ate fibrosis, quantitative measurement of fibrosis area by computer-assisted morphometric image analysis is becoming readily available.

Recently several reports questioned the generally accepted supposition that LS is determined entirely by hepatic fibrosis. In these studies, the association between LS and necroinflammatory activity was demonstrated. Coco *et al.* reported that, in patients with biochemical remission, either spontaneous or after antiviral therapy, LS was lower than in those with an identical fibrosis stage but elevated ALT.[25] In the studies of Sagir *et al.* and Arena *et al.*, patients with acute liver damage showed high values of LS suggestive of cirrhosis, while none of them had any other signs of cirrhosis, and they showed a decrease in LS values below the cut-off value of cirrhosis in the convalescent period.[26,27] The significant correlation between aminotransferases and LS at the onset of acute viral hepatitis was also described. In the present study, ALT was shown to correlate with LS in multiple regression analysis with all patients, and in the analysis with those with F2. Inflammatory grade also correlated with LS in univariate analysis, but not in multiple regression analysis. These results confirmed that inflammatory activity affects LS values in chronic hepatitis, especially in those with F2. Thus the interpretation of LS in patients with high levels of ALT should be made cautiously. Inflammatory activity is associated with inflammatory infiltrate, tissue edema and hepatocyte swelling, all of which are likely to affect LS,[25–27] although the exact mechanism by which inflammatory activity or elevation of ALT affects LS has not been elucidated.

In the present study, the positive predictive value for F4 with a cut-off value of 11.6 kPa was low (41.5%). When the higher cut-off values such as 16.9 kPa was adopted, the positive predictive value became higher (55.6%), while the sensitivity became lower (62.5%). Thus it should be noted that the false positive rate for the diagnosis of F4 is high, even if the higher cut-off value is adopted.

Ganne-Carrie *et al.* reported that most false negative diagnoses of cirrhosis by FibroScan are attributable to inactive or macronodular cirrhosis.[11] 29% of patients with cirrhosis with LS less than the cut-off value for cirrhosis were reported to have macronodular cirrhosis with limited amount of fibrosis tissue, characterized by large nodules with very thin septa. The present study demonstrated that the fibrosis area significantly correlated with LS in F4; LS was low in those with a small fibrosis area and was high in those with a large fibrosis area. When the cut-off value of 16.9 kPa is adopted for the diagnosis of F4, only 62.5% of patients with cirrhosis are correctly diagnosed as cirrhosis. 33% of the false negative patients with cirrhosis had a small fibrosis area of less than 6.4% (data not shown). Thus it should be noted that the patients with cirrhosis and a small fibrosis area may be misdiagnosed as not being cirrhosis when the higher cut-off value is adopted.

γ-GTP, prothrombin time and hyaluronic acid were also shown to correlate with LS in multiple regression analysis in all patients. Albumin in F2 and in F3, γ-globulin in F3, and hyaluronic acid in F4 were also shown to correlate with LS. These factors have been shown to bear direct or indirect correlation with fibrosis,[28–32] which is probably the reason why they correlated with LS.

The correlation between LS and liver fibrosis was confirmed by the objective measurement of fibrosis area. ALT was significantly correlated with LS, suggesting that inflammatory activity also affects LS values. The positive predictive value for F4 was low, even if the higher cut-off value is adopted. Despite some limitation, LS measurement is a useful method for the diagnosis of liver fibrosis.

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J Gastroenterol (2009) 44:952–963
DOI 10.1007/s00535-009-0087-x

ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

# 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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Received: 31 March 2009 / Accepted: 20 April 2009 / Published online: 11 June 2009


## 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 $15 \times 10^4/mm^3$ 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 μg/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 ≥ 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 NA5A 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%≤ 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 assesment-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 ($\times 10^4/mm^3$) | | | |
| 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≤ | 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%≤ | 190 (82.3%) | 96 (69.6%) | |
| Ribavirin adherence | | | |
| <80% | 54 (23.6%) | 73 (52.1%) | <0.00001 |
| 80%≤ | 175 (76.4%) | 67 (47.9%) | |
| Age: <60 years | | | |
| PEG adherence | | | |
| <80% | 30 (17.8%) | 23 (31.5%) | 0.027 |
| 80%≤ | 139 (82.2%) | 50 (68.5%) | |
| Ribavirin adherence | | | |
| <80% | 27 (16.2%) | 31 (42.5%) | 0.000029 |
| 80%≤ | 140 (83.8%) | 42 (57.5%) | |
| Age: 60 years≤ | | | |
| PEG adherence | | | |
| <80% | 11 (17.7%) | 19 (29.2%) | 0.147 |
| 80%≤ | 51 (82.3%) | 46 (70.8%) | |
| Ribavirin adherence | | | |
| <80% | 27 (43.5%) | 42 (62.7%) | 0.035 |
| 80%≤ | 35 (56.5%) | 25 (37.3%) | |

