-year-old man with nucleoside-naive CHB. ETV treatment reduced ALT level to below the upper normal limit at week 30 and reduced serum HBV DNA level to a nadir of 2.9 $\log_{10}$ copies/ml at week 124. However, HBV DNA level re-elevated to 4.7 $\log_{10}$ copies/ml (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.

Acknowledgments This work was carried out with a grant from Bristol-Myers Squibb. Taku Seriu, Hiroki Ishikawa, and Ronald Rose are employees of Bristol-Myers Squibb. Masao Omata serves as an advisor for Bristol-Myers Squibb. We acknowledge Jie Fang and Kevin Pokornowski, employees of Bristol-Myers Squibb, for genotypic analysis of the patient samples.

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doi:10.1111/j.1440-1746.2008.05593.x

HEPATOLOGY

Efficacy and safety of entecavir in nucleoside-naive, chronic hepatitis B patients: Phase II clinical study in Japan

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Key words
chronic hepatitis B, entecavir, Japanese.

Accepted for publication 2 July 2008.

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Abstract

Background and Aim: Entecavir has demonstrated clinical efficacy for chronic hepatitis B. This study evaluated the efficacy and safety of entecavir in nucleoside-naive Japanese chronic hepatitis B patients.

Methods: In this multicenter, double-blind study, 66 nucleoside-naive Japanese chronic hepatitis B patients were randomized to 0.1 mg entecavir ($n = 32$) or 0.5 mg entecavir ($n = 34$) daily for 52 weeks. The primary endpoint was the proportion of patients whose serum hepatitis B virus (HBV) DNA decreased from baseline by ≥ 2 $\log_{10}$ copies/mL or became undetectable (<400 copies/mL by polymerase chain reaction assay) at week 48.

Results: One hundred percent of patients in both treatment groups achieved the primary efficacy endpoint, with 81% and 68% of patients achieving undetectable HBV DNA in the 0.1 mg and 0.5 mg treatment groups, respectively. Mean changes from baseline in HBV DNA were -4.49 $\log_{10}$ and -4.84 $\log_{10}$ copies/mL for the 0.1 mg and 0.5 mg groups, respectively. Significant improvements in necroinflammation were seen in both groups, as assessed by Knodell and New Inuyama classifications. Most adverse events were transient and classified as grade 1 or 2. There were no clinically significant differences in adverse events across the two treatment groups and no discontinuations due to adverse events in either group.

Conclusions: In Japanese nucleoside-naive patients with chronic hepatitis B, 0.1 mg or 0.5 mg entecavir daily provided excellent efficacy and was well tolerated. The 0.5 mg dose was selected for the treatment of nucleoside-naive patients.

Introduction

It has been reported that 350–400 million people worldwide are chronically infected with hepatitis B virus (HBV)[1,2] despite the widespread use of HBV vaccination for prevention of this disease. HBV infection is particularly prevalent in Asia–Pacific countries, with an estimated 75% of all chronically infected patients living in the region.[3] Prevalence rates reported in 2000 indicated that 0.8% of the Japanese population were hepatitis B virus surface antigen (HBsAg) positive with 36% of infected individuals being chronically infected.[4] Among those chronically infected, 20–40% will develop cirrhosis, decompensated liver disease or hepatocellular carcinoma.[5] In Asia, HBV is the leading cause of chronic hepatitis, cirrhosis and hepatocellular carcinoma.[4]

Treatment for chronic hepatitis B has evolved markedly over the last decade. Interferon-α was the only available treatment for many years, but this cytokine is efficacious in only approximately 35% of patients,[6] and is poorly tolerated by many patients due to adverse effects. Lamivudine, a cytosine analog, was the first oral anti-HBV nucleoside analog developed, and has demonstrated efficacy for treatment of chronic hepatitis B during short-term administration.[7,8] However, viral breakthrough due to emergence of lamivudine-resistant strains of HBV with amino acid substitutions in the YMDD (tyrosine–methionine–aspartate–aspartate) motif of reverse transcriptase result in loss of clinical benefit.[9–11] Long-term follow up of hepatitis B e antigen (HBeAg)-negative patients treated with adefovir, the second approved oral anti-HBV, have shown cumulative probabilities of genotypic

resistance of 29% at 5 years[12] and some studies have reported that 20–50% of patients receiving a 10 mg dose of adefovir have primary non-response[13] indicating that the approved dose of adefovir may be suboptimal.[14]

