

Table 1 Patients with breakthrough hepatitis induced by lamivudine-resistant hepatitis B virus (HBV) mutants who were treated with two doses of entecavir during the initial 52 weeks

	Total (n = 19)	Initial daily dose of entecavir	
		0.5 mg (n = 10)	1.0 mg (n = 9)
Duration of entecavir (weeks)	120–144	120–144	124–140
Age (years)	38 (29–65)	37 (29–65)	39 (30–49)
Men	17 (89%)	9 (90%)	8 (89%)
ALT (IU/L)	119 (46–1708)	111 (46–1708)	275 (49–442)
Platelets ($\times 10^3/\text{mm}^3$)	190 (93–265)	180 (93–235)	190 (108–265)
HBeAg	14 (74%)	7 (70%)	7 (78%)
Genotypes (A : B : C)	1:0:18	1:0:9	0:0:9
HBV-DNA (LGE/mL)	7.2 (5.2–8.6)	7.2 (5.2–8.6)	6.6 (5.7–8.2)
YMDD mutants (I : V : I/V)	11:3:5	6:2:2	5:1:3

Median values are shown with the range in parentheses, and the ratio of HBV genotypes, as well as YIDD, YVDD and both YMDD mutants, is indicated. ALT, alanine aminotransferase; HBeAg, hepatitis e antigen; LGE, log geometric equivalents.

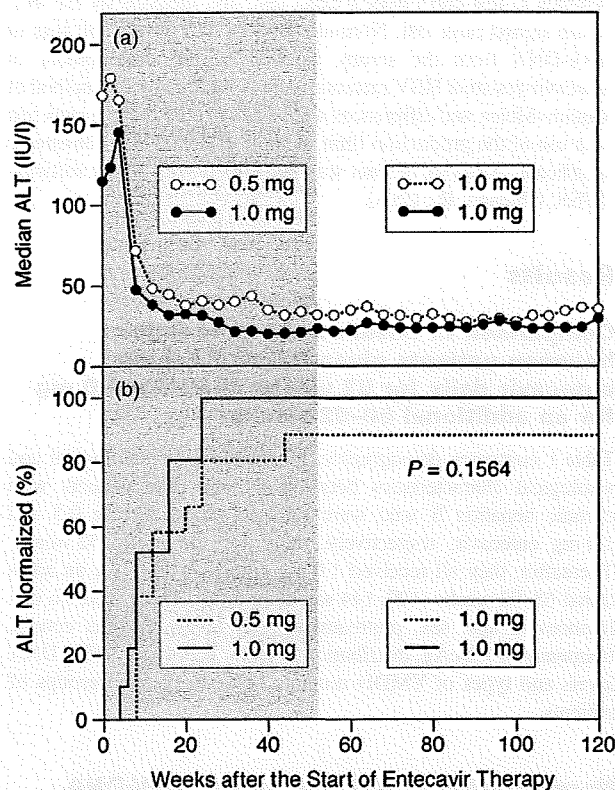


Figure 1 Alanine aminotransferase (ALT) levels in the 19 patients with breakthrough hepatitis induced by lamivudine-resistant hepatitis B virus mutants who received entecavir for 120 weeks. Of them, 10 patients received 0.5 mg and the remaining nine 1.0 mg entecavir daily during the initial 52 weeks (shaded), and thereafter both groups received 1.0 mg entecavir daily. The mean ALT levels (a) and the normalization of serum ALT (≤ 50 IU/L) (b) are illustrated.

normal limit among patients in both groups. Collectively in the 19 patients, the ALT level was normalized in 47% at week 12 and in 83% at week 24. Figure 1(b) compares the normalization of ALT levels between patients who received 0.5 and 1.0 mg entecavir daily during the initial 52 weeks. There were no statistical differences in the normalization of ALT levels between patients given 0.5 and 1.0 mg entecavir. Of the 14 patients positive for HBeAg at the start of entecavir, two (14%) lost HBeAg and seroconverted to anti-HBe, while HBsAg was not cleared from the serum in any of the 19 patients.

The loss of HBV-DNA from serum was compared between patients given 0.5 and 1.0 mg entecavir daily during the initial 52 weeks. A sharp decrease in HBV-DNA by more than 2 logs was achieved at 4 weeks in patients given the initial 0.5 mg entecavir daily, and at 8 weeks in those receiving the initial 1.0 mg entecavir daily. Twenty-four weeks after the start, HBV-DNA levels stabilized and stayed approximately 1 log lower in the patients with the initial 0.5 than 1.0 mg entecavir daily. The loss of HBV-DNA detectable by the quantitative method varied in patients with two different initial entecavir doses. At 24 weeks after the start of entecavir therapy, HBV-DNA became undetectable in 20% and 11%, respectively, of the patients with the initial 0.5 and 1.0 mg entecavir daily; the loss increased to 50% and 33% at 120 weeks, respectively. However, there were no significant differences in the loss of HBV-DNA between the patients receiving 0.5 and 1.0 mg entecavir daily during the initial 25 weeks.

Improvement of liver histology in the patients who were switched to entecavir after the development of breakthrough hepatitis during long-term lamivudine treatment

Of the 19 patients switched to receive entecavir, 12 (63%) underwent serial liver biopsies at the baseline and annually for 3 years while they were treated with entecavir. METAVIR scores for fibrosis stages at the start of entecavir were: F1 in six (50%) patients; F2 in three (25%); and F3 in three (25%). Activity grades were: A1 in six (50%) patients and A2 in six (50%). After they had received entecavir for 1 year, the fibrosis stage improved in two (17%), was

Table 2 Improvement in histology activity scores after entecavir during 3 years in the 12 patients who had developed breakthrough hepatitis induced by lamivudine-resistant HBV mutants

Features		Before	After	Decrement	Differences (<i>P</i> -value)
Periportal and/or bridging necrosis	Median (range)	1 (0–3)	0 (0–1)	1 (0–3)	0.003
	Mean \pm SD	1.2 \pm 0.9	0.1 \pm 0.3	1.1 \pm 0.8	
Lobular degeneration and focal necrosis	Median (range)	2 (0–3)	1 (0–1)	1 (0–2)	0.014
	Mean \pm SD	2.0 \pm 1.0	0.9 \pm 0.3	1.0 \pm 1.0	
Portal inflammation	Median (range)	1 (0–3)	1 (0–1)	1 (0–2)	0.015
	Mean \pm SD	1.8 \pm 1.0	0.8 \pm 0.4	0.9 \pm 0.9	
Fibrosis	Median (range)	2 (1–3)	1 (1–3)	0 (0–2)	0.059
	Mean \pm SD	2.0 \pm 1.0	1.4 \pm 0.8	0.5 \pm 1.1	
Total HAI score	Median (range)	6 (3–12)	3 (2–5)	3 (1–8)	0.002
	Mean \pm SD	7.0 \pm 2.7	3.2 \pm 0.9	3.5 \pm 2.4	

HAI, histology activity index; SD, standard deviation.

unchanged in nine (75%), and worsened in the remaining one (8%). The activity grade improved in nine (75%) patients and was unchanged in the remaining three (25%); it did not worsen in any patient.

One of the 12 patients could not receive liver biopsy 3 years after the start of therapy, because entecavir-resistant HBV mutants developed. Table 2 summarizes changes in HAI scores in the 11 patients who had received long-term entecavir treatment. After 3 years on entecavir therapy, improvement in HAI scores by 2 points or greater was achieved in nine (82%) of the 11 patients. Significant improvement was gained in the total HAI score, as well as scores for periportal and/or bridging necrosis, lobular degeneration/focal necrosis, and portal inflammation. Fibrosis score did not improve significantly ($P = 0.059$); it increased in two patients.

Clinical and virological courses of the representative patient are illustrated in Figure 2 and histological findings in yearly biopsies in Figure 3. The patient developed resistance to lamivudine and was switched to IFN. Hepatitis was exacerbated in him, however, and he was started on lamivudine again. IFN was given intermittently to him when ALT levels were elevated. Because he did not respond to IFN, entecavir was given to him. At that time, he had a HBV-DNA level of more than 7.6 LGE/mL and an HAI score of 8 in the liver biopsy. Soon after entecavir was started, HBV-DNA levels decreased sharply along with the normalization of ALT levels. He seroconverted from HBeAg to anti-HBe 1 year after the start of entecavir treatment. Histological improvement, increasing in parallel with the duration of entecavir treatment, was demonstrated by yearly liver biopsies in comparison with the baseline findings (Fig. 3). Necroinflammatory signs decreased remarkably along with narrowed portal areas, although the stage of fibrosis did not improve appreciably.

