RNA was extracted from the serum samples and converted to cDNA and two nested rounds of polymerase chain reaction (PCR) were performed. Primers used in the PCR were as follows: the first PCR was performed using cc11 (sense, 5′-GCC ATA GTG GTC TGC GGA AC-3′) and e14 (antisense, 5′-GGA GCA GTC CTT CGT GAC ATG-3′) primers. The second PCR was performed using cc9 (sense, 5′-GCT AGC CGA GTA GTG TT-3′) and e14 (antisense) primers. All samples were denatured initially at 95°C for 15 min. The 35 cycles of amplification were set as follows: denaturation for 1 min at 94°C, annealing of primers for 2 min at 55°C, and extension for 3 min at 72°C with an additional 7 min for extension. Then 1 μl of the first PCR product was transferred to the second PCR reaction. The conditions for the second PCR were the same as the first PCR, except that the second PCR primers were used instead of the first PCR primers. The amplified PCR products were purified by the QIA quick PCR Purification Kit (Qiagen, Tokyo, Japan) after agarose gel electrophoresis and then used for direct sequencing. Dideoxynucleotide termination sequencing was performed with the Big Dye Deoxy Terminator Cycle Sequencing Kit (Perkin–Elmer, Tokyo, Japan). The obtained nucleotide and amino acid sequences were compared with the prototype sequence of genotype 1b HCV-J (GenBank Accession No. D90208) [Kato et al., 1990]. Wild types virus encoded arginine (Arg) and leucine (Leu) at aa 70 and 91, respectively, and the aa substitutions were glutamine (Gln) or histidine (His) at aa 70 and methionine (Met) at aa 91. If the intensities of the band were similar, the case was regarded as competitive. Two patterns of mutant and competitive were labeled as mutant. In this study, patients were categorized into four groups according to aa substitution patterns: double-wild group, Arg70/Leu91; 70-mutant group, Gln or His70/Leu91; 91-mutant group, Arg70/Met91; and double-mutant group, Gln or His70/Met91.

### Virological Tests

Serum HCV RNA level was quantified by PCR assay (COBAS Amplicor HCV Monitor Test v2.0, Chugai-Roche Diagnostics, Tokyo, Japan), with a sensitivity limit of 5,000 IU/ml and a dynamic range from 5,000 to 5,000,000 IU/ml.

Serum HCV RNA was assessed by qualitative PCR assay (COBAS Amplicor HCV Test v2.0, Chugai-Roche Diagnostics), with a detection limit of 50 IU/ml.

### Efficacy Assessments

Patients who achieved negative HCV RNA at week 12 were defined as having a complete early virological response. Patients who became HCV RNA negative between weeks 13 and 24 were defined as having a late virological response. According to the established guidelines, the treatment was considered to have failed if the patients showed an insufficient virological response at week 12 (a detectable HCV RNA and a decrease of <2 log from the baseline level) or at week 24 (a detectable HCV RNA), and therapy was discontinued. The end-of-treatment response was defined as undetectable HCV RNA at week 48. Patients with end-of-treatment response and undetectable HCV RNA 24 weeks after completion of therapy were defined as having sustained virological response. Relapse was defined as a case in which HCV RNA had been undetectable at the end-of-treatment, but detectable during the 24-week follow-up after the treatment.

### Drug Exposure

The amounts of Peg-IFN alpha-2b and ribavirin actually taken by each patient during the treatment period were evaluated by reviewing the medical records. The mean doses of both drugs were calculated individually as averages on the basis of body weight at baseline; Peg-IFN alpha-2b expressed as μg/kg/week and ribavirin as mg/kg/day.

### Data Collection

The medical records were retrospectively reviewed and the factors necessary for this examination were extracted: age, sex, body weight, body mass index (BMI), basic laboratory assessments, liver histology, quantitative and qualitative HCV RNA, dose of Peg-IFN alpha-2b and ribavirin received at each administration, and the response to treatment.

### Statistical Analysis

Continuous variables are reported as the mean with standard deviation (SD) or median level, while categorical variables are shown as the count and proportion. In univariate analysis, the Mann–Whitney *U*-test (between two groups) or Kruskal–Wallis test (among more than three groups) was used to analyze continuous variables, while chi-squared and Fisher's exact tests were used for analysis of categorical data. For all tests, two-sided *P* values were calculated, and the results were considered statistically significant if $P < 0.05$. Variables that achieved statistical significance ($P < 0.05$) or marginal significance ($P < 0.10$) on univariate analysis were subjected to multivariate logistic regression analysis. Stepwise and multivariate logistic regression models were used to explore the independent factors that could be used to predict a virological response. Statistical analysis was performed using the SPSS program for Windows, version 15.0J (SPSS, Chicago, IL).

## RESULTS

### Baseline Characteristics of Study Groups

The total study population was predominately male (55.6%), with a mean age of 56.2 years. The baseline characteristics of all patients and the four study groups according to core aa substitution patterns are shown in Table I. Mean age of patients in the double-mutant group was higher than the other three groups ($P = 0.003$). More patients in the double-wild group had

TABLE I. Baseline Demographic and Viral Characteristics of Patients

| Characteristic | Total (n = 187) | Double-wild (n = 92) | 70-Mutant (n = 42) | 91-Mutant (n = 31) | Double-mutant (n = 22) | *P* value[a] |
|---|---|---|---|---|---|---|
| Age (years) | 56.2 ± 9.3 | 55.7 ± 9.2 | 57.0 ± 9.8 | 52.4 ± 9.9 | 61.8 ± 4.7 | 0.003 |
| Sex (male/female) | 104/83 | 51/41 | 26/16 | 18/13 | 9/13 | 0.444 |
| Body weight (kg) | 60.9 ± 11.6 | 60.9 ± 11.7 | 62.2 ± 11.7 | 62.5 ± 13.2 | 56.0 ± 7.5 | 0.193 |
| Body mass index (kg/m$^2$) | 22.8 ± 3.1 | 22.8 ± 3.0 | 22.8 ± 3.1 | 23.1 ± 3.6 | 22.1 ± 2.4 | 0.627 |
| Past IFN therapy (naïve/experienced) | 118/69 | 45/47 | 34/8 | 20/11 | 19/3 | <0.001 |
| HCV RNA (×10$^3$ IU/ml)[b] | 1,700 | 2,100 | 1,400 | 1,500 | 1,230 | 0.122 |
| Fibrosis (0–2/3–4)[c] | 105/29 | 56/11 | 22/6 | 14/7 | 13/5 | 0.366 |
| Activity (0–1/2–3)[d] | 83/50 | 42/24 | 18/10 | 11/10 | 12/6 | 0.771 |
| White blood cell (×10$^6$/l) | 4,980 ± 1,520 | 4,990 ± 1,420 | 5,180 ± 1,760 | 4,890 ± 1,430 | 4,660 ± 1,560 | 0.795 |
| Red blood cell (×10$^{12}$/l) | 4.34 ± 0.46 | 4.33 ± 0.46 | 4.41 ± 0.52 | 4.39 ± 0.42 | 4.18 ± 0.32 | 0.145 |
| Hemoglobin (g/dl) | 13.9 ± 1.4 | 13.9 ± 1.4 | 14.0 ± 1.7 | 14.2 ± 1.4 | 13.5 ± 1.1 | 0.253 |
| Platelet (×10$^9$/l) | 161 ± 54 | 167 ± 49 | 165 ± 65 | 154 ± 60 | 138 ± 30 | 0.067 |
| ALT (IU/l) | 74 ± 61 | 73 ± 67 | 79 ± 56 | 81 ± 64 | 57 ± 37 | 0.263 |
| γ-GTP (IU/l) | 62 ± 74 | 47 ± 54 | 81 ± 89 | 70 ± 93 | 78 ± 78 | 0.032 |

IFN, interferon; HCV, hepatitis C virus; ALT, alanine aminotransferase; γ-GTP, gamma-glutamyl transpeptidase.
[a]*P* value for comparison among double-wild, 70-mutant, 91-mutant, and double-mutant.
[b]Values expressed as median.
[c]Data for 53 patients are missing.
[d]Data for 54 patients are missing.

been treated previously for HCV infection ($P < 0.001$). Patients in the double-wild group had significantly lower gamma-glutamyl transpeptidase (γ-GTP) levels ($P = 0.032$).