Entecavir is a deoxyguanosine analog that has more than 300 times greater potency than lamivudine *in vitro*.[15,16] Entecavir inhibits all three steps of HBV DNA replication: (i) priming of the HBV DNA polymerase; (ii) reverse transcription of negative-strand HBV DNA from pre-genomic messenger RNA; and (iii) synthesis of positive-strand HBV DNA.[17] In woodchuck models of HBV infection, entecavir reduced viral loads by up to 9 $\log_{10}$ copies/mL and prevented the onset of hepatocellular carcinoma.[18] In early clinical studies, entecavir was demonstrated to be safe and efficacious when given for 28 days.[19] In a 24-week, phase II international clinical trial, Lai *et al.* demonstrated a dose–response relationship for entecavir, and showed that entecavir was superior to lamivudine at doses of 0.1 mg and 0.5 mg for HBV DNA reduction.[20] Subsequently, two phase III international trials showed that 0.5 mg entecavir daily for 48 weeks achieved superior histological, virological and biochemical improvement in HBeAg-positive and HBeAg-negative nucleoside-naive patients compared with lamivudine, with comparable safety and no emergence of resistance without prior existence of the amino acid substitutions rtL180M and rtM204V/I/S which are associated with lamivudine resistance.[21,22] Entecavir was approved by the US regulatory authorities in March 2005. Study AI463047 evaluated 0.01 mg, 0.1 mg and 0.5 mg entecavir and 100 mg lamivudine in nucleoside-naive Japanese patients and established the 0.5 mg dose of entecavir as the optimal dose in this patient population. The current phase II dose-ranging trial, which commenced before the completion of AI463047, evaluated the efficacy and safety of 0.1 mg and 0.5 mg entecavir daily for 52 weeks in nucleoside-naive chronic hepatitis B patients in Japan. This study's primary objective was to demonstrate that entecavir has antiviral activity as indicated by the proportion of subjects who achieve a reduction from baseline in HBV DNA by ≥ 2 $\log_{10}$ copies/mL or to <400 copies/ml at week 48.

Methods

Study design

This was a randomized, double-blind, multicenter trial of 0.1 mg entecavir once daily and 0.5 mg entecavir once daily for 52 weeks in nucleoside-naive patients with HBeAg-positive or -negative chronic hepatitis B. Patients were randomized via a central registration procedure. A total of 66 patients were enrolled in this study, including men and women ranging in age from 27–68 years who were determined to be eligible for the study during the 6-week screening period. Following randomization, patients received either a 0.1 mg entecavir tablet plus a 0.5 mg placebo tablet ($n = 32$) or a 0.5 mg entecavir tablet plus a 0.1 mg placebo tablet ($n = 34$) orally once daily for 52 weeks. After 52 weeks of blinded dosing, patients were given the option of enrolling in an entecavir rollover study. All patients who discontinued blinded dosing early, or who completed the protocol but did not enroll in the entecavir rollover study, were followed for 24 weeks post-dosing, and could receive marketed anti-HBV therapy as recommended by their physician.

The study was conducted in compliance with the ethical principles of the Declaration of Helsinki, Good Clinical Practice guidelines, and Articles/Notifications of the Ministry of Health, Labor and Welfare in Japan. Written informed consent was obtained from all patients.

The study's primary efficacy objective was to demonstrate that 0.1 mg and 0.5 mg doses of entecavir had antiviral activity as indicated by the proportion of patients who achieve a reduction in HBV DNA of ≥ 2 $\log_{10}$ copies/mL or to below the limit of quantification (LOQ, 400 copies/mL) by polymerase chain reaction (PCR) assay (Roche Amplicor, Hoffmann-La Roche Ltd, Basel, Switzerland) at week 48. Secondary endpoints included the mean change from baseline in HBV DNA by PCR assay at week 48, and proportions of patients who achieved the following at week 48: (i) HBV DNA less than 400 copies/mL; (ii) serum alanine aminotransferase (ALT) normalization (<1.25 times the upper limit of normal [ULN], World Health Organization [WHO] toxicity grade 0); (iii) HBeAg loss and HBeAg seroconversion (HBeAg loss and appearance of anti-HBe) among patients who were HBeAg-positive at baseline; and (iv) complete response, defined as HBV DNA less than 400 copies/mL by PCR assay plus ALT less than 1.25 × ULN plus HBeAg negativity for those who were HBeAg-positive at baseline. The incidence of amino acid substitutions associated with entecavir resistance in patients who experienced a virological breakthrough, defined as an increase in HBV DNA of ≥ 1 $\log_{10}$ copies/mL from nadir, was also determined. Among patients with evaluable baseline and week 48 liver biopsies, the proportion of patients with histological improvement was determined. Histological improvement was defined as a $\geq$2-point decrease in the Knodell necroinflammatory score and no worsening of fibrosis (worsening was defined as a $\geq$1-point increase in the Knodell fibrosis score) from baseline to week 48. Liver biopsies were also evaluated using the New Inuyama classification system. The biopsy reading committee was blinded to treatment and sequence.

The primary safety endpoint was the proportion of patients in each treatment group who discontinued study medication due to adverse events. Secondary safety endpoints included incidence of adverse events, serious adverse events, laboratory abnormalities, grade 3–4 clinical adverse events and grade 3–4 laboratory abnormalities. ALT flares were defined as ALT >2 × baseline and >10 × ULN.

Study population

Patients were eligible for enrollment if they met the following inclusion criteria: (i) hepatitis B surface antigen (HBsAg)-positive for 24 weeks or more prior to screening or HBsAg-positive for less than 24 weeks prior to screening, negative for immunoglobulin M anti-hepatitis B core antibody and confirmation of chronic hepatitis on liver biopsy; (ii) HBeAg-positive for more than 12 weeks prior to screening or HBeAg-negative and positive for anti-HBe; (iii) active viral replication as evidenced by HBV DNA of $\geq 10^5$ copies/mL by PCR assay at screening; (iv) serum ALT ranging 1.3–10 times the ULN; and (v) compensated liver disease, as indicated by a prothrombin time $\leq$3 s longer than the normal control value or international normalized ratio of $\leq$1.5, serum albumin of $\geq$3.0 g/dL (30 g/L) and total bilirubin of $\leq$2.5 mg/dL (42.75 μmol/L). Women of childbearing potential underwent

contraceptive procedures as appropriate to avoid pregnancy during the trial period and for up to 8 weeks after completion of the trial.