HBV mutants resistant to entecavir

Figure 4 illustrates the development of entecavir-resistant HBV mutants that increased in parallel with the duration of treatment. Entecavir-resistant HBV mutants developed in three of the 10 (30%) patients by 18, 84 and 120 weeks; and two of the nine (22%) patients by 132 and 148 weeks, respectively, who received 0.5 and 1.0 mg entecavir daily during the first year; thereafter, they all were given 1.0 mg entecavir daily for the next 68–92 weeks.

During the initial 130 weeks (~2.5 years), therefore, entecavir-resistant HBV mutants developed in three of the 10 (30%) patients with the initial entecavir dose of 0.5 mg daily, in remarkable contrast to no emergence of such mutants in any of the nine patients that received 1.0 mg daily.

Alanine aminotransferase levels were elevated in only two of the five (40%) patients infected with entecavir-resistant HBV mutants, however. These two patients were switched to receive adefovir in combination with lamivudine, and breakthrough hepatitis resolved in them both. All the five patients who developed entecavir-resistant HBV mutants had been infected with lamivudine-resistant YMDD mutants with M204V in the presence or absence of M204I. In outstanding contrast, entecavir-resistant HBV mutants did not develop in any of the 11 patients who had been infected with YMDD mutants with M204I alone.

No adverse effects developed in any of the 19 patients. Breakthrough hepatitis occurred in only one of the five (20%) patients in whom entecavir-resistant mutants emerged.

Discussion

We have previously reported in the *Journal* that entecavir suppresses serum HBV-DNA to undetectable levels and normalizes ALT levels in more than 30%, respectively, in lamivudine-resistance patients with chronic hepatitis B at 52 weeks.¹² In the present report, we have followed 19 patients from one of the participating centers for 3 years so as to establish longer-term histological efficacy and rates of viral resistance with entecavir treatment of lamivudine-resistant chronic hepatitis B.

As in the earlier report,¹² among the 19 patients described here, ALT levels were normalized in more than 90% of them 8–12 weeks after the start of entecavir until the end of treatment. Although the median HBV-DNA level dropped by 3 logs and remained low during the entecavir therapy, they became undetectable in only 20–40% of the 19 patients. In a previous report, also, the loss of detectable HBV-DNA from the serum was achieved in only 27 of the 141 (19%) patients with lamivudine-resistant HBV mutants after they had received 1.0 mg entecavir daily for 52 weeks.¹⁴ In a remarkable contrast, entecavir is much more efficient in treatment-naïve patients who had received it for 1–2 years; HBV-DNA disappeared from the serum in 67–90% of them.^{10,11,18} These differences could be attributed to some lamivudine-resistant

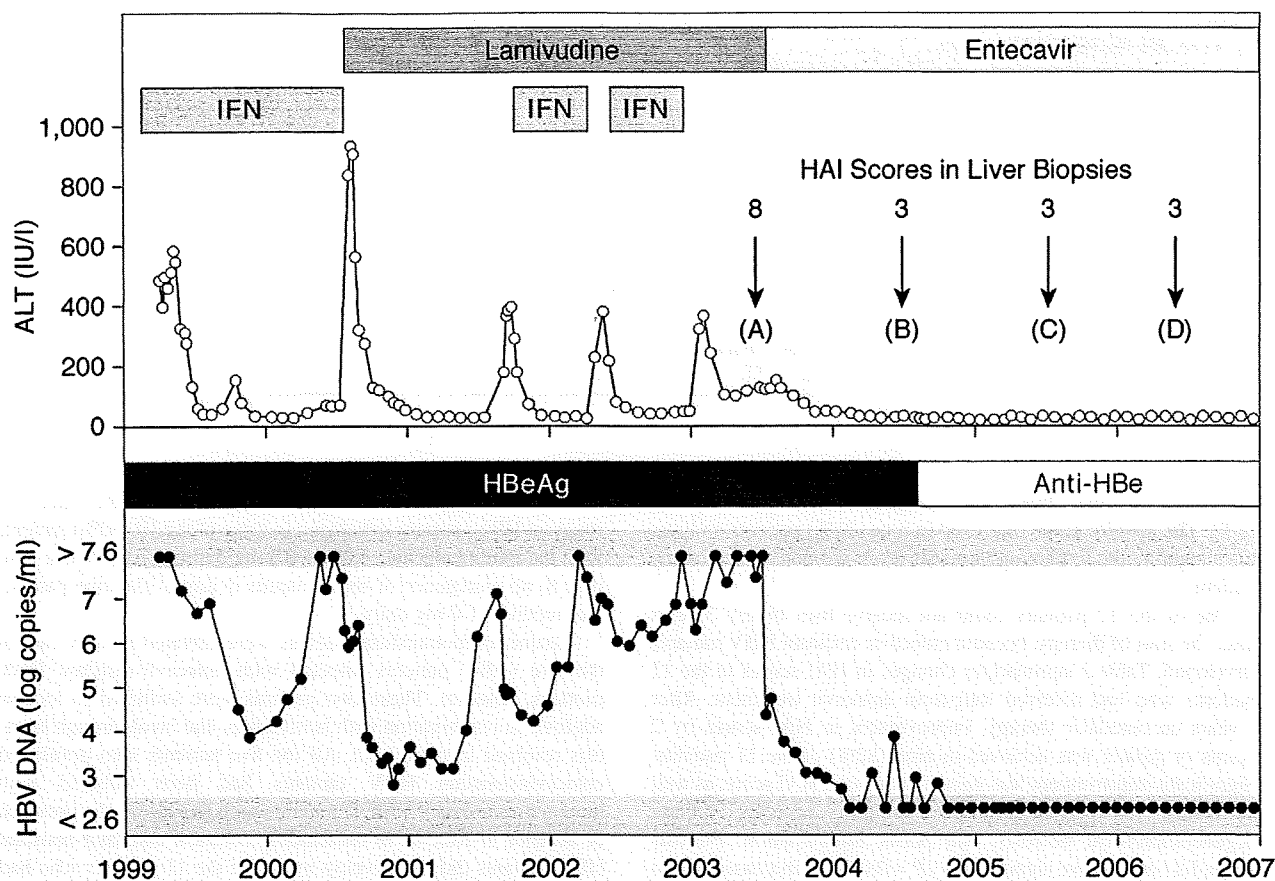


Figure 2 Clinical course of the representative patient. Fluctuating levels of alanine aminotransferase (ALT) and hepatitis B virus (HBV)-DNA are illustrated. Antiviral treatments as well as duration of hepatitis B e antigen (HBeAg) and anti-HBe are indicated by horizontal bars. Also given are time points when four liver biopsies were undertaken, along with histological activity index scores on the top. IFN, interferon.

HBV mutants contributing to the development of entecavir-resistance.^{13,18}

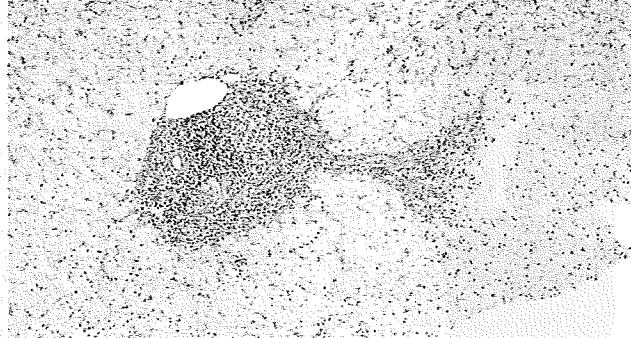
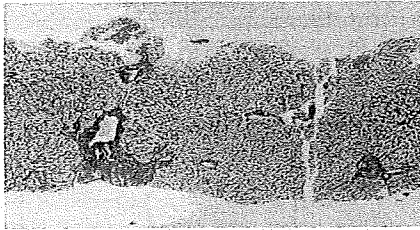
Entecavir is a cyclopentyl guanosine analog and can inhibit the polymerase of hepadnaviridae selectively by interfering with priming and reverse transcription, as well as synthesis of minus- and plus-stranded HBV-DNA species.¹⁹ In an *in vitro* expression system with HepG2 cells, entecavir exhibited an antiviral activity with EC_{50} of 0.00375 μ M, which is 1500-fold higher than 10 μ M of lamivudine.²⁰ Dose-dependent pharmacological activity of entecavir was evident in a randomized double-blind trial.²¹ Although 0.01 mg entecavir daily decreased HBV-DNA by 2.41 logs at 22 weeks, the antiviral activity was significantly lower than 4.31 and 4.72 logs, respectively, of 0.1 and 0.5 mg daily; they were both higher than 3.36 logs by 100 mg lamivudine daily, however. Accordingly, normalization of ALT was more frequent by treatments with 0.1 and 0.5 mg entecavir daily (69% and 83%, respectively) than with 100 mg lamivudine daily (59%).

