

## Original Article

## Efficacy and anticarcinogenic activity of interferon for hepatitis C virus-related compensated cirrhosis in patients with genotype 1b low viral load or genotype 2

Eiko Hasegawa, Masahiro Kobayashi, Yusuke Kawamura, Hiromi Yatsuji, Hitomi Sezaki, Tetsuya Hosaka, Norio Akuta, Fumitaka Suzuki, Yoshiyuki Suzuki, Yasuji Arase, Kenji Ikeda and Hiromitsu Kumada

Department of Hepatology, Toranomon Hospital, Tokyo, Japan

**Background:** We assessed the efficacy and anticarcinogenic effects of interferon (IFN) therapy in patients with hepatitis C virus (HCV)-related cirrhosis.

**Methods:** The study subjects were 123 Japanese patients with HCV-related cirrhosis with genotype 1b low viral load or genotype 2 who received IFN from 1989 to 2005 (18 patients continue to receive IFN therapy). They included 81 men and 42 women aged 29–74 years (median, 56 years).

**Results:** Univariate analysis identified four parameters that significantly influenced SVR; viral load (low HCV concentration,  $P < 0.001$ ), duration of IFN therapy ( $\geq 52$  weeks,  $P = 0.029$ ), daily dose of IFN ( $\geq 6$  million units,  $P = 0.018$ ), induction therapy (presence,  $P = 0.010$ ) and choline esterase ( $> 1.0 \Delta\text{pH}$ ,  $P = 0.037$ ). Multivariate analysis identified viral load (risk ratio = 6.329,  $P < 0.001$ ) and daily dose of IFN (risk ratio = 2.62,  $P = 0.042$ ) as two independent parameters that

influenced SVR. During the observation period, newly developed hepatocellular carcinoma (HCC) was detected in 22 patients. The rates of development of HCC in patients with SVR were 5.8% at the fifth year and 10.3% at the 10th year, compared with 25.8% at the fifth year and 42.5% at the 10th year in non-SVR patients. Multivariate analysis showed that IFN efficacy (SVR) was the only independent factor of hepatocarcinogenesis (hazard ratio: 0.185, 95% confidence interval: 0.042–0.810,  $P = 0.025$ ).

**Conclusion:** Among patients with HCV-related cirrhosis, the rate of development of HCC is significantly less in patients with SVR.

**Key words:** cancer prevention, cirrhosis, hepatitis C virus, hepatocellular carcinoma, interferon

## INTRODUCTION

HEPATITIS C VIRUS (HCV)-associated hepatocellular carcinoma (HCC) typically develops through a sequence of events that progresses from chronic inflammation through fibrosis and cirrhosis accompanied by dysplasia and ultimately HCC. In a cohort study of Japanese patients with HCV-related cirrhosis,<sup>1</sup> the cumulative appearance rates of HCC at 5, 10 and 15 years were 32.5, 59.6 and 77.4%, respectively. Furthermore, the life expectancy of patients with HCV-related cirrhosis is

largely influenced by the development of HCC during the clinical course.<sup>2</sup> Because an effective and curative therapy for HCC remains limited at best, primary prevention of HCC in patients with chronic liver disease is of great importance at present.

Interferon (IFN) is effective in eliminating HCV and reducing the serum level of alanine aminotransferase (ALT) in some patients with chronic hepatitis C.<sup>3–5</sup> The response to IFN therapy is related to various factors such as HCV subtype, serum concentration of HCV, IFN treatment and liver histology.<sup>6–8</sup> Kasahara *et al.* reported that the presence of a persistently normal level of serum aminotransferase after IFN therapy was associated with significant reduction in the incidence of HCC in patients with chronic hepatitis C.<sup>9</sup> Furthermore, reduced incidence of HCC in HCV-associated cirrhosis by IFN has been reported by many investigators,<sup>10–13</sup> in fact, only a few studies have failed to find its benefit.<sup>14,15</sup>

Correspondence: Dr Masahiro Kobayashi, Department of Hepatology, Toranomon Hospital, 2-2-2 Toranomon, Minato-ku, Tokyo 105-8470, Japan. Email: mshkobayashi@toranomon.gr.jp  
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In order to evaluate whether IFN can reduce the rate of hepatocarcinogenesis and to analyze the pretreatment predictive factors associated with response to IFN and carcinogenesis in patients with HCV-related cirrhosis, we retrospectively analyzed 123 patients with HCV-related cirrhosis.

## PATIENTS AND METHODS

### Study population

A TOTAL OF 634 patients were diagnosed with HCV-related cirrhosis from 1989 to 2005 at the Department of Hepatology at Toranomon Hospital, Tokyo, Japan. Of these, 267 (42.1%) patients were treated with IFN. They included 140 patients with genotype 1b-high HCV concentration, 24 patients with genotype 1b-low HCV concentration, 38 patients with genotype 2a-high HCV concentration, 42 patients with genotype 2a-low HCV concentration, 18 patients with genotype 2b-high HCV concentration, and one patient with genotype 2b-low HCV concentration. A total of 123 patients with HCV-related cirrhosis with genotype 1b low viral load or genotype 2 were enrolled in this analysis, whose initial sera tested negative for hepatitis B surface antigen by radioimmunoassay (Ausria; Dainabot, Tokyo, Japan) and positive for anti-HCV by the second or third-generation enzyme-linked immunosorbent assay (Dainabot). They included 81 men and 42 women aged 29–74 years (median, 56 years). The diagnosis of liver cirrhosis was based on clinical features, laboratory tests, and peritoneoscopy or liver biopsy. In order to investigate hepatocarcinogenesis in HCV-related cirrhosis, patients coinfecting with HBV were excluded. Our institution does not require informed consent for retrospective studies.

### Background and laboratory findings

Table 1 shows demographic profiles and results of laboratory tests for the 123 patients at baseline (before treatment with IFN). Quantitative analysis of HCV-RNA was performed using a branched DNA probe assay (bDNA probe assay, version 2.0; Chiron, Dai-ichi Kagaku, Tokyo) and polymerase chain reaction (PCR)-based assay using the protocol provided by the manufacturer (Amplicor HCV Monitor assay version 2.0; Roche Diagnostics, Tokyo, Japan). HCV genotype was classified by PCR, using a mixture of primers for six subtypes known to exist in Japan, as reported previously.<sup>16</sup>

**Table 1** Demographics and baseline characteristics of 105 patients in the present study

Age (years)	56 (29–74)†
Sex (M/F)	81/42
BMI (kg/m <sup>2</sup> )	23.9 (16.9–35.7)†
Albumin (g/dL)	3.7 (1.8–4.7)†
AST (IU/L)	70 (26–338)†
ALT (IU/L)	80 (11–434)†
Cholesterol (mg/dL)	159 (93–272)†
Choline esterase ( $\Delta$ pH)	0.8 (0.3–1.5)†
AFP ( $\mu$ g/L)	11 (2–631)†
Ferritin ( $\mu$ g/L)	178 (<10–2076)†
Hyaluronic acid ( $\mu$ L)	184 (30–1000)†
FBS (mg/mL)	94 (65–338)†
Platelet ( $\times 10^4/\mu$ L)	9.8 (2.5–22.3)†
HCV genotype	
1b	24
2a	80
2b	19
HCV-RNA	
High viral load	56
Low viral load	67

†Data expressed as median (range).

High viral load > 100 KIU/mL or > 1 Meq/mL; low viral load < 100 KIU/mL or < 1 Meq/mL.

AFP, alpha fetoprotein; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; FBS, fasting blood sugar; HCV, hepatitis C virus.

### IFN treatment and evaluation of response to therapy

Among the 123 patients, 85 (69.1%) received IFN for the first time, while the remaining 38 (30.9%) patients had received IFN prior to this protocol. In the present study, IFN treatment involved the use of natural or recombinant IFN- $\alpha$  ( $n = 83$ ), natural IFN- $\beta$  ( $n = 38$ ), or both ( $n = 2$ ). The dosage of IFN varied in this study; 22 (17.9%) patients received 3–9 million units (MU) IFN daily for 4–8 weeks; 38 (30.9%) patients received 3–9 MU IFN daily for 2–8 weeks followed by two or three times per week; 57 (46.3%) patients received intermittent IFN two to three times per week; six patients received pegylated IFN. Among the 123 patients, 10 (8.1%) were treated with both IFN and ribavirin. The median dose of IFN was 399 MU (18–14 778 MU) during a median period of 25 weeks (1.9–602 weeks), and the daily dose was < 6 MU ( $n = 59$ ) and  $\geq 6$  MU ( $n = 58$ ). In this study, the initial daily administration of IFN for two or more weeks was defined as induction therapy.

The response to IFN was evaluated by clearance of HCV-RNA from serum and serum levels of ALT. Sustained virological response (SVR) was defined as persistent disappearance of HCV-RNA after therapy, while biochemical response (BR) was defined as normalization of ALT levels without elimination of HCV-RNA for at least 6 months after therapy. No response (NR) was defined as elevation or a transient decrease in serum ALT levels with persistent HCV-RNA levels in the serum.

### Follow up of patients and diagnosis of HCC

Patients were followed up monthly after diagnosis of liver cirrhosis in our outpatient clinic and monitored clinically by hematological, biochemical and virological tests. In addition to admission to receive IFN treatment, biweekly or monthly follow up was performed in almost all patients who received IFN. Imaging studies were conducted every 3 months in the majority of patients using ultrasonography or computed tomography (CT). Angiography was considered only when HCC was suspected on ultrasonography or CT. The diagnosis of HCC was made by characteristic hypervascular stain on hepatic angiography. When the hepatic nodule did not show hypervascular stain, a fine-needle biopsy was carried out to exclude or diagnose HCC.

### Statistical analysis

We used univariate and multivariate logistic regression analyses to determine those factors that contributed to SVR. We also calculated the odds ratios and 95% confidence intervals (95% CI). All *P*-values of less than 0.05 by the two-tailed test were considered significant. Variables that achieved statistical significance ( $P < 0.05$ ) or marginal significance ( $P < 0.15$ ) on univariate analysis were entered into multiple logistic regression analysis to identify significant independent factors. Potential predictive factors associated with SVR included the following 20 variables: age, sex, body mass index (BMI), serum albumin, cholinesterase, total cholesterol, platelet count,  $\alpha$ -fetoprotein (AFP), indocyanine green retention rate at 15 min (ICG R15), fasting blood glucose, aspartate aminotransferase (AST), ALT, level of viremia, genotype, combination therapy with ribavirin, duration of IFN therapy, total dose of IFN, daily dose of IFN, method of IFN administration and type of IFN. The incidence of hepatocarcinogenesis was calculated by the Kaplan–Meier method; it was based on the duration between the start of IFN therapy and detection of HCC. Differences in slopes of carcinogenesis curves were evaluated by the log-rank test. Independent factors associated with the development of HCC were studied

using stepwise Cox regression analysis. The following 21 variables were analyzed: age, sex, BMI, serum albumin, cholinesterase, total cholesterol, platelet count, AFP, ICG R15, fasting blood glucose, AST, ALT, level of viremia, genotype, combination therapy with ribavirin, duration of IFN therapy, total dose of IFN, daily dose of IFN, method of IFN administration, type of IFN and the effect of IFN (SVR). Statistical analysis was conducted by using SPSS software (version 10; SPSS, Chicago, IL, USA).