The following patients were excluded from the study: pregnant and nursing women; patients diagnosed with cirrhosis, or with a history or evidence of variceal bleeding, encephalopathy or ascites requiring diuretics or paracentesis; patients with other forms of liver disease or suspected hepatic tumors; patients diagnosed with HIV infection; patients with a history of pancreatitis within 24 weeks prior to initiation of protocol therapy; and patients with an increased risk of hepatic toxicity or pancreatitis. In addition, patients who received immunosuppressive therapy (including systemic administration of corticosteroid-derivative agents) or who were treated with interferon-α or -β, within 24 weeks prior to initiation of protocol therapy, were excluded from the study. Patients treated with anti-HBV nucleoside analogs for more than 12 weeks were also excluded.

Assay methods

Serum HBV DNA was determined by Roche Amplicor PCR assay (LOQ, 400 copies/mL; Roche Diagnostics, Tokyo, Japan)[23] in a central laboratory. Clinical laboratory tests, PCR assays for HBV DNA, and serological tests for HBV were performed at SRL Inc. (Tokyo, Japan), the central clinical laboratory designated by the trial sponsor. Liver biopsy was performed within 6 weeks of initiation of study therapy; or, if a liver biopsy had been previously obtained within 52 weeks before initiation of protocol therapy, it was used as the baseline specimen for histological evaluation. Baseline biopsies were evaluated using the Knodell Histological Activity Index (HAI) and Knodell fibrosis scores,[24] and the New Inuyama classifications.[25] Genotype analysis of HBV strains was performed on samples from all patients at baseline using a PCR-restriction fragment length polymorphism assay (SRL). All samples were also analyzed at baseline for evidence of amino acid substitutions associated with lamivudine resistance (rtM204V/I) using a PCR-enzyme-linked minisequence assay (Medical & Biological Laboratories, Aichi, Japan). In addition, patients who experienced virological breakthrough (increase in HBV DNA of ≥1 $\log_{10}$ copies/mL from nadir of treatment) had baseline and on-treatment samples analyzed for amino acid substitutions associated with entecavir resistance (rtT184, rtS202 and rtM250) using a HBV DNA polymerase sequence assay at SRL.

Statistical analysis

Analyses of efficacy endpoints were based on treated patients. The primary objective would be demonstrated if the lower limit of the 95% confidence interval for the proportion of patients who achieved a reduction in HBV DNA from baseline by ≥2 $\log_{10}$ copies/mL or to less than 400 copies/mL by PCR assay at week 48 in either treatment arm was at least 60%. Parameters represented by continuous variables were summarized by the mean and standard error. In the analysis of binary endpoints, patients with missing week 48 measurements were treated as missing (non-completer = missing). All reported *P*-values are two-sided. For comparison of liver biopsy specimens before and after treatment, a Wilcoxin signed-rank test was performed.

Table 1 Baseline demographics and characteristics

	0.1 mg entecavir $n = 32$	0.5 mg entecavir $n = 34$
Men, *n* (%)	24 (75)	26 (76)
Women, *n* (%)	8 (25)	8 (24)
Age (years), mean ± SD	44.1 ± 11.4	46.6 ± 10.1
Weight (kg), mean ± SD	67.6 ± 18.2	65.6 ± 13.1
Ethnicity		
Japanese, *n* (%)	32 (100)	34 (100)
HBV DNA, mean ± SD		
$\log_{10}$ copies/mL by PCR	7.26 ± 1.08	7.68 ± 0.97
HBeAg-positive, *n* (%)	26 (81)	27 (79)
ALT (IU/L), mean ± SD	159.6 ± 210.4	141.0 ± 91.9
AST (IU/L), mean ± SD	97.7 ± 103.2	93.1 ± 60.6
Total bilirubin (mg/dL), mean ± SD	0.58 ± 0.22	0.62 ± 0.25
Knodell HAI score, no. of biopsy	31	31
pairs performed, mean ± SE	8.5 ± 0.5	8.7 ± 0.5
Prior treatment		
Interferon, *n* (%)	0	0
Lamivudine, *n* (%)	0	1[†]
Nucleside/nucleotide analogs, *n* (%)	0	0
HBV genotype *n* (%)		
C	29 (91)	33 (97)
B	2 (6.3)	0
Unknown	1 (3)	1 (3)

[†]Less than 12 weeks of therapy, consistent with protocol

ALT, alanine aminotransferase; AST, aspartate aminotransferase; HAI, Histological Activity Index; HBeAg, hepatitis B e antigen; HBV, hepatitis B virus; PCR, polymerase chain reaction; SD, standard deviation; SE, standard error.

Results

Study population

Of 102 patients enrolled and screened, 66 were randomized and treated. Thirty-two patients were assigned 0.1 mg entecavir and 34 patients 0.5 mg entecavir. The two treatment groups were well balanced at baseline for demographic and disease-related characteristics (Table 1). Approximately 80% of patients in both groups were HBeAg-positive, and mean HBV DNA at baseline were 7.26 and 7.68 $\log_{10}$ copies/mL for the 0.1 mg and 0.5 mg groups, respectively. Overall, 62 patients were infected with HBV genotype C, and two patients were infected with HBV genotype B. In two patients, HBV genotype was not identified. All patients completed protocol therapy for 52 weeks and were assessed for efficacy and safety. Compliance, measured by the volume of unused product returned from subjects to the institution, was reported to be between 95% and 100%. After completion of the protocol therapy, all patients entered an entecavir rollover study.