Significant decrease in HAI scores has been reported in patients with chronic hepatitis B who had received lamivudine for 1–3 years.^{22,23} Furthermore, decreases in hepatic inflammation may improve the fibrosis stage. Entecavir therapy for 52 weeks has achieved histological improvement in 55–72% of patients in phase

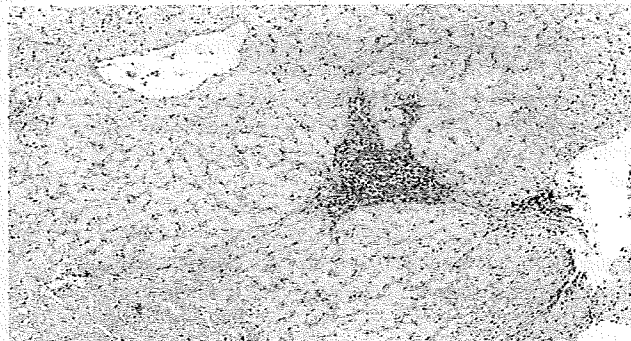
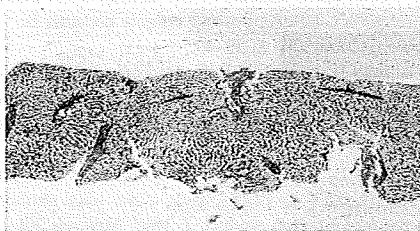
III clinical trials.^{10,11,14} In corroboration of these results, fibrosis stage and inflammation grade improved in the present series of patients who had received entecavir for 3 years, with a significant decrease in the HAI score (Table 2). Histological improvement would have been gained by long-term entecavir therapy, and it may further increase, should entecavir be continued further.

Long-term entecavir treatment, however, may be hampered by the development of drug-resistant mutants. Although entecavir-resistant HBV mutants rarely occur in treatment-naive patients,¹⁸ they can emerge rather frequently in the patients infected with lamivudine-resistant HBV mutants.^{14,24} In the present study, entecavir-resistant HBV mutants developed in five of the 19 (26%) lamivudine-resistant patients during 144 weeks of treatment. The incidence was comparable with 32% in the lamivudine-resistant patients who had received entecavir for 3 years.²⁴ Only two (40%) of them developed hepatitis flares and they were switched to receive adefovir in combination with lamivudine. Entecavir-resistant HBV mutants emerging in patients with lamivudine-resistant mutants are reported to be replication-impaired and rarely induce breakthrough hepatitis.²⁵ It should be found out how entecavir-resistant HBV mutants can be managed with antiviral nucleos(t)ide analogs other than lamivudine and entecavir, or

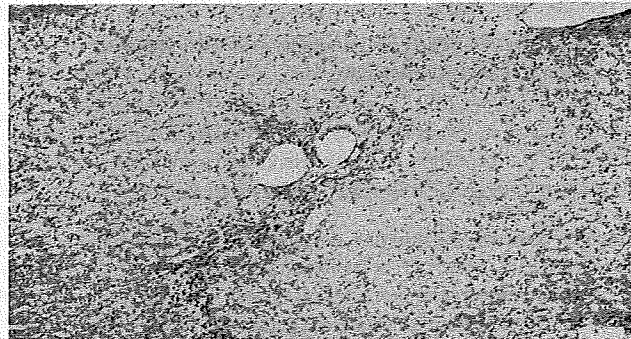
(a) Before the start of entecavir



(b) One year after the start of entecavir



(c) Two years after the start of entecavir



(D) Three years after the start of entecavir

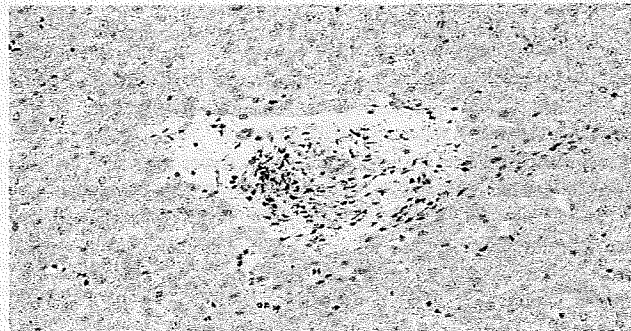
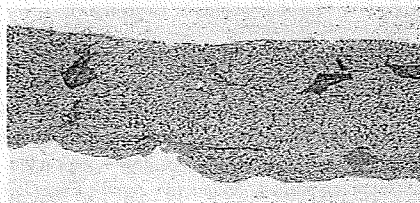


Figure 3 Histological changes in the representative patient during 3-year entecavir treatment (Fig. 2). With hematoxylin–eosin stain on the left, marked enlargement of portal areas is evident along with infiltration of mononuclear cells before the switch from lamivudine to entecavir (a). They decreased increasingly during the 3-year treatment with entecavir (b–d). Stage of fibrosis did not change appreciably by the staining for silver on the right.

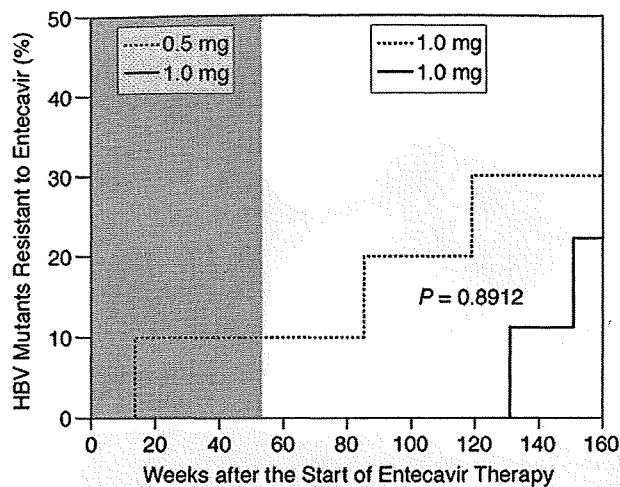


Figure 4 Development of entecavir-resistant hepatitis B virus (HBV) mutants during the 3-year treatment. The 10 patients with the initial entecavir dose of 0.5 mg daily and the nine with that of 1.0 mg daily are compared.

combination thereof. It has been proposed that adefovir add-on lamivudine is efficacious with negligible drug resistance over 3 years.^{26,27}

Acknowledgment

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Predictive values of amino acid sequences of the core and NS5A regions in antiviral therapy for hepatitis C: a Japanese multi-center study

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Abstract

Background Chronic hepatitis C (CHC) genotype 1b patients with high viral load are resistant to peginterferon (PEG-IFN) and ribavirin (RBV) combination therapy, especially older and female patients.

Methods To elucidate the factors affecting early and sustained viral responses (EVR and SVR), 409 genotype 1b patients CHC with high viral loads who had received 48 weeks of PEG-IFN/RBV therapy were enrolled. The amino acid (aa) sequences of the HCV core at positions 70 and 91 and of the interferon sensitivity determining region (ISDR) were analyzed. Host factors, viral factors, and

treatment-related factors were subjected to multivariate analysis.

Results Male gender, low HCV RNA load, high platelet count, two or more aa mutations of ISDR, and wild type of core aa 70 were independent predictive factors for SVR. In patients with over 80% adherences to both PEG-IFN and RBV, male gender, mild fibrosis stage, and wild type of core aa 70 were independent predictors for SVR.

Conclusions Independent predictive factors for SVR were: no aa substitution at core aa 70, two or more aa mutations in the ISDR, low viral load, high values of platelet count, mild liver fibrosis and male gender.

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Keywords Chronic hepatitis C · Peginterferon and ribavirin · Core amino acid · Interferon sensitivity determining region

Abbreviations

CHC	Chronic hepatitis C
PEG-IFN	Peginterferon
RBV	Ribavirin
RVR	Rapid viral response
cEVR	Complete early viral response
LVR	Late viral response
ETR	End of treatment response
NR	Non response
SVR	Sustained viral response
ISDR	Interferon sensitivity determining region
Aa	Amino acid
ALT	Alanine aminotransferase
PLT	Platelet
HCC	Hepatocellular carcinoma

Introduction

A combination of pegylated interferon (PEG-IFN) and ribavirin (RBV) therapy for 48 weeks achieves a sustained viral response (SVR) rate of 40–50% in chronic hepatitis C (CHC) patients with a high viral load of genotype 1 [1–4]. The dose-reduction rate and the frequency of discontinuation of this treatment are high in aged patients [5]. The SVR rate of the therapy is lower in females than males, especially in older patients in Japan [6].

Around 30% of HCV carriers have serum alanine aminotransferase (ALT) levels within the upper limit of normal ranges [7, 8] and HCV carriers with persistently normal serum ALT (PNALT) and serum platelet (PLT) counts of over $15 \times 10^4/\text{mm}^3$ show low grade hepatic fibrosis and good prognosis [9]. Before treating HCV carriers, it is very important to predict non-response to PEG-IFN plus RBV therapy because of its medical cost, adverse effects, and its impact on the long term quality of life.

