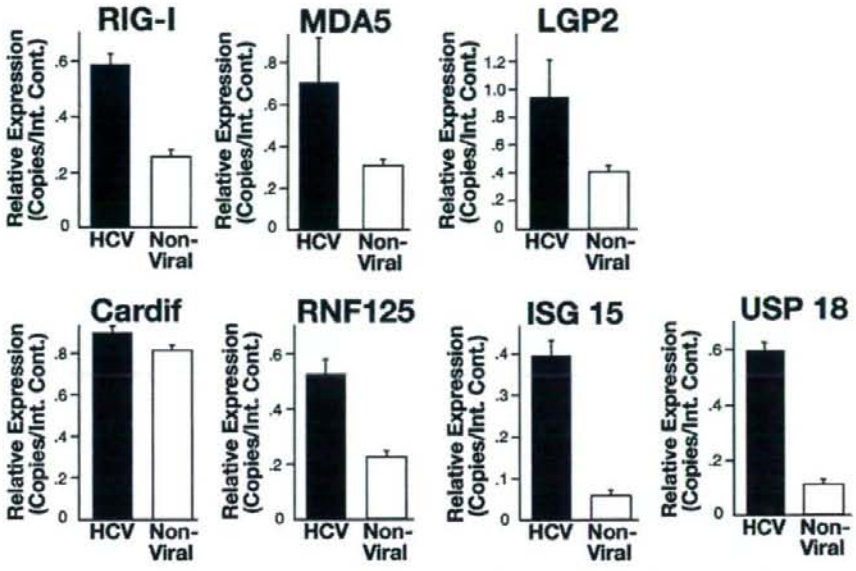


**Figure 1.** Comparison of hepatic gene expression levels between chronic hepatitis C patients (n = 74) and nonviral liver disease patients (n = 5). Expression levels of RIG-I, MDA5, LGP2, Cardif, RNF125, ISG15, and USP18 are shown. *Error bars* indicate the standard error. Upon HCV infection, expression of these genes except Cardif was stimulated. The *P* values determined by Mann–Whitney *U* test between 2 groups were as follows: RIG-I, *P* .02; MDA5, *P* .01; LGP2, *P* .005; Cardif, *P* .7; RNF125, *P* .06; ISG15, *P* .007; USP18, *P* .004.



## Results

### *Patient Characteristics*

According to the final virologic response, patients were classified into 3 groups: 30 were SVR, 24 were TR, and the remaining 20 were NVR, as shown in Table 1. Viral decline rates in NVR were significantly lower in both the first and second phases of HCV dynamics. It should be noted that most NVR patients exhibited no second-phase viral decline.

Data on factors that were available before starting the treatment were compared according to virologic response by univariate analysis. As shown in Table 1, only age and platelet count were associated with viral response, and no other clinical factors were predictive of NVR before initiation of the therapy.

### *Gene Expression Involving Innate Immunity in the Liver*

First, we compared basal hepatic gene expression between the chronic hepatitis C patients (n = 74) and the nonviral liver disease patients (n = 5). As shown in Figure 1, levels of RIG-I, MDA5, LGP2, ISG15, and USP18 expression were significantly higher in the chronic hepatitis C patients than in the nonviral liver disease patients. However, there was no significant difference in levels of Cardif expression between the chronic hepatitis C and nonviral-related liver disease patients.

Next, to assess the relationship between baseline hepatic gene expression and treatment efficacy, levels of gene expression were compared based on the final virologic response. As shown in Figure 2, the hepatic expression levels of RIG-I, MDA5, and LGP2 were significantly higher in NVR than in SVR and TR. In marked contrast, hepatic Cardif expression was significantly lower in the NVR group. The hepatic expression of RNF125, which is specific E3-ubiquitin ligase for RIG-I, MDA5, and Cardif, was also significantly lower in the NVR group. Because negative correlation was found between RIG-I and Cardif or RNF125 expression, we calculated the ratio of RIG-I to Cardif or RNF125 expression levels. As shown in Figure 2, the difference among the groups was conspicuous when comparison was made with the RIG-I/Cardif ratio or RIG-I/RNF125 ratio. Moreover, the RIG-I/Cardif expression ratio before treatment was negatively and significantly correlated with the exponential viral decline rate in both the first and the second phases of HCV dynamics (first phase, $r = -0.4$, $P < .0005$; second phase, $r = -0.5$, $P < .0001$). Similar correlation was found between RIG-I/RNF125 ratio and viral decline rate (first phase, $r = -0.4$, $P = .004$; second phase, $r = -0.2$, $P = .09$, data not shown).

Like RIG-I and MDA5, intrahepatic expression levels of ISG15 and USP18 were significantly higher in NVR than in SVR and TR (Figure 2). When we assessed the correlation of these 2 genes in individual patients, we found a strong and significant correlation between ISG15 and USP18 ($r^2 = 0.88$, $P < .0001$). Levels of ISG15 and USP18 expression before treatment were negatively correlated with the exponential viral decline rates calculated from

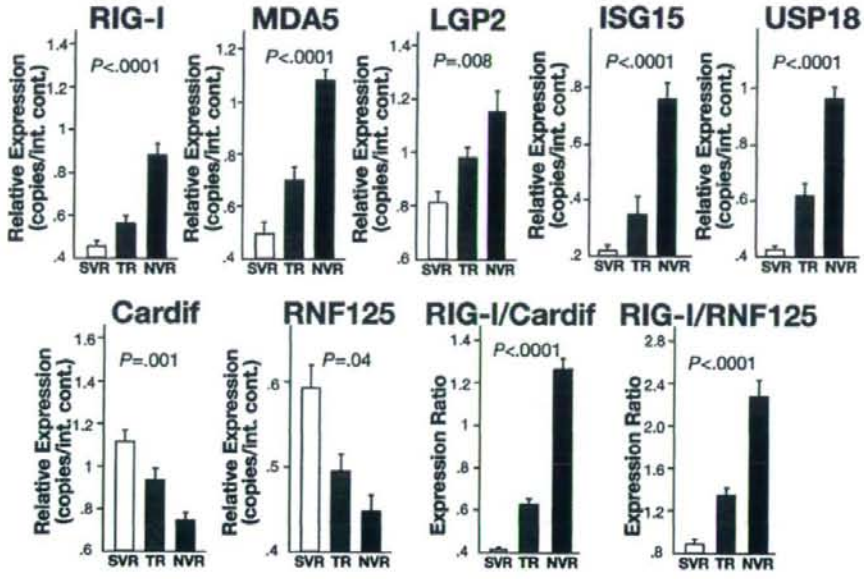


**Figure 2.** Comparison of hepatic gene expression levels according to final virologic outcome. Expression levels of RIG-I, MDA5, LGP2, ISG15, USP18, Cardif, RNF125, RIG-I/Cardif ratio, and RIG-I/RNF125 ratio are shown. *Open columns* indicate SVR (n = 30), *shaded columns* indicate TR (n = 24), and *solid columns* indicate NVR (n = 20). *Error bars* indicate the standard error. The *P* values were analyzed by the Kruskal–Wallis test.



the first and the second phases of HCV dynamics (ISG15, first phase, $r = -0.5$, $P < .0001$; ISG15, second phase, $r = -0.3$, $P = .02$; USP18, first phase, $r = -0.5$, $P < .0001$; USP18, second phase, $r = -0.3$, $P = .01$).

### *Receiver Operator Characteristic Analysis*

To determine the usefulness of these gene quantifications as predictors, receiver operator characteristic (ROC) analysis was conducted (Figure 3). The area under the ROC curve for the RIG-I/Cardif ratio, ISG15, and USP18 was 0.91, 0.90, and 0.91, respectively, suggesting that quantification of these gene transcripts is of use for the prediction of NVR (Table 2). In addition, this analysis also suggested that RIG-I/Cardif ratio would be more specific for prediction of NVR, whereas ISG15 and USP18 would be more sensitive (Table 2).

### *Multivariate Analysis*

Multivariate analysis for factors that were available before initiating therapy indicated that a higher ratio of RIG-I/Cardif and higher expression of ISG15 were independent factors that were associated with NVR (Table 3). In this analysis, USP18 was excluded because of its strong correlation with ISG15.

### *Protein Levels of Cardif in the Liver*

Because hepatic expression of Cardif mRNA was significantly lower in NVR patients than in SVR patients,

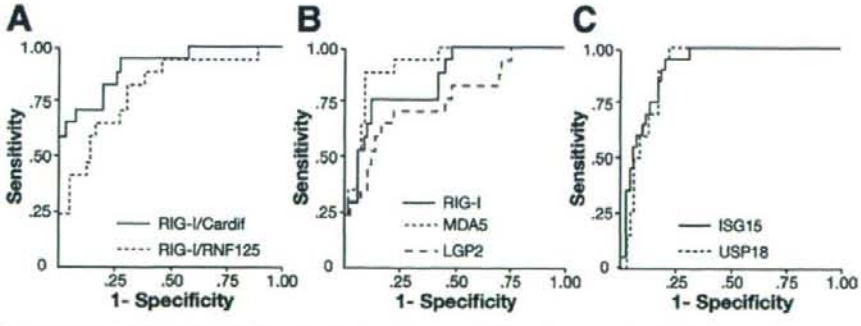


**Figure 3.** Receiver operator characteristic (ROC) curve for prediction of nonvirologic response. ROC curves were generated to compare (*A*) RIG-I/Cardif ratio (*solid line*) and RIG-I/RNF125 ratio (*shaded line*); (*B*) RIG-I (*solid line*), MDA5 (*shaded line*), and LGP2 (*dotted line*); and (*C*) ISG15 (*solid line*) and USP18 (*shaded line*).