Virological response

One hundred percent of patients in both treatment groups achieved the primary efficacy endpoint (a reduction from baseline in HBV DNA of ≥2 $\log_{10}$ copies/mL or to <400 copies/mL by PCR assay

Table 2 Virological, biochemical and serological responses at weeks 24 and 48

Response	0.1 mg entecavir $n = 32$	0.5 mg entecavir $n = 34$
Primary efficacy endpoint		
Reduction in HBV DNA >2.0 $\log_{10}$ copies/mL or to >400 copies/mL by PCR assay at week 48	32 (100)	34 (100)
Other virological endpoints		
Mean change from baseline by PCR ($\log_{10}$ copies/mL), mean ± SE		
Week 24	−4.43 ± 0.16	−4.79 ± 0.14
Week 48	−4.49 ± 0.16	−4.84 ± 0.14
HBV DNA <400 copies/mL by PCR, *n* (%)		
Week 24	20 (63)	19 (56)
Week 48	26 (81)	23 (68)
Normalization of ALT[†]		
Week 24, *n*/*n* with abnormal baseline (%)	24/28 (86)	27/32 (84)
Week 48, *n*/*n* with abnormal baseline (%)	27/28 (96)	30/32 (94)
HBeAg seroconversion at week 48[‡]	8/26 (31)	8/27 (30)
Complete response[§] at week 48, *n* (%)	12 (38)	13 (38)

[†]World Health Organization grade 0: ALT of <1.25 × ULN.
[‡]Loss of HBeAg and gain of anti-HBe.
[§]HBV DNA <400 copies/mL, HBeAg-negative and ALT of <1.25 × ULN.
ALT, alanine aminotransferase; HBeAg, hepatitis B e antigen; HBV, hepatitis B virus; PCR, polymerase chain reaction; SE, standard error.

at week 48; Table 2). By week 4, 94% and 91% of patients in the 0.1 mg and 0.5 mg groups, respectively, achieved the primary endpoint; 100% of patients in both groups had achieved it by week 8 and that proportion was maintained through to the end of treatment (week 48). Mean serum HBV DNA declined rapidly in both groups through week 4, and thereafter declined more slowly (Fig. 1). Mean change from baseline in HBV DNA at week 24 was −4.43 $\log_{10}$ copies/mL for the 0.1 mg group and −4.79 $\log_{10}$ copies/mL for the 0.5 mg group. Between weeks 24 and 48, across both groups, only slight decreases in mean HBV DNA occurred. At week 48, mean change from baseline in HBV DNA was −4.49 $\log_{10}$ copies/mL for the 0.1 mg group and −4.84 $\log_{10}$ copies/mL for the 0.5 mg group (P = non-significant [NS]; Fig. 1, Table 2). Eighty-one percent of patients receiving 0.1 mg entecavir and 68% of patients receiving 0.5 mg entecavir achieved HBV DNA of less than 400 copies/mL by PCR assay at week 48 (P = NS, Table 2).

Biochemical response

Approximately 90% of patients demonstrated abnormal ALT (≥1.25 × ULN) at baseline. Among patients with abnormal baseline ALT, the proportions achieving ALT normalization (<1.25 × ULN; WHO toxicity grade 0) at week 48 were 96% (27/28) for patients receiving 0.1 mg entecavir and 94% (30/32) for patients receiving 0.5 mg entecavir (P = NS, Table 2).

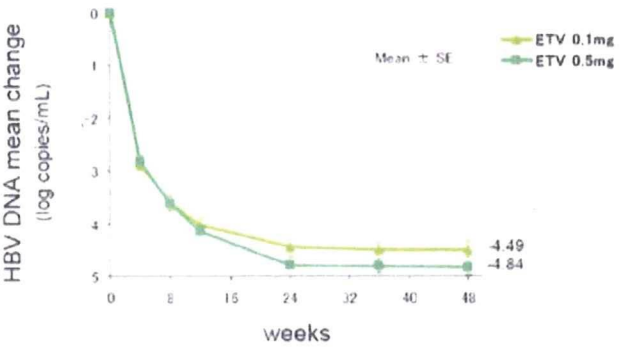

Figure 1 Mean change from baseline in hepatitis B virus (HBV) DNA through week 48 by polymerase chain reaction assay ($\log_{10}$ copies/mL) in patients treated with 0.1 mg and 0.5 mg entecavir (ETV). Data expressed as mean ± standard error.

Serological response

Among patients who were HBeAg-positive at baseline, the proportions achieving HBeAg loss at week 48 were 31% (8/26) in the 0.1 mg group and 30% (8/27) in the 0.5 mg group (Table 2). All patients who demonstrated HBeAg loss also showed acquisition of anti-HBe, thus rates of HBeAg seroconversion at week 48 were also 31% and 30% for the 0.1 mg group and 0.5 mg groups, respectively (Table 2).

Complete response

At week 48, the proportions of patients achieving complete response (defined as HBV DNA <400 copies/mL by PCR assay plus ALT <1.25 × ULN plus HBeAg negativity if they were HBeAg-positive at baseline) were 38% (12/32) for patients receiving 0.1 mg entecavir and 38% (13/34) for patients receiving 0.5 mg entecavir (P = NS, Table 2).