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There are many factors affecting response to IFN monotherapy and PEG-IFN/RBV therapy, including body mass index (BMI) [10, 11], steatosis [12, 13], insulin resistance [14], stage of liver fibrosis [15, 16], total cholesterol (T. Chol), triglyceride (TG), adherence to both PEG-IFN and RBV [17], race [18, 19], age [1, 2, 20], and viral factors including serum quantity of HCV RNA, HCV genotype and substitution of amino acids (aa) in the interferon sensitivity determining region (ISDR, 2209–2248) of the nonstructural protein 5A (NS5A) [21] and in the core protein [22, 23]. Early viral response is an important predictive factor in PEG-IFN/RBV therapy for CHC patients with genotype 1 and high viral loads [24–27].

The aim of this study was to elucidate the valuable predictive factors of SVR in Japanese patients with HCV genotype 1b high viral loads following 48 weeks of PEG-IFN/RBV therapy, focusing on the relationship between aa substitutions in the ISDR and at core aa 70 and 91 and early viral kinetics.

Patients and methods

Selection of patients

This retrospective study was conducted at 15 clinical sites in Japan which are part of the Study Group of Optimal Treatment of Viral Hepatitis supported by the Ministry of Health, Labor and Welfare, Japan. Eligible subjects were CHC patients, who (1) had received liver biopsy; (2) were genotype 1b with high viral load (≥ 100 KIU/ml by Cobas Amplicor Hepatitis C Virus Test, version 2.0) at the start of PEG-IFN/RBV therapy; (3) received weekly injections of PEG-IFN- α -2b (PEG-INTRON; Shering-Plough, Kenilworth, NJ) of 1.5 $\mu\text{g}/\text{kg}$ bw and oral administration of RBV (Rebetol; Shering-Plough) for 48 weeks. The amount of RBV was adjusted based on the subject's body weight; (600 mg for ≤ 60 kg bw, 800 mg for 60–80 kg bw, 1,000 mg for > 80 kg bw); (4) were examined serially for quantitative and qualitative HCV RNA; and (5) the aa sequences at positions 70 and 91 in the core region and of the ISDR in the NS5A had been determined in pretreatment sera.

Hepatitis B virus (HBV) infection, human immunodeficiency virus (HIV) infection, autoimmune hepatitis, primary biliary cirrhosis, hemochromatosis, and Wilson's disease were excluded. Histopathological diagnosis was based on the scoring system of Desmet et al. [28]. The definition of alcohol abuse included patients having a history of more than 100 kg of total ethanol intake. Complete blood counts, liver function tests, serum lipids, serum ferritin, serum fibrosis markers, fasting plasma glucose (FPG), and immune reactive insulin (IRI) were examined in most cases. Written informed consent was obtained from all

patients before treatment, and the protocol was approved by the ethics committees in each site.

Study design

Four hundred and nine patients who completed 48 weeks of treatment and were followed for more than 24 weeks after treatment were enrolled in the first study (*Study design 1*).

To elucidate the effect of aa substitution of HCV core and in the ISDR on HCV dynamics, including a rapid viral response (RVR), complete early viral response (cEVR), a late viral response (LVR) and SVR, according to gender and age (<60 years \geq 60 years), 201 of the 409 patients maintaining over 80% adherences to both PEG-IFN and RBV were enrolled in the second study (*Study design 2*).

Nucleotide sequencing of the core and NS5A gene

The nucleotide sequences encoding aa 1–191 (HCV core) and aa 2209–2248 (ISDR) were analyzed by direct sequencing as described by Akuta et al. [22, 27] and Enomoto et al. [21]. In brief, RNA was extracted from the sera and converted to cDNA and two nested rounds of polymerase chain reaction (PCR) were performed. Primers used in the PCR were as follows; (a) Nucleotide sequences of the core region: the first-round PCR was performed with CC11 (sense) and e14 (antisense) primers [22, 27], and the second-round PCR with CC9 (sense) and e14 (antisense) primers [22, 27]. (b) Nucleotide sequences of the ISDR in NS5A: the first-round PCR was performed with ISDR1 (sense) and ISDR2 (antisense) primers [21], and the second-round PCR with ISDR3 (sense) and ISDR4 (antisense) primers [21]. These sequences were compared with the consensus sequence of genotype 1b (HCV-J) [29]. Wild types virus encoded arginine and leucine at aa 70 and 91, respectively, and the aa substitutions were glutamine or histidine at aa 70 and methionine at aa 91.

Viral kinetic study

Serum HCV RNA levels were measured by PCR (Amplicor HCV RNA kit, version 2.0, Roche Diagnostics) using samples taken before treatment and at 4, 12, 24, and 48 weeks after the therapy. SVR was defined as HCV RNA negativity by qualitative analysis by PCR at 24 weeks after the treatment. RVR was defined as HCV RNA negativity at 4 weeks, cEVR as HCV RNA negativity at 12 weeks, LVR as HCV RNA negativity during 13–24 weeks and an end of treatment response (ETR) as HCV RNA negativity at the end of treatment. Patients who remained positive for HCV RNA at the end of the treatment and at 24 weeks after the therapy were defined as non-responders (NR).

Adherences to PEG-IFN and RBV

Adherences to PEG-IFN and RBV were assessed by separately calculating the actual doses of PEG-IFN and RBV received as percentages of the intended dosages. Adherences to PEG-IFN and RBV were divided into two groups; $80\% \leq$ and $<80\%$.

Statistical analysis

All data analyses were conducted using the SAS version 9.1.3 statistical analysis packages (SAS Institute, Cary, NC, USA). Individual characteristics between groups were evaluated by Mann–Whitney *U* test for numerical variables or Fisher's exact test for categorical variables. Variables exhibiting values of $p < 0.1$ in the univariate analysis were subjected to stepwise multivariate logistic regression analysis. The grade of steatosis and iron deposition in liver tissue, BMI, albumin (Alb), low density lipoprotein-cholesterol (LDL-C), homeostasis model assessment-insulin resistance (HOMA-IR), ferritin, and hyaluronic acid were excluded from multivariate logistic regression analysis because of the absence of those data in more than 10% of the patients. All p values of $p < 0.05$ by the two-tailed test were considered statistically significant.

Results

Study design 1

Baseline backgrounds, characteristics and adherences of peginterferon and ribavirin in males and females

The treatment outcome of PEG-IFN and RBV combination therapy depends on gender in Japanese patients, so in addition to aa substitutions in the ISDR in NS5A [21] or at HCV core 70 and 91 [22, 27], we compared the baseline characteristics according to gender (Table 1). Males were younger and the grade of hepatic inflammation was milder in males. The serum levels of LDL-C, PLT count, and aa substitutions of ISDR and at core 70 and 91 did not differ significantly different between males and females. The frequency of no alcohol abuse was significantly ($p < 0.0001$) higher in females than males (Some of them are not described in Table 1).

The rates of over 80% adherences to PEG-IFN and RBV were significantly lower ($p = 0.0066$, $p < 0.00001$, respectively) in females than males. Only in those above 60 years did the rate of over 80% adherence to PEG-IFN not differ significantly between males and females, but the rate of over 80% adherence to RBV was significantly lower ($p = 0.035$) in females than males (Table 1).