## RESULTS

### Response to IFN

**A**MONG 123 PATIENTS who received IFN therapy, the response to IFN therapy could be evaluated in 105 who completed the treatment protocol until December 2005, while the remaining 18 patients continue to receive IFN therapy. The dose of IFN was reduced from 6 to 3 MU per day in 20 patients. In 14 of the 20 patients, the IFN dose was reduced according to the study protocol; it was reduced at 1 week after starting IFN in 10 patients and at two weeks in four patients. In the other six patients, the dose was reduced due to thrombocytopenia. Among the 105 patients, 48 (45.7%) showed SVR, 14 (13.3%) showed BR and 43 (41%) were NR.

### Efficacy of IFN treatment according to baseline viral load and genotype

For this part of the study, pretreatment viral load was measured in 123 cases and subjects were divided into two groups using a cut-off viral load of 1 Meq/mL or  $10^6$  copies/mL. Table 2 shows the treatment efficacy estimated by baseline (pretreatment) viral load and genotype among the 105 cases. Of 21 patients with genotype 1b (low HCV concentration), nine (42.9%) showed SVR. Among 31 patients with genotype 2a (high HCV concentration), eight (25.8%) showed SVR and six (19.4%) showed BR. Among 35 patients with genotype 2a (low HCV concentration), 26 (74.3%) showed SVR and two (5.7%) showed BR. Among 17 patients with genotype 2b (high HCV concentration), four (23.5%) showed SVR and two (11.8%) showed BR. The single patient with genotype 2b (low HCV concentration) showed SVR. In summary, among 57 patients with a low viremia level, 36 (63.2%) achieved SVR and six (10.9%) achieved BR, while of 48 patients with a high viremia level, 12 (25.0%) showed SVR and eight (16.7%) patients showed BR.

**Table 2** Proportion of patients with SVR among the 105 patients who were treated with IFN for HCV-related cirrhosis

	HCV genotype			Total
	1b	2a	2b	
HCV-RNA high viral load	–	8/31 (25.8)	4/17 (23.5)	12/48 (25.0)
HCV-RNA low viral load	9/21 (42.9)	26/35 (74.3)	1/1 (100.0)	36/57 (63.2)
Total	8/20 (42.9)	34/66 (51.5)	5/18 (27.8)	48/105 (48.0)

Numbers in parentheses are percentages of patients.

High viral load > 100 KIU/mL or > 1 Meq/mL; low viral load < 100 KIU/mL or < 1 Meq/mL.

HCV, hepatitis C virus; IFN, interferon; SVR, sustained virological response.

### Side-effects

Almost all patients treated with IFN showed a variable degree of fever, chills, myalgia, headache, and general malaise after the first injection of IFN. Most patients developed a variable degree of leukocytopenia and thrombocytopenia. IFN therapy was discontinued due to anemia in one patient, thrombocytopenia in one patient, skin eruption in one patient, worsening of diabetes mellitus in one patient, retinopathy in one patient, bleeding from the ocular fundus in one patient, and interstitial pneumonia in one patient (total seven patients, 5.7%).

### Predictive factors associated with SVR in multivariate analysis

We then analyzed the data for the entire population sample to determine those factors that could predict SVR. Univariate analysis identified four parameters that significantly influenced the SVR. These included viral load (low HCV concentration,  $P < 0.001$ ), daily dose of IFN ( $\geq 6$  MU,  $P = 0.018$ ), induction therapy (present,  $P = 0.010$ ) and choline esterase level ( $> 1.0$  ΔpH,  $P = 0.037$ ). AFP ( $< 20$  μg/L,  $P = 0.058$ ) and duration of IFN therapy ( $\geq 52$  weeks,  $P = 0.064$ ) were marginally associated with SVR (Table 3). Multivariate analysis identified two parameters that independently influenced SVR, including viral load (risk ratio = 6.99,  $P < 0.001$ ) and daily dose of IFN (risk ratio = 2.62,  $P = 0.042$ ) (Table 4).

### Crude rates of hepatocarcinogenesis

Four of the 123 patients received IFN therapy after removal of HCC by either surgical resection or locoregional ablation. Therefore, these four patients were excluded from the following analysis. During the observation period (median: 4.6 years, range: 0.3–14.0 years), HCC developed in 22 (18.5%) of the 119 patients. Of these, three patients showed SVR, 16

patients showed NR and the remaining three patients developed HCC while still receiving IFN therapy and their ALT were below the upper limit of normal. One patient continued IFN therapy after the diagnosis of HCC. The crude rates of hepatocarcinogenesis were 16.8% at the fifth year, 29.1% at the 10th year and 34.2% at the 15th year. The rates of hepatocarcinogenesis in patients with SVR were 5.8% at the fifth year, and 10.3% at the 10th year, and in patients with non-SVR were 25.8% at the fifth year, and 42.5% at the 10th year (Fig. 1). Hepatocarcinogenesis was significantly less frequent in patients with SVR than in patients with non-SVR. (log-rank test,  $P = 0.007$ ).

### Predictive factors of hepatocarcinogenesis

Univariate analysis identified three factors that correlated significantly with hepatocarcinogenesis (Table 5). They were the response to IFN therapy (SVR,  $P = 0.007$ ), serum albumin level ( $> 4.0$  g/dL,  $P = 0.043$ ) and choline esterase ( $> 1.0$  ΔpH,  $P = 0.009$ ). Age ( $> 56$  years,  $P = 0.080$ ) and daily dose of IFN ( $> 6$  MU,  $P = 0.100$ ) were marginally associated with hepatocarcinogenesis. Multivariate analysis showed that efficacy of IFN therapy independently influenced the development of HCC in the cohort; SVR was associated with a significant decrease in risk of hepatocarcinogenesis (hazard ratio: 0.185, 95%CI: 0.042–0.810), compared with non-SVR (Table 6).

### DISCUSSION

**P**ROGNOSIS OF PATIENTS with HCV-related cirrhosis is greatly affected by the development of HCC, especially during the compensation period.<sup>2</sup> Kasahara *et al.* reported previously that the development of HCC could be suppressed by IFN therapy and elimination of HCV in patients with chronic hepatitis C.<sup>9</sup> Likewise, hepatocarcinogenesis is significantly inhibited by IFN

Table 3 Results of univariate analysis for SVR to IFN therapy in patients with HCV-related cirrhosis

Factor	Category	SVR (n = 48)	Non-SVR (n = 58)	P-value
Age	> 56 years	29 (60.4%)	32 (55.2%)	NS
Sex	Male	31 (64.6%)	38 (65.5%)	NS
BMI	≥ 25 kg/m <sup>2</sup>	14 (29.2%)	17 (29.3%)	NS
Albumin	> 4.0 g/dL	17 (35.4%)	18 (31.0%)	NS
AST	> 76 IU/l	23 (47.9%)	29 (50.0%)	NS
ALT	> 100 IU/l	14 (29.2%)	22 (37.9%)	NS
Cholesterol	> 160 mg/dL	26 (54.2%)	27 (46.6%)	NS
Choline esterase	> 1.0 ΔpH	22 (46.8%)	14 (25.5%)	0.037
AFP	> 20 μg/L	11 (23.9%)	24 (43.6%)	0.058
FBS	> 126 mg/mL	10 (23.3%)	6 (11.1%)	NS
Platelets	> 10 × 10 <sup>4</sup> /mL	27 (56.3%)	27 (46.6%)	NS
HCV genotype	1b	9 (18.8%)	12 (20.7%)	NS
HCV-RNA level	High	12 (25.0%)	36 (62.1%)	< 0.001
Total dose of IFN†	≥ 400 MU	21 (45.7%)	26 (44.8%)	NS
Daily dose of IFN†	≥ 6 MU	31 (67.4%)	25 (43.1%)	0.018
Duration of IFN	≥ 52 weeks	4 (8.3%)	13 (22.4%)	0.064
Induction therapy†	Yes	31 (67.4%)	24 (41.4%)	0.010
Type of IFN	Alpha	29 (60.4%)	38 (65.5%)	NS
Combination with ribavirin	Yes	5 (10.4%)	2 (3.4%)	NS

†Patients who were treated with pegylated IFN were excluded from analysis.

High viral load ≥ 100 KIU/mL or ≥ 1 Meq/mL.

AFP, alpha fetoprotein; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; FBS, fasting blood sugar; HCV, hepatitis C virus; IFN, interferon; NS, not significant; SVR, sustained virological response.

therapy in patients with HCV-related cirrhosis.<sup>10–13</sup> In addition, the incidence of hepatocarcinogenesis is reduced in patients with SVR to IFN.<sup>17</sup>

Previous studies of patients with chronic hepatitis C indicated that HCV genotype and HCV-RNA level are the most significant factors that contribute to SVR and it is assumed that patients with genotype 1b and high viral load respond poorly to IFN therapy.<sup>7</sup> In addition, IFN is less effective in patients with advanced fibrosis than those without.<sup>7,18</sup> In the present study, we retrospectively reviewed the efficacy of IFN therapy and subsequent cancer prevention effect among patients with HCV-related compensated cirrhosis. Patients with both HCV

genotype 1b and high viral load were excluded because the rate of HCV elimination among these patients was extremely low and the anticancer effect of such therapy was expected to be low.

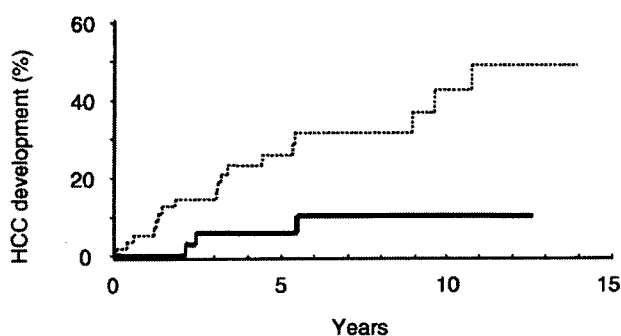
In our study, even in patients in whom fibrosis progressed to liver cirrhosis, the SVR ratio was 42.9% with genotype 1b-low HCV concentration, 25.8% with genotype 2a-high HCV concentration, 74.3% with genotype 2a-low HCV concentration, and 23.5% with genotype 2b-high HCV concentration. Thus, the therapeutic efficacy of IFN was approximately equal for liver cirrhosis and chronic hepatitis. Univariate analysis identified four parameters that significantly influenced the SVR. These

Table 4 Results of multivariate analysis for SVR to IFN for HCV-related cirrhosis

Factors	Category	Risk ratio (95% CI)	P-value
HCV-RNA level	1: low viral load	6.99 (2.72–17.9)	< 0.001
	2: high viral load	1	
Daily dose of IFN	1: ≥ 6 MU	2.62 (1.04–6.67)	0.042
	2: < 6 MU	1	

High viral load > 100 KIU/mL or > 1 Meq/mL; low viral load < 100 KIU/mL or < 1 Meq/mL.