Histological response

Ninety-one percent (29/32) of patients in the 0.1 mg entecavir group and 88% (30/34) of patients in the 0.5 mg entecavir group had evaluable biopsy pairs from baseline and week 48 (Table 3). Histological improvement, defined using the Knodell classification system, occurred in 72% (21/29) and 80% (24/30) of patients in the 0.1 mg and the 0.5 mg groups, respectively. Mean change in Knodell HAI scores were −3.2 and −4.6 for the 0.1 mg and the 0.5 mg groups, respectively. For both groups, the change from baseline in HAI score was significant ($P < 0.0001$ for both groups). In patients who received 0.5 mg entecavir, 29% (9/31) of patients experienced an improvement or no worsening of Knodell fibrosis score, and the mean change from baseline in Knodell fibrosis score from baseline was significant ($P = 0.004$). According to New Inuyama classification, grading of necrotic/inflammatory findings improved for 64% (20/31) of patients in the 0.1 mg group and 74% (23/31) of patients in the 0.5 mg group, while no patient demonstrated worsening. For both groups, the improvement from baseline was significant ($P < 0.0001$ for both comparisons)

Table 3 Histological improvement at week 48 by Knodell scores and New Inuyama classification, relative to baseline

	0.1 mg entecavir $n = 32$	0.5 mg entecavir $n = 34$
Performed biopsy pairs, *n* (%)	31 (97)	31 (91)
Biopsy pairs evaluable for fibrosis, *n* (%)	29 (91)	30 (88)
Histological improvement (Knodell scores), *n* (%)†	21/29 (72)	24/30 (80)
Knodell HAI scores, reduction from baseline at Week 48, mean ± SE	−3.2 ± 0.5*	−4.6 ± 0.5*
New Inuyama classification, *n* (%)		
Grading (necroinflammation)‡	20/31 (64)*	23/31 (74)*
Staging (fibrosis)		
Improvement	7/29 (24)**	12/30 (40)***
No change	18/29 (62)**	17/30 (57)***
Worsening	4/29 (14)**	1/30 (3)***

Statistical significance relative to baseline. *$P < 0.0001$, Wilcoxin signed-rank test (two patients in the 0.1 mg group and one in the 0.5 mg group are evaluable for necroinflammation but not fibrosis). **$P = 0.432$, Wilcoxin signed-rank test. ***$P = 0.003$, Wilcoxin signed-rank test.

†≥2-point decrease in Knodell necroinflammatory score with no worsening of Knodell fibrosis score.

‡Proportions with improvement.

HAI, Histological Activity Index; SE, standard error.

Table 4 Summary of safety

	No. of subjects (%)	
	0.1 mg entecavir $n = 32$	0.5 mg entecavir $n = 34$
Any adverse event *n* (%)	32 (100)	34 (100)
Clinical adverse events *n* (%)	31 (97)	34 (100)
Most frequent clinical adverse events,† *n* (%)		
Nasopharyngitis	15 (47)	17 (50)
Headache	9 (28)‡	10 (29)§
Abdominal pain upper	0	7 (21)
Tachycardia	0	5 (15)
Diarrhea	3 (9)	5 (15)
Laboratory adverse events *n* (%)	30 (94)	28 (82)
Grade 3–4 clinical adverse events *n* (%)	2 (6)	2 (6)
Grade 3–4 laboratory adverse events *n* (%)	5 (16)	6 (18)
Serious adverse events *n* (%)	3 (9.4)	3 (8.8)
Discontinuations due to adverse events *n* (%)	0	0¶
Death *n* (%)	0	0
ALT flare *n* (%)	2 (6)	2 (6)

†Occurring in at least 15% of patients of either treatment group.

‡Five cases related to study drug.

§Eight cases related to study drug.

¶One patient temporarily discontinued, but treatment resumed after 5 days when adverse event was judged unrelated to study drug.

ALT, alanine aminotransferase.

(Table 3). According to the New Inuyama fibrosis staging system, improvement in fibrosis occurred in 24% (7/29) of patients in the 0.1 mg group (P = NS) and in 40% (12/30) of patients in the 0.5 mg group ($P = 0.003$, Table 3).

Resistance analysis

During the treatment period, two patients who received 0.5 mg entecavir experienced virological breakthrough at week 36. Both patients achieved undetectable HBV DNA. The first patient, with a baseline HBV DNA of 8.4 $\log_{10}$ copies/mL, experienced an increase in HBV DNA to 3.6 $\log_{10}$ copies/mL which was maintained at 48 weeks. The second patient, with a baseline HBV DNA of 8.5 $\log_{10}$ copies/mL, experienced an increase in HBV DNA to 4.5 $\log_{10}$ copies/mL and a subsequent decrease to 3.1 $\log_{10}$ copies/mL at week 48. Neither of the patients experienced ALT flares or other clinically relevant events. Genotypic analysis of HBV DNA polymerase was performed on samples from these two patients. Neither L180M nor M204V/I/S (which are associated with lamivudine resistance)[17,26,27] nor amino acid substitutions associated with entecavir resistance (at positions T184, S202 and M250) were detected.[28] Genotypic analysis of virus from all patients was carried out at baseline using methods with a sensitivity cut-off of 25%. Neither rtL180M nor rtM204V/I/S was detected in any patient. At week 48, the amino acid substitution rtM204I (associated with lamivudine resistance) was detected in two patients, one in the 0.1 mg group and one in the 0.5 mg group. However, entecavir was efficacious in the presence of the rtM204I variants and neither patient demonstrated virological breakthrough (HBV DNA decreased by 3.1 $\log_{10}$ copies/mL in the patient in the 0.1 mg group, and reduced to <2.6 $\log_{10}$ copies/mL in one patient in the 0.5 mg group at week 48) or elevation of ALT.