**Table 2.** Area Under the ROC Curves, Sensitivity, Specificity, and Negative and Positive Predictive Values of Non-Virologic Responses

| Variables | Az | 95% CI | Cut-off | Sensitivity | Specificity | NPV[a] | PPV[b] |
|---|---|---|---|---|---|---|---|
| RIG-I | 0.89 | 0.78–0.95 | 0.68 | 0.80 | 0.87 | 0.92 | 0.70 |
| MDA5 | 0.92 | 0.86–0.98 | 0.84 | 0.82 | 0.89 | 0.93 | 0.74 |
| LGP2 | 0.76 | 0.63–0.90 | 1.03 | 0.65 | 0.72 | 0.85 | 0.46 |
| RIG-I/Cardif | 0.91 | 0.84–0.99 | 0.88 | 0.75 | 0.91 | 0.91 | 0.75 |
| RIG-I/RNF125 | 0.81 | 0.69–0.93 | 1.05 | 0.82 | 0.62 | 0.91 | 0.43 |
| ISG15 | 0.91 | 0.85–0.97 | 0.36 | 0.90 | 0.81 | 0.96 | 0.64 |
| USP18 | 0.90 | 0.84–0.96 | 0.67 | 0.90 | 0.83 | 0.96 | 0.67 |

[a]NPV, negative predictive value.
[b]PPV, positive predictive value.

we determined the basal protein expression levels of Cardif in the liver in NVR and SVR patients. Western blot analysis demonstrated a single Cardif product in all samples (Figure 4*A*). Similar to Cardif mRNA expression, mean Cardif expression in NVR patients was significantly lower than that in SVR (Figure 4*B*, $P = .01$). The cleavage product of Cardif, which has been reported by Loo et al,[23] was not detected in our analyses.

### *Transcriptional Responses to PEG-IFN-α-2b and Ribavirin Therapy in PBMC*

Sequential analysis in response to PEG-IFN-α-2b and ribavirin demonstrated a rapid and strong induction of RIG-I, ISG15, and USP18 mRNA expression, which peaked 8 hours after PEG-IFN-α-2b administration (Figure 5). A greater fold change of these peak inductions was observed in SVR patients compared with NVR patients, although statistical significance was not achieved. In marked contrast, RNF125 expression profile in response to PEG-IFN-α-2b was triphasic, and consisted of (1) rapid and strong suppression peaked at 8 hours after administration, (2) increased 1.5- to 2-fold above baseline level during 24–48 hours after the administration, and (3) gradually decreased to baseline level (Figure 5). The rapid suppression and subsequent increase following PEG-IFN-α-2b administration tended to have a greater fold change in NVR patients compared with those in SVR patients. In contrast from RIG-I, ISG15, USP18, and RNF125, Cardif expression profile was relatively constitutive, and transcriptional response to PEG-IFN was weak (Figure 5).

## Discussion

In the present study, we found that baseline expression levels of intrahepatic viral sensors and related regulatory molecules were significantly associated with the final virologic outcome in patients with chronic hepatitis C who were treated with PEG-IFN-α-2b and ribavirin combination therapy: up-regulation of RIG-I, MDA5, LGP2, ISG15, and USP18 and lower expression of Cardif and RNF125 could predict nonresponse to subsequent treatment with PEG-IFN-α-2b and ribavirin. The positive predictive value of a high ratio of expression of RIG-I to Cardif (>0.88) for NVR was the highest at a value of 0.75, and the negative predictive values of high expression of ISG15 (>0.36/internal control) and USP18 (>0.67/internal control) were the highest at values of both 0.96. These data may be of use in predicting clinical responses to the PEG-IFN-α and ribavirin combination before initiating therapy.

**Table 3.** Multivariate Analysis for the Factors Associated With Non-Virologic Response

| Variable | Odds ratio | 95% CI | *P* value |
|---|---|---|---|
| RIG-I/Cardif Ratio (by 0.1) | 1.5 | 1.1–2.1 | .008 |
| RIG-I/RNF125 Ratio (by 0.1) | 1.2 | 1.0–2.5 | .1 |
| ISG15 (by 0.1/internal control) | 1.5 | 1.1–2.0 | .01 |
| Age (by 1 y) | 1.0 | 0.9–1.1 | .6 |
| Platelet count (by $1 \times 10^4/\mu L$) | 1.2 | 0.9–1.5 | .07 |

Previously, large randomized controlled trials identified several pretreatment factors associated with the final virologic outcome, such as genotype, HCV RNA level, degree of fibrosis, age, body weight, ethnicity, and steatosis.[24] However, these findings lead us to believe that predicting the final virologic response before initiating PEG-IFN-α and ribavirin is difficult. Indeed, only age and platelet count were associated with the outcome in our patients with genotype 1b and a high viral load. Currently, the final response can be gauged only after treatment has been initiated. Although an early viral response at 12 weeks suggests the eventual outcome with 60%–90% accuracy,[25] a 12-week regimen is associated with adverse effects and is expensive. Therefore, this study investigated the baseline expression of genes involving innate immunity that may have significant effects on clinical outcomes.

In the present study, we demonstrated that RIG-I and MDA5 were inducible upon HCV infection and that expression of these intrahepatic positive viral sensors was up-regulated in NVR. In vitro studies have suggested that RIG-I and MDA5 play a pivotal role in the regulation of IFN production and augment the production of IFN via an amplification circuit. These results suggest that expression of RIG-I and MDA5 and related amplification system may be up-regulated by endogenous IFN at a higher baseline level in NVR patients. However, HCV elimination by subsequent exogenous IFN is insufficient

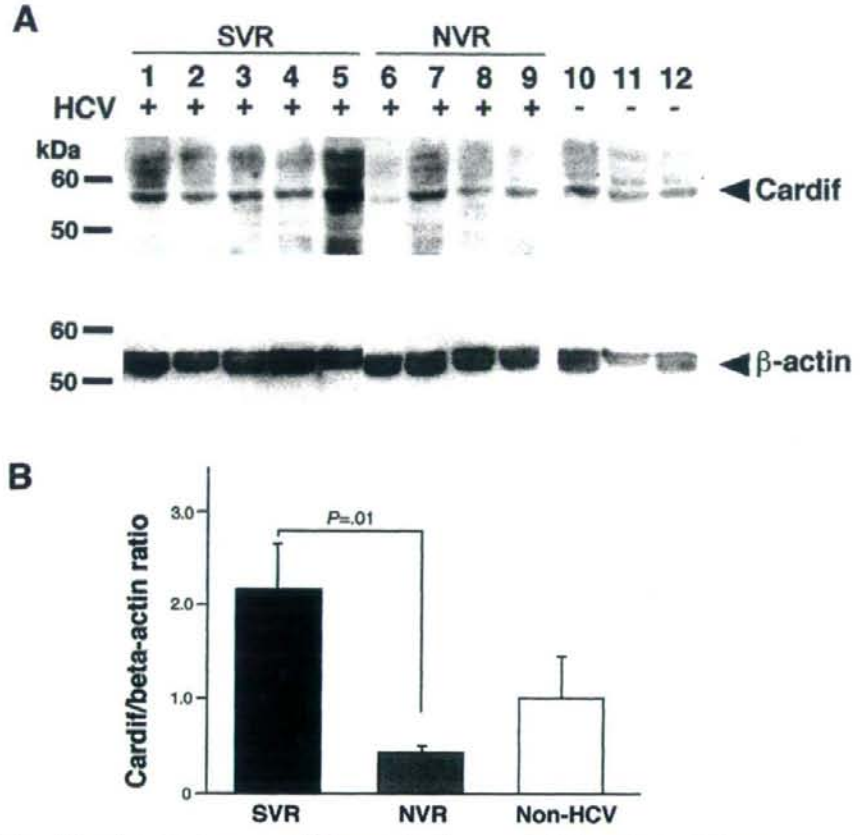


**Figure 4.** (*A*) Western blot analysis. Five lanes were SVR (*lanes 1–5*), 4 lanes were NVR (*lanes 6–9*), and 3 lanes were non-HCV control (*lanes 10–12*). Specific bands for Cardif and β-actin are indicated by *arrows*. (*B*) Expression level of Cardif protein normalized to β-actin in the liver biopsy specimens according to ultimate treatment response. *Error bars* indicate the standard error.

in these patients, suggesting that NVR patients may have adopted a different equilibrium in their immune response to the virus. In contrast to the expression of RIG-I and MDA5, Cardif mRNA, which was expressed in a relatively constitutive fashion, was significantly lower in NVR. Our ROC analysis highlights that lower expression of Cardif relative to that of RIG-I was one of the strongest predictors for NVR. Moreover, Western blot analysis further confirmed the down-regulation of Cardif in NVR patients, as demonstrated by its protein level. Because Cardif is one of the substantial target molecules of HCV evasion,[11,20] it is likely that Cardif expression is suppressed by HCV with resistant phenotype or is inadequate in NVR patients. Loo et al have demonstrated a Cardif cleavage product in 2 of 4 liver tissue samples of chronic HCV infection.[23] In our study, however, the Cardif cleavage product was not detected, presumably because the product could be unstable in vivo, resulting in rapid degradation. Although further studies are necessary to elucidate mechanisms of Cardif down-regulation, our findings of lower expression of Cardif in NVR suggested that the status of Cardif expression in the liver might have a significant effect on the ultimate outcome of antiviral treatment.

The antiviral effect brought by RIG-I/Cardif signaling is regulated by the coordination of negative and positive regulators. It has been shown that RNF125 functions as a negative regulator of RIG-I/Cardif signaling. RNF125 is an ubiquitin E3-ligase with activity against protein containing CARD domains, such as RIG-I, MDA5, and Cardif, and these ubiquitinated molecules undergo proteasomal degradation. In contrast, RNF125 do not have negative function against LGP2, a negative regulator of RIG-I signaling, because LGP2 lacks CARD domain. In contrast to RIG-I, RNF125 expression was rapidly suppressed by exogenous IFN; therefore, observed lower basal hepatic level of RNF125 in NVR could be explained by the suppressive effect of endogenous IFN, which may be up-regulated in NVR patients. Hence, RNF125 may constitute a negative regulatory circuit for IFN production and is responsible for responsiveness to PEG-IFN and ribavirin therapy.