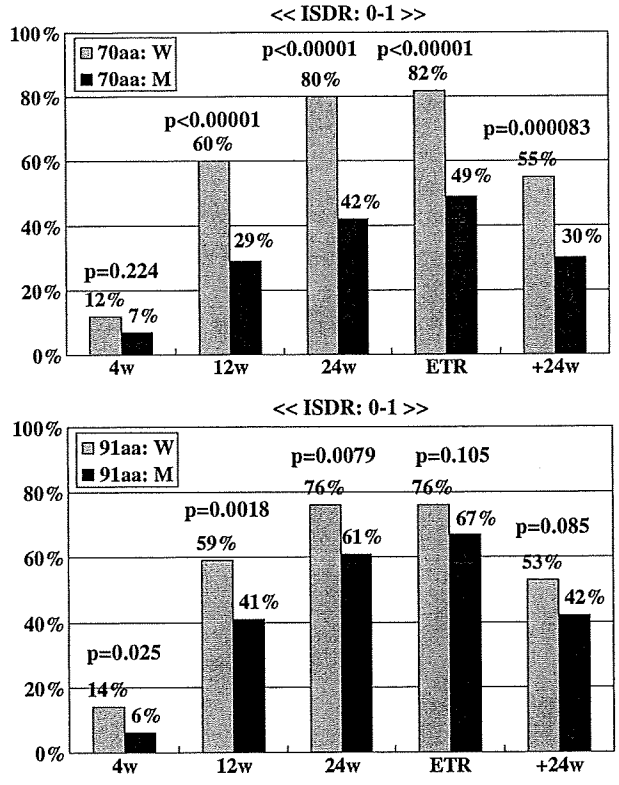


**Fig. 1** Relationship between time course of serum HCV RNA negativity and amino acid substitutions in the ISDR and core amino acids 70 and 91. For cases with no or only one amino acid (aa) change in the ISDR, the rates of cEVR, LVR, ETR and SVR were significantly higher in patients with wild type core 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 $<15 \times 10^4/mm^3$, 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 | | *p* 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 ($\times 10^4$/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 ($\times 10^4$/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 load0 week (KIU/mL) | | | |
| <1000/1000≤ | 2.024 | 1.163–3.534 | 0.013 |
| PLT count ($\times 10^4$/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 $<15 \times 10^4$/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 $\geq 15 \times 10^4$/mm$^3$. In this study, the combination analysis of PLT count, ISDR, and core aa substitution was useful for predicting non-SVR.