Safety

All 66 patients treated with the study drug completed 52 weeks of dosing. Adverse events were reported for all patients, but most were transient and mild or moderate (grade 1–2) in severity (Table 4). Adverse events were not considered to be related to the study drug, except for a number of cases of headache (five in the 0.1 mg arm and eight in the 0.5 mg arm), all of which were grade 1 or 2. Grade 3–4 clinical adverse events were observed in two patients (6%) in each treatment group, none of which was related to the study drug (one case of enteritis and one of spondylolisthesis in the 0.1 mg arm; one case of enteritis and one of intervertebral disc herniation in the 0.5 mg arm). Grade 3–4 laboratory adverse events occurred in five (16%) and six (18%) of patients in the 0.1 mg and 0.5 mg groups, respectively (two cases of ALT and AST elevations, two of lipase elevations and one of blood glucose elevation in the 0.1 mg arm; two cases of ALT and AST elevations, one of ALT elevation, one of lipase elevation, one of blood glucose elevation and one of amylase elevation in the 0.5 mg arm). There were no deaths in the study.

Serious adverse events occurred in three (9%) of patients in each treatment group (one case of infectious enterocolitis, one of acquired spondylolisthesis and one of ALT elevation in the 0.1 mg arm; one case of duodenal ulcer hemorrhage, one of ligament damage and one of intervertebral disc protrusion in the 0.5 mg arm). No serious adverse event was judged by the investigator

to be related to study medication. The protocol therapy was temporarily discontinued for one patient who developed duodenal ulcer hemorrhage, but therapy was restarted after 5 days when causal relationship with the test medication was ruled out. Except for this temporary interruption, no patient discontinued therapy for adverse events.

Alanine aminotransferase flares (defined as ALT of >2 times baseline and of >10 × ULN: grade 4) occurred in two patients (6%) in the 0.1 mg entecavir group and two patients (6%) in the 0.5 mg entecavir group. All ALT flares were transient, resolved on treatment, and were associated with a ≥2 $\log_{10}$ copies/mL reduction in HBV DNA. No ALT flare was associated with signs or symptoms of hepatic decompensation.

Discussion

The current study demonstrates that entecavir dosing for 52 weeks in Japanese patients was highly effective in reducing HBV DNA and normalizing ALT. The primary objective of this study was to demonstrate that entecavir has antiviral activity as indicated by the proportion of subjects who achieve a reduction from baseline in HBV DNA by ≥2 $\log_{10}$ copies/mL or to <400 copies/mL at week 48. All patients in both treatment groups achieved this primary efficacy endpoint, underscoring entecavir's potent anti-HBV efficacy. In both treatment groups, serum HBV DNA declined rapidly through week 4, then declined more slowly through week 24, and continued to decline through week 48 (mean change from baseline of −4.49 ± 0.16 and −4.84 ± 0.14 $\log_{10}$ copies/mL for 0.1 mg and 0.5 mg entecavir, respectively). This profile confirms the typical multiphasic pattern of antiviral action against HBV, similar to that observed in an international phase II 24-week trial of entecavir.[20]

Entecavir treatment also resulted in ALT normalization (ALT of <1.25 × ULN: WHO grade 0 toxicity) and HBeAg seroconversion concurrent with the observed declines in HBV DNA. Overall (across both treatment groups), approximately 95% of patients achieved ALT normalization, and approximately 30% of HBeAg-positive patients achieved HBeAg seroconversion. These results are similar to those reported in other international clinical trials of entecavir.[21,22] Specifically, Chang *et al.* reported that 48 weeks of entecavir 0.5 mg daily in nucleoside-naive, HBeAg-positive patients resulted in ALT normalization (ALT of ≤1.0 × ULN) in 68% and HBeAg seroconversion in 21% of these patients.[21] In the same study, 67% of patients achieved undetectable HBV DNA at week 48, which is comparable to the results of the present study. However, the mean change from baseline in HBV DNA in the study by Chang *et al.* (−6.9 $\log_{10}$ copies/mL) was greater than was observed in the current study (−4.84 $\log_{10}$ copies/mL for the entecavir 0.5 mg group). This difference is accounted for by the higher baseline HBV DNA of patients in the international trial (9.6 $\log_{10}$ copies/mL vs 7.7 $\log_{10}$ copies/mL for the 0.5 mg group in the present study).

The ultimate goal of chronic hepatitis B treatment is to arrest or reverse liver disease progression associated with HBV infection. This parameter is most directly and reliably measured by histological evaluation. In the current study, entecavir at doses of both 0.1 mg and 0.5 mg daily resulted in high rates of histological improvement (72% and 80%, respectively) when assessed by the Knodell scoring system. As the New Inuyama classification system is most often used to grade and stage liver disease progression in Japan, we also employed this method of histological evaluation, and obtained results consistent with the results of the Knodell evaluations; that is, there was significant improvement in necrosis/inflammation across both entecavir treatment groups and significant improvement in fibrosis for the 0.5 mg entecavir group. Entecavir's demonstrated histological benefit after 1 year of treatment suggests that its potent viral suppression might also reduce the risk of progression to cirrhosis and end-stage liver disease among chronic hepatitis B patients.