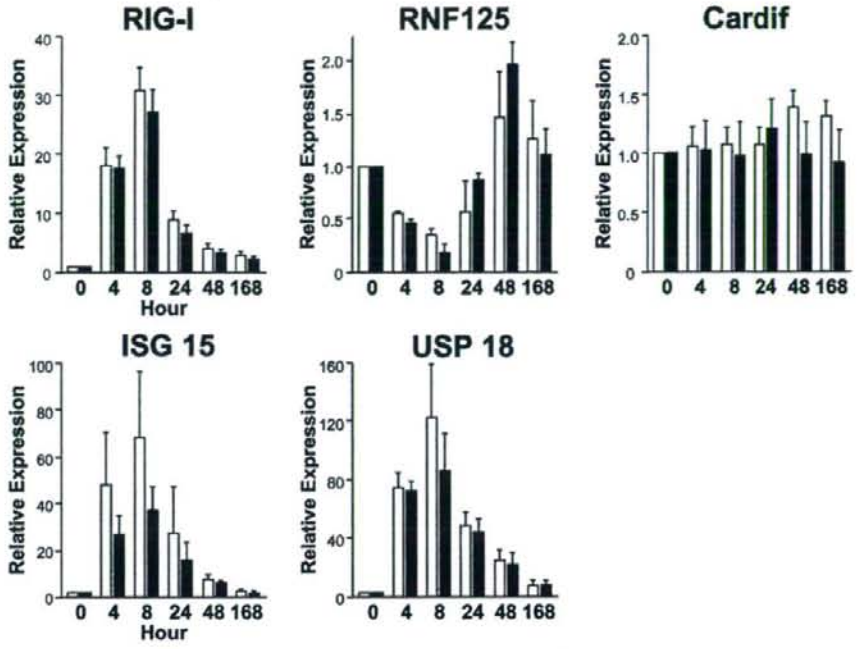


**Figure 5.** Transcriptional responses during PEG-IFN-α-2b and ribavirin therapy in PBMC (n = 14). *Open columns* indicate SVR (n = 7), and *solid columns* indicate NVR (n = 7). *Error bars* indicate the standard error. The *P* values determined by Mann–Whitney *U* test between 2 groups at 8 hours were as follows: RIG-I, *P* .3; RNF125, *P* .3; Cardif, *P* .7; ISG15, *P* .3; USP18, *P* .2.



It has been shown that RIG-I function is modified by ISG15 via ISGylation.[17] Consistent with our data, Chen et al identified 18 genes, including ISG15 and USP18, whose expression differed between responders and non-responders.[26] Interestingly, a recent study has shown that USP18 negatively regulates IFN signaling independently of its isopeptidase activity toward ISG15 by binding to the IFNAR2 receptor subunit and blocking the interaction between Janus kinase and the IFN receptor.[27] Moreover, the siRNA knockdown of USP18 in human cells has consistently been shown to potentiate the ability of IFN to inhibit HCV RNA replication.[28] Therefore, USP18 is suggested as a novel in vivo inhibitor of signal transduction pathways that are specifically triggered by type I IFN. Consistent with a role for USP18 in down-regulating the antiviral IFN response, we confirmed that up-regulation of USP18 was one of the factors predicting a lack of response to treatment with IFN.

The mechanism underlying the association of gene expression involving innate immunity with resistance to therapy is not well understood. Our human study with HCV patients treated by PEG-IFN and ribavirin highlights RIG-I/Cardif, RIG-I/RNF125, and ISG15/USP18, which is partly responsible for the clinical responsiveness to antiviral therapy. RIG-I signaling by viral pathogens may affect a wide variety of responses in not only innate but also acquired immunity. Our study is the first to demonstrate the potential relevance between molecules involving innate immunity and the clinical response to antiviral therapy.

In addition, sequential analysis of expression profile during PEG-IFN-α-2b and ribavirin treatment was also performed in this study. Lanford et al demonstrated transcriptional response to IFN-α in chimpanzee by genome microarray analysis, which included RIG-I, ISG15, and USP18.[29] An association of transcriptional response with early phase of virologic response has been also reported in PBMC or liver biopsy specimen.[30–32] We recently reported that the transcriptional double-stranded RNA-activated protein kinase response during treatment with PEG-IFN-α-2b and ribavirin was associated with the ultimate clinical response.[30] Similarly, the present study demonstrated a strong and rapid increase of RIG-I, ISG15, and USP18 mRNA in response to clinical PEG-IFN treatment especially in SVR patients, although few patients were available to achieve statistical significance between SVR and NVR. In marked contrast, transcriptional response of RNF125 exhibited a triphasic pattern. Rapid suppression seen in the first phase was presumably because of a negative regulatory effect of IFN. However, increase of RNF125 mRNA in the second phase, which tended to be grater in NVR, may be responsible for inhibiting RIG-I expression seen 8–48 hours after PEG-IFN-α-2b administration. Although limitations includ-

ing the use of PBMC and small sample size still deserve mention, the sequential expression profile during treatment may provide further valuable information regarding the prediction of the clinical response to the therapy and the mechanism of action of antiviral treatment.

In the present study, we have included patients with genotype 1b because it is imperative to designate a virologically homogeneous patient group to associate individual treatment responses with different gene expression profiles that direct innate immune responses. We have preliminarily studied genotype 2 patients and found that Cardif and RNF125 gene expression levels in NVR patients were significantly lower than those with SVR patients ($P = .03$ and $P = .04$, respectively) and that RIG-I/Cardif and RIG-I/RNF125 ratios were significantly higher in NVR patients ($P = .02$ and $P = .009$, respectively, see Supplementary Figure 2 online at www.gastrojournal.org). These findings suggest that the differences in gene expression profiles between SVR and NVR were almost identical to those demonstrated in patients with genotype 1b. However, the correlation between treatment responses in all the genotypes and the different status of innate immune responses needs to be explored. Further studies may be necessary to clarify this issue.

In conclusion, the results of the present study offer potentially important clinical implications for patients with chronic hepatitis C who are treated with PEG-IFN-$\alpha$ and ribavirin. Quantifying hepatic gene expression of the RIG-I/Cardif system, including its regulators before treatment, is useful in identifying patients who are at a higher risk for NVR. The data from these assays can provide valuable information that may influence the decision about the treatment strategy in each individual patient. Finally, this clinical human study demonstrates the potential relevance of the molecules involving innate immunity to the clinical response to therapy. Our data will help understand the pathogenesis of HCV resistance and development of new antiviral therapy targeted toward the innate immune system.



## Supplementary Data

Note: To access the supplementary material accompanying this article, visit the online version of *Gastroenterology* at www.gastrojournal.org, and at doi: 10.1053/j.gastro.2008.02.019.

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# The presence of steatosis and elevation of alanine aminotransferase levels are associated with fibrosis progression in chronic hepatitis C with non-response to interferon therapy☆

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***Background/Aims*: Interferon (IFN) therapy leads to regression of hepatic fibrosis in chronic hepatitis C patients who achieve a sustained virologic response (SVR), while the beneficial effect is limited in those who fail to do so. The aim of the present study was to define factors associated with progression of fibrosis in patients who do not achieve a SVR.**

***Methods*: Fibrosis staging scores were compared between paired liver biopsies before and after IFN in 97 chronic hepatitis C patients who failed therapy. The mean interval between biopsies was 5.9 years. Factors associated with progression of fibrosis were analyzed.**

***Results*: Fibrosis progressed in 23%, remained unchanged in 47% and regressed in 29%. Steatosis and a high average alanine aminotransferase (ALT) between biopsies were independent factors for progression of fibrosis with risk ratios of 5.53 and 4.48, respectively. Incidence and yearly rate of progression of fibrosis was 64% and $0.22 \pm 0.29$ fibrosis units per year in those with both risk factors compared to 8% and $-0.04 \pm 0.17$ fibrosis units per year in those negative for both factors.**

***Conclusions*: Hepatic steatosis and elevated ALT levels are risk factors for progression of fibrosis in chronic hepatitis C patients who fail to achieve a SVR to IFN therapy and therefore may be therapeutic targets to halt the potentially progressive disease.**



***Keywords*: Steatosis; ALT; Fibrosis**

## 1. Introduction

Hepatitis C virus (HCV) is a major cause of chronic liver disease worldwide. Mortality associated with HCV infection results from the development of liver cirrhosis and hepatocellular carcinoma, which now is the leading indication for liver transplantation [1]. Treatment with interferon (IFN), alone or in combination with ribavirin (RBV), can eradicate HCV infection in some patients, leading to sustained nor-

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malization of liver function, improvement of hepatic inflammation and fibrosis and a decreased risk of the development of hepatocellular carcinoma [2,3]. The problem is that only 50% of patients achieve a sustained virological response (SVR) to therapy even with the most highly developed regimens of IFN [4,5]. The remaining patients who fail to clear the virus are left with the risk of progressive disease. In order to halt this potentially progressive disease, there is a need to establish an effective target of therapeutic intervention independent of antiviral therapy. Therefore, it is important to define risk factors for the progression of fibrosis among chronic hepatitis C patients who do not achieve a SVR to IFN therapy.

Several factors that may affect the rate of progression of fibrosis have been investigated extensively, including older age at infection, male gender, obesity, heavy alcohol consumption, and a high grade of necroinflammation [6–8]. Several cross-sectional and longitudinal studies suggest that hepatic steatosis, which is a common histological feature of chronic hepatitis C [9], influences the progression of hepatic fibrosis [10–14], while other studies did not find such an association [15–18]. Besides these conflicting results, no study to date has reported the effect of steatosis on longitudinal progression of fibrosis among patients who fail to respond to IFN therapy. Therefore, we studied factors associated with progression of fibrosis in those who failed IFN therapy by comparing paired pre-treatment and post-treatment liver biopsies.

## 2. Methods

### 2.1. Patients

The aim of the study was to identify risk factors associated with progression of fibrosis in chronic hepatitis C patients who failed to achieve a SVR to IFN therapy. To be included in this retrospective study, patients had to have undergone liver biopsy before and after therapy, been treated with IFN and not achieved a SVR. Patients with alcohol consumption of more than 20 g per day, co-infected with HBV or HIV, and those with another known aetiology of liver disease, such as autoimmune hepatitis or metabolic disorders, were excluded. A database of patients who had undergone liver biopsy at Musashino Red Cross Hospital between 1990 and 2004 was reviewed retrospectively and a total of 1241 chronic hepatitis C patients treated with IFN were identified; of these, 407 had a SVR and 834 had not achieved a SVR. Among those with treatment failure, 104 fulfilled the above criteria but seven patients with cirrhosis before treatment were excluded because the endpoint of the study was progression of fibrosis. Therefore, this study cohort consisted of 97 patients. In these patients, second liver biopsies were performed before the second course of IFN therapy. Otherwise, there were no standardized indications for the second liver biopsy which may be the limitation of our study. Demographic characteristics of patients at the time of initial biopsy are shown in Table 1. The time between the paired biopsies was 5.9 years on average, with a range of 1.2–11.6 years. The median interval between first biopsy and IFN therapy was 3 days (range 2–93 days), and that between completion of IFN therapy and second biopsy was 5.4 years (range 0.8–11.2 years). Laboratory tests were performed monthly or bimonthly in all patients and all measurements were taken at our single hospital.