Table 1 Backgrounds and characteristics of male and female patients

Factors	Gender		p value
	Male	Female	
No. of patients	256 (62.6%)	153 (37.4%)	
Age			
Median (range)	53 (18–73)	59 (23–75)	0.00001
F stage			
F0–2	206 (80.5%)	119 (77.8%)	0.592
F3–4	50 (19.5%)	34 (22.2%)	
Grade (A factor)			
A0–1	163 (63.7%)	79 (51.6%)	0.026
A2–3	93 (36.3%)	74 (48.4%)	
HCV RNA load 0 week (KIU/mL)			
Median (range)	1500 (100–5000 <)	1280 (100–5000<)	0.384
ALT 0 week (IU/L)			
Median (range)	74.5 (16–504)	59 (19–391)	0.001
BMI			
Median (range)	23.6 (17.5–31.2)	22.1 (16.1–33.9)	0.00033
Alb (g/dL)			
Median (range)	4.0 (3.0–5.2)	3.8 (3.0–4.8)	0.011
LDL-C (mg/dL)			
Median (range)	97 (30–185)	90 (34–174)	0.612
T-Chol (mg/dL)			
Median (range)	167 (85–273)	176 (114–261)	0.0016
PLT count ($\times 10^4/\text{mm}^3$)			
Median (range)	17.0 (8.0–31.9)	16.4 (8.1–39.9)	0.350
Amino acid mutation of ISDR			
0–1	200 (78.1%)	121 (79.1%)	0.608
2 \leq	56 (21.9%)	32 (20.9%)	
Amino acid substitution of core 70			
Wild	177 (69.1%)	114 (74.5%)	0.261
Mutant	79 (30.9%)	39 (25.5%)	
Amino acid substitution of core 91			
Wild	153 (59.8%)	98 (64.1%)	0.403
Mutant	103 (40.2%)	55 (35.9%)	
PEG-IFN adherence			
<80%	41 (17.7%)	42 (30.4%)	0.0066
80% \leq	190 (82.3%)	96 (69.6%)	
Ribavirin adherence			
<80%	54 (23.6%)	73 (52.1%)	<0.00001
80% \leq	175 (76.4%)	67 (47.9%)	
Age: <60 years			
PEG adherence			
<80%	30 (17.8%)	23 (31.5%)	0.027
80% \leq	139 (82.2%)	50 (68.5%)	
Ribavirin adherence			
<80%	27 (16.2%)	31 (42.5%)	0.000029
80% \leq	140 (83.8%)	42 (57.5%)	
Age: 60 years \leq			
PEG adherence			
<80%	11 (17.7%)	19 (29.2%)	0.147
80% \leq	51 (82.3%)	46 (70.8%)	
Ribavirin adherence			
<80%	27 (43.5%)	42 (62.7%)	0.035
80% \leq	35 (56.5%)	25 (37.3%)	

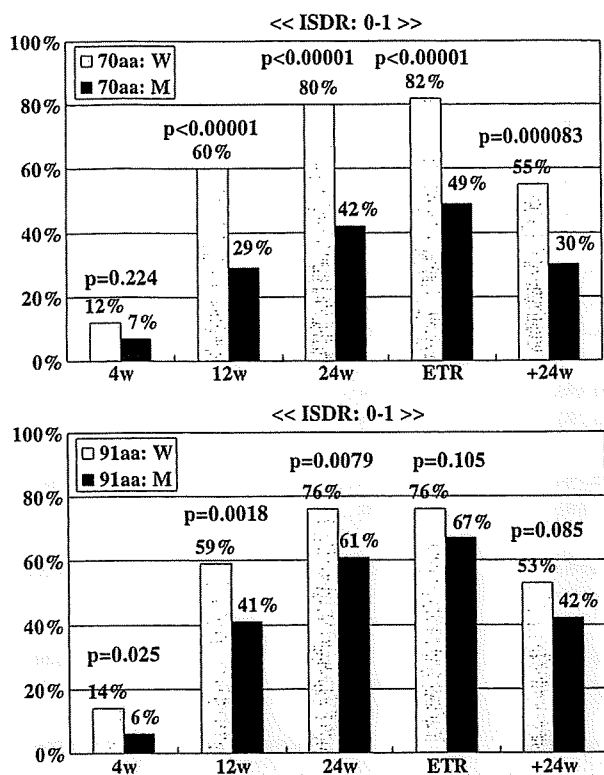


Fig. 1 Relationship between time course of serum HCV RNA negativity and amino acid substitutions in the ISDR and core amino acids 70 and 91. For cases with no or only one amino acid (aa) change in the ISDR, the rates of cEVR, LVR, ETR and SVR were significantly higher in patients with wild type core aa 70 but only the rates of RVR, cEVR, and LVR were significantly higher in patients with wild type core aa 91

Amino acid substitutions

There were no significant differences in the frequency of aa substitutions in the ISDR between males and females. Core aa substitutions at positions 70 and 91 were as follows; 291 (71.1%) were wild type and 118 (28.9%) were mutant at core aa 70, and 251 (61.4%) were wild type and 158 (38.6%) were mutant at core aa 91. There were no significant differences between males and females and between patients below and above 60 years of age.

Virological responses and aa substitutions

The rate of RVR did not differ significantly between males and females. However, more male patients showed HCV RNA negativity at 12 weeks (males vs. females; 60.7 vs. 48.4%, $p = 0.018$), 24 weeks (76.8 vs. 64.2%, $p = 0.0078$) and 48 weeks (78.2 vs. 68.6%, $p = 0.049$), and the proportion of male patients in SVR was significantly higher than that of females (61.3 vs. 37.3%, $p < 0.00001$).

RVR, cEVR and SVR rates were significantly higher in patients with two or more aa mutations in the ISDR compared to patients having no or one aa substitution in that region (20 vs. 11%, $p = 0.044$; 71 vs. 52%, $p = 0.0021$; 66 vs. 49%, $p = 0.0054$, respectively). AA substitution at core position 70 resulted in significantly lower rate of cEVR, LVR, ETR, and SVR (40 vs. 63%, $p = 0.000037$; 51 vs. 81%, $p < 0.00001$; 56 vs. 83%, 41 vs. 57%; $p < 0.00001$, $p = 0.0031$, respectively). Although the patients with the wild type aa at core 91 showed significantly higher rates of RVR and cEVR, the rate of SVR was not significantly higher in those patients ($p = 0.054$).

SVR rates were 30% for patients with no or one aa substitution in the ISDR and the core aa 70 substitution, and were significantly lower compared to those with the wild type aa core 70 (Fig. 1). These findings were not confirmed in patients with no or one aa substitution in the ISDR and the core aa 91 substitution (Fig. 1).

Factors affecting SVR by univariate analysis

Univariate analysis identified nine parameters that influenced non-SVR significantly: female gender, older age, advanced staged liver fibrosis, high viral load, low serum Alb level, low PLT count, no or one aa substitution in the ISDR, aa substitution at core aa 70, and low adherence to RBV (Table 2). The frequency of steatosis and HOMA-IR were significantly ($p = 0.0057$, $p < 0.00001$, respectively) lower in patients with SVR compared with non-SVR (data not shown). However, these factors were not entered in the multivariate analysis because of the absence of the data in many cases.

Factors affecting RVR, cEVR, and SVR by multivariate logistic regression analysis

Multivariate analysis identified four parameters that influenced RVR independently: low HCV RNA load, low serum ALT level, two or more aa mutations in the ISDR and the wild type aa at core position 91 (Table 3).

Concerning cEVR, male gender, mild fibrosis stage, low HCV RNA load, two or more aa mutations in the ISDR, and the wild type aa at core positions 70 and 91 were independent predictors (Table 3).

Concerning SVR, male gender ($p < 0.0001$), low HCV RNA load ($p = 0.013$), high PLT count ($p = 0.0019$), two or more aa mutations in the ISDR ($p = 0.024$), and wild type core aa 70 ($p = 0.0045$) were found to be independent predictors (Table 3).

The predictive values of the combination of gender, PLT count, ISDR and core aa 70 are shown in Fig. 2a. In male patients having PLT of $<15 \times 10^4/\text{mm}^3$, and, no or one aa substitution in the ISDR, the SVR rate was 68% when core 70

Table 2 Univariate analysis to identify the factors of SVR

Factors	Negative of HCV RNA after 24 weeks		<i>p</i> value
	(-)	(+)	
No. of patients	214 (52.3%)	195	
Gender			
Male	157 (61.3%)	99	<0.00001
Female	57 (37.3%)	96	
Age			
Median (range)	52.5 (18–75)	58 (20–74)	<0.00001
<60 years	155 (58.1%)	112	0.0018
60 years≤	59 (41.5%)	83	
Age: <60 years			
Male	118 (63.4%)	68	0.010
Female	37 (45.7%)	44	
Age: 60 years≤			
Male	39 (55.7%)	31	0.0011
Female	20 (27.8%)	52	
F stage			
F0–2	190 (58.5%)	135	0.000013
F3–4	25 (29.8%)	59	
Grade (A factor)			
A0–1	138 (56.8%)	104	0.130
A2–3	81 (48.5%)	86	
HCV RNA load 0 week (KIU/mL)			
Median (range)	1300 (100–5000<)	1700 (130–5000<)	0.016
ALT 0 week (IU/L)			
Median (range)	66 (16–391)	67 (19–504)	0.892
BMI			
Median (range)	23.0 (17.3–32.4)	23.25 (16.1–33.9)	0.714
Alb (g/dL)			
Median (range)	4.0 (3.2–5.2)	3.8 (3.0–4.8)	0.0088
LDL-C (mg/dL)			
Median (range)	94.5 (31–185)	97.5 (30–182)	0.611
T-Chol (mg/dL)			
Median (range)	169.5 (85–257)	170 (103–273)	0.511
PLT count ($\times 10^4/\text{mm}^3$)			
Median (range)	18.2 (8.7–39.9)	15.1 (8.0–31.9)	<0.00001
<15	54 (36.5%)	94	<0.00001
15≤	160 (61.3%)	101	
Amino acid mutation of ISDR			
0–1	156 (48.6%)	165	0.0054
2≤	58 (65.9%)	30	
Amino acid substitution of core 70			
Wild	166 (57.0%)	125	0.0031
Mutant	48 (40.7%)	70	
Amino acid substitution of core 91			
Wild	141 (56.2%)	110	0.054
Mutant	73 (46.2%)	85	
PEG-IFN adherence			
<80%	35 (42.2%)	48	0.063
80%≤	154 (53.8%)	132	
Ribavirin adherence			
<80%	55 (43.3%)	72	0.048
80%≤	132 (54.5%)	110	