A high barrier to resistance among nucleoside-naive patients has been demonstrated with entecavir.[28,29] The combination of potent viral suppression and the requirement for multiple amino acid substitutions in the HBV reverse transcriptase to confer resistance to entecavir suggests that resistance emergence will be a rare event during long-term administration of entecavir. In phase III international clinical trials, less than 1% of patients treated with entecavir through 2 years experienced a virological breakthrough due to the emergence of entecavir resistance.[29] Phenotypic analyses have demonstrated that entecavir-resistant strains do not emerge in the absence of amino acid substitutions associated with lamivudine resistance (rtL180M and/or rtM204V/I/S).[29] In the present study, no amino acid substitutions at T184, S202 or M250 (all of which can mediate resistance in the presence of rtL180M + rtM204V/I/S) were detected. rtM204I emerged in two patients, one in the 0.1 mg entecavir group and one in the 0.5 mg entecavir group. In both patients, entecavir continued to suppress HBV replication and virological breakthroughs were not observed.

Entecavir was generally well tolerated in the current study. There were no clinically significant differences in the incidence or severity of adverse events between the two treatment groups, indicating that entecavir was well tolerated at a daily dose of 0.5 mg. ALT flares were infrequent, and those flares that did occur were associated with reductions in HBV DNA, and resolved without treatment interruption. These results are consistent with the safety and tolerability profile of entecavir reported in international trials.[20–22]

Entecavir's potent antiviral efficacy, good tolerability and high barrier to resistance offer the potential for long-term treatment of chronic hepatitis B with the objective of halting or reversing liver disease progression. The mean reduction in HBV DNA from baseline at week 48 and histological improvement observed in this trial, together with the results of previously published international trials, support the selection of the 0.5 mg dose of entecavir as an appropriate choice of primary therapy for treatment of nucleoside-naive Japanese patients with chronic hepatitis B infection.

Acknowledgments

This work was carried out with a grant from Bristol-Myers Squibb Company. T. S. is an employee of Bristol-Myers Squibb Company. M. O. serves as an advisor for Bristol-Myers Squibb Company. Thanks go to Namiki Izumi, Etsuro Orito, Takashi Kumada, Mitsuhiko Kawaguchi, Masanori Miyake, Kouzo Fujio, Yasuyuki Araki and Masaharu Ando who were investigators in this study and Chifumi Sato, Kendo Kiyosawa, Kyuichi Tanikawa who participated on the efficacy and safety committee for this study. The work was carried out at: Chiba University Hospital, Chiba; Musashino Red Cross Hospital, Tokyo; Nagoya City University Hospital, Aichi; Ogaki Municipal Hospital, Gifu; Okayama University

Hospital, Okayama; Okayama Saiseikai General Hospital, Okayama; Tsuyama Chuo Hospital, Okayama; Kurashiki Central Hospital, Okayama; Fukuyama City Hospital, Hiroshima; Hiroshima City Hospital, Hiroshima; Kagawa Prefectural Central Hospital, Kagawa; and Mitoyo General Hospital, Kagawa, Japan.

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Scandinavian Journal of Gastroenterology, 2009; 44: 872–877

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ORIGINAL ARTICLE

Quantification of hepatitis C amino acid substitutions 70 and 91 in the core coding region by real-time amplification refractory mutation system reverse transcription-polymerase chain reaction

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Abstract

Objective. The effects of hepatitis C virus (HCV) sequence variations on the success of antiviral therapy or the development of hepatocellular carcinoma (HCC) are complex for many reasons. Recently, there have been several reports on the effects of genotype 1b HCV core amino acid substitutions 70 and/or 91 on the outcome of antiviral therapies and the clinical course. The purpose of this study was to establish real-time amplification refractory mutation system (ARMS) reverse transcription (RT)-polymerase chain reaction (PCR) assays for easy detection of these HCV mutations. ***Material and methods.*** Plasmids p-core-W, including the wild-type HCV core coding region (70R and 91L), and p-core-M, including the mutant-type HCV core (70Q and 91M), were constructed by cloning and PCR-based mutagenesis for control vector of the wild-type core and that of the mutant core, respectively. Using serially diluted forms of these vectors, SyBr Green-based real-time ARMS RT-PCR detection with each of the specific primer pairs was performed. ***Results.*** Each primer could clearly distinguish the difference between p-core-W and p-core-M at the same copy numbers. Concerning substitution 70, the ratios 100:1, 10:1, 1:1, 1:10, and 1:100 of p-core-W versus p-core-M could be distinguished, while for substitution 91, the ratios 100:1, 10:1, 1:1, 1:10, 1:100, and 1:1000 could be distinguished, confirming the sensitivity and specificity of the assay. ***Conclusions.*** This method could be a useful alternative for the detection of genotype 1b HCV core amino acid substitutions 70 and 91 and be reliably applied for rapid screening.