CI, confidence interval; HCV, hepatitis C virus; IFN, interferon; SVR, sustained virological response.



**Figure 1** Cumulative rate of development of hepatocellular carcinoma (HCC) in patients treated with interferon. The rates of development of HCC in sustained virological response (SVR) patients at 5 years (5.8%) and 10 years (10.3%) were significantly lower than the respective rates in non-SVR patients (25.8%, 42.5%,  $P = 0.007$ ). —, SVR; ·····, non-SVR.

were viral load, daily dose of IFN, presence of induction therapy and choline esterase.

According to a previous study, the SVR rate was higher among patients who received long-term and high daily dose of IFN.<sup>13</sup> Although the duration of IFN

therapy was marginally longer in that study compared to the present study, the results were almost similar to those reported here. In addition, as the advantage of induction therapy among patients with chronic hepatitis has been emphasized in Japan, our result indicates that induction therapy is also important for cirrhotic patients. While the total dose of IFN was also reported previously to correlate with SVR,<sup>19</sup> no significant effect for the dose was recognized in our study. Because the design of our study was not prospective, the treatment schedule varied among patients, and therefore many biases may exist in our results. Further prospective studies are needed to confirm our findings. Multivariate analysis showed that viral load ( $< 100$  KIU/mL) independently influenced SVR, and the SVR rate was as high as 63.2% in those patients with low viral load. As patients with genotype 1b-high viral load were not included in our study, HCV genotype was not significant. When we compared only patients with low viral load, 9/21 (42.9%) patients with genotype 1b, 26/35 patients (74.3%) with genotype 2a, and one patient with genotype 2b showed SVR. Thus, the therapeutic effect of IFN in patients with genotype 2 was significantly high ( $P = 0.023$ ).

**Table 5** Results of univariate analysis for development of hepatocellular carcinoma after IFN therapy

Factor	Category	HCC (+) <i>n</i> = 22	HCC (-) <i>n</i> = 97	<i>P</i> -value
Age	> 56 years	16 (72.7%)	50 (51.5%)	0.080
Sex	Male	16 (72.7%)	63 (64.9%)	NS
BMI	$\geq 25$ kg/m <sup>2</sup>	4 (18.2%)	32 (33.0%)	NS
Albumin	> 4.0 g/dL	3 (13.6%)	49 (50.5%)	0.043
AST	> 76 IU/L	11 (50.0%)	47 (48.5%)	NS
ALT	> 100 IU/L	11 (50.0%)	44 (45.4%)	NS
Cholesterol	> 160 mg/dL	13 (59.1%)	49 (50.5%)	NS
Choline esterase	> 1.0 ΔpH	3 (14.3%)	40 (42.1%)	0.009
AFP	> 20 μg/L	9 (42.9%)	31 (32.6%)	NS
FBS	> 126 mg/mL	2 (9.5%)	13 (14.4%)	NS
Platelets	> $10 \times 10^4$ /mL	11 (50.0%)	47 (48.5%)	NS
HCV genotype	1b	16 (72.7%)	81 (83.5%)	NS
HCV-RNA level	High	10 (45.5%)	51 (52.6%)	NS
IFN efficacy	SVR	3 (14.3%)	43 (52.4%)	0.007
Total dose of IFN	$\geq 400$ MU	10 (45.5%)	44 (48.9%)	NS
Daily dose of IFN	> 6 MU	8 (36.4%)	48 (52.7%)	0.100
Duration of IFN	$\geq 52$ weeks	7 (31.9%)	22 (22.7%)	NS
Induction therapy	Yes	8 (36.4%)	50 (54.9%)	NS
Type of IFN	Alpha	15 (68.2%)	65 (67.0%)	NS
Combination with ribavirin	Yes	0 (0.0%)	10 (10.3%)	NS

High viral load  $\geq 100$  KIU/mL or  $\geq 1$  Meq/mL.

AFP, alpha fetoprotein; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; FBS, fasting blood sugar; HCV, hepatitis C virus; IFN, interferon; NS, not significant; SVR, sustained virological response.

Table 6 Results of multivariate analysis for development of hepatocellular carcinoma after IFN therapy

Factor	Category	Hazard ratio (95% CI)	P-value
IFN efficacy	1: SVR	0.185 (0.042–0.810)	0.025
	2: non-SVR	1	

CI, confidence interval; IFN, interferon; SVR, sustained viral response.

Our results showed that the incidence of HCC increased in a time-dependent manner after induction of IFN (16.8% at the fifth year, 29.1% at the 10th year, and 34.2% at the 15th year). Compared with the rates in Child A cirrhosis patients in our institution who had not been treated with IFN (32.5, 59.6 and 77.4%, respectively),<sup>1</sup> the rate of hepatocarcinogenesis after induction of IFN was half. These results suggest that hepatocarcinogenesis is significantly reduced in patients treated with IFN.

Our retrospective analysis of 123 patients with HCV-related cirrhosis who received IFN treatment showed that the outcome of IFN therapy was significantly associated with carcinogenesis. SVR was associated with a significantly reduced risk of carcinogenesis (hazard ratio: 0.185) compared with non-SVR. Three patients with SVR developed HCC; Case 1 with genotype 2a-low viral load showed SVR after 6 weeks of 3MU IFN- $\beta$  therapy, but developed HCC 28 months later. Case 2 with genotype 2a-low viral load showed SVR after 6 weeks of 3MU IFN- $\beta$  therapy but developed HCC 5 years later. Case 3 was also genotype 2a-low viral load who developed HCC at the end of 24 months of 6 MU IFN- $\alpha$  three times per week therapy. According to our previous study, the tumor-doubling time of small HCC ranges from 227 to 607 days (median, 392).<sup>20</sup> The tumor diameter at the time of diagnosis of HCC was 20 mm in Case 1, 26 mm in Case 2 and 10 mm in Case 3. Therefore, it is not clear whether hepatocarcinogenesis developed before or after eradication of HCV.

There was no relationship between the duration of IFN therapy and the rate of hepatocarcinogenesis. Likewise, the duration of IFN therapy and the total dose of IFN did not correlate with hepatocarcinogenesis. It should be noted that 38 patients had been treated with IFN prior to the present study and, thus, we cannot exclude the influence of previous IFN therapy on the development of HCC. Unfortunately, we could not collect data on the type, dose or duration of IFN for these patients because such treatment was carried out in other hospitals. To compensate for this, we analyzed those 85 patients who received IFN for the first time. In

these patients, neither the duration of IFN therapy nor the total dose of IFN was significantly associated with the development of HCC.

Our results indicated that long-term IFN therapy in non-SVR patients does not seem to improve long-term prognosis, such as the development of HCC and progression to decompensation. We reported previously that the rate of hepatocarcinogenesis was approximately equal in non-responders and untreated patients with genotype 1b-high virus load.<sup>21</sup> However, the rate was lower in BR cases who were maintained on long-term IFN therapy.<sup>21</sup> In three patients who developed HCC during IFN therapy in the present study, ALT normalization was noted after giving twice or thrice per week IFN- $\alpha$  (3MU), a therapeutic course aimed at reducing the risk of hepatocarcinogenesis. Although the duration of IFN therapy was 3.3, 4.2 and 5.3 years and serum ALT was almost below the upper limit of normal, HCC appeared in these patients during the course of IFN therapy. It is difficult to conclude from the present study that SVR is the only factor that reduced the risk of hepatocarcinogenesis. Although the result of statistical analysis was not significant in our patients, hepatocarcinogenesis was less frequent in patients with BR than in those with NR (the rate of HCC development at 5 years was 17.5% vs 29.4%, respectively, data not shown). A larger study is needed to determine the impact of BR on hepatocarcinogenesis among non-SVR patients.

In the present study, only seven of 123 patients (5.7%) discontinued IFN therapy. None developed life-threatening serious adverse effects. This outcome is probably similar to that reported in chronic hepatitis.<sup>21</sup> However, careful follow up is necessary in such cirrhotic patients including liver function tests, platelet and leukocyte counts.

In summary, the SVR rate to IFN therapy was 48.0% in patients with HCV-related compensated cirrhosis except for those patients infected with genotype 1b-high virus load. The SVR was especially high (63.2%) among patients with low viral loads. The risks of hepatocarcinogenesis decreased to almost 50% in all patients treated with IFN and to only 20% among patients with SVR.

Therefore, IFN therapy is strongly recommended for patients with compensated cirrhosis with genotype 1b-low viral load or genotype 2.

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# Loss of Hepatitis B Surface Antigen From the Serum of Patients With Chronic Hepatitis Treated With Lamivudine

Mariko Kobayashi,<sup>1\*</sup> Fumitaka Suzuki,<sup>2</sup> Norio Akuta,<sup>2</sup> Tetsuya Hosaka,<sup>2</sup> Hitomi Sezaki,<sup>2</sup> Hiromi Yatsuji,<sup>2</sup> Hiromi Yatsuji,<sup>2</sup> Masahiro Kobayashi,<sup>2</sup> Yoshiyuki Suzuki,<sup>2</sup> Yasuji Arase,<sup>2</sup> Kenji Ikeda,<sup>2</sup> Sachiyo Watahiki,<sup>1</sup> Satomi Iwasaki,<sup>1</sup> Yuzo Miyakawa,<sup>3</sup> and Hiromitsu Kumada<sup>2</sup>

<sup>1</sup>Research Institute for Hepatology, Toranomon Hospital, Tokyo, Japan

<sup>2</sup>Department of Hepatology, Toranomon Hospital, Tokyo, Japan

<sup>3</sup>Miyakawa Memorial Research Foundation, Tokyo, Japan

Although loss of hepatitis B e antigen (HBeAg) from the serum is sought by treatment with lamivudine, clearance of hepatitis B surface antigen (HBsAg) is the eventual goal of any antiviral therapy. In a single hepatology center in the Metropolitan Tokyo, 486 patients with chronic hepatitis B were followed up for longer than 3 years after they started treatment with lamivudine. HBsAg disappeared from the serum in 17 (3.5%). Age  $\geq 50$  years and low HBsAg levels (hemagglutination titer  $\leq 2^7$ ) at the start of lamivudine were significantly more frequent in the patients who did than did not lose HBsAg from the serum. Except for these two factors, there were no differences between the two groups of patients in the prevalence of HBeAg and HBV DNA levels at the baseline, as well as the development of YMDD mutants and breakthrough hepatitis during lamivudine treatment. Using multivariate analysis, age  $\geq 50$  years at the start of lamivudine was the only factor predicting the loss of HBsAg (hazard ratio: 2.96 [95% confidence interval: 1.14–7.68],  $P=0.028$ ). By the method of Kaplan–Meier performed on the 486 patients, the loss of HBsAg was estimated to occur in 3% and 13% of patients, respectively, who had received lamivudine therapy for 5 and 10 years. These results indicate that loss of HBsAg occurs in a minority (3.5%) of patients with chronic hepatitis B who receive lamivudine therapy and more frequently in those with lower HBsAg titers and older ages at the start of treatment. *J. Med. Virol.* 79:1472–1477, 2007. © 2007 Wiley-Liss, Inc.