### Progress of Patients

The progress of patients in this study is shown in Figure 1. Of the 187 patients, 183 completed 4 weeks of treatment. Among them, 133 were assessed based on HCV RNA dynamics between baseline and week 4. Those completing 12 weeks of treatment totaled 181, of which 154 were assessed for HCV RNA dynamics between baseline and week 12. Those completing 24 weeks of treatment totaled 153, and all were assessed for HCV RNA quantitatively or qualitatively at week 24. Those completing 48 weeks of treatment totaled 114. These 114 patients and the 55 patients who had discontinued treatment because of treatment failure entered a follow-up period. Among these 169 patients, 164 completed 24 weeks follow-up and the sustained virological response (SVR) rate

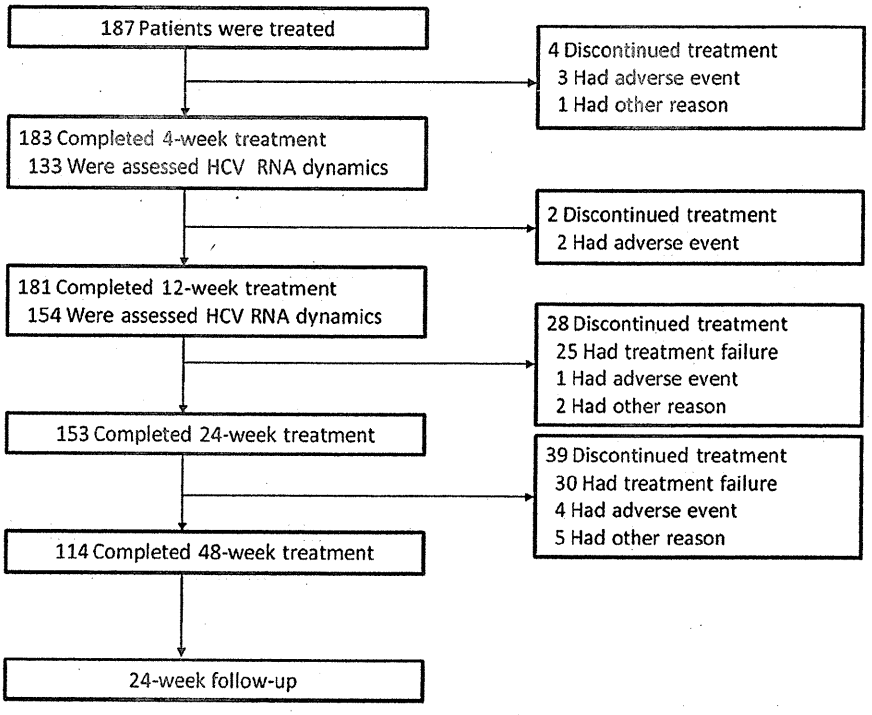


Fig. 1. Treatment and follow-up of the study patients. Treatment was discontinued for patients with <2 log decrease from the baseline HCV RNA level at week 12 or detectable HCV RNA at week 24.

TABLE II. Multivariate Analysis for Factors Associated With <1 log Decrease in HCV RNA Level at Week 4, <2 log Decrease at Week 12, Detectable HCV RNA at Week 24, and Relapse After Treatment

| Factor | Category | Odds Ratio | 95% CI | *P* value |
|---|---|---|---|---|
| HCV RNA <1 log decrease at week 4 | | | | |
| White blood cells ($\times 10^6$/l) | <5,000/5,000≤ | — | — | NS |
| γ-GTP (IU/l) | <40/40≤ | — | — | NS |
| Peg-IFN dose (μg/kg/week) | By 0.1 μg/kg/week | 0.80 | 0.67–0.97 | 0.020 |
| Core aa 70 | Wild/mutant | 1/2.80 | 1.16–6.75 | 0.022 |
| HCV RNA <2 log decrease at week 12 | | | | |
| γ-GTP (IU/l) | <40/40≤ | — | — | NS |
| Peg-IFN dose (μg/kg/week) | By 0.1 μg/kg/week | — | — | NS |
| Core aa 70 | Wild/mutant | 1/2.72 | 1.09–6.78 | 0.032 |
| Detectable HCV RNA at week 24 | | | | |
| Platelet ($\times 10^9$/l) | <150/150≤ | — | — | NS |
| γ-GTP (IU/l) | <40/40≤ | 1/2.46 | 1.02–5.95 | 0.045 |
| Core aa 91 | Wild/mutant | 1/4.11 | 1.73–9.78 | 0.001 |
| Relapse after treatment | | | | |
| Ribavirin dose (mg/kg/day) | By 1 mg/kg/day | 0.77 | 0.60–0.98 | 0.036 |
| Virological response | Complete early virological response/late virological response | 1/23.69 | 5.44–103.08 | <0.001 |

CI, confidence interval; NS, not significant difference; γ-GTP, gamma-glutamyl transpeptidase; Peg-IFN, pegylated interferon; aa, amino acid.

was 48.2% (79/164), based on per-protocol set. Among the 106 patients who had an end-of-treatment response and completed follow-up, 27 showed relapse during the follow-up period; the relapse rate was 25.5% (27/106).

## IMPACT OF CORE-RELAPSE AFTER TREATMENT (TABLE II)

Impact of core aa substitutions on <1 log viral decrease rate at week 4, <2 log at week 12, detectable HCV RNA at week 24, and virological relapse after treatment (Table II).

The impact of core aa substitutions on <1 log viral decrease rate at week 4, <2 log at week 12, detectable HCV RNA at week 24, and virological relapse after treatment (Table II).

The impact of the core aa substitutions on <1 log viral decrease at week 4, which is a predictor of non-sustained virological response; fewer than 5% of patients without 1 log decrease at week 4 had an sustained virological response [McHutchison et al., 2009] was examined. Among the 133 patients who completed 4 weeks of treatment, 31 failed to show a ≥1 log decrease of HCV RNA level at week 4. Univariate analysis for factors associated with <1 log decrease of HCV RNA level at week 4 was performed on the following variables: age, sex, body weight, BMI, history of past IFN therapy, baseline HCV RNA level, histological fibrosis and activity, white blood cell count, red blood cell count, hemoglobin level, platelet count, alanine aminotransferase (ALT) level, γ-GTP level, dose exposure of Peg-IFN and ribavirin, and aa substitutions in the HCV core protein. The results indicated that pretreatment white blood cell count, γ-GTP level, the mean dose of Peg-IFN during the first 4 weeks of treatment and single-spot substitution in the HCV RNA core position at aa 70 contributed to a <1 log decrease of HCV RNA level at week 4. Analysis of these factors by multivariate logistic regression analysis showed that substitution of aa 70 (odds ratio (OR) 2.80, 95% confidence interval (CI) 1.16–6.75, $P = 0.022$) as well as the mean dose of Peg-IFN (OR 0.80, 95% CI 0.67–0.97, $P = 0.020$) was independently associated with viral decline (<1 log) at week 4.

Next, the impact of the core aa substitutions on <2 log viral decrease rate at week 12, which is presently considered to be the most reliable predictor of non-sustained virological response [Fried et al., 2002; Davis et al., 2003] was examined. Among the 154 patients who completed 12 weeks of treatment, 25 failed to show a ≥2 log decrease of HCV RNA level at week 12. Univariate analysis was performed on the same factors in the preceding examination. As a result, pretreatment γ-GTP level, the mean dose of Peg-IFN during the first 12 weeks of treatment and single-spot substitution in the HCV RNA core position at aa 70 contributed to a <2 log decrease of the HCV RNA level. These factors were then analyzed by multivariate logistic regression analysis; only substitution of aa 70 (OR 2.72, 95% CI 1.09–6.78, $P = 0.032$) was found to be independently associated with an insufficient virological response (<2 log HCV RNA decrease from baseline level) at week 12.