**Table 1**
**Demographic characteristics of patients**

| | |
|---|---|
| Number of patients | 97 |
| Age (years) | 52 ± 9 |
| Gender: male/female | 50/47 |
| BMI (kg/m$^2$) | 23.9 ± 3.2 (median 24.0, range19–33) |
| BMI <25/25–30/30 ⩽ (kg/m$^2$) | 55/37/5 |
| *Route of transmission* | |
| Blood transfusion/unknown | 38/59 |
| Duration of infection (years) | 30.4 ± 9.2 (median 33.5, range 3–48) |
| Genotype 1b/2a/2b | 85/4/8 |
| Serum HCV-RNA (Meq/ml) | 7.7 ± 9.7 |
| Pretreatment AST (IU/l) | 73 ± 40 |
| Pretreatment ALT (IU/l) | 104 ± 69 |
| Pretreatment GGT (IU/l) | 51 ± 44 |
| *Histological variables at first biopsy* | |
| Stage of fibrosis 1/2/3 | 33/38/26 |
| Grade of activity 0/1/2/3 | 15/36/41/5 |
| Grade of steatosis 0/1/2/3 | 21/37/25/14 |
| Size of steatosis macro/micro/mixed | 16/17/64 |
| Localization of steatosis centrilobular/diffuse | 3/94 |

BMI, body mass index; AST, aspartate aminotransferase, normal range is 7–38 IU; ALT, alanine aminotransferase, normal range is 4–43 IU/l; GGT, gamma-glutamyltransferase, normal range is 0–73 IU/l; macro, macro-vesicular steatosis; micro, micro-vesicular steatosis.

### 2.2. Histological evaluation

Median length of biopsy specimen and number of portal tracts were 13.0 mm (range 10–40 mm) and 12 (range 6–34). All liver biopsy specimens were evaluated separately by three independent pathologists who were blinded to the clinical data. If there was discordance, the scores assigned by two pathologists were used for the analysis. Fibrosis and activity were scored according to the METAVIR scoring system [19]. Fibrosis was staged on a scale of 0–4: F0 (no fibrosis), F1 (mild fibrosis: portal fibrosis without septa), F2 (moderate fibrosis: few septa), F3 (severe fibrosis: numerous septa without cirrhosis) and F4 (cirrhosis). Activity of necroinflammation was graded on a scale of 0–3: A0 (no activity), A1 (mild activity), A2 (moderate activity) and A3 (severe activity). Percentage of steatosis was quantified by determining the average proportion of hepatocytes affected by steatosis and was graded on a scale of 0–3: grade 0 (no steatosis), grade 1 (0–9%), grade 2 (10–29%), and grade 3 (over 30%). Size of steatosis was categorized into micro-vesicular, macro-vesicular and mixed types. Localization of steatosis was categorized into either centrilobular or diffuse pattern. Definition of changes in the grade of steatosis was as follows: worsening as 1 point or more increase, improvement as 1 point or more decrease, and stability as no change.

### 2.3. Changes in fibrosis-staging score overtime

Changes in progression of fibrosis were defined as follows: progression of fibrosis was defined as a 1 point or more increase, regression as a 1 point or more decrease and stability as no change in the METAVIR fibrosis-staging score. In addition, because the time between paired biopsies was variable, the yearly rate of progression of fibrosis was calculated as the change in fibrosis-staging score divided by the time between paired biopsies, as originally described by Poynard et al. [6].

### 2.4. Statistical analysis

The STAT View software package was used for statistical analysis. Categorical data were analyzed using the Fisher's exact test. Continuous variables were compared with the Student's $t$ test. Variables that were statistically significant in univariate analysis were included in multivariate analysis using logistic regression analysis. The Kaplan–Meier method and log-rank test were used to analyze the time to occurrence of fibrosis progression. A $p$-value of less than 0.05 was considered statistically significant.

## 3. Results

### 3.1. Factors associated with the initial stage of fibrosis (cross-sectional study)

All three pathologists assigned the same score in 85% of patients for fibrosis staging and 95% of patients for steatosis-grading. In cases with discordance, at least two pathologists assigned the same scale. The stage of fibrosis in the initial liver biopsy was F1 in 33, F2 in 38 and F3 in 26 patients. Various clinical factors were analyzed in association with the advanced stage of fibrosis. As a result, the presence of F3 fibrosis was associated with older age, ($51 \pm 9$ in F1–2 vs. $55 \pm 9$ in F3, $p = 0.03$), higher grade of histological activity (A2–3 was 35% in F1–2 vs. 84% in F3, $p = 0.0001$) and higher grade of steatosis (steatosis grade 2–3 was 34% in F1–2 vs. 58% in F3, $p = 0.04$).

The grade of steatosis was 0 in 21, 1 in 37, 2 in 25 and 3 in 14 patients. A higher grade of steatosis was associated with female gender (the male/female ratio was 35/23 in grade 0–1 vs. 15/24 in grade 2–3, $p = 0.04$), increased BMI (BMI over 25 kg/m$^2$ was 31% in grade 0–1 vs. 62% in grade 2–3, $p = 0.006$), and higher grade of histological activity (A2–3 was 38% in grade 0–1 vs. 62% in grade 2–3, $p = 0.03$). Multivariate logistic regression analysis revealed that increased BMI and female gender were independent factors associated with a high grade of steatosis (Table 2).

**Table 2**
**Multivariate logistic regression analysis of factors associated with hepatic steatosis**

| | Odds | 95% C.I. | $p$ Value |
|---|---|---|---|
| BMI | | | |
| ⩾25 kg/m$^2$ | 4.23 | 1.63–10.95 | 0.003 |
| Gender | | | |
| Female | 2.75 | 1.06–7.14 | 0.04 |
| Activity grade | | | |
| 2–3 | 2.30 | 0.85–6.26 | 0.10 |
| Fibrosis stage | | | |
| 3 | 1.63 | 0.53–4.97 | 0.39 |

### 3.2. Change in fibrosis-staging scores over time (longitudinal study)

Fibrosis staging progressed in 23% (progression by 2 points in 5% and 1 point in 18%), remained unchanged in 47% and regressed in 29% (regression by 2 points in 2% and 1 point in 27%). At first liver biopsy, laparoscopy was performed in 73 patients and the presence of cirrhosis (F4) was carefully excluded. In another 24 patients, the possibility of mis-diagnosis of F4 as F3 remains. However, the incidence of fibrosis progression did not differ according to the initial stage of fibrosis (21.2% in F1, 26.3% in F2 and 19.2% in F3, $p = 0.78$) which indicates that misdiagnosis of F4 as F3 at initial biopsy is unlikely.

Among various factors, as shown in Table 3, a higher grade of steatosis, higher levels of ALT and AST (average value for the period between the paired liver biopsies) were associated with progression of fibrosis. Since there was significant correlation between ALT and AST levels ($r = 0.684$, $p < 0.0001$), these two variables could not be analyzed together in multivariate analysis.

**Table 3**
**Factors associated with the progression of fibrosis over time**

| | Progression $n = 22$ | Non-progression $n = 75$ | $p$ Value |
|---|---|---|---|
| Gender: male/female | 9/13 | 41/34 | 0.33 |
| Age at biopsy: <60/⩾60 years | 14/8 | 59/16 | 0.17 |
| HCV genotype: 1b/non-1b | 19/3 | 66/9 | 0.99 |
| BMI: <25/⩾25 kg/m$^2$ | 11/11 | 44/31 | 0.48 |
| Duration of infection (years) | $32.1 \pm 5.2$ | $29.9 \pm 10.0$ | 0.56 |
| *Activity on first biopsy* | | | |
| Grade: 0–1/2–3 | 8/14 | 43/32 | 0.10 |
| *Steatosis on first biopsy* | | | |
| Grade: 0–1/2–3 | 6/16 | 52/23 | 0.001 |
| Size: macro/micro/mixed | 4/4/14 | 12/13/50 | 0.96 |
| Location: centrilobular/diffuse | 1/21 | 2/73 | 0.54 |
| *Evolution of steatosis* | | | |
| Worsening/improvement/stable | 2/2/18 | 9/8/58 | 0.09 |
| Average ALT: <100/⩾100 IU/l | 13/9 | 67/8 | 0.003 |
| Average AST: <75/⩾75 IU/l | 10/12 | 61/14 | 0.002 |
| Interval between biopsies (years) | $5.1 \pm 3.2$ | $6.2 \pm 2.4$ | 0.09 |
| Interval between completion of IFN and second biopsy (years) | $4.6 \pm 3.2$ | $5.7 \pm 2.4$ | 0.10 |
| *Treatment regimen* | | | |
| RBV–/RBV+ | 22/0 | 71/4 | 0.27 |
| *Response to IFN* | | | |
| Relapser/non-responder | 16/6 | 53/22 | 0.99 |
| *Evolution of weight* | | | |
| Gain/loss/stable | 5/8/9 | 29/21/25 | 0.38 |

macro, macro-vesicular steatosis; micro, micro-vesicular steatosis; RBV–, interferon monotherapy; RBV+, interferon plus ribavirin combination therapy.
Duration of infection was determined in 38 patients whose source of infection was blood transfusion.