Key Words: *ARMS, core, HCV, interferon response, real-time PCR*

Introduction

More than 170 million people world-wide are chronically infected with hepatitis C virus (HCV), which can lead to hepatic cirrhosis and hepatocellular carcinoma (HCC) [1]. Treatment with peginterferon and ribavirin for 24–48 weeks can result in a sustained loss of serum HCV-RNA (termed a sustained virological response (SVR)), with resolution of chronic hepatitis in approximately half of the patients [2]. Several new, potent HCV protease and polymerase inhibitors have been described recently, but none of them are available for therapeutic use.

The genomic region encoding the HCV core protein is located between amino acids 1 and 191 and is likely to be the first gene product synthesized due to its localization at the 5′ end of the HCV polyprotein transcript [3]. The core protein has an ability to interact with the viral genomic region to form nucleocapsids [4], and the presence of a putative DNA-binding motif, nuclear localization signals, phosphorylation sites, and a nucleocytoplasmic localization of the core protein suggest its possible function as a gene regulatory protein [3,5]. In many previous studies it has been suggested that the HCV core protein may be important in hepatocarcinogenesis and interferon signaling [3,6–8].

HCV genotype 1b is a major genotype (~70%) in Japan. HCV genotype 1 is one of the most refractory to interferon treatment with or without ribavirin. It has been reported that its response to interferon

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(Received 5 February 2009; accepted 29 March 2009)

ISSN 0036-5521 print/ISSN 1502-7708 online
DOI: 10.1080/00365520902937362

monotherapy is affected by HCV NS5A gene diversity [9]. Thus, sequence diversity may predict the response to the combination therapy of peginterferon and ribavirin. Furthermore, ribavirin has different antiviral effects from those of interferon [10]. An approach to the prediction of treatment against hepatitis C in patients who do not have SVR is urgently needed. Several reports suggest that HCV amino acid substitutions 70 and 91 in the core coding region affect the results of combination therapies of interferon and ribavirin [11–13], but most of these studies were retrospective, and we do not know whether these substitutions already existed before treatment or were selected by the treatment. A sensitive, real-time polymerase chain reaction (PCR)-based assay for the detection of these mutations in the presence of high levels of wild-type virus is described here. The method is based on the amplification refractory mutation system (ARMS) reverse transcription (RT)-PCR for detection of single base mutations [14,15].

Material and methods

Plasmid DNA controls

Plasmids carrying HCV genotype 1 b core wild-type and mutant clones were made as described previously [16,17] and are summarized in Table I. Plasmid DNA was purified using the QIAprep spin miniprep kit (Qiagen, Hilden, Germany). Plasmids were serially diluted 1:10 in EASY dilution (for real-time PCR) (Takara, Ohtsu, Shiga, Japan) to give a dilution range of 1–1 $\times 10^9$ copies for controls of real-time PCR.

Extraction of HCV-RNA from serum

Serum samples (100 µl) were extracted using the high pure viral RNA kit (Roche Diagnostics, Indianapolis, Ind., USA) according to the manufacturer's protocol. The RNA was eluted in RNase-free water. Written informed consent was obtained from each patient included in this study.

cDNA synthesis and SyBr Green real-time PCR

Reverse transcription was carried out using random hexamers to make HCV cDNA by superscript cDNA synthesis kit (Invitrogen, Carlsbad, Calif., USA). ARMS primers were designed so that the 3′ base matched either the wild-type or mutant sequence [18] (Table II). Each 25-µl reaction contained 2× Power SYBR Green PCR Master Mix (Applied Biosystems, Tokyo, Japan), 2.5 pmol of each primer (Table II). Reactions were run on the Step One real-time PCR system (Applied Biosystems). Cycling conditions were: denaturation at 95°C for 10 min, then 40 cycles at 95°C for 15 s and 60°C for 1 min, followed by a melting curve analysis, confirming their specificity. A plasmid DNA standard was included in each run.

Cloning of clinical HCV sequences and site-directed mutagenesis

To make the plasmid p-core-mutant, PCR products were cloned into pCR-TOPO2.1 vector (Invitrogen). To make the plasmid p-core-wild, PCR-based *in vitro* site-directed mutagenesis was performed using the Quick Change site-directed mutagenesis kit (Stratagene, La Jolla, Calif., USA). DNA sequences of clones were confirmed by direct sequencing.

Results

Optimization of real-time PCR

For this study, real-time PCR using the SYBR Green I detection system (Applied Biosystems) was implemented to detect the HCV amplicon. ARMS PCR specificity is conferred by direct placement of the 3′ end of one of the primers (Figure 1). Cross-reactivity was tested to ensure that the primer sets specifically bound their targets.

When 10^8 copies of the CAA (codon c70) template were amplified using the primer with a base mismatch, approximately 15 cycles were required before the crossing threshold was reached. This compares with 8 cycles for the matching primer. On the other hand, when 10^8 copies of the CGA (codon c70) template were amplified using the primer with a base mismatch, approximately 16 cycles were required before the crossing threshold was reached. This compares with 6 cycles for the matching primer (Figure 1A and B).

When 10^8 copies of the ATG (codon c91) template were amplified using the primer with a base mismatch, approximately 25 cycles were required before the crossing threshold was reached. This compares

Table I. Plasmid DNA used as standard in this study.

Plasmid	Amino acid c70	Codon c70	Amino acid c91	Codon c91
p-core-wild	Arginine	CGA	Leucine	CTG
p-core-mutant	Glutamine	CAA	Methionine	ATG