The impact of the core aa substitutions on detectable HCV RNA at week 24, which is another non-sustained virological response predictor [Davis et al., 2003] was also examined. Among 153 patients who completed 24 weeks of treatment, 30 still had detectable HCV RNA at week 24. Univariate analysis revealed that pretreatment platelet count, γ-GTP level, and single-spot substitution in the HCV RNA core position at aa 91 contributed to the HCV RNA remaining positive. Multivariate logistic regression analysis, using these factors, indicated that substitution of aa 91 (OR 4.11, 95% CI 1.73–9.78, $P = 0.001$) as well as γ-GTP level (>40 IU/l) (OR 2.46, 95% CI 1.02–5.95, $P = 0.045$) was

independently associated with detectable HCV RNA at week 24.

Next, the factors associated with virological relapse after the treatment was examined. Univariate analysis was performed on the virological response (complete early virologival response or late virological response) in addition to the factors in the preceding examination, revealing the mean dose of ribavirin during the full treatment period and a late virological response, but not aa substitutions (single-spot substitution in the HCV RNA core position at aa 70, $P = 0.467$; aa 91, $P = 0.776$).

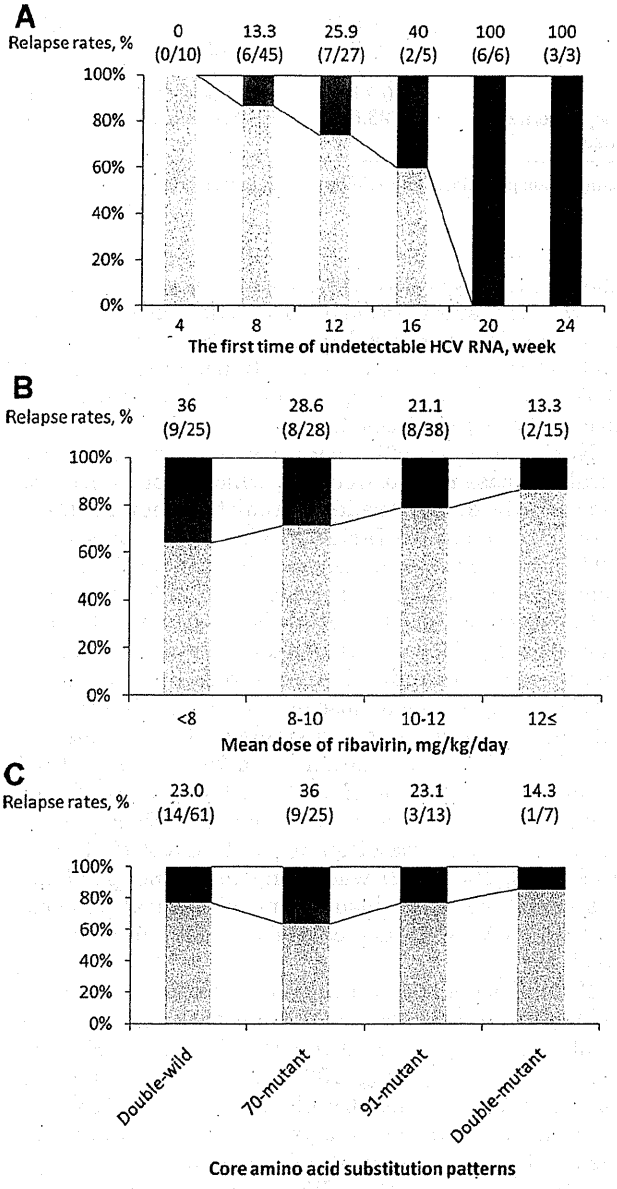


Fig. 2. Relapse rates according to the timing of HCV RNA disappearance (**A**), mean ribavirin dose (**B**), and core amino acid substitution patterns (**C**) in patients who had end-of-treatment response and completed 24-week follow-up. Relapse rates are shown as percentages and the number of patients with relapse in relation to the total number of patients examined is shown at the top of each column. Gray bar, sustained virological response; black bar, relapse.

These factors were analyzed by multivariate logistic regression analysis. This analysis revealed that the mean ribavirin dose (OR 0.77, 95% CI 0.60–0.98, $P = 0.036$) and a late virological response (OR 23.69, 95% CI 5.44–103.08, $P < 0.001$) were independently associated with relapse.

### Relapse Rates According to the Timing of HCV RNA Disappearance, Ribavirin Dose, and Core aa Substitution Patterns

The relapse rates were indicated according to the time to the first non-detection of HCV RNA, mean ribavirin dose and core aa substitution patterns (Fig. 2). The relapse rate was 0% (0/10) in patients with undetectable HCV RNA during 1–4 weeks, and increased 13.3% (6/45) during 5–8 weeks, 25.9% (7/27) during 9–12 weeks, 40% (2/5) during 13–16 weeks, 100% (6/6) during 17–20 weeks, and 100% (3/3) during 21–24 weeks (Fig. 2A). Similarly, the relapse rates increased as the mean ribavirin dose decreased; 13.3% (2/15) in patients receiving ≥12 mg/kg/day of ribavirin, 21.1% (8/38) at 10–12 mg/kg/day, 28.6% (8/28) at 8–12 mg/kg/day, and 36% (9/25) at <8 mg/kg/day (Fig. 2B). On the other hand, the relapse rates were similar among the four core aa substitution patterns; 23.0% (14/61) in patients in the double-wild group, 36% (9/25) in 70-mutant group, 23.1% (3/13) in 91-mutant group, and 14.3% (1/7) in double-mutant group (Fig. 2C). In the subgroup of patients receiving <10 mg/kg/day of ribavirin, no significant difference of the relapse rates was observed between double-wild group and 70-mutant and/or 91-mutant group (31.3% (10/32) in double-wild group vs. 33.3% (7/21) in 70-mutant and/or 91-mutant group), and also in the patients receiving ≥10 mg/kg/day of ribavirin (13.8% (4/29) in double-wild group vs. 25% (6/24) in 70-mutant and/or 91-mutant group) (Fig. 3). Among patients with complete early virological response, the relapse rates were also similar between double-wild group and 70-mutant and/or 91-mutant group (13.7% (7/51) in double-wild vs. 18.4% (7/38) in 70-mutant and/or 91-mutant group). The impact of core aa substitutions on relapse rates in patients with late virological response could not be assessed because of the small number of patients.

## DISCUSSION

Kobayashi et al. [2010] investigated the clinical and virological factors influencing these core aa substitutions in patients infected with HCV genotype 1 who had not received antiviral therapy, and found that HCV variants with wild type of core aa 70 and 91 significantly decreased with age, while those with the mutant type of core aa 70 and/or 91 significantly increased with age. Furthermore, they demonstrated that the proportion of patients with the mutant type of core aa 70 HCV variant significantly increased with an elevated γ-GTP level and a decrease in platelet counts. In this study, the significant differences of baseline demographics between patient groups according to core aa substitution pat-