**KEY WORDS:** chronic hepatitis; hepatitis B virus; hepatitis B surface antigen; hepatitis B e antigen; lamivudine

## INTRODUCTION

Over the world, an estimated 400 million people are infected persistently with hepatitis B virus (HBV), which may progress to chronic hepatitis, cirrhosis and hepatocellular carcinoma [Lai et al., 2003]. Many antiviral drugs have been used for preventing the development of liver disease. Among these, lamivudine was approved for clinical use in 1995 and has gained popularity for the treatment of chronic hepatitis B [Lai et al., 1998; Dienstag et al., 1999; Schalm et al., 2000]. The major goal of lamivudine therapy is loss of hepatitis B e antigen (HBeAg) from the serum, because it reflects decreased HBV replication in the liver [Magnius and Espmark, 1972]. HBV mutants resistant to lamivudine, however, elicits in recipients in parallel with the duration of treatment; they have mutations in the tyrosine-methionine-aspartate-aspartate (YMDD) motif in the DNA polymerase/reverse transcriptase [Honkoop et al., 1997; Chayama et al., 1998; Liaw et al., 1999]. Although YMDD mutants induce breakthrough hepatitis, they can be treated by rescue treatments with other antiviral drugs [Suzuki et al., 2002; Hosaka et al., 2004], thereby enabling long-term treatment with lamivudine for 7 years or longer [Kumada, 2003; Akuta et al., 2005].

Loss of hepatitis B surface antigen (HBsAg) from the serum is regarded as the eventual goal of antiviral therapy, because it improves long-term clinical outcomes of HBV infection [Fattovich et al., 1998; de Franchis et al., 2003; Lok and McMahon, 2004]. Data

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\*Correspondence to: Mariko Kobayashi, Research Institute for Hepatology, Toranomon Hospital, 1-3-1, Kajigaya, Takatsu-ku, Kawasaki City 213-8587, Japan. E-mail: vj7m-kbys@asahi-net.or.jp

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are lacking, however, on the efficacy of lamivudine in achieving loss of HBsAg from the sera of patients. In a single hepatology center in Metropolitan Tokyo, 486 patients with chronic hepatitis B were followed-up for the loss of HBsAg. Pretreatment factors influencing the loss of HBsAg were evaluated by univariate and multivariate analyses.

## MAERIALS AND METHODS

### Patients

During 10 years from 1995 to 2004, 486 patients with chronic hepatitis B received 100 mg of lamivudine daily, and had been followed for 3 years or longer at the Department of Hepatology, Toranomon Hospital in Tokyo, Japan. They had a median age of 43 years (range: 18–76 years) and included 399 (82.1%) men. Chronic hepatitis was diagnosed in 385 (79.2%) by liver biopsies performed under laparoscopy and/or ultrasonic imaging, and cirrhosis in the remaining 101 (20.7%) by liver biopsy and/or ultrasonography plus laparoscopic findings. They had a median serum level of HBV DNA of 7.2 log genome equivalents (LGE) per milliliter, and 338 (69.5%) of them were positive for HBeAg. They received lamivudine for the median of 4.8 years (range: 0.1–15.8 years), and were followed up for the median of 5.0 years (3–15) after the treatment had been started. Lamivudine was discontinued in only 95 (19.5%) patients. Reasons for withdrawal were a change to another antiviral drug in 41, the wish of patients in 29, loss of HBeAg accompanied by normalized ALT levels in 19 and side effects in 6. HBV genotypes were A in 10 (2.1%) patients, B in 32 (6.6%), C in 438 (90.1%), F in 2 (0.4%) and not typeable in the remaining 4 (0.8%) patients.

The 502 patients were followed-up for loss of HBsAg, at least 3 years or longer after treatment with lamivudine had been started, and pretreatment factors predictive of the loss were evaluated by univariate and multivariate analyses. The study design conformed to the 1975 Declaration of Helsinki, and was approved by the Ethics Committee of the institution. Every patient gave an informed consent on the purpose of this study.

### Markers of HBV Infection

HBsAg and the corresponding antibody (anti-HBs) were determined by hemagglutination (MyCell; Institute of Immunology Co., Ltd., Tokyo, Japan) and HBeAg by enzyme-linked immunosorbent assay (ELISA) (F-HBe; Sysmex, Kobe, Japan). HBV DNA was determined by transcription-mediated amplification (TMA; Chugai Diagnostics, Tokyo, Japan) and the results were expressed in LGE/ml over a range from 3.7 to 8.7. The six major genotypes of HBV (A–F) were determined serologically by ELISA (HBV GENOTYPE EIA; Institute of Immunology) and the PCR-Invader method with genotype-specific probes [Tadokoro et al., 2006]. YMDD mutants were determined by polymerase chain reaction

(PCR) followed by restriction fragment length polymorphism (RFLP) after the method of Chayama et al. [1998].

### Statistical Analysis

Factors influencing the loss of HBsAg were evaluated by the log-rank test. Independent factors associated with clearance of HBsAg from the serum by lamivudine treatment were analyzed with a stepwise Cox regression analysis. The relationship between loss of HBsAg and duration of lamivudine therapy was assessed by the method of Kaplan–Meier. Analysis of all data was performed with the computer program SPSS software (SPSS, Inc., Chicago, IL).

## RESULTS

### Comparison of Baseline Characteristics Between Patients Who Did and Did Not Lose HBsAg From the Serum

During follow-ups for 3 years or longer after the start of lamivudine treatment, 17 of the 486 (3.5%) patients lost HBsAg from the serum. All the 17 patients had lost HBV DNA detectable by a semiquantitative method before HBsAg loss, while only two (12%) patients seroconverted to anti-HBs. Table I compares demographical, clinical and virological characteristics between the patients who did and did not lose HBsAg from the serum by univariate analysis. Older age ( $\geq 50$  years) and lower HBsAg levels (hemagglutination titer  $\leq 2^7$ ) at the start of lamivudine were significantly more frequent in the patients who did than did not lose HBsAg from the serum. Except for these two factors, there were no differences between them in ALT levels, HBeAg, HBV DNA, and genotypes, and emergence of YMDD mutants as well as breakthrough hepatitis during lamivudine treatment. Among the 14 factors listed in Table I, however, only the age  $\geq 50$  years at the start of lamivudine therapy was found to increase the chance for HBsAg loss by multivariate analysis (Table II).

### Loss of HBsAg From the Serum of the 17 Patients

Figure 1 illustrates loss of HBsAg from the serum of the 17 patients who received treatment with lamivudine. HBsAg was cleared after the withdrawal of lamivudine treatment in three (18%) of them (cases 1–3). In the remaining 14 patients, HBsAg was cleared from the serum during 3.2–10.8 years while they received lamivudine. Lamivudine was withdrawn in one patient (case 4) after he had received it for 3 years, but it was resumed 6 months thereafter. Anti-HBs developed in sera of cases 2 and 6 after they lost HBsAg.

Within 3 years after the start of lamivudine therapy, HBsAg was cleared from the serum more frequently in the patients who did than did not possess HBeAg at the baseline (57% [4/7] vs. 10% [1/10],  $P = 0.036$ ). Genotypes of HBV were B in two, C in 14 and D in the remaining one; none were infected with HBV genotype A.

TABLE I. Univariate Analysis for Factors Influencing the Loss of HBsAg in Patients Treated With Lamivudine

Factors	Category	HBsAg		Differences ( <i>P</i> -value) <sup>a</sup>
		Lost (n = 17)	Persisted (n = 469)	
Age	≥50 years	9 (83%)	121 (26%)	0.017
Gender	Male	16 (94%)	382 (82%)	NS
HBV infection in mother	Positive	6 (32%)	180 (38%)	NS
History of liver disease	Chronic hepatitis	14 (82%)	371 (79%)	NS
ALT (IU/l)	≥60 IU/l	13 (76%)	360 (77%)	NS
Total bilirubin	≥0.7 mg/dl	14 (82%)	292 (62%)	NS
Cholin esterase	≥1.2 ΔpH	7 (41%)	181 (34%)	NS
HBsAg titer (2 <sup>N</sup> ) <sup>b</sup>	<2 <sup>7</sup>	8 (47%)	84 (18%)	0.032
HBeAg	Positive	7 (41%)	240 (51%)	NS
HBV DNA	≥7.1 LGE <sup>c</sup> /ml	8 (47%)	270 (58%)	NS
HBV genotypes	Genotype C	14 (82%)	438 (93%)	NS
Duration of lamivudine	≥4.1 years	13 (76%)	348 (74%)	NS
YMDD mutants	Present	9 (53%)	264 (56%)	NS
Breakthrough hepatitis	Occurred	4 (24%)	158 (34%)	NS

NS, Not significant.

<sup>a</sup>Evaluated by the log-rank test.<sup>b</sup>Determined by the passive hemagglutination method.<sup>c</sup>Log genome equivalents.

### Time Course of HBsAg Loss During Treatment With Lamivudine

Figure 2 shows the loss of HBsAg in the 486 patients who had been treated with lamivudine by the method of Kaplan–Meier. HBsAg was estimated to be cleared from the serum in 3% and 13% of the patients, respectively, at 5 and 10 years.

### DISCUSSION

Conventionally, the therapeutic efficacy of antiviral treatment in patients with chronic liver disease has been evaluated by loss of HBeAg from the serum [Wong et al., 1993]. However, HBeAg reappears in the sera of some patients from whom lamivudine therapy had been withdrawn [Song et al., 2000; Lee et al., 2002; van Nunen et al., 2003]. Hence, loss of HBeAg from the serum is not a reliable indicator of the antiviral response to lamivudine. Loss of HBsAg from the serum, instead, is usually durable and would be the gold standard for valid virological responses to lamivudine treatment. It has been absent or rare in studies on small series of patients treated with lamivudine [Lai et al., 1998; Dienstag et al., 1999].