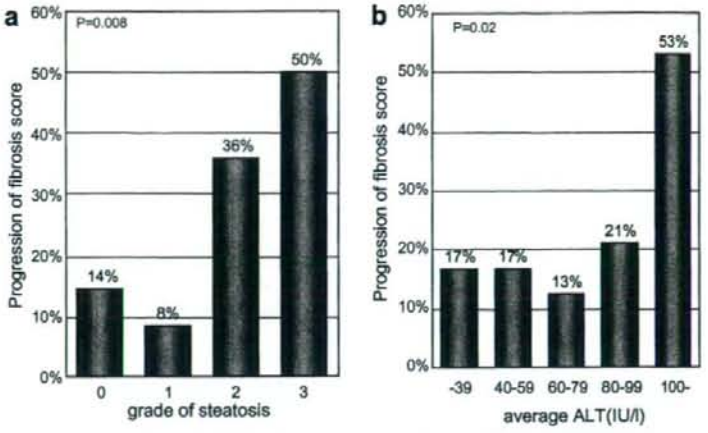


**Fig. 1. Progression of fibrosis stage, hepatic steatosis and the average level of ALT. The progression of the fibrosis score over time is illustrated using bar charts. (a) Steatosis grades of 2 or 3 at initial liver biopsy were associated with the increased progression of fibrosis over time. (b) High average ALT levels during the observation period were associated with progression of fibrosis at the threshold of 100 IU/l.**

Thus, average level of ALT was used for the following analysis. The probability of progression of fibrosis was 14%, 8%, 36% and 50% in patients with steatosis grades of 0, 1, 2 and 3, respectively ($p = 0.008$), and 17%, 17%, 13%, 21% and 53% in patients with average ALT values of <40, 40–59, 60–79, 80–99 and over 100 IU/l, respectively ($p = 0.02$) (Fig. 1). Multivariate logistic regression analysis revealed that these two were independent risk factors associated with the progression of fibrosis with risk ratios of 5.14 for steatosis ($p = 0.004$) and 5.21 for ALT ($p = 0.01$) (Table 4).

When patients were categorized in terms of these two risk factors, the incidence of progression of fibrosis was as high as 64% in those with both risk factors, compared to 8% in those negative for these factors. Conversely, the incidence of fibrosis regression was only 9% in those with both risk factors, compared to 37% in those negative for these factors ($p = 0.0003$) (Fig. 2).

In order to adjust for the effect of variable intervals between paired biopsies, the yearly rate of progression of fibrosis was calculated as the change in the fibrosis-staging score divided by the time between paired biopsies. The average of all patients was $0.02 \pm 0.22$ fibrosis units per year. Again, a higher grade of steatosis ($p = 0.004$) and higher average level of ALT ($p = 0.0005$) were associated with a higher rate of progression of fibrosis (Table 5). In addition, the yearly rate of progression of fibrosis was $0.22 \pm 0.29$ fibrosis units per year in those with both risk factors, $0.12 \pm 0.37$ in those with elevated ALT alone, $0.05 \pm 0.16$ in those with steatosis alone and $-0.05 \pm 0.17$ in those negative for these two factors ($p = 0.001$). Time to progression of fibrosis at second biopsy was also analyzed by the Kaplan–Meier method. The cumulative probabilities of progression of fibrosis at five years were 58% in those with both risk factors, 33% in those with elevated ALT alone, 18% in those with steatosis alone and 2% in those negative for these two factors ($p < 0.0001$) (Fig. 3).

**Table 4**
**Multivariate logistic regression analysis of factors associated with progression of fibrosis over time**

| | Odds | 95% C.I. | $p$ Value |
|---|---|---|---|
| Steatosis grade ⩾2 | 5.14 | 1.67–15.77 | 0.004 |
| Average ALT ⩾100 IU/l | 5.21 | 1.49–18.20 | 0.01 |

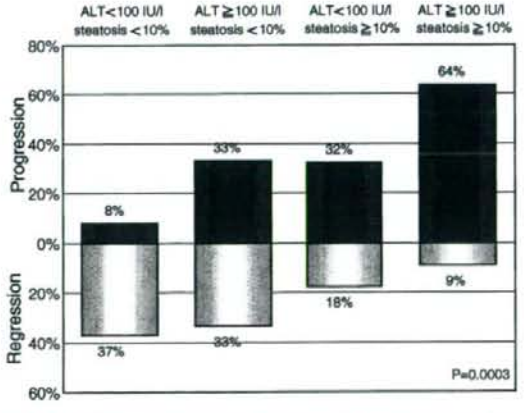


**Fig. 2. Evolution of fibrosis stage in terms of risk factors. Patients were categorized into four groups according to the presence or absence of two risk factors. The upper bar chart (dark gray) indicates the progression of fibrosis while the lower bar chart (light gray) indicates the regression of fibrosis.**

**Table 5**
**Factors associated with the yearly rate of fibrosis progression**

| | *n* | Mean | SD | *p* Value |
|---|---|---|---|---|
| Gender | | | | |
| Male | 50 | −0.01 | 0.19 | 0.12 |
| Female | 47 | 0.06 | 0.23 | |
| Age at biopsy | | | | |
| <60 years | 73 | −0.0002 | 0.21 | 0.06 |
| ⩾60 years | 24 | 0.10 | 0.23 | |
| HCV genotype | | | | |
| 1b | 83 | 0.02 | 0.20 | 0.37 |
| non-1b | 14 | 0.08 | 0.32 | |
| BMI | | | | |
| <25 kg/m$^2$ | 53 | 0.004 | 0.24 | 0.32 |
| ⩾25 kg/m$^2$ | 44 | 0.05 | 0.19 | |
| Steatosis on first biopsy | | | | |
| 0–1 | 58 | −0.03 | 0.20 | 0.004 |
| 2–3 | 39 | 0.10 | 0.21 | |
| Activity on first biopsy | | | | |
| 0–1 | 51 | −0.001 | 0.21 | 0.24 |
| 2–3 | 46 | 0.05 | 0.22 | |
| Fibrosis on first biopsy | | | | |
| 1–2 | 71 | 0.03 | 0.20 | 0.43 |
| 3 | 26 | −0.01 | 0.25 | |
| Average ALT between paired biopsies | | | | |
| <100 IU/l | 80 | −0.01 | 0.17 | 0.0005 |
| ⩾100 IU/l | 17 | 0.18 | 0.31 | |

## 4. Discussion

In the present study, we found that a higher grade of hepatic steatosis at baseline and a higher average value of ALT are independent risk factors for the progression of fibrosis over time in chronic hepatitis C patients who fail to achieve a SVR to IFN therapy. These two factors may be involved in promoting the progression of fibrosis. The association between steatosis and progression of fibrosis in untreated patients had been suggested by previous studies but this study is the first to demonstrate a similar association for treated patients. These findings are particularly important to establish a rationale for identifying therapeutic targets to halt potentially progressive disease independent of antiviral therapy.

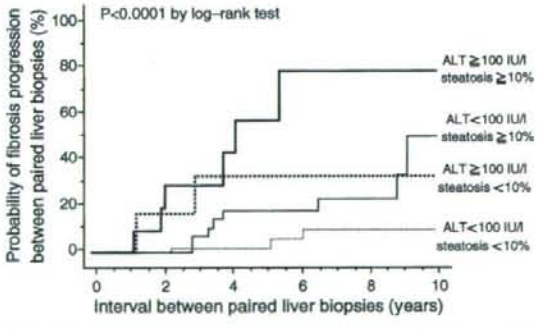


**Fig. 3. Probability of fibrosis progression according to the presence of risk factors. Patients were categorized into four groups according to the presence or absence of two risk factors and the time to progression of fibrosis was analyzed.**

There have been many studies that analyzed the association between steatosis and progression of liver fibrosis in HCV-infected patients, and the majority have shown a positive association [10–13], including a large scale meta-analysis [14]. However, some studies did not report this association [15–18]. There are two possible reasons for these conflicting results. First, longitudinal studies, rather than cross-sectional studies, are particularly important in the analysis of the role of steatosis in time-dependent progression of hepatic fibrosis, because cross-sectional studies involve patients with an unknown duration of steatosis. Three of four longitudinal studies that analyzed the progression of fibrosis through paired biopsies in untreated patients showed that the presence or worsening of steatosis was associated with the progression of fibrosis [12,13,20], and the probability of progression of fibrosis was significantly related to the grade of steatosis [13]. In one study, however, progression of fibrosis was correlated with older age, periportal necroinflammation and ALT elevations but not with steatosis [17]. Interestingly, steatosis was associated with older age, higher body mass index and ALT elevations in that study, indicating an indirect association of steatosis and fibrosis progression. The authors assumed that steatosis was the result rather than the cause of inflammation. This observation highlights the second reason for the controversies over a correlation between the presence of steatosis and progression of fibrosis, that is, there are so many confounding factors associated with both steatosis and fibrosis progression such as older age, advanced stage of fibrosis, higher degree of inflammation, elevated ALT, increased body mass index and insulin resistance. Because it is very difficult to prove a causal relationship between these confounding factors through clinical observations, steatosis may be a hallmark of the progression of fibrosis but it is unclear whether the effect of steatosis on progression of fibrosis is direct or mediated by other confounding factors.

Hepatic steatosis is a common pathological finding in patients with chronic hepatitis C [9]. Because the proportion of patients with steatosis is higher than would be expected from a chance association, a direct role of HCV in the pathogenesis of steatosis is suggested, at least in some patients with genotype 3 infection [21]. Furthermore, other observations suggest that steatosis may be metabolic; it is correlated with a high body mass index, visceral adiposity and insulin resistance, especially in non-3a genotypes and metabolic steatosis also is correlated with progression of fibrosis [11,22]. The

most reliable evidence that metabolic steatosis is associated with progression of fibrosis is shown by a study indicating that weight reduction in patients with chronic hepatitis C leads to a reduction in steatosis and an improvement in fibrosis, despite the persistence of HCV infection. A reduction in steatosis was significantly associated with a decrease in stellate cell activation and regression of hepatic fibrosis in 56% of patients. Thus, weight reduction may provide an important new adjunct treatment strategy for patients with chronic hepatitis C [23]. A recent study showed that the administration of pioglitazone led to metabolic and histological improvement in subjects with non-alcoholic steatohepatitis [24]. Whether amelioration of insulin resistance could improve steatosis and fibrosis in chronic hepatitis C awaits future investigation.