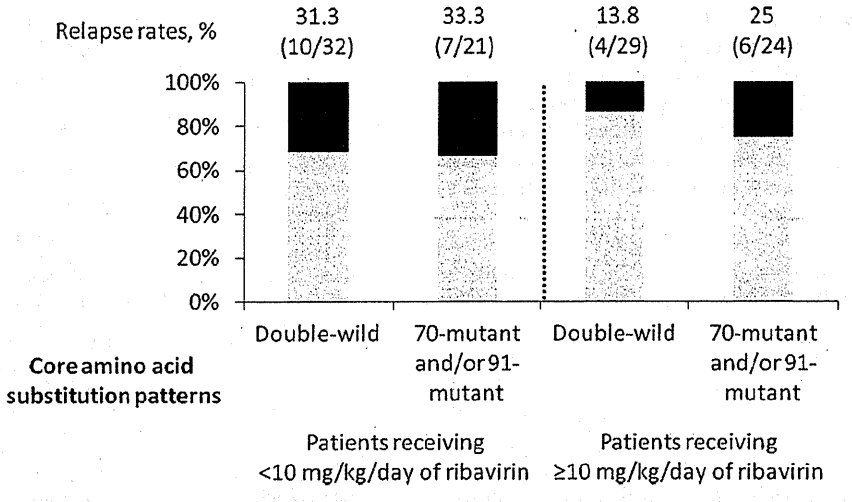


Fig. 3. Relapse rates according to core amino acid substitution patterns in patients receiving <10 mg/kg/day and receiving ≥10 mg/kg/day of ribavirin. Relapse rates are shown as percentages and the number of patients with relapse in relation to the total number of patients examined is shown at the top of each column. Gray bar, sustained virological response; black bar, relapse.

terns were similarly found in age, platelet count, and γ-GTP level. Accordingly, this study cohort had no specific bias and seems to reflect the natural background of the patients according to the HCV variance. In this study, the impact of HCV core aa substitutions on the virological response were evaluated by multivariate analysis, in order to resolve the bias of patient background factors among the groups classified according to the core aa substitution patterns. Recently, Abe et al. [2010] reported that the human genotype of the rs8099917 SNP at the IL28B locus was associated with lower γ-GTP level and viral wild type of core aa 70 and 91. Possibly these differences of IL28B genotype may influence the difference of patient background factors. Further studies are needed to clarify the relationship between human genetic variation and HCV core amino acid substitutions.

The HCV core protein has been reported to have an effect on a variety of cellular functions [Lai and Ware, 2000; Joo et al., 2005; Ariumi et al., 2007; Waris et al., 2007; Osna et al., 2008]. Currently, aa substitutions in the HCV core region has been thought to be related with outcome of antiviral therapy [Akuta et al., 2005; Donlin et al., 2007] and also the development of hepatocellular carcinoma [Akuta et al., 2007a; Hu et al., 2009]. Importance of core aa substitutions, especially at aa 70 and 91, comes to be recognized, and the new method to detect these substitutions easily has been proposed [Nakamoto et al., 2009]. As for the mechanism of antiviral activity on core aa substitutions, Ikeda et al. [2010] showed that core aa substitutions were not associated with intracellular antiviral response to IFN-alpha by in vitro analysis. The mechanism of antiviral activity and hepatocarcinogenesis on core aa substitutions has not been elucidated enough, so far. Further in vitro studies will be needed to clarify this.

Previous studies showed that patients with substitution of core aa 70 often had slow or no decrease in HCV RNA levels during the early phase of IFN-alpha treatment [Akuta et al., 2005, 2007b,c; Donlin et al., 2007]. Consistent with these reports, multivariate analysis in this study revealed that substitution of core aa 70 could be independently associated with insufficient viral decline during the first 12 weeks after the treatment (decline of <1 log from baseline at week 4, <2 log at week 12). This suggests that patients with substitution of core aa 70 are likely to fail to have a sustained virological response. On the other hand, dose exposure of Peg-IFN during the first 4 weeks of treatment was also independently linked to a minimal decline in HCV RNA (<1 log) at week 4 in this study. This suggests that maintaining the dose of Peg-IFN as high as possible until the disappearance of HCV RNA can help avoid treatment failure [McHutchison et al., 2002; Oze et al., 2009], especially in patients with substitution of core aa 70. On the other hand, substitution of core aa 91 was independently associated with detectable HCV RNA at week 24. This suggests that patients with substitution of core aa 91 are likely to achieve non-sustained virological response even if they had a ≥2 log decline in the HCV RNA level at week 12. The reason for the difference of the impact on virological response is not yet clear.

Multivariate logistic regression analysis also showed that the dose exposure of ribavirin during the full treatment period and having late virological response were independently associated with relapse. As for ribavirin exposure, it has been previously demonstrated that the relapse rate among patients responding to the treatment showed a decline in relation to the increase in the dose of ribavirin [Hiramatsu et al., 2009]. In this study, relapse rates were also decreased from 36% to 13.3% with increasing dose exposure of ribavirin among patients with end-of-treatment response. These results

confirm that maintaining a sufficient dose of ribavirin during the full treatment period could reduce the possibility of relapse, and that an extended duration of therapy for patients with late virological response could increase the chance of achieving sustained virological response, regardless of core aa substitution patterns [Berg et al., 2006; Pearlman et al., 2007; Ferenci et al., 2010].

In this study, the COBAS Amplicor HCV Test v2.0, with a lower limit of detection of 50 IU/ml, was used to assess the serum HCV RNA. Recently, real-time PCR-based HCV RNA assays with a higher sensitivity, COBAS TaqMan HCV assay (Chugai-Roche Diagnostics), with a lower limit of detection of 15 IU/ml, have been introduced. Sarrazin et al. [2010] compared virological response rates that were originally tested by COBAS Amplicor assay with those retested by COBAS TaqMan assay, using the same cohort. Among genotype 1 patients, complete early virological response and sustained virological response rates were similar when virological responses were defined as <50 IU/ml by Amplicor assay (77% and 87%) and <15 IU/ml by TaqMan assay (76% and 88%). Therefore, measuring HCV RNA by the Amplicor assay in this study would have little affects on the results.

In conclusion, the results have demonstrated that substitution of core aa 70 could be independently associated with an insufficient decline in HCV RNA level during first 12 weeks, and substitution of core aa 91 was independently associated with detectable HCV RNA at week 24, all of which were considered to be important negative predictors of attaining sustained virological response in patients with HCV genotype 1 treated with Peg-IFN plus ribavirin. On the other hand, only dose exposure of ribavirin and no complete early virological response was independent predictors of virological relapse among patients with end-of-treatment response, not substitution of core aa 70 or 91. The aa substitution patterns of the HCV core protein can be an important pretreatment predictor for non-response in patients with HCV genotype 1 treated with Peg-IFN plus ribavirin, but not for relapse after the completion of therapy.

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***Original Article***

# Hepatitis C virus-specific CD8+ T cell frequencies are associated with the responses of pegylated interferon-α and ribavirin combination therapy in patients with chronic hepatitis C virus infection

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*Aim:* Hepatitis C virus (HCV)-specific cytotoxic T lymphocytes (CTLs) play critical roles in elimination of the HCV-infected hepatocytes. However, the mechanism of HCV elimination by pegylated interferon-α (peg-IFNα) plus ribavirin is not fully understood. We examined HCV-specific CTL responses during this combination therapy.

*Methods:* CD8+ T cells were isolated from 16 HCV infected patients treated by this combination therapy and were subjected to IFN-γ enzyme-linked immunospot (ELISPOT) assay.

*Results:* The numbers of IFN-γ spots against HCV Core or NS3 protein-derived peptides in HCV patients before treatment were similar to those in healthy donors, and those in HCV patients significantly increased 4 weeks after the initiation of combination therapy. All HCV Core or NS3 proteins-derived peptides specific CD8+ T cells responses in pre-treated patients were not associated with ALT levels and HCV viral loads of HCV patients before treatment. And those in pre-treated patients were similar between sustained virologic responder (SVR) patients and non-SVR patients. Significant increase of HCV Core or NS3 proteins-derived peptides specific CD8+ T cells responses between before and 4 weeks after this combination therapy were observed in SVR patients, but not in non-SVR patients.

*Conclusions:* These results demonstrated that significant increase of HCV-specific CD8+ T cells at 4 weeks after the initiation of IFN treatment might be associated with the elimination of HCV. Our findings suggest that the reactivity against HCV Core and NS3 proteins-derived peptides might be useful in predicting the clinical outcome of the combination therapy of peg-IFNα and ribavirin.