TABLE II. Multivariate Analysis for Factors Influencing the Loss of HBsAg<sup>a</sup>

Factor	Category	Hazard ratio	Significance
Age at the start of lamivudine	1: <49 years	1	<i>P</i> = 0.028
	2: ≥50 years	2.96 (1.14–7.68) <sup>b</sup>	

<sup>a</sup>Evaluated by a stepwise Cox regression analysis on factors listed in Table I.<sup>b</sup>95% confidence interval.

In the present study, HBsAg was lost from the serum, during or after treatment with lamivudine, in 17 of the 487 (3.5%) patients who had been followed up at least 3 years after the start of treatment. Three patients lost HBsAg after lamivudine had been withdrawn; it is not certain whether or not lamivudine influenced HBsAg loss. The observed incidence is much lower than that in the six patients with de novo HBV reactivator, five (83%) of whom cleared HBsAg from the serum and developed anti-HBs after short-term lamivudine treatment [Umeda et al., 2006]. The efficacy of lamivudine would be much different between patients with ongoing HBV infection and those who have resolved infection and in whom HBV is reactivated following immunosuppressive treatments.

Older age and lower HBsAg levels at the start of lamivudine therapy increased significantly the likelihood of the loss of HBsAg from serum. Anti-HBs developed in only two of the 17 (12%) patients who had cleared HBsAg from the serum. It is not certain, therefore, whether HBsAg disappeared from the circulation, or complexed with anti-HBs and escaped detection, in the patients who lost HBsAg from the serum without developing detectable anti-HBs. When lamivudine treatment can be withdrawn is a matter of conjecture. It would be reasonable to stop lamivudine therapy in the patients who seroconverted to anti-HBs. The patients who lost HBsAg but remain negative for anti-HBs, however, would need to receive lamivudine for at least 6 months, and lamivudine therapy may be stopped in those in whom HBsAg remains negative, provided that they are followed for virological markers at regular intervals.

In previous studies, HBsAg was cleared from the serum in none of the 272 patients treated with lamivudine for 48 weeks and followed for an additional

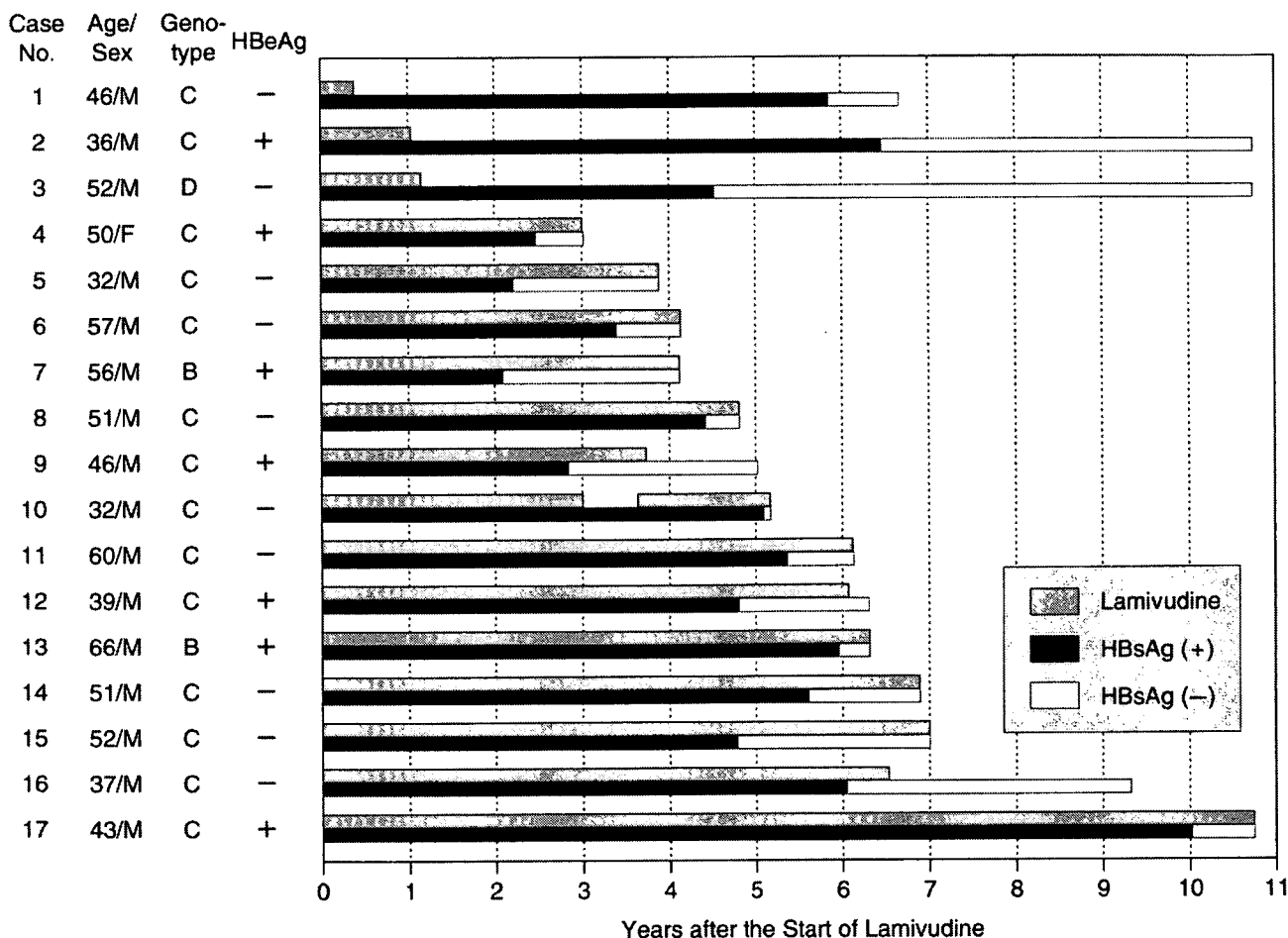


Fig. 1. Loss of HBsAg from the serum in the 17 patients followed for longer than 3 years after the start of lamivudine. Age and sex of patients, as well as genotypes of HBV and HBeAg at the baseline, are described on the left. Duration of lamivudine is indicated above, and that of HBsAg below, in columns for each patient. White columns represent periods negative for HBsAg by the hemagglutination method.

24 weeks [Lau et al., 2005], in only 1 of the 313 (0.3%) patients [Lai et al., 2006] and in 4 of the 355 (1%) patients who had received lamivudine therapy for at least 52 weeks [Chang et al., 2006]. By contrast, HBsAg was lost from the serum in 17 of the 487 (3.5%) patients treated with lamivudine in the present study. Such differences may be explained by the long-term treatment with lamivudine given to patients with chronic hepatitis B since 1995 in the Department of Hepatology, Toranomon Hospital [Kumada, 2003; Akuta et al., 2005]; lamivudine treatment has been continued in some patients for longer than 10 years. It is naturally presumed that chances for HBsAg seroconversion will increase in parallel with the duration of lamivudine therapy. This view would be supported by the loss of HBsAg in the 486 patients simulated by the method of Kaplan-Meier (Fig. 2).

With careful monitoring for YMDD mutants and breakthrough hepatitis, as well as intervention with rescue therapy by other antiviral drugs as required [Suzuki et al., 2002; Hosaka et al., 2004], long-term treatment with lamivudine has been carried out in

Toranomon Hospital [Suzuki et al., 2002; Kumada, 2003; Akuta et al., 2005]. Some patients had histological improvement [Suzuki et al., 1999, 2003]. Long-term treatment with lamivudine would be justified, because antiviral effects are shown only during therapy, and rebound of HBV DNA in the serum accompanied by hepatitis flare can develop after the withdrawal of lamivudine. Furthermore, it can suppress the development of hepatocellular carcinoma [Liaw et al., 2004; Matsumoto et al., 2005]. Combined with an excellent tolerability [Lok et al., 2003], the merit of long-term treatment with lamivudine would far outweigh its demerits [Kumada, 2003; Ryu et al., 2003].

The loss of HBsAg would be influenced by the route by which patients were infected with HBV, as well as the genotype. Loss of HBsAg from the serum may occur less frequently in the patients who were infected perinatally and were infected with HBV for many decades than in those who contracted infection during the adulthood by sexual contacts or intravenous drug use. Most Oriental carriers have been infected perinatally and possess HBV genotype B or C, in contrast to those with the adult

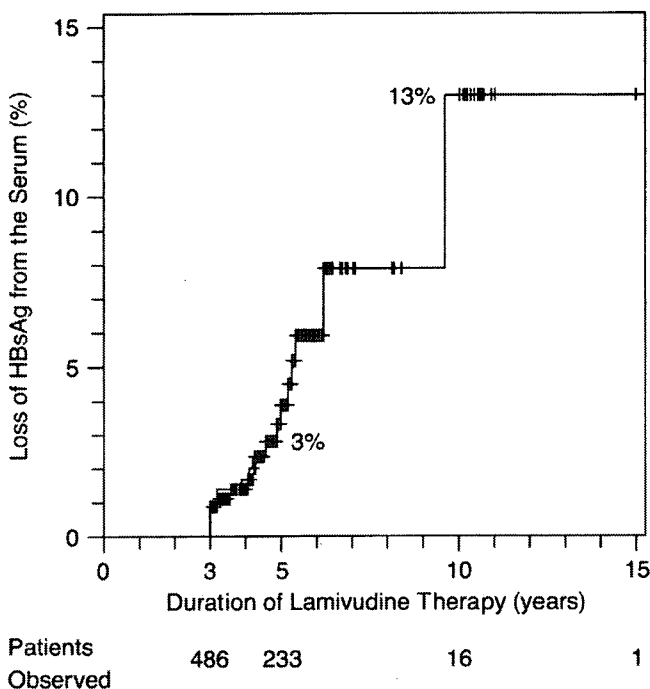


Fig. 2. Kaplan-Meier curve for the loss of HBsAg in the 486 patients who had been treated with lamivudine and followed for 3 years or longer. Numbers of patients observed at 3, 5, 10, and 15 years are indicated below.

infection who are infected frequently with HBV genotype A [Kobayashi et al., 2002]. None of the 17 patients who lost HBsAg from the serum were infected with genotype A. It is presumed, therefore, that chances for loss of HBsAg from the serum would be higher than 3.5% in the present study, among the patients in Western countries where genotype A is prevalent [Miyakawa and Mizokami, 2003].

The incidence of HBsAg clearance during long-term lamivudine, as well as factors influencing it, will provide the basis for comparison with those of other antiviral treatments including adefovir [Hadziyannis et al., 2003; Marcellin et al., 2003], entecavir [Chang et al., 2006; Lai et al., 2006], and standard as well as pegylated interferons [Wong et al., 1993; Chan et al., 2005; Lau et al., 2005], for achieving the eventual goal of clearing HBsAg from the serum. HBsAg would be useful as a practical marker, for evaluating the efficacy of antiviral therapies, since HBV DNA will rarely, if ever be cleared from the circulation of patients, once they are infected with this enduring blood-borne virus [Rehermann et al., 1996; Yotsuyanagi et al., 1998].