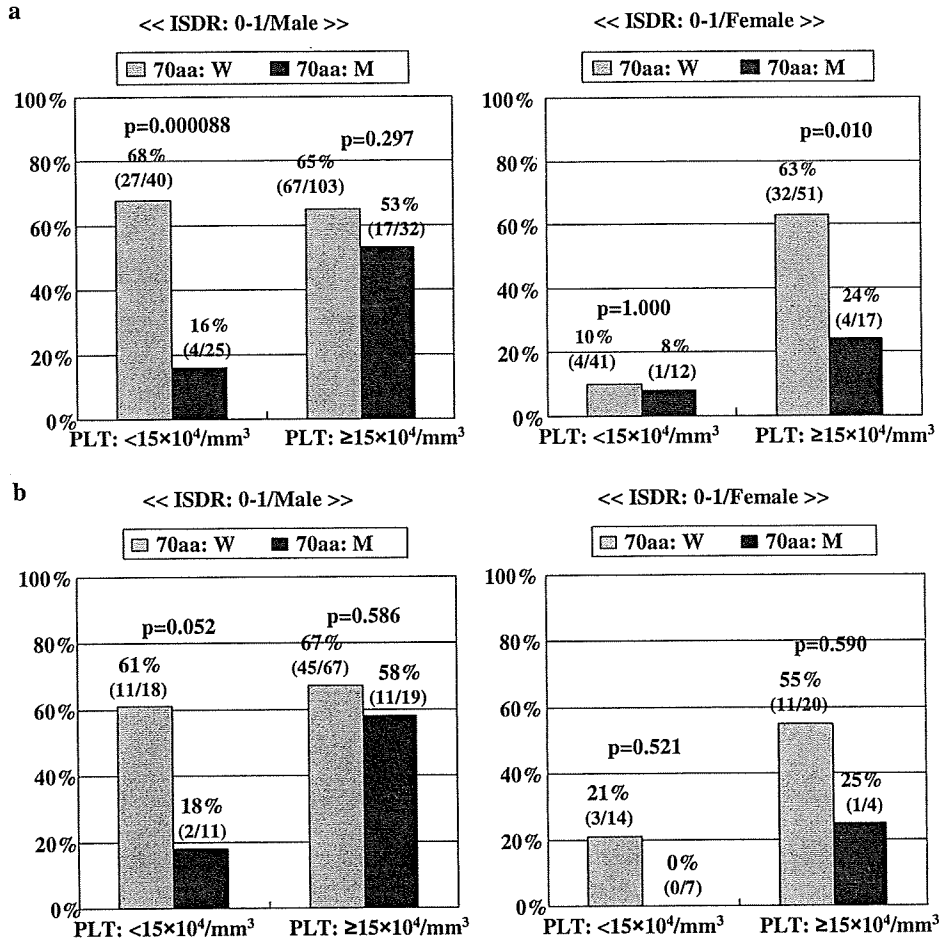


**Fig. 2** Relationship between SVR rate and amino acid substitutions in the ISDR and core amino acids 70 and 91, PLT counts and gender difference. The two figures of **a** show the results of *Study 1* and the two figures of **b** show the results of *Study 2*. In male patients with no or only one amino acid (aa) substitution in the ISDR and PLT count of less than $15 \times 10^4/mm^3$, 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 $15 \times 10^4/mm^3$. 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 $15 \times 10^4/mm^3$, but there were significant differences between wild type and mutant type of core aa 70 with PLT counts of less than $15 \times 10^4/mm^3$ (**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 $15 \times 10^4/mm^3$, 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 $15 \times 10^4/mm^3$, 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 ($\times 10^4/mm^3$) | | | |
| 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 ($<15 \times 10^4/mm^3$ vs. $15 \times 10^4/mm^3 \leq$; $p = 0.00024$), 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 (≤10% vs. 11–33% vs. 34%≤; $p = 0.046$) 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, $\geq 15 \times 10^4/mm^3$ 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 $<15 \times 10^4/mm^3$, 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 ≥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-$\gamma$ 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.

**Acknowledgments** We express our thanks to other members of the Study Group of Optimal Treatment of Viral Hepatitis; Hideyuki Nomura, Shin-Kokura Hospital; Yoshiyuki Ueno, University of Tohoku; Hisataka Moriwaki, Gifu University; Makoto Oketani, Kagoshima University Graduate School of Medical and Dental Sciences; Masataka Seike, Oita University; Hiroshi Yotsuyanagi, The University of Tokyo. This study was supported in part by a Grant-in-Aid from the Ministry of Health, Labor and Welfare, Japan.

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Journal of Medical Virology 81:459–466 (2009)

# Mutations in the Interferon Sensitivity-Determining Region of Hepatitis C Virus Genotype 2a Correlate With Response to Pegylated-Interferon-Alpha 2a Monotherapy

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The interferon sensitivity-determining region (ISDR) is thought to be inhibited by the double-stranded RNA-dependent protein kinase (PKR). Several studies have reported a relationship between the ISDR and interferon (IFN) responsiveness. However, this relationship is controversial. The aim of this study was to investigate whether genomic heterogeneity of the ISDR among patients with hepatitis C virus (HCV) genotype 2a affects the response to pegylated-IFN-alpha 2a monotherapy. Eighty patients (47 men, 33 women; mean age: 54.2 ± 12.9 years) infected with HCV genotype 2a were evaluated. HCV viral loads were determined by real-time PCR. The ISDR (amino acids 2193–2228) was examined by direct sequencing. Thirty-one patients received subcutaneous injections of pegylated-IFN-alpha 2a (180 μg) once weekly for 24 weeks, and 35 patients received injections for 48 weeks. Fourteen patients withdrew from treatment. Of the remaining 66 patients, 51 (77.3%) showed a sustained virologic response. Factors related to sustained virologic response on multivariate analysis were rapid virologic response (negative HCV at 4 weeks; odds ratio: 0.033; 95% confidence interval (95% CI) 0.003–0.363; $P = 0.0052$) and the number of mutations in the ISDR (odds ratio: 0.025; 95% CI 0.001–0.476; $P = 0.0141$). There were no significant differences in other factors, including sex, age, aspartate aminotransferase, alanine aminotransferase, platelet count, duration of treatment, and HCV viral load. Rapid virologic response and the ISDR sequence variations are significantly associated with response to pegylated-IFN-alpha 2a monotherapy in Japanese patients with HCV genotype 2a. ***J. Med. Virol. 81:459–466, 2009.*** 

**KEY WORDS:** sustained virologic response; rapid virologic response; chronic hepatitis C

## INTRODUCTION

Hepatitis C virus (HCV) is a member of the *Flaviviridae* family and causes chronic hepatitis that can develop into cirrhosis and hepatocellular carcinoma (HCC) [Seeff, 2002]. HCV infection is a significant global health problem, affecting 170 million individuals worldwide. HCV consists of three structural proteins (core, envelope 1, and envelope 2) and six non-structural proteins (NS2, NS3, NS4A, NS4B, NS5A, and NS5B). HCV NS5A protein was reported to have a domain associated with interferon (IFN) response. This domain, located in the NS5A region of HCV, is closely associated with response to IFN therapy and is known as the IFN sensitivity-determining region (ISDR) [Enomoto et al., 1996; Murakami et al., 1999; Nakano et al., 1999; Pascu et al., 2004]. There are several modes of IFN action

Grant sponsor: Hori Information Science Promotion Foundation.

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Accepted 21 October 2008
DOI 10.1002/jmv.21407
Published online in Wiley InterScience (www.interscience.wiley.com)

against HCV infection, and the final mode is still under debate. However, one mechanism of IFN action involves inhibition of viral replication by inducing the double-stranded RNA-dependent protein kinase (PKR). The ISDR is located in the 5′ end of the PKR-binding domain and is inhibited by PKR in vitro [Gale et al., 1998]. Therefore, ISDR heterogeneity is an important factor that may affect response to IFN. The utility of ISDR sequences for predicting IFN responsiveness has been investigated for HCV genotype 1b, as well as for genotypes 2 and 3, because HCV genotypes, which vary in prevalence around the world, influence IFN responsiveness [Manns et al., 2001; Fried et al., 2002; Simmonds et al., 2005]. HCV genotype 2a is relatively common in Japan [Enomoto et al., 1990; Hayashi et al., 2003]. However, there are few reports regarding the ISDR and IFN responsiveness in HCV genotype 2a [Murakami et al., 1999; Kobayashi et al., 2002; Akuta et al., 2005], and the association of mutations in the ISDR and response to IFN therapy among patients with HCV genotype 2a is not well understood. The aim of the present study was to determine whether genomic heterogeneity of the ISDR among patients with HCV genotype 2a affects the response to pegylated-IFN-alpha 2a monotherapy.