**Key words:** chronic hepatitis C, HCV-specific CTL, IFN-γ ELISPOT, peg-IFNα, ribavirin

## INTRODUCTION

CHRONIC INFECTION OF Hepatitis C virus (HCV) often leads to cirrhosis and hepatocellular carcinoma (HCC), which causes the poor prognosis of HCV-infected patients.[1,2] Combination therapy of pegylated interferon-α (Peg-IFNα) plus ribavirin is standard treatment for patients with chronic hepatitis C (CH-C), and sustained virologic response (SVR) in this combination therapy occurs in about 40–60% of genotype 1 patients,[1,2] which can improve the prognosis of HCV-infected patients. HCV-specific cytotoxic T lymphocytes (CTLs) is believed to play essential roles in determining the course of chronic infection,[3] and the insufficient activation, dysfunction, suppression of CTLs may cause persistent infection of HCV.[4–6] The elimination of HCV by HCV-specific CTLs is believed to consist of second slope of decay after viral decay during the first 24–48 h of IFN therapy.[7] However, the detail immune mechanism of HCV elimination by this combination therapy is not fully understood. In addition to direct antiviral

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property of Peg-IFNα and ribavirin against HCV infection, this combination therapy might have immunomodulatory activity. IFN-α enhances the maturation of antigen-presenting cells and CD4+ T cell function, but with little effect on CTLs. In contrast, ribavirin could induce a switch from Th2 to Th1 profile.[8] Although the base line immune responses of CTLs have been reported to be associated with the achievement of SVR in a few reports[7,8] , even now there are relatively little reports examining the detail of HCV-specific CTL responses during this combination therapy.

IFN-γ enzyme-linked immunospot (ELISPOT) assay allows detection of finally differentiated effector CTLs, which means the ELISPOT data reflect the in vivo situation.[9–11] In the current study, we evaluated the HCV Core and NS3 proteins-derived peptides specific CD8+ T cells responses of the HCV infected patients by IFN-γ ELISPOT assay and examined the relationship between CTL activity and the clinical outcome of the combination therapy of Peg-IFNα plus ribavirin. The frequencies of HCV-specific CD8+ T cells in pre-treated HCV patients were not associated with antiviral activity of this combination therapy in SVR. However, the significant increase of HCV-specific CD8+ T cells at 4 weeks after the starting of IFN treatment could be observed in SVR patients, but not in non-SVR patients. Our findings suggest that the reactivity against HCV Core and NS3 proteins-derived peptides might be useful in predicting the clinical outcome of this combination therapy.

## MATERIALS AND METHODS

### Patients

SIXTEEN PATIENTS CHRONICALLY infected with HCV were examined for HCV specific CTL responses during the combination therapy of Peg-IFNα plus ribavirin. All patients enrolled in this study were infected with HCV genotype 1b with a high viral load and were HLA-A2 positive. The patients who were infected with other viruses (Hepatitis B virus, Human immunodeficiency virus) or had other forms of liver disease (alcohol liver disease, autoimmune hepatitis) were excluded from this study. Informed consent, under an Institutional Review Board-approved protocol, was obtained from each patient. All patients received Peg-IFNα-2b (PEGINTRON, Schering-Plough, Kenilworth, NJ) plus ribavirin (REBETOL, Schering-Plough) for the duration of the study of 48–72 weeks. In only one patient (Patients#11), treatment was stopped at 24 weeks because this patient remained HCV-RNA positive after 24 weeks and developed significant side effect. To evaluate the antiviral activity, serum HCV RNA levels were quantified during the combination treatment. Serum HCV RNA level was quantified using the COBAS AMPLICORE HCV MONITOR test (version 2.0; Roche Diagnostics, Branchburg, NJ). SVR was defined as the absence of detectable serum HCV RNA at 24 weeks after the end of the combination therapy. All treated patients were assessed the antiviral responses (SVR or non-SVR) as previously described.[12]The characteristics of patients with chronic HCV infection were summarized in Table 1.

### CD8+ T cells isolation from peripheral blood mononuclear cells (PBMC)

PBMC was obtained from 16 treated HCV infected patients before IFN treatment (pre-IFN) and 4 weeks after starting of this combination therapy (IFN-4week) and six healthy donors. CD8+ T cells were isolated from PBMC by magnetic cell sorting using CD8 MicroBeads according to the manufacturer's instructions (Miltenyl Biotech, Auburn, CA). More than 95% of the cells were CD8+ lymphocytes.

### IFN-γ ELISPOT assays for HCV Core and NS3 protein-derived peptide-specific CD8+ T cells responses

To evaluate the frequencies of CD8+ T cells recognizing peptide epitopes, IFN-γ ELISPOT assay were performed as previously described.[11] Briefly, 96-well multiscreen hemagglutinin antigen plates (Millipore, Billerica, MA) were coated with 10 μg/mL of anti-human IFN-γmAb (1-D1K; Mabtech, Stockholm) in phosphate-buffered saline (PBS) overnight at 4°C. Unbound antibody was removed by four successive washing with PBS. After blocking the plates with RPMI 1640/10% human serum (1 h, 37°C), $1 \times 10^5$ CD8+ T cells were co-cultured with $2 \times 10^4$ T2.DR4 cells (HLA-A2 positive peptide-presenting cells generously provided from Dr Walter J. Storkus, University of Pittsburgh, School of Medicine, Pittsburgh, PA) pulsed with HCV Core and NS3 derived peptides (a final concentration of 10 μg/mL). HLA-A2-restricted HCV Core protein derived peptides ($Core_{35-44}$, YLLPRPGPRL, $Core_{131-140}$, ADLMGYIPLV) or NS3 protein derived peptides ($NS3_{1073-1081}$, CINGVCWTV, $NS3_{1406-1415}$, KLVALGINAV) were synthesized as previously described.[13] Negative control wells contained CD8+ T cells with T2.DR4 cells pulsed with HIV-$nef_{190-198}$ peptide (AFHHVAREL). After 24 h incubation of the plates, cells were removed from the ELISPOT well by washing and captured cytokine was detected at sites of their secretion

Table 1 Characteristics of patients with chronic hepatitis C virus (HCV) infection

| Subject | Age | Sex | HCV-RNA (KIU) | ALT (U/l) | Treatment duration | SVR |
|---|---|---|---|---|---|---|
| 1 | 43 | F | 440 | 17 | 48 week | SVR |
| 2 | 56 | M | 2000 | 146 | 48 week | non |
| 3 | 49 | F | 1200 | 31 | 72 week | SVR |
| 4 | 49 | M | 340 | 106 | 48 week | SVR |
| 5 | 65 | F | 3800 | 24 | 72 week | SVR |
| 6 | 58 | M | 320 | 25 | 48 week | SVR |
| 7 | 56 | M | 2551 | 24 | 48 week | non |
| 8 | 55 | M | 939 | 43 | 48 week | SVR |
| 9 | 46 | M | 1200 | 64 | 48 week | SVR |
| 10 | 46 | M | 1059 | 42 | 48 week | SVR |
| 11 | 43 | M | 407 | 91 | 24 week | non |
| 12 | 63 | F | 1621 | 61 | 48 week | non |
| 13 | 63 | F | 1841 | 63 | 48 week | non |
| 14 | 47 | M | 458 | 41 | 48 week | SVR |
| 15 | 36 | M | 1024 | 79 | 48 week | non |
| 16 | 61 | F | 677 | 148 | 48 week | non |

ALT, alanine aminotransferase; F, female; M, male; non, non-SVR; SVR, sustained virologic response.

by incubation for 2 h with biotinylated mAb anti-human IFN-γ (7-6B-1, Mabtech) at 2 μg/mL. Plates were washed six times and avidin-peroxidase complex (Vectastain Elite Kit, Vector Laboratories, Burlingame, CA) were added for 1 h. Unbound complex was removed by washing and 3-Amino-9-ethylcarbazole substrate (Sigma, St Louis, MO) was added for 5 min. The data are represented as mean IFN-γ spots per 100 000 T cells analyzed.