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# Predictors of Viral Kinetics to Peginterferon Plus Ribavirin Combination Therapy in Japanese Patients Infected With Hepatitis C Virus Genotype 1b

Norio Akuta,<sup>1\*</sup> Fumitaka Suzuki,<sup>1</sup> Yusuke Kawamura,<sup>1</sup> Hiromi Yatsuji,<sup>1</sup> Hitomi Sezaki,<sup>1</sup> Yoshiyuki Suzuki,<sup>1</sup> Tetsuya Hosaka,<sup>1</sup> Masahiro Kobayashi,<sup>1</sup> Mariko Kobayashi,<sup>2</sup> Yasuji Arase,<sup>1</sup> Kenji Ikeda,<sup>1</sup> and Hiromitsu Kumada<sup>1</sup>

<sup>1</sup>Department of Hepatology, Toranomon Hospital, Tokyo, Japan

<sup>2</sup>Liver Research Laboratory, Toranomon Hospital, Tokyo, Japan

For chronic hepatitis C virus (HCV) infection, evaluation of response to peginterferon (PEG-IFN) plus ribavirin (RBV) therapy based on viral kinetics is useful as an early predictor of treatment efficacy, but the underlying mechanisms of the different viral kinetics to treatment are still unclear. The response to 48-week PEG-IFN-RBV combination therapy was evaluated in 160 Japanese adult patients infected with HCV genotype 1b and determined the rapid virological response (at 4 weeks), early virological response (at 12 weeks), end-of treatment response, and sustained virological response (6 months after end of treatment). The proportion of patients who showed rapid, early and sustained virological, and end-of treatment responses were 50%, 73%, 47%, and 71%, respectively. Furthermore, 66% of patients who achieved early virological response also showed sustained virological response. Multivariate analysis identified substitutions of amino acid (aa) 70 and 91 in the HCV core region (double-wild-type) as a predictor of early HCV-RNA negativity, rapid, early, and sustained virological responses and end-of treatment response, and lipid metabolic factors (high levels of LDL cholesterol and total cholesterol) as predictors of early and rapid virological responses and end-of treatment response. Male sex and low levels of alpha-fetoprotein were other predictors of sustained virological response. Furthermore, female sex and severity of liver fibrosis were determinants of lack of sustained virological response in spite of early virological response. This study identified predictors of efficacy of PEG-IFN-RBV therapy based on viral kinetics in Japanese patients infected with HCV genotype 1b. *J. Med. Virol.* 79:1686–1695, 2007. © 2007 Wiley-Liss, Inc.

**KEY WORDS:** HCV; viral kinetics; peginterferon; ribavirin; core region; lipid metabolism; sex; alpha-fetoprotein

## INTRODUCTION

Treatment of chronic hepatitis C virus (HCV) infection with peginterferon (PEG-IFN) combined with ribavirin (RBV) carries potential serious side effects and is costly especially when used long enough to achieve a high sustained virological response. For these reasons, we need to identify those patients who do not achieve sustained virological response to free them of unnecessary side effects and reduce costs, preferably as early as possible after the start of the combination therapy. In this regard, previous studies showed that the rapid virological response at 4 weeks and the early virological response at 12 weeks after the commencement of 48-week treatment with PEG-IFN plus RBV are important predictors of sustained virological response [Fried et al., 2002; Jensen et al., 2006]. In fact, the observation that patients lacking early virological response following PEG-IFN- $\alpha$ -2a-RBV combination therapy are highly unlikely to develop sustained virological response was adopted as an assessment criterion by the National Institutes of Health Consensus Development Conference [National Institutes of Health Consensus Development Conference Statement, 2002]. The predictive potential of early virological response

\*Correspondence to: Norio Akuta, MD, Department of Hepatology, Toranomon Hospital, 2-2-2 Toranomon, Minato-ku, Tokyo 105-0001, Japan. E-mail: akuta-gi@umin.ac.jp

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was also confirmed in patients treated with PEG-IFN- $\alpha$ -2b-RBV [Davis et al., 2003]. Thus, it is useful to evaluate the response to treatment based on viral kinetics as an early predictor of treatment efficacy. However, the underlying mechanisms of the different viral kinetics to treatment are still unclear.

Determinants of the response to the PEG-IFN-RBV therapy were studied previously in patients with high titers of genotype 1b ( $\geq 100$  kiloIU [KIU]/ml), which is dominant in Japan [Akuta et al., 2005, 2006, 2007a,b]. The results identified aa substitutions of aa 70 and/or 91 in the HCV core region, LDL cholesterol (LDL-C), and sex as independent and significant pretreatment predictors of the response to PEG-IFN-RBV therapy. Whether these factors are also useful as pretreatment predictors of viral kinetics await further investigation.

The aim of the present study was to analyze the pretreatment viral kinetics in order to determine those factors that could predict the early response to 48-week PEG-IFN-RBV therapy in Japanese patients with HCV genotype 1b.

## PATIENTS AND METHODS

### Study Population

A total of 342 HCV-infected Japanese patients were recruited consecutively into the study protocol between December of 2001 and August of 2005 at Toranomon Hospital, Tokyo. Among these, 160 patients were selected based on the following criteria. (1) Negativity for hepatitis B surface antigen (radioimmunoassay, Dainabot, Tokyo, Japan), positivity for anti-HCV (third-generation enzyme immunoassay, Chiron Corp., Emerville, CA), and positivity for HCV RNA qualitative analysis with PCR (Amplicor, Roche Diagnostic Systems, CA). (2) Infection with HCV genotype 1b only. (3) A high viral load ( $\geq 100$  KIU/ml) by quantitative analysis of HCV RNA with PCR (Cobas Amplicor HCV monitor v 2.0 using the 10-fold dilution method, Roche Diagnostics, Tokyo, Japan) within the preceding 2 months of enrolment. (4) No hepatocellular carcinoma. (5) Body weight  $> 40$  kg. (6) Lack of coinfection with human immunodeficiency virus. (7) No previous treatment with antiviral or immunosuppressive agents within the preceding 3 months of enrolment. (8) None was an alcoholic; lifetime cumulative alcohol intake was  $< 500$  kg. (9) None had other forms of liver diseases, such as hemochromatosis, Wilson disease, primary biliary cirrhosis, alcoholic liver disease, or autoimmune liver disease. (10) None of the females was pregnant or a lactating mother. (11) All accepted treatment for  $\geq 24$  weeks as outlined in the study protocol, as well as repeated evaluation of HCV-RNA levels during the treatment (at least once every month). (12) All patients have completed a 24-week follow-up program after cessation of treatment, and sustained virological response could be evaluated. (13) Each signed a consent form of the study protocol that had been approved by the Human Ethics Review Committee of Toranomon Hospital.

Patients received PEG-IFN $\alpha$ -2b at a median dose of 1.5  $\mu$ g/kg (range, 0.8–1.8  $\mu$ g/kg) subcutaneously

each week plus oral RBV at a median dose of 11.0 mg/kg (range, 3.4–14.2 mg/kg) daily for 48 weeks. The RBV dose was adjusted according to body weight (600 mg for  $\leq 60$  kg, 800 mg for  $> 60$  kg and  $\leq 80$  kg, and 1000 mg for  $> 80$  kg), except for 40 patients who started at a reduced dose based on low pretreatment levels of hemoglobin (Hb). Furthermore, the dose of RBV was reduced during treatment in another group of 47 patients due to falls in Hb concentration.

Table I summarizes the profiles of the patients. They included 103 men and 57 women. The median duration of treatment was 48 weeks (range, 24–48 weeks). The efficacy of the combination therapy was evaluated by HCV-RNA negativity based on qualitative PCR analysis at the end of treatment (end-of treatment response) and 6 months after the completion of therapy (sustained virological response). The dynamics of on-treatment HCV was assessed by the rapid virological response defined as a decrease in HCV RNA of  $> 2.0$  log based on quantitative PCR analysis or HCV-RNA negativity based on qualitative PCR analysis at 4 weeks, and by that of  $> 2.0$  log or HCV-RNA negativity at 12 weeks (early virological response) since the commencement of combination therapy. The rapid virological response, early virological response, end-of treatment response, and sustained virological response could be evaluated in 115 (71.9%), 151 (94.4%), 160 (100%), 160 (100%) of the 160 patients, respectively.

### 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  $-80^{\circ}\text{C}$  within 4 hr of collection and then thawed at the time of measurement. HCV genotype was determined by PCR using a mixed primer set derived from the nucleotide sequences of NS5 region [Chayama et al., 1993]. HCV-RNA level was measured quantitatively by PCR (Cobas Amplicor HCV monitor v 2.0 using the 10-fold dilution method, Roche) before, during, and after therapy. The lower detection limit of the assay was 5 KIU/ml. Samples collected during and after therapy that showed no detectable levels of HCV-RNA ( $< 5$  KIU/ml) were checked also by qualitative PCR (Amplicor, Roche), which has a higher sensitivity than quantitative analysis, and the results were labeled as positive or negative. The lower limit of this assay was 50 IU/ml. For evaluation of the rapid virological response and early virological response, we used the  $\log_{10}$  of the cut-off value (5 KIU/ml) was used for HCV-RNA values below the limit of detection.