The mechanism by which steatosis could aggravate hepatic fibrosis in chronic hepatitis C patients remains largely hypothetical. Steatosis related insulin resistance may contribute to hyperinsulinemia and increased hepatic expression of connective tissue growth factor leading to progression of fibrosis [25]. Alternatively, a steatohepatitis-like pathway may be involved where steatosis requires a second hit for progression to fibrosis [26]. The most likely candidate is an oxidative stress with subsequent lipid peroxidation which is reported to correlate with the stage of fibrosis [27]. Another important candidate is an antiviral inflammatory response. It is reported that steatotic liver has increased susceptibility to inflammatory response [28] and that a higher grade of steatosis is correlated with a higher degree of inflammation or elevated ALT [14,15,17]. Higher degree of inflammation or elevated ALTs are associated with the progression of fibrosis [29,30], but hepatic steatosis may be responsible for the amplification of hepatic inflammation and vice versa, and the coexistence of these two factors may lead to further progression of fibrosis, as in patients with non-alcoholic steatohepatitis. In our study, average value of ALT between two biopsies was associated with fibrosis progression, whereas histological inflammation at first liver biopsy was not. The reason for this discordance may be explained by the dynamic process of hepatic necroinflammation. Severity of histological inflammation at the time of biopsy may not reflect subsequent inflammation process, whereas average value of regularly determined ALT may reflect entire fluctuation of hepatic inflammation. If so, our finding may support the hypothesis that co-operation of steatosis as the first hit and dynamic process of hepatic inflammation as the second hit promotes fibrosis progression. On the other hand, elevation of ALT may not be a mere reflection of hepatic inflammation so much as hepatocellular death such as apoptosis. Since it is reported that apoptotic caspase activation is elevated in HCV-associated steatosis [31] and that steatotic liver has increased susceptibility to apoptosis [28], elevation of ALT may also reflect an apoptosis amplified by steatosis which may lead to fibrosis progression.

Regardless of the precise mechanism, the results of the present study suggest that lowering of ALT levels may be beneficial in preventing progression of fibrosis in patients who failed to achieve a SVR. In our population, all patients received 24 weeks of IFN therapy and none received long-term maintenance therapy aiming to ameliorate hepatic inflammation. However, we speculate that amelioration of hepatic inflammation and lowering ALT levels by long-term IFN may prevent fibrosis progression in patients who remain viremic since it has been reported that IFN slowed the natural progression of fibrosis in patients who failed IFN therapy when the rate of progression of fibrosis after IFN therapy was compared to the estimated rate of progression before therapy [2,32], and that treatment duration was associated with the reduction of fibrosis independent of virological response [2]. Another possible approach to lower ALT levels may be the use of ursodeoxycholic acid, which has been reported to induce an almost 30% decrease in serum ALT levels [33,34]. The long-term efficacy of therapies targeted to the reduction of hepatic fibrosis needs future verification.

Some factors related to fibrosis progression in previous studies such as obesity [35] and worsening of steatosis [20] were not significant in our study. In our study where the majority of the population had normal body weight and very few had obesity (BMI $\geqslant 30\ kg/m^2$), impact of increased BMI on fibrosis progression may not be evaluated. Also, a smaller number of patients with worsening of steatosis (11.3% in present study and 34% in previous study [20]) may be the reason for the discrepancy. This may be due to difference in patient selection since no patients in that study had antiviral treatment between two biopsies.

In conclusion, the presence of hepatic steatosis and elevated ALT levels are risk factors for progression of fibrosis in chronic hepatitis C patients who failed to achieve a SVR to IFN therapy. These two factors may be a therapeutic target to halt the potentially progressive disease independent of antiviral therapy.

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# Pretreatment Prediction of Virological Response to Peginterferon Plus Ribavirin Therapy in Chronic Hepatitis C Patients Using Viral and Host Factors

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**The interferon sensitivity determining region (ISDR) of the hepatitis C virus (HCV) and T-helper type 1 and type 2 (Th1/Th2) ratio were analyzed along with other host and viral factors for their ability to predict the response of patients with chronic hepatitis C to pegylated interferon alpha-2b (Peg-IFN) and ribavirin (RBV) combination therapy. A total of 120 chronic hepatitis C patients with genotype 1 HCV and high baseline viral loads who were to undergo combination therapy scheduled for 48 weeks were enrolled. Sustained virologic response (SVR) was achieved in 54 (45%) of the 120 patients. The pretreatment factors significantly associated with SVR by logistic regression analysis were ISDR mutant [odds ratio (OR) = 86.0, $P = 0.0008$], Th1/Th2 ratio ≤ 15.5 (OR = 9.6, $P = 0.0021$), body weight 59 kg, and neutrophil count 2,300/$\mu$L. A logistic regression model to estimate SVR before combination therapy was constructed using these four factors. Patients fell into three groups when plotted according to estimated and actual SVR rates: actual SVR rate was 91% (32/35) in the high sensitivity group, 41% (15/37) in the intermediate sensitivity group, and 15% (7/48) in the low sensitivity group. Rapid or early virological responses were seen in 80% of patients with high sensitivity and who achieved SVR but were found in only 40% of patients with intermediate or low sensitivity. Null- and very late virological responses were quite rare in the high sensitivity group. In conclusion, a logistic regression model that includes the sequence of ISDR of the HCV, Th1/Th2 ratio, body weight, and neutrophil count can be useful for accurately predicting actual SVR rate before combination therapy.** (HEPATOLOGY **2008;48:000-000.**)

Chronic infection with hepatitis C virus (HCV) can lead to chronic hepatitis and eventually liver cirrhosis and hepatocellular carcinoma.[1] Administration of antiviral agents such as interferon (IFN) can eradicate HCV in some patients with chronic hepatitis C, and the risk of complicating hepatocellular carcinoma has been reported to decrease remarkably once this is achieved.[2-6]

HCV genotype and viral load are two major factors used to predict the response of patients with chronic hepatitis C to IFN. Patients who have genotype 1 HCV and high viral loads are relatively resistant to IFN therapy.[7] Peg-IFN and RBV combination therapy is currently the first line of therapy for these cases.[8] However, although the sustained virologic response (SVR) rate has been improved with the advent of combination therapy, it remains approximately 50%. The velocity of decrease in viral load during combination therapy is also a good indicator for predicting SVR; high SVR rates are predicted in rapid and early virological responders, whereas low SVR rates are predicted in late and nonvirological responders.[9-12]

It is considered beneficial to predict the response of patients with genotype 1 HCV and high viral load to Peg-IFN and RBV combination therapy before starting treatment because therapy can be long, costly, and have many side effects. However, prediction is often difficult in

*Abbreviations: BMI, body mass index; EVR, early virologic response; HCV, hepatitis C virus; IFN, interferon; ISDR, interferon sensitivity determining region; Null-R, null-response; Peg-IFN, peginterferon-alpha-2b; RBV, ribavirin; RVR, rapid virological response; SVR, sustained virological response; Th1/Th2 ratio, T-helper type 1 and type 2 ratio; VLVR, very late virological response.*

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these patients because they have already been selected as being poor responders to IFN therapy by the major prediction factors of HCV genotype and viral load.

Amino acid substitutions in the interferon sensitivity determining region (ISDR), located in HCV nonstructural region 5A, have also been reported as useful for predicting the response of patients with genotype 1 to IFN therapy.[13] Several host factors, such as immunological responses, are suggested to be associated with viral response as well.[14-22] Among them, we chose the T-helper type 1 and type 2 (Th1/Th2) ratio as a representative host factor for analysis.

In the current study, we studied whether a combination of viral and host factors, including the presence of ISDR mutants and Th1/Th2 ratio, could predict response to Peg-IFN and RBV therapy in chronic hepatitis C patients with genotype 1 HCV and high viral load.

## Patients and Methods

***Patients.*** A total of 120 patients with chronic hepatitis C were treated with Peg-IFN and RBV combination therapy at Shinshu University Hospital and the 21 member hospitals of the Shinshu Interferon Treatment Research Group. The cohort included 65 men and 55 women, ranging from 17 to 75 years of age, who were registered prospectively from December 2004 to December 2005. All patients had HCV genotype 1b and had shown high viral load for at least 6 months. High viral load was defined as serum HCV RNA equal to or greater than $10^5$ international units/mL as measured by quantitative Cobas Amplicor assays (Roche Diagnostics Co. Ltd, Tokyo, Japan). Of the 120 patients, 86 had undergone a liver biopsy before combination therapy, and seven of the 86 patients were diagnosed as having cirrhosis. Exclusion criteria for patients not eligible for Peg-IFN and RBV combination therapy were as follows: (1) pregnant women or women of child-bearing potential, nursing mothers, or male patients whose partner might become pregnant; (2) patients with anemia (hemoglobin concentration of 10 g/dL or less), leukopenia (1,500/$\mu$L or less), or thrombocytopenia (80,000/$\mu$L or less); (3) patients with depression; (4) patients with serious complications in the heart, kidneys, or lungs; (5) patients with autoimmune diseases, such as autoimmune hepatitis; (6) patients infected with hepatitis B virus or human immunodeficiency virus; and (7) patients with hypersensitivity to Peg-IFN or RBV.

This study was approved by the ethics committee of Shinshu University and performed in accordance with the internationally accepted ethical standards for human experimentation. The purpose and the protocol of this study were explained to all patients, and written informed consent was obtained from each participant.

***Peg-IFN and RBV Combination Therapy.*** Peg-IFN-$\alpha$-2b (Schering-Plough K.K., Tokyo, Japan) was given in weekly doses adjusted to body weight according to manufacturer's instructions (45 kg or less; 60 $\mu$g/dose, 46 to 60 kg; 80 $\mu$g/dose, 61 to 75 kg; 100 $\mu$g/dose, 76 to 90 kg; 120 $\mu$g/dose, 91 kg or more; 150 $\mu$g/dose). Similarly, RBV (Schering-Plough K.K.) was given in daily doses adjusted to body weight according to manufacturer's instructions (60 kg or less; 600 mg/day, 61 kg to 80 kg; 800 mg/day, 81 kg or more; 1,000 mg/day). The duration of the combination therapy was set at a standard 48 weeks, but treatment extension was permitted to up to 72 weeks if the patient requested.

A rapid virologic response (RVR) was defined as undetectable serum HCV RNA at 4 weeks as measured by qualitative Cobas Amplicor assays (Roche Diagnostics Co. Ltd., Tokyo, Japan). An early virological response (EVR) was defined as detectable serum HCV RNA at 4 weeks but undetectable by 12 weeks, a late virological response was defined as serum HCV RNA detectable at 12 weeks but undetectable by 24 weeks, a very late virological response (VLVR) was defined as serum HCV RNA detectable at 24 weeks but undetectable by 48 weeks, and a null-response (Null-R) was defined as serum HCV RNA not becoming undetectable during the treatment course. An end of treatment response was defined as negative serum HCV RNA by the end of treatment. An SVR was defined as serum HCV RNA becoming undetectable during therapy and remaining so for at least 24 weeks afterwards. Responses other than SVR were regarded as non-SVR.

Achieved rates of Peg-IFN and RBV administration were calculated as the percentage of actual total dose administered of a standard total dose of 48 weeks calculated according to body weight before therapy.