### Statistics

All values were expressed as the mean and standard deviation (SD). The statistical significance of differences between the groups was determined by applying Mann–Whitney *U*-test. We defined statistical significance as $P < 0.05$.

## RESULTS

### Analysis of HCV derived peptide-specific IFN-γ release of peripheral blood CD8+ T cells in ELISPOT assay

WE ASSESSED PERIPHERAL blood CD8+ T cell responses against HCV derived peptides ($Core_{35-44}$, $Core_{131-140}$, $NS3_{1073-1081}$, $NS3_{1406-1415}$) in 16 HLA-A2+ HCV patients and 6 healthy donors. As shown in Figure 1, the numbers of IFN-γ spots (per 100 000 CD8+ T cells) observed for T cell responses against HCV peptides in pre-IFN patients were as low as those observed in healthy HLA-A2+ donors. In contrast, significant elevations of ELISPOT reactivity to three peptides ($Core_{131-140}$, $NS3_{1073-1081}$, $NS3_{1406-1415}$) were observed in IFN-4week patients compared with healthy donors. The number of IFN-γ spots against $Core_{35-44}$ peptides in IFN-4week patients also tended to be higher than those in healthy donors. In treated HCV patients, the numbers of IFN-γ spots against all four HCV derived peptides in IFN-4week patients were significantly higher than those in pre-IFN patients (Fig. 1). We also examined whether the frequencies of HCV-specific CD8+ T cell responses were associated with sex difference. The frequencies of CTLs against all four peptides were similar between males and females before and 4 weeks after starting treatment (data not shown).

### HCV-specific CD8+ T cell responses in pre-IFN patients were not associated with the antiviral activity of the combination therapy of Peg-IFNα-2b plus ribavirin

We examined the association between HCV-specific CD8+ T cell responses in pre-IFN patients and ALT levels or HCV viral load before treatment. No association was observed between the frequencies of HCV-specific CD8+ T cells in pre-IFN patients and ALT levels or HCV viral load of pre-treated patients (Fig. 2).

We next examined whether HCV-specific CD8+ T cell responses in pre-IFN patients were associated with the antiviral activity of this combination therapy. As shown in Figure 3, the frequencies of CD8+ T cell responses against all four HCV proteins-derived peptides in

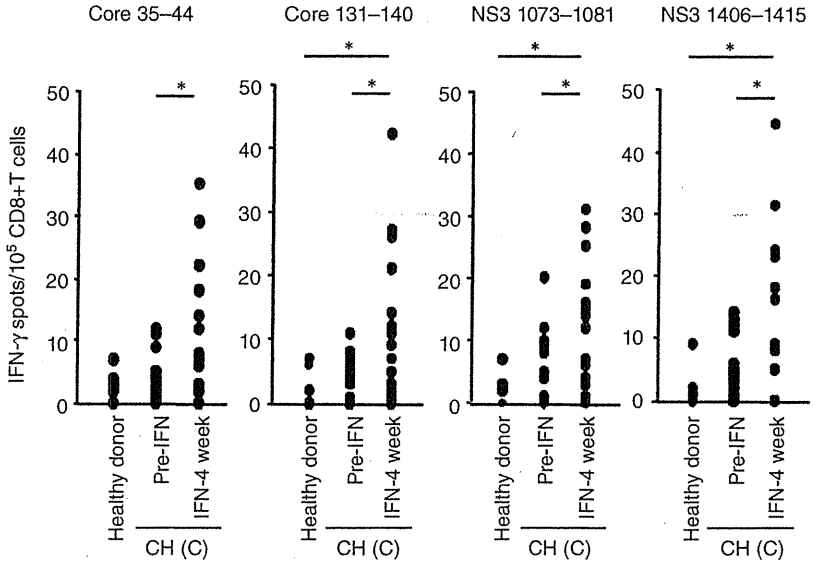


**Figure 1** Interferon (IFN)-γ enzyme-linked immunospot (ELISPOT) analysis of hepatitis C virus (HCV)-specific CD8+ T cell responses in HCV patients treated with the combination therapy of peg-IFNα plus ribavirin. Peripheral blood CD8+ T cells were isolated from HLA-A2+ healthy donors and chronic hepatitis C (CH-C) patients. The CH-C patients were treated with the combination therapy of peg-IFNα plus ribavirin and PBMC were isolated from pre-treated patients (Pre-IFN) and treated patients 4 weeks after starting treatment (IFN-4week). HCV-specific CD8+ T cell responses were evaluated by IFN-γ ELISPOT as outlined in "Materials and Methods". Data are reported as IFN-γ spots/ 100 000 CD8+ T cells and represent the mean of triplicate determinations. T cell reactivity against T2.DR4 cells pulsed with HLA-A2-presenting HIV-nef$_{190-196}$ epitope served as the negative control in all cases, and this value was subtracted from all experimental determinations to determine HCV specific spots numbers. Each symbol within a panel represents the response of an individual donor to the indicated HLA-A2-presenting HCV Core- or NS3-peptides. *$P < 0.05$.

pre-IFN patients were not significantly different between SVR, the group of the patients who were observed SVR, and non-SVR, the group of the patients who were not observed SVR. These results suggested that the baseline HCV-specific CD8+ T cell responses in HCV patients were not associated with the antiviral activity of this combination therapy.

### Significant early elevation of HCV-specific CD8+ T cell responses were associated with the antiviral activity of the combination therapy of Peg-IFNα plus ribavirin

We examined the association between early elevation of HCV-specific CD8+ T cell responses and the antiviral activity of this combination therapy. We evaluated the frequencies of CD8+ T cell responses against HCV proteins-derived peptides before and 4 weeks after starting treatment. As shown in Figure 4, in SVR patients, the frequencies of CD8+ T cell responses against all four HCV peptides (Core$_{35-44}$, Core$_{131-140}$, NS3$_{1073-1081}$, NS3$_{1406-1415}$) increased significantly 4 weeks after starting treatment. In contrast, the frequencies of CD8+ T cell responses against all four HCV peptides did not increase in non-SVR patients. These results demonstrated that significant early elevation of HCV-specific CD8+ T cell responses were associated with the antiviral activity of this combination therapy.

## DISCUSSION

HCV-SPECIFIC CD8+ CTLs have been reported to play a significant role in the elimination of HCV in acute hepatitis of HCV.[4,9] In contrast, in chronic infection of HCV, HCV-specific CD8+ T cell responses were weak and were directed against a limited series of epitopes compared with acute hepatitis.[9] These might cause persistent infection of HCV in the HCV infected host. However, conflicting results have been reported with respect to HCV-specific CD8+ T cell responses on the antiviral activity of IFN therapy. IFNα monotherapy may promote viral clearance by enhancing the host CTL responses.[14,15] But Rehermann et al. reported that CTL