### Histopathological Examination

Liver biopsy specimens were obtained percutaneously or at peritoneoscopy using a modified Vim Silverman needle (Tohoku University style, Kakinuma Factory, Tokyo), fixed in 10% formalin, and subsequently stained



TABLE I. Patient Profile and Laboratory Data at Commencement of 48-Week Combination Therapy of Peginterferon Plus Ribavirin in 160 Patients Infected With HCV Genotype 1b

Demographic data	
Number of patients	160
Sex (M/F)	103/57
Age (years) <sup>a</sup>	54 (24–70)
History of blood transfusion	56 (35.0%)
Family history of liver disease	43 (26.9%)
Body mass index (kg/m <sup>2</sup> ) <sup>a</sup>	23.1 (17.6–32.7)
Laboratory data <sup>a</sup>	
Serum aspartate aminotransferase (IU/L)	56 (17–266)
Serum alanine aminotransferase (IU/L)	77 (24–504)
Serum albumin (g/dl)	3.8 (3.0–4.5)
Gamma-glutamyl transpeptidase (IU/L)	50 (14–393)
Leukocytes (/mm <sup>3</sup> )	4,600 (2,200–9,400)
Hemoglobin (g/dl)	14.4 (10.6–17.6)
Platelets (×10 <sup>4</sup> /mm <sup>3</sup> )	16.9 (6.6–40.2)
ICG R15 (%)	15 (3–49)
Serum iron (μg/dl)	138 (18–308)
Serum ferritin (μg/L)	150 (<10–1,104)
Creatinine clearance (ml/min)	99 (51–146)
Level of viremia (KIU/ml)	1,800 (12 to >5,000)
Alpha-fetoprotein (μg/L)	6 (2–167)
Total cholesterol (mg/dl)	168 (98–236)
High density lipoprotein cholesterol (mg/dl)	45 (10–83)
Low density lipoprotein cholesterol (mg/dl)	97 (46–162)
Triglycerides (mg/dl)	98 (33–362)
Uric acid (mg/dl)	5.6 (2.3–8.8)
Fasting blood sugar (mg/dl)	97 (67–257)
Histological findings	
Stage of fibrosis (F1/F2/F3/F4/ND)	79/34/22/1/24
Hepatocyte steatosis (none to mild/moderate to severe/ND)	116/15/29
Treatment	
PEG-IFN $\alpha$ -2b dose (μg/kg)	1.5 (0.8–1.8)
Ribavirin dose (mg/kg)	11.0 (3.4–14.2)
Amino acid substitutions in the core region <sup>b</sup>	
aa 70 (wild/non wild/ND)	79/53/9
aa 91 (wild/non wild/ND)	85/53/3
aa 70 and aa 91 (double wild/non double wild/ND)	53/84/4

Two patterns of mutant and competitive are indicated as non-wild. The pattern of wild at aa 70 and wild at aa 91 was evaluated as double wild-type, and the other patterns were non-double wild-type.

ND, not determined.

<sup>a</sup>Data are number and percentages of patients, except those denoted by, which represent the median (range) values.

<sup>b</sup>Amino acid substitutions were evaluated in 141 patients using pretreatment sera by PCR method with mutation specific primers.

with hematoxylin and eosin, Masson's trichrome, silver impregnation, and periodic acid-Schiff after diastase digestion. All specimens contained 6 or more 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 the histological scoring system of Desmet et al. [1994]. Hepatocyte steatosis was graded as none (absent), mild (<33% of hepatocytes involved), moderate (>33% but <66% of hepatocytes involved), or severe (>67% of hepatocytes involved) [D'Alessandro et al., 1991].

### Detection of Amino Acid Substitutions in the Core Region

We developed a simple and low-cost PCR method for detecting substitutions of aa 70 or aa 91 in the HCV core region of genotype 1b using mutation-specific primer, as an alternative to the direct sequencing method. The major protein type was determined based on the relative

intensity of the bands for wild (aa 70: arginine, aa 91: leucine) and mutant (aa 70: glutamine/histidine, aa 91: methionine) HCV in agarose gel electrophoresis. If the intensities of the bands were similar, the case was regarded as competitive. The detection rate was 94.4%, the sensitivity was 10 KIU/ml, the reproducibility was high, and consistency with direct sequencing was 97.1% in positive cases [Okamoto et al., 2007]. In this study, the pattern of arginine (wild) at aa 70 and leucine (wild) at aa 91 was evaluated as double wild-type, while the other patterns were non-double-wild-type. The mutation in this study refers to substitution from consensus sequence. In previous studies, HCV-J was considered as a prototype and the aa substitution was evaluated by comparison with the consensus sequence prepared from 50 clinical trial samples [Kato et al., 1990; Akuta et al., 2005]. In the present study, the PCR genotyping could be performed in 141 patients; the remaining 19 patients could not be analyzed due to the lack of adequate serum samples obtained before treatment.

### Statistical Analysis

The sustained virological response was analyzed on an intention to treat basis. Non-parametric tests were used to compare variables between groups (Mann-Whitney *U* test, chi-squared test and Fisher's exact probability test). The cumulative HCV-RNA negative rates were calculated using the Kaplan-Meier technique, and differences between the curves were tested using the log-rank test. Statistical analyses of HCV-RNA negative periods according to variables were calculated using the period from the commencement of the combination treatment. Univariate and multivariate logistic regression analyses were used to determine the predictors of rapid virological response, early virological response, end-of treatment response, and sustained virological response. Univariate analysis and multivariate Cox proportional hazard model were used to determine the predictors of early HCV-RNA negativity. We also calculated the odds ratios and 95% confidence intervals (95%CI). All *P* values less than 0.05 by the two-tailed test were considered significant. Variables that achieved statistical significance ( $P < 0.05$ ) or marginal significance ( $P < 0.10$ ) on univariate analysis were entered into multiple logistic regression analysis and multivariate Cox proportional hazard model to identify significant independent factors. Potential predictive factors associated with rapid virological response, early virological response, end-of treatment response, sustained virological response, and early HCV-RNA negativity included the following variables: sex, age, history of blood transfusion, familial history of liver disease, BMI, aspartate aminotransferase (AST), ALT, albumin,  $\gamma$ -glutamyl transpeptidase (GGT), leukocyte count, Hb, platelets, indocyanine green retention rate at 15 min (ICG R15), serum iron, serum ferritin, creatinine clearance, viremia level, alpha-fetoprotein (AFP), TC, high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), TG, uric acid (UA), FBS, hepatocyte steatosis, pathological staging, PEG-IFN dose/body weight, RBV dose/body weight, and aa substitutions in HCV core region. Statistical analyses were performed using the SPSS software (SPSS, Inc., Chicago, IL).

## RESULTS

### Response to Therapy

A rapid virological response was achieved by 57 of 115 (49.6%) patients, early virological response by 110 of 151 (72.8%), end-of treatment response by 113 of 160 (70.6%), and sustained virological response by 75 of 160 (46.9%). Furthermore, 47.7% (72/151 patients) achieved both early and sustained virological responses, 26.5% (40/151) were considered to have neither achieved an early nor sustained virological response, 25.2% (38/151) achieved early virological response but not a sustained virological response, and 0.7% (1/151) did not achieve an early virological response but showed sustained virological response. Thus, 65.5% (72/110) of

those who achieved early virological responses also achieved sustained virological responses, and 2.4% (1/41) of those who did not show early virological response later achieved sustained virological responses.

### Predictors of Early HCV-RNA Negativity as Determined by Univariate and Multivariate Analyses

Overall, the cumulative HCV-RNA negative rates were 3.8%, 30.8%, 65.4%, and 71.0% at the end of 4, 12, 24, and 48 weeks after the start of treatment. The potential predictive factors associated with early HCV-RNA negativity during treatment were explored in all 160 patients. In univariate analyses, the following 13 factors tended to or significantly influenced the early HCV-RNA negativity: HCV core region ( $P < 0.001$ ), AFP ( $P < 0.001$ ), sex ( $P = 0.002$ ), LDL-C ( $P = 0.002$ ), leukocyte count ( $P = 0.015$ ), UA ( $P = 0.015$ ), AST ( $P = 0.026$ ), age ( $P = 0.035$ ), TC ( $P = 0.044$ ), stage of fibrosis ( $P = 0.050$ ), ICG R15 ( $P = 0.071$ ), RBV dose/body weight ( $P = 0.076$ ), and Hb ( $P = 0.077$ ). In multivariate analysis using these factors, aa substitutions of the HCV core region (double wild-type;  $P < 0.001$ ), AFP ( $< 11 \mu\text{g/L}$ ;  $P = 0.014$ ), and age ( $< 55$  years;  $P = 0.028$ ) were independent significant predictors of early HCV-RNA negativity during treatment (Fig. 1; Table II).

### Predictors of Rapid Virological Response as Determined by Univariate and Multivariate Analyses

Univariate analysis identified 10 parameters that correlated with the rapid virological response: AFP ( $< 11 \mu\text{g/L}$ ;  $P < 0.001$ ), aa substitutions of the HCV core region (double wild-type;  $P = 0.001$ ), TC ( $\geq 170 \text{ mg/dl}$ ;  $P = 0.005$ ), RBV dose/body weight ( $\geq 11.0 \text{ mg/kg}$ ;  $P = 0.009$ ), AST ( $< 60 \text{ IU/L}$ ;  $P = 0.024$ ), LDL-C ( $\geq 86 \text{ mg/dl}$ ;  $P = 0.027$ ), ICG R15 ( $< 10\%$ ;  $P = 0.053$ ), gender (male sex;  $P = 0.054$ ), leukocyte count ( $\geq 4,500/\text{mm}^3$ ;  $P = 0.058$ ), and age ( $< 55$  years;  $P = 0.063$ ). Multivariate analysis that included the above variables identified three parameters that independently influenced the rapid virological response: aa substitutions of the HCV core region (double-wild-type;  $P = 0.001$ ), TC ( $\geq 170 \text{ mg/dl}$ ;  $P = 0.003$ ), and age ( $< 55$  years;  $P = 0.042$ ). Especially, aa substitutions of HCV core region (double-wild-type) and TC ( $\geq 170 \text{ mg/dl}$ ) were three parameters that increased the likelihood of a rapid virological response fivefold or more (Table III).

### Predictors of Early Virological Response as Determined by Univariate and Multivariate Analyses

Univariate analysis identified eight parameters that influenced the early virological response: LDL-C ( $\geq 86 \text{ mg/dl}$ ;  $P < 0.001$ ), AFP ( $< 11 \mu\text{g/L}$ ;  $P < 0.001$ ), aa substitutions of the HCV core region (double wild-type;  $P = 0.001$ ), TC ( $\geq 170 \text{ mg/dl}$ ;  $P = 0.005$ ), leukocyte count ( $\geq 4,500/\text{mm}^3$ ;  $P = 0.009$ ), viremia level ( $\geq 2,000 \text{ KIU/ml}$ ;

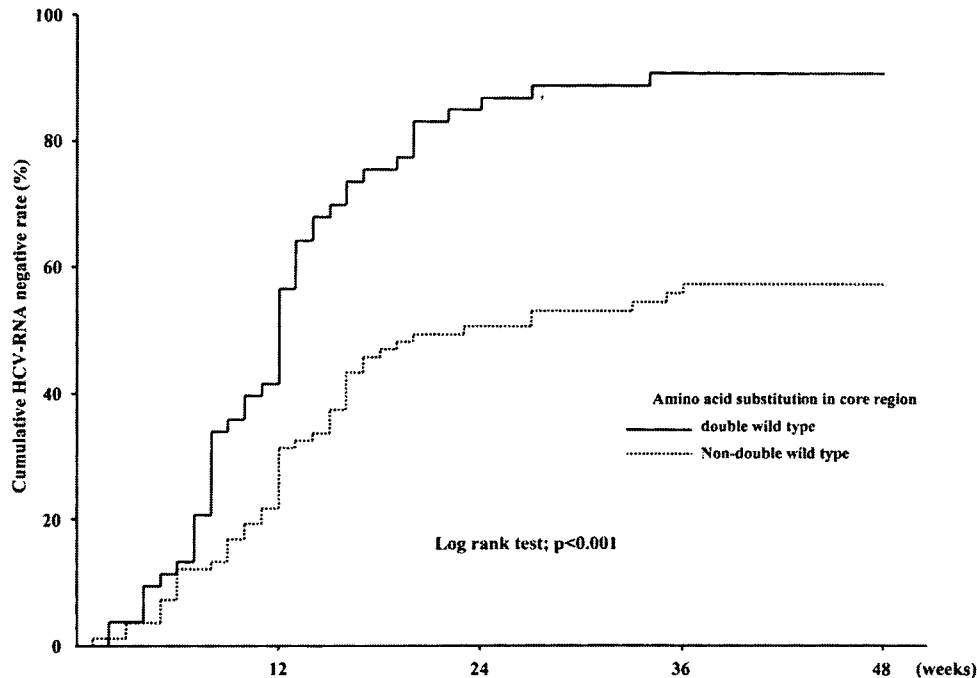


Fig. 1. Cumulative HCV-RNA negative rates during treatment, according to amino acid substitutions of the HCV core region.