## MATERIALS AND METHODS

This prospective analysis involved 80 patients with chronic hepatitis C who received pegylated-IFN-alpha 2a monotherapy between January 2004 and December 2005. Patients who were previously treated with IFN were excluded. All patients were positive for serum anti-HCV antibody on a commercial enzyme-linked immunosorbent assay (Dinabot, Tokyo, Japan) and for HCV-RNA on a commercial polymerase chain reaction (PCR) test (Roche Diagnostic Systems, Tokyo, Japan). No patients had hepatitis B surface antigen, coinfection with human immunodeficiency virus, autoimmune disease, or chronic alcohol abuse.

### Schedule of IFN Therapy

Patients received pegylated-IFN-alpha 2a (Pegasys Roche, Tokyo, Japan) at a dose of 180 μg injected subcutaneously once weekly for 24 or 48 weeks. The patients were allocated, at the discretion of the physician in charge, to a protocol lasting either 24 or 48 weeks. Laboratory tests and evaluation of adverse events were performed once weekly during treatment. The pegylated-IFN-alpha 2a dose was dropped to 90 μg when clinically significant adverse events or laboratory abnormalities such as neutropenia (<750 cells/mm$^3$) or thrombocytopenia (<50,000 cells/mm$^3$) occurred. Pegylated-IFN-alpha 2a was discontinued when neutropenia (<250 cells/mm$^3$) or a platelet count below 25,000 cells/mm$^3$ was observed. Patients who did not receive 80% of the ideal total dose of IFN were defined as the reduced-dose group. Serum HCV-RNA levels were examined at 4, 12 weeks, at the end of IFN therapy, and 6 months after the end of treatment. Serum was stored at −80°C for virologic examination. Patients who were persistently negative for serum HCV-RNA and who had a normal serum alanine aminotransferase (ALT) level 24 weeks after withdrawal of IFN treatment were considered to have a sustained virologic response. Patients who were HCV-negative at the end of the treatment but returned to HCV-positive status after withdrawal of IFN were defined as virologic relapsers. Patients who did not become HCV-negative with IFN therapy were defined as virologic non-responders. Informed consent was obtained from each patient, and the study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki.

### Virologic Tests

The HCV-RNA quantitative viremia load was determined using real-time PCR [Takeuchi et al., 1999]. HCV was genotyped by direct sequencing of the 5′-untranslated region and/or E1 regions, as described previously [Otagiri et al., 2002; Hayashi et al., 2003]. The genotypes were classified according to the nomenclature proposed by a previous report [Simmonds et al., 2005]. Direct sequencing of the ISDR region was performed using serum samples taken within 2 days before the first administration of pegylated-IFN-alpha 2a. In brief, RNA was extracted from 140 μl of sera with a commercial kit (QIAamp Viral RNA Kit; Qiagen, Valencia, CA) and dissolved in 50 μl of diethylpyrocarbonate-treated water. Ten nanograms of the RNA was used for reverse transcription using the oligo and random hexamer primers of a commercial kit (iScript cDNA Synthesis Kit; Bio-Rad, Hercules, CA). The ISDR was amplified by hemi-nested PCR. In brief, each 50-μl PCR reaction contained 100 nM of each primer, 1 ng template cDNA, 5 μl of GeneAmp 10× PCR buffer, 2 μl of dNTPs, and 1.25 U of AmpliTaq Gold (Applied Biosystems, Foster City, CA). Primer sequences were sense, 5′-ACGTCCATGCTAACAGACCC-3′ and antisense, 5′-GGGAATCTCTTCTTGGGGAG-3′. Amplification conditions consisted of 10 min at 94°C followed by 40 cycles of 94°C for 10 sec, 55°C for 30 sec, and 72°C for 30 sec in a thermal cycler (GeneAmp PCR System 9700; Applied Biosystems). The second PCR was done in the same reaction buffer with the first-round PCR product as the template, the sense primer from the first-round PCR, and a new antisense primer, 5′-CGAGAGAGTCCAGAACGACC-3′. PCR products were separated by electrophoresis on 2% agarose gels, stained with ethidium bromide, and visualized under ultraviolet light. PCR products were then purified and sequenced with the second-round PCR primers using a dye terminator sequencing kit (BigDye Terminator v1.1 Cycle Sequencing Kit; Applied Biosystems) and an ABI 310 DNA Sequencer (Applied Biosystems). The neighbor-joining method was used for phylogenetic analysis of the ISDR (amino acids 2193–2228) in the NS5A region [Saitou and Nei, 1987], and bootstrap analysis (1,000 replicates) was performed [Felsenstein, 1985].

### Statistical Analysis

The data are expressed as mean ± standard deviation (SD). The paired *t*-test was used to analyze differences in the variables. A *P*-value of $<0.05$ was considered statistically significant. Multiple logistic regression models were used to identify factors predictive of sustained virologic response. Statview 5.0 software (SAS Institute Inc., Cary, NC) was used for all analyses.