Table 3 Multivariate logistic regression analysis to identify independent predictive factors of RVR, cEVR, and SVR

	Odds ratio	95% CI	p value
RVR factors selected by stepwise method			
F stage			
F0–2/F3–4	2.924	0.988–8.696	0.053
HCV RNA load 0 week (KIU/mL)			
<1000/1000≤	2.151	1.130–4.082	0.020
ALT 0 week (IU/L)			
<60/60≤	2.165	1.127–4.149	0.020
Amino acid mutation of ISDR			
2≤/0–1	2.371	1.187–4.735	0.014
Amino acid substitution of core 91			
W/M	2.137	1.021–4.464	0.044
cEVR factors selected by stepwise method			
Gender			
Male/female	1.912	1.209–3.021	0.0055
F stage			
F0–2/F3–4	2.079	1.133–3.817	0.018
HCV RNA load 0 week (KIU/mL)			
<1000/1000≤	1.608	1.002–2.577	0.049
PLT count ($\times 10^4/\text{mm}^3$)			
15≤/<15	1.427	0.882–2.309	0.148
Amino acid mutation of ISDR			
2≤/0–1	2.512	1.407–4.485	0.0018
Amino acid substitution of core 70			
W/M	2.513	1.508–4.184	0.0004
Amino acid substitution of core 91			
W/M	1.965	1.241–3.115	0.004
SVR factors selected by stepwise method			
Gender			
Male/female	3.704	2.132–6.410	<0.0001
F stage			
F0–2/F3–4	1.812	0.888–3.690	0.103
HCV RNA load 0 week (KIU/mL)			
<1000/1000≤	2.024	1.163–3.534	0.013
PLT count ($\times 10^4/\text{mm}^3$)			
15≤/<15	2.469	1.394–4.372	0.0019
Amino acid mutation of ISDR			
2≤/0–1	2.148	1.107–4.170	0.024
Amino acid substitution of core 70			
W/M	2.415	1.316–4.444	0.0045
Amino acid substitution of core 91			
W/M	1.433	0.828–2.481	0.199
PEG adherence (%)			
80≤/<80	1.562	0.834–2.926	0.164

W Wild, M Mutant

was a wild type but only 16% in patients with mutant at core 70. In female patients, no or one aa substitution in ISDR and $<15 \times 10^4/\text{mm}^3$ of PLT count, the SVR rates were as low as 10 or 8%, irrespective of aa substitution at core 70. SVR was

only 24% in patients with substitution of core aa 70 even when the PLT count was $\geq 15 \times 10^4/\text{mm}^3$. In this study, the combination analysis of PLT count, ISDR, and core aa substitution was useful for predicting non-SVR.

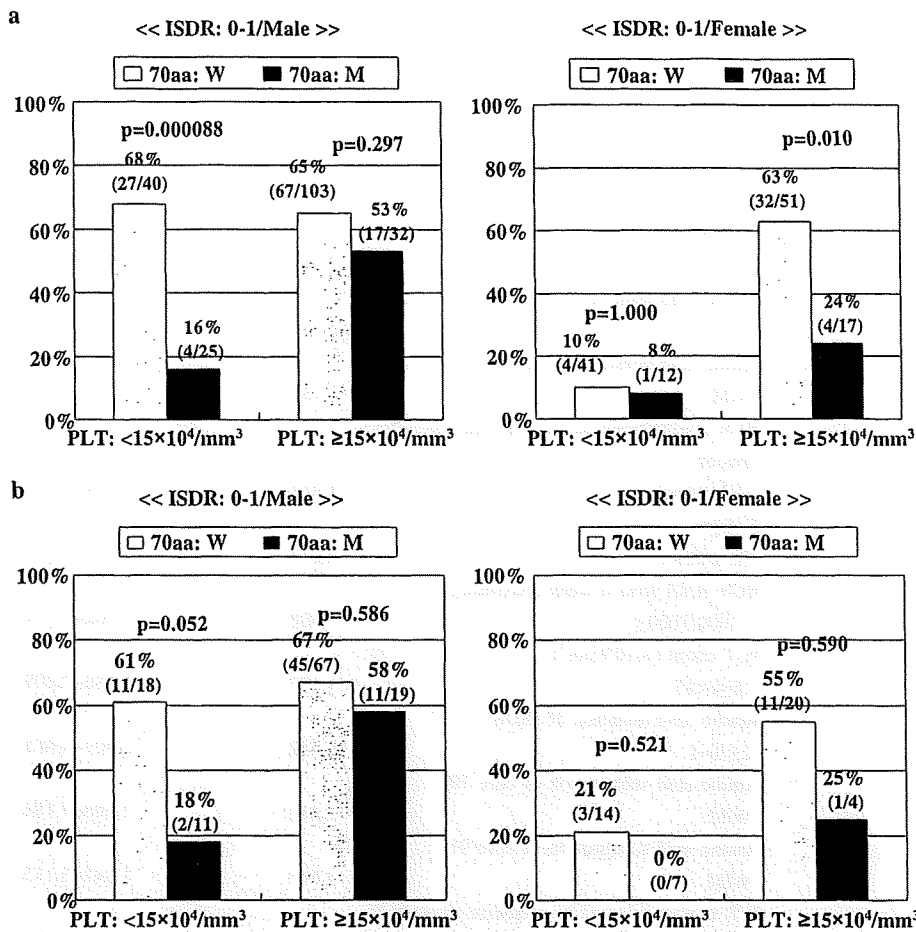


Fig. 2 Relationship between SVR rate and amino acid substitutions in the ISDR and core amino acids 70 and 91, PLT counts and gender difference. The two figures of a show the results of *Study 1* and the two figures of b show the results of *Study 2*. In male patients with no or only one amino acid (aa) substitution in the ISDR and PLT count of less than $15 \times 10^4/\text{mm}^3$, the SVR rate was 68% in those with wild type core aa 70, but only 16% in patients with mutant type of core aa 70, which is significantly different ($p = 0.000088$). There were no significant differences between wild type and mutant type of core aa 70 in the patients with no or one aa substitution in the ISDR and PLT count of over $15 \times 10^4/\text{mm}^3$. By contrast, in female patients with no or one aa substitution in the ISDR, there were no significant differences between wild type and mutant type of core aa 70 with PLT

count of less than $15 \times 10^4/\text{mm}^3$, but there were significant differences between wild type and mutant type of core aa 70 with PLT counts of less than $15 \times 10^4/\text{mm}^3$ (a). For the patients maintaining over 80% adherences to both PEG-IFN and RBV, in males having no or one aa substitution in the ISDR and PLT counts of less than $15 \times 10^4/\text{mm}^3$, a wild type of core aa 70 could predict SVR with a positive predictive value (PPV) of 61% and negative predictive value (NPV) of 82% ($p = 0.052$). However, in male patients with PLT counts of over $15 \times 10^4/\text{mm}^3$, core aa 70 was not a useful marker for predicting SVR and non-SVR. The number of female patients with no or one aa substitution in ISDR was too small to reach a definite conclusion (b)

Study design 2

The basic features of 201 patients achieving 80% adherences to both PEG-IFN and RBV are as follows: the females were significantly ($p = 0.00006$) older than the males. Iron deposition in liver tissue, alcohol abuse, BMI, serum albumin level, serum ferritin level, and PLT count were significantly higher in males than females. Inflammatory activity was significantly ($p = 0.046$) higher in females than males (data not shown).

AA substitutions in the ISDR were as follows; in males 33 (22.3%) had two or more aa substitutions, in females 8 (15.1%) had two or more aa substitutions. The analysis of core aa position 70 and 91 sequences showed no significant differences in aa substitutions of either core aa 70 or 91 between males and females (data not shown).

In patients less than 60 years of age, SVR rate was significantly higher ($p = 0.0042$) in males than females, but no significant difference was noted between males and females over 60 years old. However, the number of patients over 60 years was small (Table 4).