***Serological Tests for HCV, Hepatitis B Virus, and Human Immunodeficiency Virus.*** Antibodies to HCV, hepatitis B virus surface antigen, and human immunodeficiency virus were measured using commercially available enzyme-linked immunosorbent assays (International Reagents Co., Kobe, Japan). Serum HCV RNA was determined using qualitative and quantitative Cobas Amplicor assays (Roche Diagnostics Co. Ltd., Tokyo, Japan), which amplify HCV RNA using reverse transcription polymerase chain reaction. HCV genotypes were determined according to the method reported by Ohno et al.[23] Serum alanine aminotransferase and other relevant biochemical tests were performed using standard methods.

***Serum Level of Ribavirin.*** Serum level of ribavirin was measured using a validated liquid chromatography/

**Table 1. Comparison of Pretreatment Factors Between Patients With and Without SVR**

| Factors | SVR (n = 54) | Non-SVR (n = 66) | P |
|---|---|---|---|
| Age (years)* | 59 (26-75) | 63 (17-74) | 0.032 |
| Sex (male %) | 74.1% | 37.9% | <0.001 |
| Body weight (kg)* | 64 (45-80) | 57 (38-92) | <0.001 |
| Body mass index (kg/m$^2$)* | 24 (18-29) | 23 (16-31) | 0.092 |
| Blood transfusion history | 37.0% | 31.8% | 0.567 |
| Past Interferon therapy | 46.3% | 50.0% | 0.716 |
| Serum ALT at baseline (IU/L)* | 57 (25-389) | 55 (21-332) | 0.516 |
| Serum creatinine (mg/dL)* | 0.71 (0.45-1.06) | 0.60 (0.47-1.09) | 0.011 |
| Serum iron (mg/dL)* | 129 (65-293) | 144 (19-222) | 0.379 |
| White blood cell count (/$\mu$L)* | 4,990 (2,800-8,770) | 4,100 (1,950-7,100) | <0.001 |
| Neutrophil count (/$\mu$L)* | 2,310 (1,100-5,800) | 1,967 (800-4,500) | 0.015 |
| Hemoglobin (g/dL)* | 15.4 (12.8-17.6) | 14.2 (10.9-17.7) | <0.001 |
| Platelets ($10^3$/$\mu$L)* | 183 (83-262) | 158 (81-319) | 0.031 |
| HCV RNA ($10^3$ IU/mL)* | 1215 (100 to >5000) | 1850 (100 to >5000) | 0.024 |
| ISDR (W:I:M:UD) | 19:11:22:2 | 46:15:4:1 | 0.002 |
| Th1/Th2 ratio* | 13.7 (3.2-63.7) | 20.1 (2.0-103.3) | 0.001 |
| Fibrosis stage (F1-2:F3-4:UD) | 29:7:18 | 32:18:16 | 0.508 |

*Data are expressed as median (range).
W, wild; I, intermediate; M, mutant; UD, undermined.

tandem mass spectrometric assay with a detection limit of 50 ng/mL.[24] Serum samples were taken at 1 and 8 weeks after starting combination therapy.

***Amino Acid Substitutions in the ISDR.*** ISDR type was determined by the method reported by Enomoto et al.,[13] in which the HCV-J strain of genotype 1b, as reported by Kato et al.,[25] was used as the wild type. Briefly, the nucleotide sequence of ISDRs in the nonstructural 5A region was determined by direct sequencing of polymerase chain reaction amplified materials to deduce amino acid sequence. Wild-type ISDR was defined as having no amino acid substitutions, intermediate-type ISDR was defined as having one amino acid substitution, and mutant-type ISDR was defined as containing two or more amino acid substitutions.

***Th1/Th2 Ratio.*** The Th1/Th2 ratio in peripheral blood was determined using flow cytometry according to the method reported by Kawakami et al.[26] Briefly, CD4-positive cells were extracted, then Th1 (IFN-$\gamma$+/interleukin-4−) and Th2 (IFN-$\gamma$−/interleukin-4+) cells were classified using monoclonal antibodies to IFN-$\gamma$ and interleukin-4. The Th1/Th2 ratio was calculated as number of Th1 cells per number of Th2 cells.

***Statistical Analyses.*** The Mann-Whitney *U*-test was used to analyze continuous variables. Chi-squared and Fisher's exact tests were used for analysis of categorical data. Multivariate analysis was performed using a logistic regression model with stepwise method. Each cutoff point for continuous variables was decided by receiver operating characteristic curve analysis. A *P*-value of less than 0.05 was considered significant. Statistical analyses were performed using SPSS for Windows v16.0J (SPSS Inc, Chicago, IL).

## Results

***Response Rate and Clinical Characteristics.*** SVR was achieved in 54 (45%) of the 120 patients enrolled in the current study. In total, 18 (15%) patients elected to extend treatment to up to 72 weeks, although SVR rate was similar between patients with (44%) and without (45%) the extension. Discontinuation of Peg-IFN and RBV combination therapy during treatment course was recorded in four (7%) of the 54 patients with SVR and 21 (32%) of the 66 patients with non-SVR. Of the 21 non-SVR patients who discontinued combination therapy, nine were because of side effects and 12 because of insufficient effects, namely serum HCV RNA remaining detectable at 24 weeks.

***Factors Associated with SVR.*** Pretreatment factors that could be associated with responses to Peg-IFN and RBV combination therapy were compared between patients with and without SVR in Table 1. Patients with SVR tended to be younger than those with non-SVR and who were male. Body weight was higher in SVR patients, but body mass index (BMI) did not differ between the two groups. Median counts of white blood cells, neutrophils, and platelets and median concentrations of creatinine and hemoglobin were significantly higher in patients with SVR than in those without. Mutant ISDR was more prevalent in patients with SVR, but the Th1/Th2 ratio was significantly lower.

Predictive factors measured during treatment were also compared between patients with and without SVR (Table 2). Serum concentrations of RBV at 1 and 8 weeks of therapy did not differ between the two groups. Total ad-

**Table 2. Comparison of Treatment Factors and Virological Responses During Peg-IFN and RBV Therapy Between Patients With and Without SVR**

| Factors | SVR (n = 55) | Non-SVR (n = 66) | P |
|---|---|---|---|
| RBV concentration during therapy | | | |
| At 1 week (ng/mL)* | 1,256 (482-3455) | 1453 (227-3496) | 0.138 |
| At 8 weeks (ng/mL)* | 2558 (1131-12,260) | 2507 (1004-6229) | 0.746 |
| Total dose administered | | | |
| Peg-IFN (μg)* | 4240 (1380-5760) | 3540 (200-7200) | 0.001 |
| RBV (g)* | 268.8 (67.2-403.2) | 166.6 (11.2-336.0) | <0.001 |
| Achieved administration rate | | | |
| Peg-IFN (%)* | 91.5 (28.0-133.0) | 87.2 (4.2-147.0) | 0.146 |
| RBV (%)* | 90.5 (24.0-157.0) | 75.8 (4.2-138.0) | <0.001 |
| Virological response | | | |
| RVR:EVR:LVR:VLVR:Null-R | 22:23:9:0:0 | 4:11:14:4:33 | <0.001 |

*Data are expressed as median (range).
Achieved administration rate for Peg-IFN and RBV was calculated as the percentage of actual dose administered of the scheduled dose for 48 weeks.
RVR, rapid virological response; EVR, early virological response; LVR, late virological response; VLVR, very late virological response; Null-R, null response.

ministered dose of Peg-IFN and RBV was significantly higher in patients with SVR. RVR and EVR were more prevalent in patients with SVR, whereas VLVR and Null-R were more prevalent in patients without.

Factors that were significantly associated with SVR by univariate analysis were then analyzed by multivariate analysis. Both pretreatment and treatment factors were analyzed together to select the pretreatment prediction factors that were independent from the treatment prediction factors. Cutoff points for continuous data were determined by receiver operating characteristic analysis and were as follows: 57 years old, body weight 59 kg, BMI 23 kg/m$^2$, creatinine 0.75 mg/dL, white blood cells 4,200 cells/μL, neutrophils 2,300 cells/μL, hemoglobin 15.0 g/dL, platelets 135,000 cells/μL, HCV RNA $6.0 \times 10^5$ international units/mL, Th1/Th2 ratio 15.5, total Peg-IFN dose 2900 μg, total RBV dose 182 g, achieved rate of Peg-IFN 73% of target amount, and achieved rate of RBV 79% of target amount. The seven factors shown in Table 3 were then evaluated by logistic regression analysis with stepwise method, indicating that mutant ISDR, Th1/Th2 ratio 15.5 or lesser, body weight 59 kg or greater, and neutrophils 2,300 cells/μL or greater were significantly associated with SVR among pretreatment factors. The odds ratio of mutant ISDR was as high as 86.0, and the odds ratios of the remaining three pretreatment factors all fell between 5.0 and 10.0. The positive predictive values of ISDR mutant, Th1/Th2 ratio 15.5 or lesser, body weight 59 kg or greater, and neutrophil count 2300 cells/μL or greater were 82.8%, 61.8%, 63.1%, and 54.1%, respectively, and negative predictive values were 67.0%, 69.2%, 76.4%, and 64.4%, respectively. As for treatment factors, RVR, EVR, and a higher dose of Peg-IFN were also found to be factors predicting SVR.

***Pretreatment Prediction of SVR by Logistic Model.*** A logistic regression model for predicting SVR was constructed using the four pretreatment factors significantly associated with SVR by multivariate analysis:

$$R = -3.615 + 3.117 \times (\text{ISDR mutant}) + 1.732 \times (\text{Th1/Th2} \leq 15.5) + 2.184 \times (\text{body weight} \geq 59\ \text{kg}) + 1.384 \times (\text{neutrophil count} \geq 2300\ \text{cells}/\mu\text{L})$$

(each variable: yes = 1, no = 0)

$$\text{Predicted SVR rate} = 1 / (1 + \exp[-R])$$

Figure 1 shows the distribution of patients according to predicted SVR rates, which are well correlated with

**Table 3. Multivariate Logistic Regression Analysis for Factors Associated with SVR**

| Factors | n | OR | (95% CI) | P |
|---|---|---|---|---|
| Pretreatment factors | | | | |
| Mutant ISDR | 29 | 86.0 | (6.4-1162.3) | 0.0008 |
| Th1/Th2 ratio ≤ 15.5 | 55 | 9.6 | (2.3-40.7) | 0.0021 |
| Body weight ≥ 59kg | 65 | 6.4 | (1.5-26.9) | 0.0106 |
| Neutrophil count ≥ 2,300/μL | 61 | 5.5 | (1.2-26.2) | 0.0031 |
| Virological response during therapy | | | | |
| Rapid virological response | 26 | 6.8 | (1.0-45.8) | 0.0491 |
| Early virological response | 60 | 12.0 | (2.4-60.0) | 0.0024 |
| Treatment factor | | | | |
| Total dose of Peg-IFN ≥ 2,900 μg | 84 | 23.1 | (2.7-194.9) | 0.0039 |

Cutoff value for each factor was determined by receiver operating characteristic curve (ROC) analysis.