## RESULTS

The patients' clinical characteristics are summarized in Table I. All patients were infected with HCV genotype 2a, and 27 of 80 (33.8%) patients had a serum HCV-RNA level higher than 1 million copies/ml. Eighty patients were initially entered, but 14 patients withdrew from IFN therapy, and 4 of these 14 patients could not be followed-up. The remaining 66 patients were followed-up for 6 months after the end of treatment. The completion rate was 82.5% (66/80). Thirty-one patients were treated with pegylated-IFN-alpha 2a for 24 weeks, and 35 patients were treated for 48 weeks. Virologic response is shown in Table II. The rapid virologic response rate, which was defined as negativity for HCV after 4 weeks of treatment, was 74.2% (49/66). The early virologic response rate, which was characterized by undetectable HCV at 12 weeks, was 92.4% (61/66). The virologic response rate at the end of the treatment was 97.0% (64/66). Finally, 51 of 66 (77.3%) patients achieved sustained virologic response. There were no significant differences in clinical characteristics and virologic response between patients treated for 24 weeks and those treated for 48 weeks. ISDR sequences were obtained in 62 patients, and the sequence alignments of the ISDR according to virologic response are shown in Figure 1. The mean number of ISDR mutations in patients with non-sustained virologic response was 1.2 ± 0.6, and that in patients with sustained virologic response was 2.8 ± 2.1. Patients with sustained virologic response had a significantly higher number of mutations in the ISDR than did patients with non-sustained virologic response ($P = 0.0090$). Codon 2205 was frequently changed. The association of this single mutation with sustained virologic response was examined; however, there was no significant relationship between a single mutation at codon 2205 and sustained virologic response. Sequences of the HCJ6 strain and the HCJ6 strain with all nucleotide substitutions in codon 2205 were defined as the wild type, and ISDR sequences that deviated from these strains were defined as mutant type. A rapid virologic response was achieved in 7 of 33 patients with wild-type ISDR and 5 of 41 patients with mutant-type ISDR. There were no correlations between rapid virologic response and ISDR sequence. Mutant-type ISDR was detected more frequently in sustained virologic response patients (66.7%) than in non-SVR patients (28.6%) (odds ratio: 0.200; 95% confidence interval (95% CI) 0.054–0.738; $P = 0.015$). Phylogenetic analyses of the ISDR (amino acids 2193–2228) of the 62 patients were performed, and the results are shown in Figure 2. There were differences in distinctive clustering between the wild type and the mutant type defined by counting the number of substitutions in the ISDR, but no distinctive clustering was observed in wild types with A2205 and with T2205 and with V2205. The phylogenetic analyses did not show a significant relationship between the ISDR sequences and sustained virologic response. The clinical characteristics of the patients who achieved sustained virologic response are compared to those without sustained virologic response in Table III. There were significant differences in four factors (age, HCV-RNA level, the number of mutations in the ISDR, and rapid virologic response) between the sustained virologic response group and the non-sustained virologic response group on univariate analysis. The results of the multivariate analyses of factors predictive of sustained virologic response are shown in Table IV. The variables were recorded categorically as ordinal data. The background factors were: age (<60 years vs. ≥60 years); sex (male vs. female); platelet count ($<15 \times 10^4/mm^3$ vs. $\geq 15 \times 10^4/mm^3$); HCV-RNA level ($<10^6$ copies/ml vs. $\geq 10^6$ copies/ml); ALT levels (<70 IU/L vs. ≥70 IU/L); AST levels (<60 IU/L vs. ≥60 IU/L); length of IFN therapy (24 weeks vs. 48 weeks); reduction of IFN dose (yes or no); ISDR (wild type vs. mutant type); and rapid virologic response (yes or no). Rapid virologic response at 4 weeks was the most influential factor ($P = 0.0052$), followed by mutations in the ISDR ($P = 0.0141$). No other factors achieved statistical significance. Analysis of rapid virologic response in combination with the ISDR revealed that 28 of 29 patients with mutant-type ISDR and rapid virologic response achieved sustained virologic response. The positive predictive value for sustained virologic response was 96.6% (28/29). IFN therapy was withdrawn from 14 patients. The reasons for discontinuing therapy, length of IFN therapy, ISDR sequences, rapid virologic response, and outcomes are shown in Figure 3. Ten patients discontinued therapy within 16 weeks, but 4 of the 10 patients achieved sustained virologic response. All sustained virologic response patients who withdrew from therapy within 16 weeks had at least three ISDR mutations.

TABLE I. Clinical Characteristics

| | N = 80 |
|---|---|
| Age (y.o.) | 54.2 ± 12.9 |
| Sex: male/female | 47/33 |
| AST (IU/L) | 57.9 ± 37.5 |
| ALT (IU/L) | 81.1 ± 65.3 |
| Platelet count ($10^4/\mu l$) | 20.7 ± 22.2 |
| HCV-RNA level (copies/ml) | 360,000 (540–63,000,000) |
| Body weight (kg) | 60.8 ± 9.8 |

Data are expressed as mean ± standard deviation. HCV-RNA level was shown by median (range). AST, aspartate aminotransferase; ALT, alanine aminotransferase; HCV, hepatitis C virus.