Table 4 Univariate analysis to identify the significantly different factors between SVR and non-SVR (201 patients received over 80% adherences of both PEG-IFN and RBV)

Factors	Negative of HCV RNA after 24 weeks		p value
	(-)	(+)	
No. of patients	111 (55.2%)	90	
Gender			
Male	93 (62.8%)	55	0.00037
Female	18 (34.0%)	35	
Age			
Median (range)	51 (18–70)	56 (23–74)	0.00025
<60 years	91 (60.3%)	60	0.014
60 years ≤	20 (40.0%)	30	
Age: <60 years			
Male	79 (66.4%)	40	0.0042
Female	12 (37.5%)	20	
Age: 60 years ≤			
Male	14 (48.3%)	15	0.243
Female	6 (28.6%)	15	
F stage			
F0–2	103 (60.9%)	67	0.0012
F3–4	8 (25.8%)	23	
Grade (A factor)			
A0–1	80 (59.3%)	55	0.189
A2–3	31 (47.0%)	35	
HCV RNA load 0 week (KIU/mL)			
Median (range)	1300 (110–5000<)	1280 (130–5000<)	0.351
ALT 0 week (IU/L)			
Median (range)	74 (16–268)	67.5 (19–504)	0.752
BMI			
Median (range)	23.1 (17.3–31.0)	23.6 (16.1–33.9)	0.626
Alb (g/dL)			
Median (range)	3.95 (3.3–5.2)	3.9 (3.0–4.8)	0.079
LDL-C (mg/dL)			
Median (range)	96 (31–185)	97.5 (30–182)	0.865
T-Chol (mg/dL)			
Median (range)	170 (85–248)	170 (105–273)	0.624
PLT count ($\times 10^4/\text{mm}^3$)			
Median (range)	18.9 (8.7–30.9)	15.55 (7.2–28.4)	0.00003
<15	23 (35.9%)	41	0.00024
15 ≤	88 (64.2%)	49	
Amino acid mutation of ISDR			
0–1	84 (52.5%)	76	0.159
2 ≤	27 (65.9%)	14	
Amino acid substitution of core 70			
Wild	91 (61.5%)	57	0.0037
Mutant	20 (37.7%)	33	
Amino acid substitution of core 91			
Wild	73 (60.3%)	48	0.083
Mutant	38 (47.5%)	42	

Virological responses and aa substitution

The rates of RVR, cEVR, LVR, ETR and SVR in males and females were 12.5 versus 11.3% ($p = 1.000$), 59.6 versus 43.4% ($p = 0.053$), 74.3 versus 50.0% ($p = 0.0018$), 76.2 versus 66.7% ($p = 0.198$), and 62.8 versus 34.0% ($p = 0.00037$), respectively (data not shown). The backgrounds and characteristics of SVR and non-SVR patients are shown in Table 4. There were significant differences in gender (male vs. female; $p = 0.00037$), age (<60 years vs. ≥ 60 years; $p = 0.014$), F stage (F0-2 vs. F3,4; $p = 0.0012$), PLT count ($<15 \times 10^4/\text{mm}^3$ vs. $15 \times 10^4/\text{mm}^3 \leq$; $p = 0.00024$), and substitution of core aa 70 (wild type vs. mutant, $p = 0.0037$) between SVR and non-SVR patients. The distribution of fatty change in liver tissue ($\leq 10\%$ vs. 11–33% vs. $34\% \leq$; $p = 0.046$) and the grade of HOMA-IR (1.7 vs. 3.9, $p = 0.0018$) were significantly different between SVR and non-SVR (data not described in Table 4).

Factors affecting SVR by multivariate logistic regression analysis

Male gender ($p = 0.0006$), mild fibrosis stage ($p = 0.027$), and wild type of core aa 70 ($p = 0.043$) were independent predictors of SVR.

Valuable markers for predictions of sustained virological response to peginterferon and ribavirin therapy

Two or more aa mutations in the ISDR, wild type core aa 70, $\geq 15 \times 10^4/\text{mm}^3$ of PLT count, and male gender were selected statistically as independent predictors of SVR. We show here SVR rates of the patients having over 80% adherences to both PEG-IFN and RBV (Fig. 2b). In males having no or one aa substitution in the ISRD and PLT count of $<15 \times 10^4/\text{mm}^3$, wild type core aa 70 could predict SVR with a positive predictive value (PPV) of 61% and negative predictive value (NPV) of 82% ($p = 0.052$). In females, the SVR rate was very low in those who had substitution of core aa 70, but there was no significant difference between patients with wild type and substitution of core aa 70. The number of female patients was too small to provide a definite conclusion.

Discussion

The present multivariate logistic regression analysis revealed that male gender, low HCV RNA load, high PLT count, and two or more aa mutations in the ISDR and wild type core aa 70 were independent predictors for SVR. PLT

count significantly decreased corresponding to the progression to the stage of liver fibrosis in CHC [9, 30, 31].

It has been considered that the low adherence level to PEG-IFN/RBV is a major cause of a significantly lower SVR rate in females and older patients [32]. The percentage of patients having over 80% adherences to both PEG-IFN and RBV was significantly lower in females than males, however, differences in the adherence to PEG-IFN/RBV between males and females were not independent predictive factors of non-SVR.

A recent report from Japan showed six or more mutations in the variable region 3 (V3) of nonstructural protein 5A (NS5A) plus upstream flanking region NS5A (aa 2334–2379), referred to as the IFN/RBV resistance determining region (IRRDR), was a useful marker for predicting SVR, but the ISDR sequence was not valuable for predicting SVR [33]. However, the number of subjects in that study was too small ($n = 45$) to reach an acceptable conclusion.

To elucidate the factors affecting low SVR rate in older female patients, we performed a multivariate logistic regression analysis using patients who achieved $\geq 80\%$ adherence to both PEG-IFN and RBV. Male gender, stage of mild liver fibrosis, and wild type core aa 70 were independent predictors of SVR. In this study, blood concentration of RBV was determined in fewer than 50% of cases during treatment. Thus we cannot exclude the possibility of the effect of the blood concentration of RBV during treatment on the low SVR rate in females and older patients.

From the present analysis, it was clear that ALT, BMI, Alb, T. Chol, and adherence to RBV differed significantly between males and females, however, these factors were not independent predictors of SVR. There is a report that steatosis is an important cofactor that reduces the SVR rate in genotype 1 infected patients [34], however, such an effect was not seen in this study. Thus we could not identify the factors associated with a significantly lower SVR rate in females than males.

In the present multivariate logistic regression analyses, patients having wild type core aa 91 had significantly higher rates of RVR and cEVR, but not SVR, and patients with wild type core aa 70 had significantly higher rates of cEVR and SVR, but not RVR. Patients having two or more aa substitutions in the ISDR had significantly higher rates of RVR, cEVR, and SVR. Although several possibilities have been considered concerning the effects of aa substitutions of core protein on SVR in PEG-IFN/RBV therapy for CHC patients, the exact mechanisms have not yet been elucidated.

Recent reports have indicated that low serum IP-10 (interferon- γ inducible protein 10 kDa) [35], a higher HCV-specific CD8 cell proliferation potential [36], and a high ratio of Th1/Th2 [37] are good predictors of SVR to

PEG-IFN/RBV therapy. These results indicate the importance of immunological status and immunological response to treatment in patients difficult to treat with PEG-IFN/RBV therapy for CHC.

The present univariate analyses revealed that there were many factors relating to RVR, cEVR, and SVR including LDL-C, HOMA-IR, fatty change in liver tissue, and hyaluronic acid, however some of these factors had not been examined in some participating institutes. We consider that we must perform a prospective mass study using many factors including immunological aspects, viral factors, disease status, and therapeutic aspects to elucidate the reason that older female patients are resistant to a combination of PEG-IFN and RBV therapy in CHC with a high viral load genotype 1b.

In conclusion, our results demonstrated that wild type core aa 70, two or more aa mutations in the ISDR, low viral load, high PLT counts, and male gender are useful markers for predicting SVR.

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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 sig-

nificant 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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long enough to achieve a high SVR. For these reasons, especially in genotype 2 infection, it is necessary to identify those patients who could achieve SVR with a shorter treatment course (≤ 16 weeks) to free them of unnecessary side effects and reduce costs, preferably as early as possible [6–8]. Furthermore, we also sometimes encounter treatment-resistant patients infected with genotype 2a [3, 10]. The underlying mechanism(s) of the different virological responses to treatment in patients infected with genotype 2a is still unclear. Hence, the pretreatment predictors of the efficacy of IFN + ribavirin combination therapy were investigated in the present study.

Amino acid (aa) substitutions at position 70 and/or 91 in the HCV core region of infected patients with genotype 1b and a high viral load (≥ 100 kIU/ml) are predictors of poor virological response to 48- and 72-week pegylated IFN (PEG-IFN) + ribavirin combination therapies [11–15], and also affect clinical outcomes, including insulin resistance and hepatocarcinogenesis [16–18]. However, it is unknown whether the aa substitutions of the core region in patients infected with genotype 2a who have high viral load might also be as useful as the pretreatment virological predictive factors apart from the genotype and viral load.