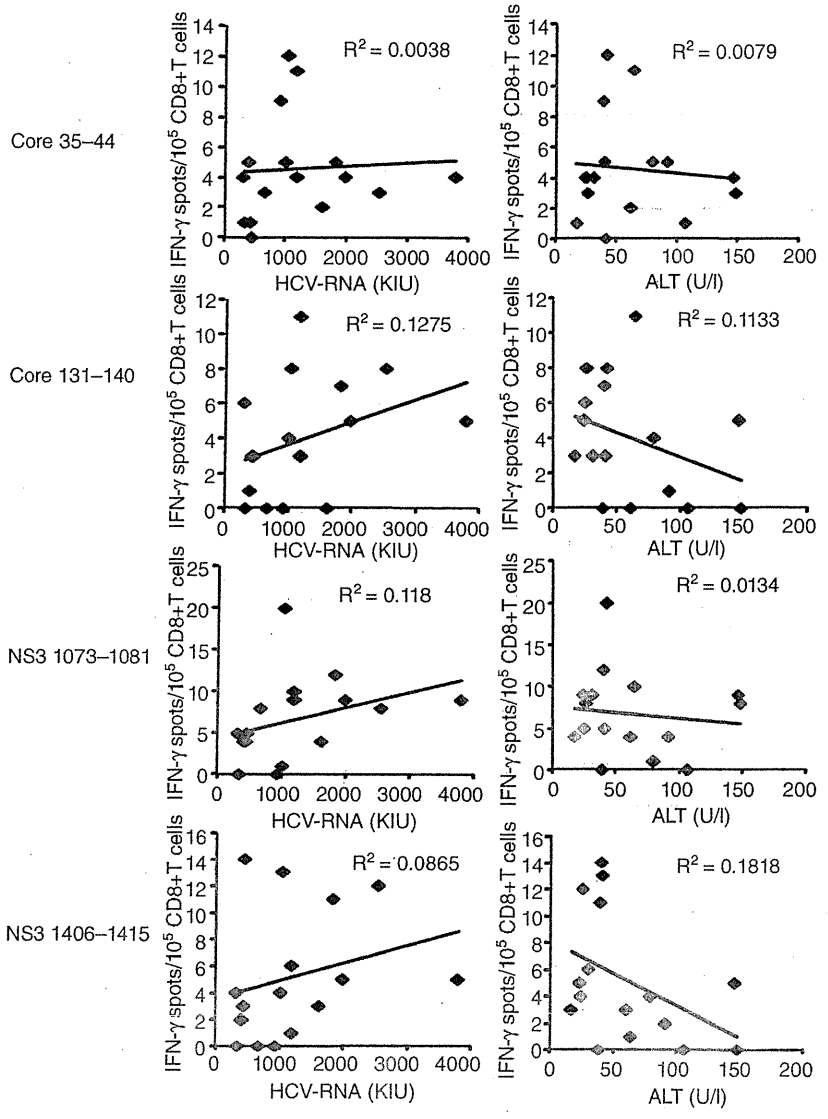


**Figure 2** The association between the hepatitis C virus (HCV)-specific CD8+T cell responses of pre-IFN patients and the serum alanine aminotransferase (ALT) levels or the HCV viral load of patients before treatment. The frequencies of HCV Core and NS3 proteins-derived peptides specific CD8+ T cell responses in pre-IFN HCV patients were evaluated by interferon (IFN)-γ enzyme-linked immunospot (ELISPOT). We examined the association between the frequencies of HCV Core and NS3 proteins-derived peptides specific CD8+ T cell responses in pre-IFN HCV patient and the serum ALT levels or HCV viral loads of patients before treatment.

precursor frequencies against a range of HCV epitopes did not change during or after the course of IFNα monotherapy.[13] Recently, the combination therapy of PegIFNα plus ribavirin is standard treatment in the treatment of HCV infected patients with the better results of viral clearance compared with IFNα monotherapy. This suggested that this combination therapy might modify the HCV specific CD8+ T cell responses. We evaluated HCV-specific CD8+ T cell responses by IFN-γ ELISPOT assay, a functional assay of T cells. Significant increase of the frequencies of HCV-specific CD8+ T cells between pre-IFN and IFN-4week could be observed in SVR patients, but not in non SVR patients. This is consistent with the previous report of evaluating the frequencies of HCV-specific CTLs by direct ex vivo staining with HCV-specific pentamers.[16] Thus the evaluation of reactivity against HCV Core and NS3 proteins-derived peptides might be useful in predicting the clinical outcome of this combination therapy.

It has been reported that complete early virologic response (cEVR), which means HCV RNA negativity at week 12, is strongly related to SVR in the combination therapy of Peg-IFNα plus ribavirin.[12,17] cEVR itself has been reported to be an independent predictive factor of

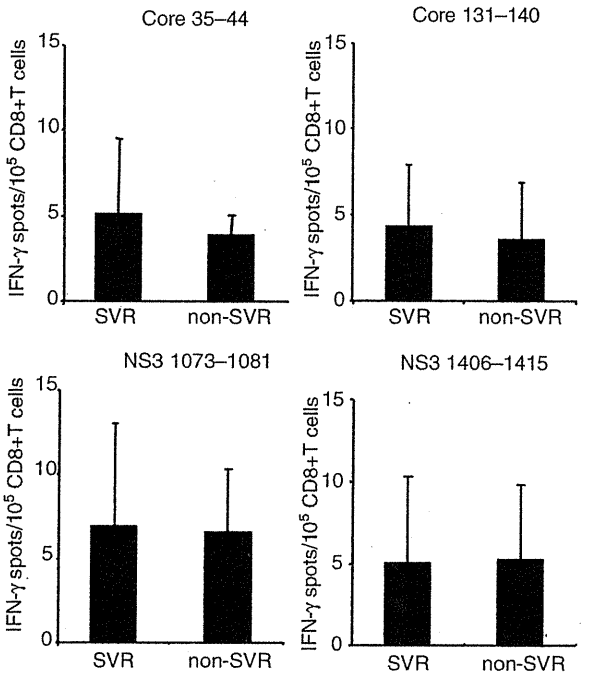


**Figure 3** Comparison of the frequencies of hepatitis C virus (HCV)-specific CD8+ T cells in pre-treated HCV patients between sustained virologic response (SVR) and non-SVR. HCV Core and NS3 proteins-derived peptides specific CD8+ T cell responses in pre-treated HCV patients were evaluated by interferon (IFN)-γ enzyme-linked immunospot (ELISPOT). We analyzed the association between the HCV-specific CD8+ T cell responses and the achieving of SVR. SVR: patients who were observed SVR, non-SVR: patients who were not observed SVR.

SVR.[1,12] We also examined the association between cEVR and early elevation of HCV-specific CD8+ T cell responses. The frequencies of CD8+ T cell responses against all four HCV derived peptides in pre-IFN patients were not significantly different between cEVR and non-cEVR (Tatsumi T, unpublished data). In cEVR patients, the frequencies of CD8+ T cell responses against three HCV peptides $Core_{35-44}$, $Core_{131-140}$, $NS3_{1406-1415}$) increased significantly 4 weeks after the starting treatment and those against $NS3_{1073-1081}$ peptide tended to increase although these were not significant. In contrast, the frequencies of CD8+ T cell responses against all four HCV peptides did not increase in non-cEVR patients (Tatsumi T, unpublished data). The cEVR results were almost similar to those of the SVR results. Although we could not evaluate the HCV RNA levels at 4 week after starting treatment, the cEVR results suggested that early elevation of the frequencies of HCV-specific CD8+ T cell responses might reflect the decrease of viral load of HCV.

CD8+ CTL activities in pre-treated HCV patients have been reported to be very low.[7,18,19] Consistent with the previous observations, the frequencies of HCV specific CD8+ T cell in pre-treated patients were also low in our study. The frequencies of HCV-specific CD8+ T cells in pre-treated patients were not associated with the HCV viral load and the serum ALT levels of patients before treatment. Several reports demonstrated that the baseline presence of HCV-specific CTLs prior to treatment was associated with viral clearance.[7,18] However, the frequencies of HCV-specific CD8+ T cells in pre-treated patients were not associated with the achievement of SVR in our study. In previous other reports, whole PBMC isolated from treated patients were used to evaluate the antiviral activity of HCV-specific CD8+ T cells. In our study, enriched CD8+ T cells obtained by magnetic sorting methods were used to enhance the sensitivity for the detection of HCV-specific CD8+ T cells. Both ELISPOT and staining with tetramers/pentamers could be applied for immunological monitoring for peptide-specific CTLs.[20] ELISPOT can detect activated functional CTLs, and tetramers/pentamers staining can detect peptide-specific CTLs.[20] In our study, we assessed the HCV-specific CD8+ T cell responses by IFN-γ ELISPOT, which is the most well-established methods and has already applied for immunological monitoring in cancer patients.[11] Recently perforin- or granzyme B-ELISPOT assays have also been reported. However, due to limitations in cell numbers of PBMC isolated from HCV patients, we were unable to apply another system of immunological monitoring and test other functional molecules. If we can apply these ELISPOT assays, we could directly evaluate the cytotoxic activity of HCV-specific CTLs.