TABLE II. Virologic Response Rates

| | All (n = 66) | 24 weeks (n = 31) | 48 weeks (n = 35) |
|---|---|---|---|
| Rapid virologic response | 74.2% (n = 49) | 77.4% (n = 24) | 71.4% (n = 25) |
| Early virologic response | 92.4% (n = 61) | 96.8% (n = 30) | 88.6% (n = 31) |
| End of treatment response | 97.0% (n = 64) | 96.8% (n = 30) | 97.1% (n = 34) |
| Sustained virologic response | 77.3% (n = 51) | 77.4% (n = 24) | 77.1% (n = 27) |

Rapid virologic response as HCV-negative at 4 weeks. Early virologic response as HCV-negative at 12 weeks. End of treatment response as HCV-negative at the end of the treatment. Sustained virologic response as HCV-negative at 24 weeks after withdrawn of treatment.

## DISCUSSION

HCV genotype is one of the most important factors that predict response to IFN therapy. Genotypes 1 and 4 respond poorly to IFN therapy, whereas genotypes 2 and 3 show a sustained virologic response to IFN therapy. However, patients infected with HCV genotype 2 respond differently to IFN therapy, suggesting that an additional viral factor associated with resistance to IFN exists. The ISDR sequence in the HCV NS5A region may influence the IFN response of patients with HCV genotype 1b [Enomoto et al., 1996; Nakano et al., 1999; Pascu et al., 2004]. The influence of the ISDR sequence in response to IFN has been investigated in patients with HCV genotypes 2a and 2b [Murakami et al., 1999; Kobayashi et al., 2002; Akuta et al., 2005]. In the present study, it was hypothesized that the amino acid variations in ISDR would explain differences in IFN resistance in patients infected with HCV genotype 2a. Multivariate analyses showed that mutation of the ISDR is one of the most influential factors for sustained virologic response (odds ratio: 0.025; 95% CI 0.001–0.476; $P = 0.0141$). The sustained virologic response rate of patients with more than three mutations in the ISDR was 100% (23/23) in the present study. The results confirmed that the number of mutations in the ISDR is an important determinant of the effectiveness of pegylated-IFN-alpha 2a monotherapy in patients with

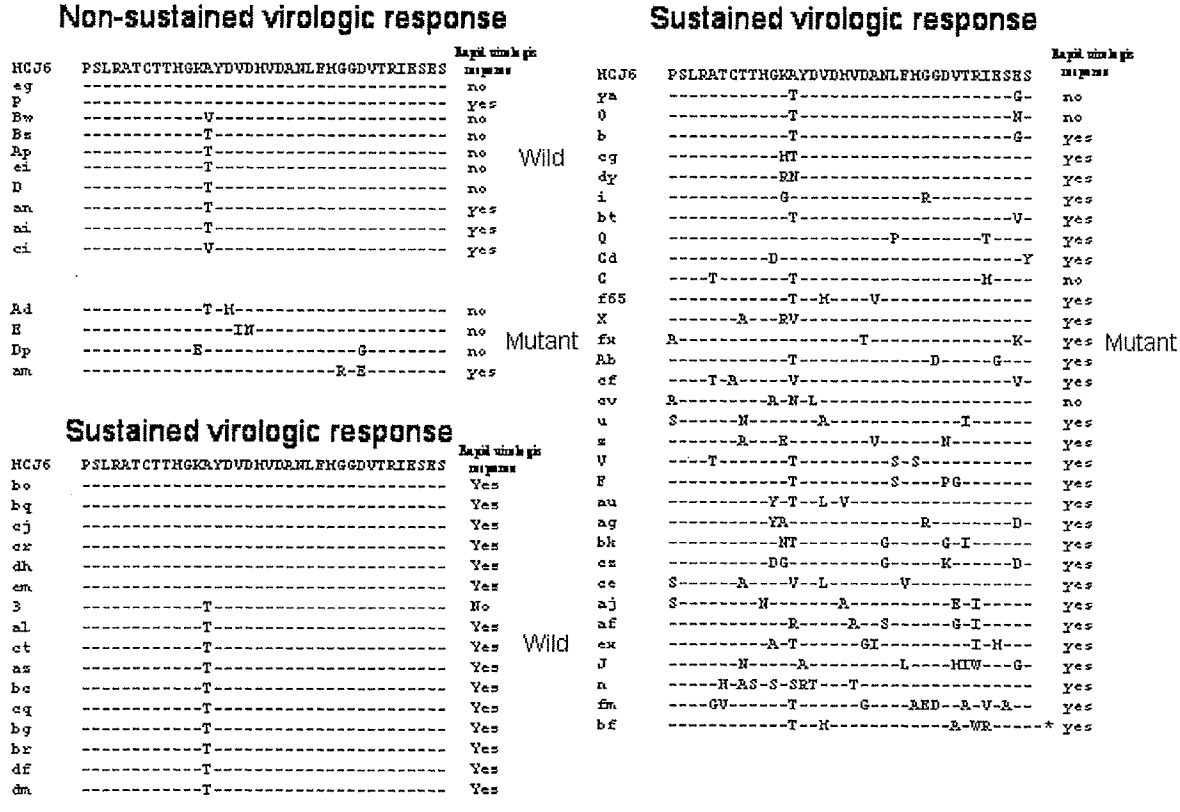


Fig. 1. Alignment of the amino acid sequence of the ISDR and response to pegylated-interferon-alpha 2a therapy. In the sequence alignment, dashes indicate amino acids identical to consensus sequence HCJ6. Sequences of the HCJ6 strain and the HCJ6 strain with all nucleotide substitutions in codon 2205 were defined as wild-type ISDR, and the other strains were defined as mutant-type ISDR. The strain marked with an asterisk had an insertion mutation. ISDR, interferon sensitivity-determining region.