The present study included 154 Japanese adults with genotype 2a and a high viral load, who received combination therapy for 24 weeks. The aim of the study was to investigate the treatment efficacy and pretreatment predictive factors including virological features.

Materials and Methods

Study Population

A total of 190 HCV genotype 2a-infected Japanese adult patients were consecutively recruited into the study protocol of the combination therapy with IFN (PEG-IFN α -2b or IFN α -2b) + ribavirin for 24 weeks between March 2002 and February 2008 at Toranomon Hospital, Tokyo, Japan. Among these, 154 patients, who could complete a total of 24 weeks of combination therapy, were enrolled in this retrospective study and fulfilled the following criteria: (1) They were negative for hepatitis B surface antigen (radioimmunoassay, Dainabot, Tokyo, Japan), positive for anti-HCV (third-generation enzyme immunoassay, Chiron Corp., Emerville, Calif., USA), and positive for HCV-RNA qualitative analysis with PCR (Amplicor, Roche Diagnostic Systems, Calif., USA). (2) They were naive to ribavirin therapy. (3) They were infected with HCV genotype 2a alone. (4) Each had a high viral load (≥ 100 kIU/ml) by quantitative analysis of HCV-RNA with PCR (Amplicor GT HCV Monitor v2.0 using the 10-fold dilution method, Roche Molecular Systems, Inc., Pleasanton, Calif., USA) within the preceding 2 months of enrolment. (5) They had no hepatocellular carcinoma. (6) Their body weight was >40 kg. (7) All

were free of coinfection with human immunodeficiency virus. (8) None had been treated with antiviral or immunosuppressive agents within the preceding 3 months of enrolment. (9) None was an alcoholic; lifetime cumulative alcohol intake was <500 kg (mild to moderate alcohol intake). (10) None had other forms of hepatitis, such as hemochromatosis, Wilson's disease, primary biliary cirrhosis, alcoholic liver disease, and autoimmune liver disease. (11) None of the females were pregnant or lactating mothers. (12) All patients had completed a 24-week follow-up program after cessation of treatment, and SVR could be evaluated. (13) Each signed a consent form of the study protocol that had been approved by the Human Ethics Review Committee.

Table 1 summarizes the profiles and data of the 154 patients at the commencement of combination therapy with IFN + ribavirin. The study included 92 men and 62 women, aged 20–70 years (median 53). In all patients, the total duration of treatment was 24 weeks. In 43 of the 154 (27.9%) patients, the dose of ribavirin was reduced during treatment due to a fall in Hb concentration.

With regard to the treatment protocol, 104 (67.5%) patients received PEG-IFN α -2b + ribavirin for 24 weeks, and the remaining 50 (32.5%) patients received IFN α -2b + ribavirin for 24 weeks. They received PEG-IFN α -2b at a median dose of 1.5 μ g/kg (range 1.0–1.8) subcutaneously each week, or IFN α -2b at a median dose of 6 million units (range 3–6) intramuscularly each day (7 times per week for the initial 2 weeks, followed by 3 times per week for 22 weeks). They also received oral ribavirin at a median dose of 11.2 mg/kg (range 5.4–14.1) daily.

The treatment efficacy was evaluated by HCV-RNA positivity based on qualitative PCR analysis at the end of treatment (non-virological response; NVR), and by HCV-RNA negativity based on qualitative PCR analysis at 24 weeks after the completion of therapy (SVR). Furthermore, rapid responders were defined as SVR patients who could achieve a negative status within 8 weeks after the start of treatment, based on qualitative PCR analysis.

Laboratory Tests

Blood samples were obtained at least once every month before, during, and after treatment, and were analyzed for alanine aminotransferase (ALT) and HCV-RNA levels. The serum samples were frozen at -80° within 4 h of collection and thawed at the time of measurement. HCV genotype was determined by PCR using a mixed primer set derived from the nucleotide sequences of the NS5 region [19]. HCV-RNA levels were measured by quantitative PCR (Amplicor GT HCV Monitor v2.0 using the 10-fold dilution method, Roche Molecular Systems, Inc.) at least once every month before, during, and after therapy. The dynamic range of the assay was 5–5,000 kIU/ml. Samples collected during and after therapy that showed undetectable levels of HCV-RNA (<5 kIU/ml) were checked by qualitative PCR (Amplicor HCV v2.0, Roche Molecular Systems, Inc.), which has a higher sensitivity than quantitative analysis, and the results were expressed as positive or negative. The lower limit of the assay was 50 IU/ml.

Histopathological Examination of Liver Biopsies

Liver biopsy specimens were obtained percutaneously or at peritoneoscopy using a modified Vim Silverman needle with an internal diameter of 2 mm (Tohoku University style, Kakinuma Factory, Tokyo, Japan), fixed in 10% formalin, and stained with hematoxylin and eosin, Masson's trichrome, silver impregnation, and periodic acid-Schiff after diastase digestion. All specimens

Table 1. Profile and laboratory data of 154 patients infected with HCV genotype 2a

Demographic data	
Number of patients	154
Sex, M/F	92/62
Age, years*	53 (20–70)
History of blood transfusion	43 (27.9%)
Family history of liver disease	26 (16.9%)
Body mass index*	22.7 (17.9–31.8)
Laboratory data*	
Serum aspartate aminotransferase, IU/l	43 (7–404)
Serum alanine aminotransferase, IU/l	54 (8–651)
Serum albumin, g/dl	3.9 (3.2–4.7)
γ -Glutamyl transpeptidase, IU/l	38 (9–406)
Leukocytes, /mm ³	4,800 (2,200–9,000)
Hemoglobin, g/dl	14.4 (9.9–17.8)
Platelet count, $\times 10^4$ /mm ³	17.9 (6.1–32.9)
Indocyanine green retention rate at 15 min, %	13 (4–35)
Serum iron, μ g/dl	136 (22–336)
Serum ferritin, μ g/l	124 (<10–820)
Level of viremia, kIU/ml	720 (5–>5,000)
α -Fetoprotein, μ g/l	4 (2–103)
Total cholesterol, mg/dl	174 (107–275)
High-density lipoprotein cholesterol, mg/dl	47 (15–109)
Low-density lipoprotein cholesterol, mg/dl	105 (48–201)
Triglycerides, mg/dl	98 (36–449)
Uric acid, mg/dl	5.6 (2.5–9.4)
Fasting plasma glucose, mg/dl	93 (75–187)
Histological findings	
Stage of fibrosis (F1/F2/F3/ND)	58/23/16/57
Grade of activity (A1/A2/ND)	57/40/57
Hepatocyte steatosis (<5% (absent)/ \geq 5% (present)/ND)	35/52/67
Treatment	
PEG-IFN α -2b/IFN α -2b	104/50
Ribavirin dose, mg/kg*	11.2 (5.4–14.1)
Total duration of treatment, weeks	24
Past history of IFN monotherapy	56 (36.4%)

Data are number and percentages of patients, except those denoted by asterisk (*), which represent the median (range) values. ND = Not determined.

for examinations contained ≥ 6 portal areas. Histopathological diagnosis was confirmed by an experienced liver pathologist (H. K.) who was blinded to the clinical data. Chronic hepatitis was diagnosed based on histological assessment according to the scoring system of Desmet et al. [20].

Nucleotide Sequencing of the Core Region

We determined the sequences of aa 1–191 in the core region by the direct sequencing method using pretreatment sera of patients who could be analyzed due to adequate serum samples obtained at the start of combination treatment. These sequences were compared with the consensus sequences of genotype 2a, which were determined by comparing the sequences obtained in this study and prototype sequence (HCV J6) [21]. HCV-RNA was extracted from serum samples at the start of treatment and reverse tran-

scribed with random primer and MMLV reverse transcriptase (Takara Syuzo, Tokyo, Japan). Nucleic acids were amplified by nested PCR using the following primers. Nucleotide sequences of the core region: the first-round PCR was performed with 2ACF5 (sense, 5'-GCA AGA CTG CTA GCC GAG TA-3') and 2ACR6 (antisense, 5'-ATC TGA GCT GCG AGC ATC AC-3') primers, and the second-round PCR with 2ACF3N (sense, 5'-CCT TGT GGT ACT GCC TGA TA-3') and 2ACR8 (antisense, 5'-CCA GGT GAT GCT GTC ATT AG-3') primers. All samples were initially denatured at 95° for 2 min. The 35 cycles of amplification were set as follows: denaturation for 30 s at 95°, annealing of primers for 30 s at 55°, and extension for 1 min at 72° with an additional 7 min for extension. Then 1 μ l of the first PCR product was transferred to the second PCR reaction. Other conditions for the second PCR were the same as the first PCR, except that the second PCR prim-