$P = 0.042$ ), AST ( $< 60$  IU/L;  $P = 0.068$ ), and gender (male sex;  $P = 0.087$ ). Multivariate analysis that included the above variables identified three parameters that independently influenced the early virological response: aa substitutions of the HCV core region (double-wild-type;  $P = 0.001$ ), LDL-C ( $\geq 86$  mg/dl;  $P = 0.002$ ), and viremia level ( $\geq 2,000$  KIU/ml;  $P = 0.027$ ). Especially, aa substitutions of the HCV core region (double-wild-type) and LDL-C ( $\geq 86$  mg/dl) were the two parameters that increased the likelihood of early virological response fivefold or more (Table IV).

#### Predictors of End-of Treatment Response as Determined by Univariate and Multivariate Analyses

Univariate analysis identified ten parameters that correlated with the end-of treatment response: aa substitutions of the HCV core region (double wild-type;  $P < 0.001$ ), AFP ( $< 11$   $\mu$ g/L;  $P < 0.001$ ), LDL-C ( $\geq 86$  mg/dl;  $P = 0.002$ ), viremia level ( $\geq 2,000$  KIU/ml;  $P = 0.009$ ),

gender (male sex;  $P = 0.011$ ), TC ( $\geq 170$  mg/dl;  $P = 0.035$ ), leukocyte count ( $\geq 4,500/\text{mm}^3$ ;  $P = 0.035$ ), UA ( $\geq 7.0$  mg/dl;  $P = 0.081$ ), age ( $< 55$  years;  $P = 0.082$ ), and AST ( $< 60$  IU/L;  $P = 0.083$ ). Multivariate analysis that included the above variables identified five parameters that independently influenced the end-of treatment response: aa substitutions of the HCV core region (double-wild-type;  $P < 0.001$ ), LDL-C ( $\geq 86$  mg/dl;  $P = 0.004$ ), AFP ( $< 11$   $\mu$ g/L;  $P = 0.007$ ), viremia level ( $\geq 2,000$  KIU/ml;  $P = 0.012$ ), and UA ( $\geq 7.0$  mg/dl;  $P = 0.020$ ). Especially, aa substitutions of the HCV core region (double-wild-type), LDL-C ( $\geq 86$  mg/dl), and AFP ( $< 11$   $\mu$ g/L) were the four parameters that increased the likelihood of end-of treatment response fivefold or more (Table V).

#### Predictors of Sustained Virological Response as Determined by Univariate and Multivariate Analyses

Univariate analysis identified 14 parameters that correlate with the sustained virological response:

TABLE II. Factors Associated With Early HCV-RNA Negativity During 48-Week Peginterferon Plus Ribavirin Combination Therapy in Patients Infected With HCV Genotype1b, Identified by Multivariate Analysis

Factor	Category	Odds ratio (95% CI)	<i>P</i>
Amino acid substitution in core region <sup>a</sup>	(1) Non-double wild-type	1	<0.001
	(2) Double wild-type	2.725 (1.686–4.405)	
Alpha-fetoprotein ( $\mu$ g/L)	(1) $\geq 11$	1	0.014
	(2) $< 11$	2.427 (1.199–4.902)	
Age (years)	(1) $\geq 55$	1	0.028
	(2) $< 55$	1.767 (1.062–2.941)	

Only variables that achieved statistical significance ( $P < 0.05$ ) on multivariate Cox proportional hazard model are shown.

95% CI, 95% confidence interval.

<sup>a</sup>The pattern of wild at aa 70 and wild at aa 91 was evaluated as double wild-type, and the other patterns as non-double wild-type.

TABLE III. Factors Associated With Rapid Virological Response to 48-Week Peginterferon Plus Ribavirin Combination Therapy in Patients Infected With HCV genotype1b, Identified by Multivariate Analysis

Factor	Category	Odds ratio (95% CI)	P
Amino acid substitution in core region <sup>a</sup>	(1) Non-double wild-type	1	0.001
	(2) Double wild-type	7.692 (2.421–24.39)	
Total cholesterol (mg/dl)	(1) <170	1	0.003
	(2) ≥170	5.459 (1.768–16.86)	
Age (years)	(1) ≥55	1	0.042
	(2) <55	3.165 (1.044–9.615)	

Only variables that achieved statistical significance ( $P < 0.05$ ) on multivariate logistic regression are shown.

<sup>a</sup>The pattern of wild at aa 70 and wild at aa 91 was evaluated as double wild-type, and the other patterns as non-double wild-type.

gender (male sex;  $P < 0.001$ ), AFP ( $<11 \mu\text{g/L}$ ;  $P < 0.001$ ), stage of fibrosis (F1;  $P = 0.001$ ), leukocyte count ( $\geq 4,500/\text{mm}^3$ ;  $P = 0.001$ ), age ( $<55$  years;  $P = 0.002$ ), aa substitutions of the HCV core region (double wild-type;  $P = 0.003$ ), PEG-IFN dose/body weight ( $\geq 1.25 \mu\text{g/kg}$ ;  $P = 0.003$ ), AST ( $<60 \text{ IU/L}$ ;  $P = 0.006$ ), platelet count ( $\geq 15 \times 10^4/\text{mm}^3$ ;  $P = 0.012$ ), UA ( $\geq 7.0 \text{ mg/dl}$ ;  $P = 0.029$ ), Hb ( $\geq 14.0 \text{ g/dl}$ ;  $P = 0.032$ ), serum albumin ( $\geq 3.9 \text{ g/dl}$ ;  $P = 0.056$ ), RBV dose/body weight ( $\geq 11.0 \text{ mg/kg}$ ;  $P = 0.059$ ), and viremia level ( $<1,000 \text{ KIU/ml}$ ;  $P = 0.084$ ). Multivariate analysis that included the above variables identified six parameters that independently influenced the sustained virological response: aa substitutions of the HCV core region (double-wild-type;  $P = 0.001$ ), gender (male sex;  $P = 0.002$ ), AFP ( $<11 \mu\text{g/L}$ ;  $P = 0.005$ ), leukocytes ( $\geq 4,500/\text{mm}^3$ ;  $P = 0.011$ ), ribavirin dose ( $\geq 11.0 \text{ mg/kg}$ ;  $P = 0.029$ ), and age ( $<55$  years;  $P = 0.030$ ). Especially, aa substitutions of the HCV core region (double-wild-type), gender (male sex), and AFP ( $<11 \mu\text{g/L}$ ) were three parameters that increased the likelihood of sustained virological response fivefold or more (Table VI).

**Predictors of Sustained Virological Response in Patients Who Achieved Early Virological Response as Determined by Univariate and Multivariate Analyses**

Univariate analysis identified ten parameters that influenced the sustained virological response in patients who were able to achieve early virological response: gender (male sex;  $P < 0.001$ ), stage of fibrosis (F1;  $P = 0.002$ ), AST ( $<60 \text{ IU/L}$ ;  $P = 0.014$ ), age ( $<55$  years;  $P = 0.015$ ), leukocyte count ( $\geq 4,500/\text{mm}^3$ ;  $P = 0.020$ ), PEG-IFN dose/body weight ( $\geq 1.25 \mu\text{g/kg}$ ;  $P = 0.025$ ),

viremia level ( $<1,000 \text{ KIU/ml}$ ;  $P = 0.027$ ), AFP ( $<11 \mu\text{g/L}$ ;  $P = 0.057$ ), and Hb ( $\geq 14.0 \text{ g/dl}$ ;  $P = 0.058$ ). Multivariate analysis that included the above variables identified four parameters that independently influenced the sustained virological response of patients who achieved early virological response: gender (male sex;  $P = 0.001$ ), stage of fibrosis (F1;  $P = 0.002$ ), leukocyte count ( $\geq 4,500/\text{mm}^3$ ;  $P = 0.020$ ), and aa substitutions of the HCV core region (double wild-type;  $P = 0.025$ ). Especially, gender (male sex) and stage of fibrosis (F1) were the two parameters that increased fivefold or more the chance for sustained virological response among the patients who achieved early virological response (Table VII).

**Comparison of Factors Associated With Each of Treatment Efficacy Identified by Multivariate Analysis**

Table VIII shows the variables that achieved excellent statistical significance ( $P < 0.01$  and Odds ratio  $>5.0$ ) on multivariate logistic regression for each evaluation of treatment efficacy. With regard to viral factors, the HCV core region was the most important predictor of rapid and early virological responses, end-of treatment response, and sustained virological response. For the host factors, lipid metabolic factors including LDL-C and TC were the two most important predictors of rapid and early virological responses as well as end-of treatment response. Thus, the HCV core region and lipid metabolic factors were important predictors of viral kinetics and treatment efficacy. Furthermore, sex and AFP were also identified as other important predictors of sustained virological response, in addition to viral and lipid metabolic factors.

TABLE IV. Factors Associated With Early Virological Response to 48-Week Peginterferon Plus Ribavirin Combination Therapy in Patients Infected With HCV Genotype1b, Identified by Multivariate Analysis

Factor	Category	Odds ratio (95% CI)	P
Amino acid substitution in core region <sup>a</sup>	(1) Non-double wild-type	1	0.001
	(2) Double wild-type	10.20 (2.674–38.46)	
LDL cholesterol (mg/dl)	(1) <86	1	0.002
	(2) ≥86	5.844 (1.911–17.87)	
Level of viremia (KIU/ml)	(1) <2,000	1	0.027
	(2) ≥2,000	3.359 (1.147–9.833)	

Only variables that achieved statistical significance ( $P < 0.05$ ) on multivariate logistic regression are shown.

<sup>a</sup>The pattern of wild at aa 70 and wild at aa 91 was evaluated as double wild-type, and the other patterns as non-double wild-type.