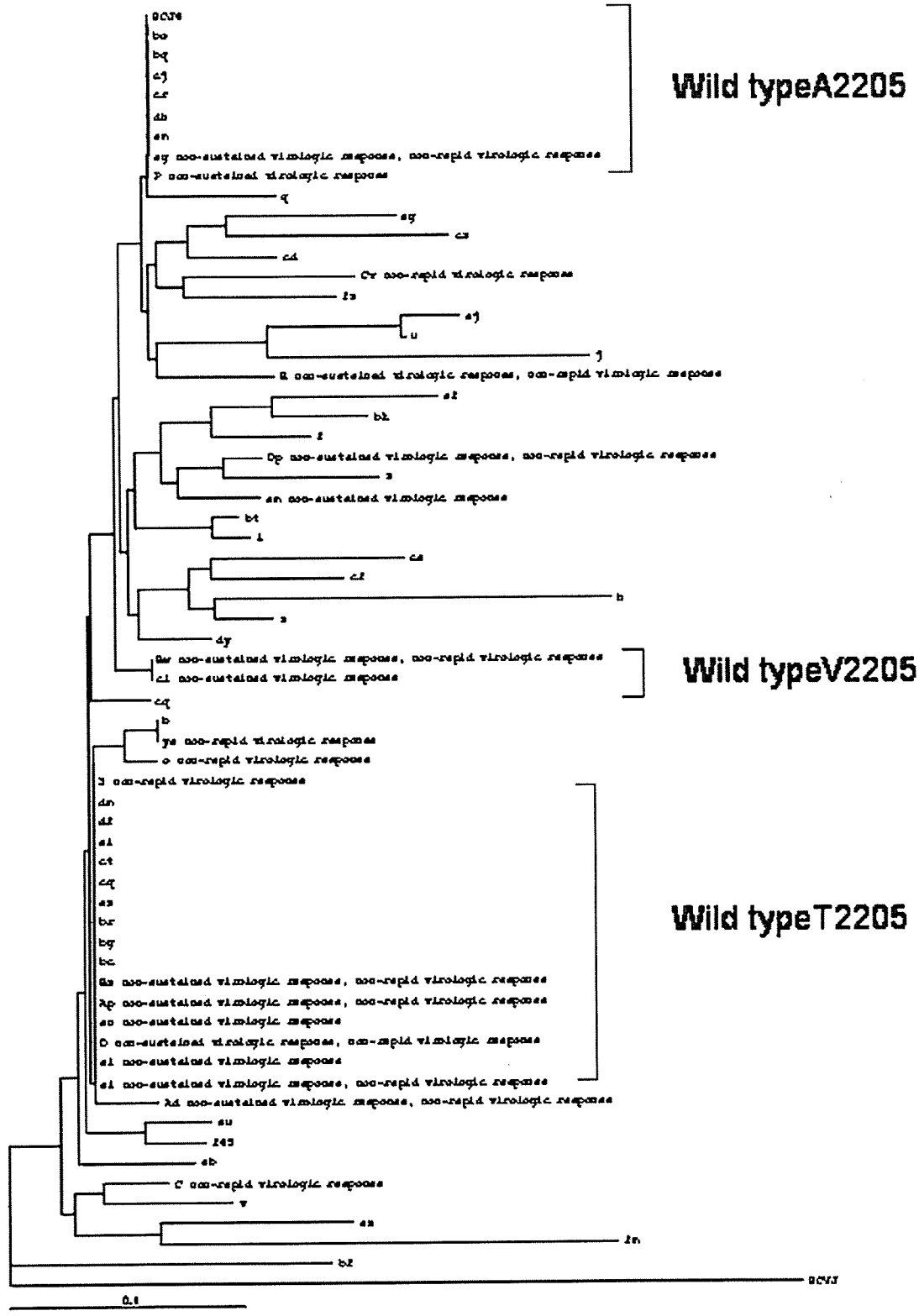


Fig. 2. Results of phylogenetic analysis of 62 sequences from the interferon sensitivity-determining region (amino acids 2193–2228) and relationship with the response to pegylated-interferon-alpha 2a therapy. Phylogenetic analysis was performed by the neighbor-joining method. HCVJ, which is the prototype of genotype 1b, was used as the outer group. The scale bar indicates genetic distance. Each strain from the present study is shown with original code followed by the virologic response. All strains without description of virologic response were rapid virologic response and sustained virologic response. Definition of wild type was counting the number of substitution in the ISDR.