In our study, the frequencies of HCV-specific CD8+ T cells in pre-treated patients were similar between SVR and non-SVR patients. In contrast, significant increase of the frequencies of HCV-specific CD8+ T cells between pre-IFN and IFN-4week could be observed in SVR patients, but not in non SVR patients. Caetano et al. evaluated the HCV-specific CD8+ T cells by HLA class I pentamers specific for the one HCV-Core epitope and one NS3 epitope which were same as we used.[16] They demonstrated that the increase of the frequencies of HCV-specific CTLs at 1 month after starting treatment was mainly due to terminally differentiated cells as well as, to a lesser extent, central memory cells in SVR patients and, in contrast, the increase of HCV-specific

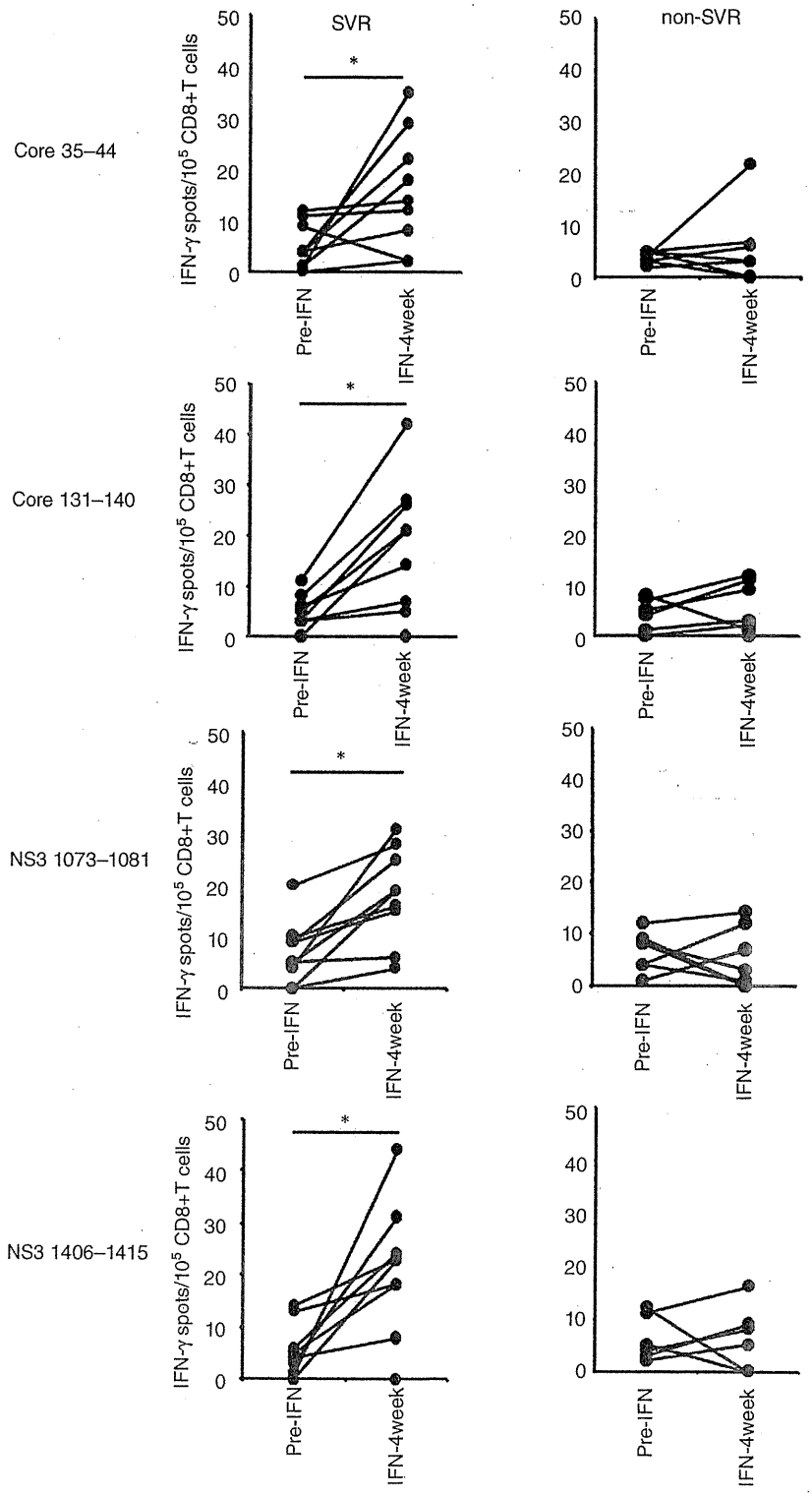


**Figure 4** Analysis of the association of the change of hepatitis C virus (HCV)-specific CD8+ T cell responses between pre-IFN and IFN-4week chronic hepatitis C (CH-C) patients with the achieving sustained virologic response (SVR). Peripheral blood CD8+ T cells were isolated from pre-IFN and IFN-4week patients. HCV-specific CD8+ T cell responses were evaluated by interferon (IFN)-γ enzyme-linked immunospot (ELISPOT) assay. We analyzed the association of HCV-specific CD8+ T cell responses in treated CH-C patients with the achieving SVR. Each symbol within a panel represents the response of an individual donor to the indicated HLA-A2-presenting HCV Core or NS3 protein-derived peptides. The treated patients were divided into two groups; SVR group and non-SVR group. $^*P < 0.05$.

pre-terminally differentiated CD8+ T cells was also observed in non-SVR patients.[16] These results suggested that CTLs maturation efficiently occurred in SVR patients. HCV or HCV-gene products have been reported to inhibit the maturation pathway of CTLs.[5,21] Thus the decrease of viral load during this combination therapy may induce CTL maturation.

We demonstrated that the achievement of SVR in this combination therapy was associated with the early elevation of HCV-specific CD8+ T cell responses, but not with the pre-treated levels of HCV-specific CD8+ T cell responses. These results suggested, at least, that the enhancement of HCV-specific CD8+ T cell responses might play critical roles in the second slope of viral clearance by this combination therapy. The increasing frequencies of HCV-specific CD8+ T cells have also been reported to be associated with SVR during the combination therapy by evaluating with pentamers of HCV-specific peptides.[16] Ribavirin has immunomodulatory effect with a switch from Th2 to Th1 cytokine profile.[22] The combined use of pegIFNα and ribavirin might have more immunomodulatory effect to generate HCV specific CTLs. However, even now, this should be elucidated to develop better treatment of chronic hepatitis C.

Although CTL responses to HCV are multi-specific,[13,23] we and others tested only small part of the known CTL epitopes of HCV, which do not comprise all potential HLA A2-restricted CTL epitopes of HCV. HCV may have mutated and escaped from the CTL responses to the corresponding epitopes in the chronically infected patients. The epitopes used in our study have been applied to the detection of HCV-specific CTLs in several other previous studies,[5,15,16] which support the usefulness of the selected epitopes. Our results demonstrated that the increases of the frequencies of CD8+ T cells against four synthesized peptides were associated with the antiviral activity of this combination therapy. Thus the selected epitopes used in our experiments were probably stable, at least, during the 4 weeks after starting treatment.

In spite of recent progress for HCV treatment, there remains significant room for improvement. To date, a variety of viral factors and host factors that correlate with SVR in the combination therapy have been noted. Recently, in addition to viral factors and host factors, response and adherence to treatment have been noted.[2] To establish the better treatment, the detail mechanism of HCV elimination should be elucidated. In the present study, we demonstrated that early enhancement of HCV-specific CD8+ T cell responses was associated with the achieving SVR in this combination therapy. These suggest that activation of antiviral CTLs might be involved in the elimination of HCV. The early elevation of HCV-specific CTL responses in treated HCV patients may be a candidate for predicting SVR in this combination therapy.

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