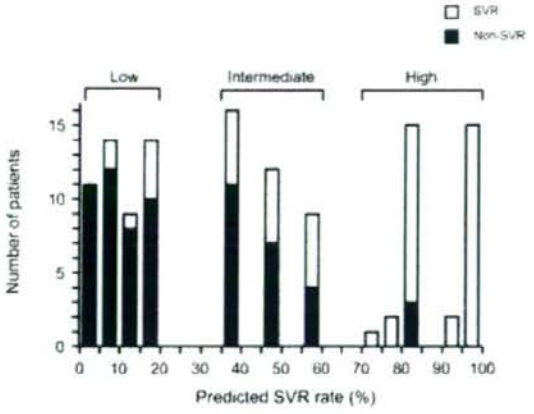


Fig. 1. Distribution of patients with and without SVR according to predicted SVR rate. Patients were classified into three groups according to distribution and named as low, intermediate, and high sensitivity groups to Peg-IFN and RBV combination therapy. Open bars indicate patients with SVR, and closed bars indicate those without SVR.

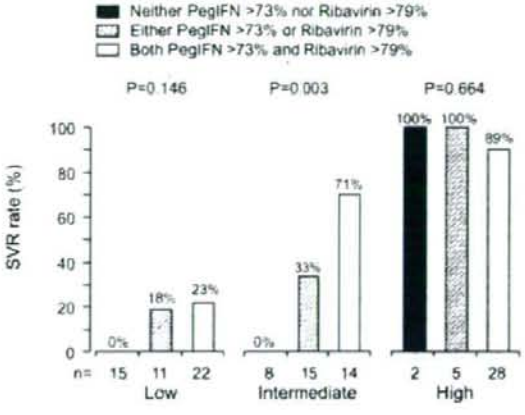


Fig. 2. Actual SVR rates were compared among the three groups of patients classified according to achieved rates of scheduled doses of Peg-IFN and RBV administration. The first group consisted of patients who were given neither a Peg-IFN dose of over 73% nor an RBV dose of over 79% of target amounts. The second group consisted of patients who received either a Peg-IFN dose of over 73% or an RBV dose of over 79% of target amounts. The third group consisted of patients who received both a Peg-IFN dose of over 73% and an RBV dose of over 79% of target amounts.

actual SVR rates. The patients were further divided into three groups: a high-sensitivity group for patients whose predicted SVR rates were in the upper one third (>66%), an intermediate group for SVR rates in the middle one third (33%-66%), and a low-sensitivity group for rates in the lower one third (<33%). The actual SVR rates were 91% (32/35), 41% (15/37), and 15% (7/48) in the high, intermediate, and low sensitivity groups, respectively.

The actual SVR rates were then compared among the three groups classified according to standard doses of Peg-IFN and RBV administration (Fig. 2). The first group consisted of patients who took lesser amounts of treatment (Peg-IFN dose less than 73% and RBV dose less than 79% of target amounts). The second group received Peg-IFN over 73% or RBV over 79% of target amounts. The third group consisted of patients who received both Peg-IFN over 73% and RBV over 79% of target amounts. SVR rates were similarly low among patients with low sensitivity and high among patients with high sensitivity, but rose with increases of Peg-IFN and RBV doses received in patients with intermediate sensitivity.

The SVR rates in patients with RVR, EVR, late virological response, VLVR, and Null-R were 85% (22/26), 68% (23/34), 39% (9/23), 0% (0/5), and 0% (0/32), respectively. Distributions of actual virological responses during therapy are shown according to the estimated sensitivity of low, intermediate, and high in Fig. 3, and are significantly associated with the estimated sensitivities. RVR and EVR were seen in 80% of patients with high sensitivity, but were less than 40% in patients with intermediate or low sensitivity. Null-R and VLVR were quite rare in the high-sensitivity group. All patients with RVR and EVR in the high-sensitivity group achieved SVR.

## Discussion

Besides HCV genotype and viral load, several factors, including age, sex, race, BMI, HCV mutations, and host

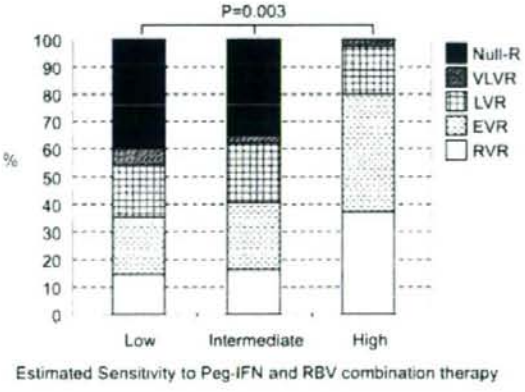


Fig. 3. Distribution of actual virological responses during combination therapy shown according to estimated sensitivities of low, intermediate, and high. Null-R, null-response; VLVR, very late virologic response; LVR, late virological response; EVR: early virological response; RVR, rapid virological response.

immunological parameters, have been reported to be associated with SVR rates in patients with chronic hepatitis C treated with Peg-IFN and RBV combination therapy. However, virological responses during therapy, such as RVR and EVR, are now widely used for predicting final virological response because such treatment factors have even higher predictive value.[9-12] Nonetheless, it is obvious that predictions made before administration of therapy are more desirable than those done during treatment course if accuracy of prediction is comparable. We therefore planned the current study to find such a way to predict SVR before starting Peg-IFN and RBV combination therapy in patients already known to be resistant.

Several regions in the HCV genome have been reported to be associated with sensitivity to interferon therapy. Enomoto et al.[13] reported that a higher number of amino acid substitutions in the ISDR (NS5A, a.a. 2209-2248) were strongly associated with a favorable response to IFN-$\alpha$ monotherapy in patients with genotype 1 HCV.[13] It is postulated that the NS5A protein, in which the ISDR exists, has transcriptional activation functions and represses interferon-induced gene expression.[27] The ISDR overlaps a putative acidic amino acid region that confers transcriptional activity.[28] Akuta et al.[29] reported that amino acid substitutions of R by Q at a.a. 70 or L by M at a.a. 91 in the core region were significantly frequent in patients who showed a null or weak response to combination treatment of 48 weeks. As such, we chose the ISDR among all HCV genetic factors for analysis because it has already been well characterized.[30]

Immunological backgrounds are known to be associated with response to IFN therapy because cellular immune functions are essential to eliminate HCV-infected hepatocytes. Masaki et al.[15] reported that a lower Th1/Th2 ratio before IFN monotherapy was a significant host factor for predicting long-term virological response in Japanese patients with chronic hepatitis C. Lee et al.[31] reported that high baseline sCD30 levels predicted an early and sustained virological response to IFN and RBV therapy, and suggested that therapy might be more effective in patients with a predominant T2 profile. Lagging et al.[32] reported that low levels of a 10-kDa IFN-$\gamma$ inducible protein predicted rapid and sustained virological response in patients with genotype 1 HCV treated with Peg-IFN and RBV combination therapy. Taken together, these results indicate that an imbalance of Th1 and Th2 subsets before IFN therapy is possibly associated with long-term therapy outcome. In the current study, we chose the Th1/Th2 ratio in peripheral blood as an immunological marker for predicting SVR because identification of helper T cell subpopulations at the cellular level has become practical with the development of intracellular cytokine assays using flow cytometry.

Of the virological and host factors analyzed in the current study, mutant ISDR, Th1/Th2 ratio 15.5 or less, body weight 59 kg, and neutrophil count 2300 cells/$\mu$L were selected as significant pretreatment factors predicting a higher rate of SVR. Although our study suggests that higher body weight is a favorable predictor for SVR in the Japanese, several studies on Caucasians have shown that higher body weight or BMI results in a lower SVR rate.[33,34] This difference may be attributed to a difference in average body weight among the studies; whereas median body weight was over 70 kg and patients with body weights of less than 59 kg were quite rare in studies reported from the United States and Europe, the median body weight in our study was 60.7 kg, and patients with body weights of less than 59 kg accounted for 45.8% of our cohort. According to manufacturer's instructions, patients with body weights equal to or less than 60 kg received 80 $\mu$g of Peg-IFN-$\alpha$-2b and 600 mg RBV as initial doses in the current study. It is possible that such doses were insufficient to achieve a high SVR rate despite having been adjusted accordingly. This possibility is further supported by our result that the distribution of BMI did not differ between patients with and without SVR.

A higher neutrophil count was also a significant pretreatment factor predicting a greater likelihood of SVR. Patients with chronic hepatitis C who have advanced fibrosis tend to show lower counts of neutrophils and platelets, which can interfere with administration of Peg-IFN, another significant treatment factor identified in this study. Indeed, pretreatment counts of neutrophils were significantly higher in patients who received sufficient (73% or more) Peg-IFN doses than in those who did not.

Interestingly, a lower Th1/Th2 ratio predicted a higher SVR rate in our study, contrary to the common knowledge that a stronger Th1 response is important to eradicate HCV; Shinohara et al.[35] reported that a higher increase in Th1 response during the early phase of interferon therapy was associated with higher SVR rates. However, these patients also showed a lower Th1/Th2 ratio before starting therapy than those without SVR. Thus, our result that a lower Th1/Th2 ratio predicts a favorable response does not necessarily refute the importance of the Th1 response in eradicating HCV. Further studies are required to clarify the significance of the Th1/Th2 ratio.

The logistic regression model for predicting SVR in this study yielded three patient groups classified according to predicted SVR rate. The actual SVR rates were 91% in the high sensitivity group, 41% in the intermediate sensitivity group, and 15% in the low sensitivity group, and all were well correlated with predicted SVR rates. Earlier