TABLE III. Clinical Characteristics of Patients With or Without Sustained Virologic Response

| Factors | Sustained virologic response (n=51) | Non-sustained virologic response (n=15) | *P*-value |
|---|---|---|---|
| Age (y.o.) | 52.7 ± 13.1 | 60.3 ± 6.8 | 0.0356 |
| Gender: male/female | 33/18 | 6/9 | 0.1346 |
| ALT (IU/L) | 75.6 ± 57.7 | 66.8 ± 14.1 | 0.6002 |
| AST (IU/L) | 51.8 ± 29.4 | 56.5 ± 40.1 | 0.6218 |
| PLT ($\times 10^4$/mm$^3$) | 18.5 ± 6.0 | 15.5 ± 5.4 | 0.0866 |
| HCV-RNA level (copies/ml) | 340,000 (2,600–63,000,000) | 1,400,000 (50,000–22,000,000) | 0.0067 |
| Reduction: yes/no | 8/43 | 6/9 | 0.0691 |
| Duration: 24 weeks/48 weeks | 24/27 | 7/8 | 0.9999 |
| Mutations in the ISDR | 2.8 ± 2.1 | 1.2 ± 0.6 | 0.0090 |
| Rapid virologic response: yes/no | 44/7 | 5/10 | 0.0001 |

Data are expressed as mean ± standard deviation. HCV-RNA level was shown by median (range). AST, aspartate aminotransferase; ALT, alanine aminotransferase; PLT, platelet count; HCV, hepatitis C virus; ISDR, interferon sensitivity-determining region.

HCV genotype 2a. In addition, 10 patients discontinued IFN therapy within 16 weeks in the present study; 4 of these 10 patients achieved sustained virologic response. All sustained virologic response patients who discontinued IFN therapy were infected with mutant-type ISDR. Thus, the mutant-type ISDR appears to be associated with good response to IFN. The ISDR sequence variation of HCV genotype 2a may also play an important role as a predictor of IFN responsiveness. However, most Western reports have not confirmed the clinical usefulness of ISDR analysis for predicting response to IFN therapy [Zeuzem et al., 1997; Chung et al., 1999; Squadrito et al., 2002]. Bias relating to the IFN therapy regimens, racial differences, and HCV strains may have produced this conflicting result. To investigate the role of the ISDR while avoiding bias, all of the patients in the present study were infected with genotype 2a and received pegylated-IFN-alpha 2a monotherapy. Most studies that did not find ISDR analysis useful had a lower dose of IFN than those that reported that ISDR analysis was useful (3 million units vs. 6–10 million units). A low IFN dose was associated with a low sustained virological response rate. The present study and the studies that confirmed the usefulness of ISDR analysis had a higher sustained virological response rate (mean 50.5%) than those that did not confirm the usefulness of ISDR analysis (mean 9.6%) [Enomoto et al., 1996; Zeuzem et al., 1997; Chung et al., 1999; Murakami et al., 1999; Nakano et al., 1999; Squadrito et al., 2002]. The low sustained virological response rate, as well as the low IFN dose, would not favor the use of ISDR analysis for predicting IFN responsiveness. The number of substitutions in the ISDR in reports with negative results was significantly smaller than in studies that confirmed the correlation between ISDR mutations and IFN responsiveness [Herion and Hoofnagle, 1997]. The present study and other studies that confirmed the association between ISDR mutations and IFN sensitivity frequently found that the patients had ISDR mutant type [Saiz et al., 1988; Murakami et al., 1999; Nakano et al., 1999]. The prevalence of patients infected with ISDR mutant type would affect the association between ISDR sequence and IFN responsiveness. Thus, a study including a large number of patients with two or more amino acid substitutions in the ISDR would be suitable for using the ISDR system to predict sustained virologic response. The original classification for the ISDR sequence of genotype 1b included three categories (wild, intermediate, and mutant) according to the number of amino acid substitutions compared to the HCVJ strain. In the present study, sequences of the HCJ6 strain and the HCJ6 strain with all amino acid substitutions in codon 2205 were defined as the wild type, and the other strains were mutant type. The classification for the ISDR sequence was minimally modified for ease of analysis

TABLE IV. Multivariate Analysis: Factors Predictive of Sustained Virologic Response

| Factors | *P*-value | Risk ratio | 95% CI | |
|---|---|---|---|---|
| Age: <60 years | 0.0554 | 8.306 | 0.952 | 72.486 |
| Sex: male | 0.8270 | 1.228 | 0.194 | 7.778 |
| ALT: <70 IU/L | 0.5065 | 0.227 | 0.003 | 17.976 |
| AST: <60 IU/L | 0.9923 | 1.020 | 0.018 | 58.089 |
| PLT: $<15 \times 10^4$/mm$^3$ | 0.1528 | 0.154 | 0.012 | 2.001 |
| HCV-RNA level: $<10^6$ copies/ml | 0.4830 | 0.437 | 0.043 | 4.425 |
| Reduction: yes | 0.2242 | 0.187 | 0.013 | 2.790 |
| Duration: 48 weeks | 0.1016 | 8.100 | 0.662 | 99.135 |
| ISDR: wild | 0.0141 | 0.025 | 0.001 | 0.476 |
| Rapid virologic response: no | 0.0052 | 0.033 | 0.003 | 0.363 |

AST, aspartate aminotransferase; ALT, alanine aminotransferase; PLT, platelet count; HCV, hepatitis C virus; ISDR, interferon sensitivity-determining region.