

Figure 3 Sustained virological response (SVR) rates according to virological response in re-treatment and treatment duration in patients with genotype 1. □, Patients treated for 48 weeks; ■, patients treated for 72 weeks. RVR, rapid virological response; cEVR, complete early virological response; LVR, late virological response. * $P < 0.05$; compared to 48 weeks of treatment.

genotype and antiviral effect of re-treatment because of their small number in this study. In this study, only one patient with the minor allele of IL-28B and NR in previous treatment could start and continue with the increased dose of PEG IFN (from 1.37 $\mu\text{g}/\text{kg}$ in the previous treatment to 1.79 $\mu\text{g}/\text{kg}$ in re-treatment) and ribavirin (from 10.3 mg/kg per day in the previous treatment to 11.1 mg/kg per day in re-treatment) and attained SVR by extended treatment. If the drug

adherence does not improve, patients with the minor allele of IL-28B who show NR in the previous treatment should be treated with new drugs.

The next question is how the patients should be re-treated in order to attain SVR on re-treatment. In this study, the patients with a low serum HCV RNA level ($<5 \log_{10}$ IU/mL) at the start of re-treatment showed a significant rate of cure on re-treatment, and this is almost the same result as that previously reported.^{16,17} In this study, the two patients with NR in the previous treatment and with less than 5 \log_{10} IU/mL of HCV RNA level (20 KIU/mL and 52 KIU/mL of HCV RNA) at the start of re-treatment attained SVR. On the other hand, even if the previous treatment response was a relapse, the SVR rates were 58% (25/43) among the patients with 5 \log_{10} IU/mL or more of HCV RNA. Because the HCV RNA level changed after the antiviral treatment, it is important to not miss the timing of when the HCV RNA level is low.

With respect to treatment duration among patients with HCV RNA negativation during re-treatment, 72 weeks of treatment significantly increased the SVR rate compared to 48 weeks. This result was almost the same as that of the REPEAT study.¹⁶ In our present study, the SVR rate among the patients with c-EVR but not RVR in re-treatment was significantly high by 72 weeks of treatment. On the other hand, the SVR rates among the

Table 4 Factors associated with a sustained virological response in re-treatment with PEG IFN plus ribavirin in patients with genotype 2

Factor	SVR	Non-SVR	P-value	
No. of patients	17	10		
Age (years)	57.7 \pm 8.8	63.7 \pm 5.1	0.06	
Sex: male/female	7/10	8/2	0.11	
Serum HCV RNA (log IU/mL)	5.4 \pm 1.4	6.1 \pm 0.8	0.15	
Serum HCV RNA: $<5 \log$ / $\geq 5 \log$	5/11	1/9	0.35	
White blood cells (/mm ³)	5049 \pm 1355	4171 \pm 910	0.10	
Neutrophils (/mm ³)	2556 \pm 1064	1999 \pm 404	0.24	
Hemoglobin (g/dL)	14.1 \pm 1.3	13.8 \pm 1.6	0.51	
Platelets ($\times 10^4/\text{mm}^3$)	17.9 \pm 5.4	14.8 \pm 4.3	0.17	
ALT (IU/L)	38 \pm 19	48 \pm 47	0.71	
IL-28B SNP: TT/TG	6/2	4/2	1.00	
ITPA SNP: CC/CA	5/1	4/0	1.00	
PEG IFN: α -2a/ α -2b	4/13	2/8	1.00	
PEG IFN dose ($\mu\text{g}/\text{kg}$ per week)	α -2a	3.23 \pm 0.34	2.24 \pm 2.25	1.00
	α -2b	1.32 \pm 0.28	1.18 \pm 0.23	0.21
Ribavirin dose (mg/kg per day)	10.4 \pm 2.21	10.1 \pm 1.31	0.44	
1st treatment virological response	RVR/non-RVR	4/13	3/7	1.00

ALT, alanine aminotransferase; HCV, hepatitis C virus; IFN, interferon; IL, interleukin; ISDR, IFN-sensitivity determining region; PEG, pegylated; RVR, rapid virological response; SNP, single nucleotide polymorphism; SVR, sustained virological response.

patients with RVR in re-treatment were similar between the patients with 48 weeks and 72 weeks of treatment. Thus, patients with c-EVR but not RVR in re-treatment should be re-treated for a longer period. In order to attain better SVR, extended treatment duration is generally recommended for patients with on-treatment LVR, whereas standard treatment duration is considered to be sufficient for patients with on-treatment c-EVR. However, the present study revealed that, even if patients achieved c-EVR on re-treatment, 72 weeks of treatment seems to be better than 48 weeks for treatment-experienced patients. The majority of naïve patients showing on-treatment c-EVR could eradicate HCV with 48 weeks of treatment while some could not. In a treatment-experienced setting, patients who are able to respond early but not eradicate HCV would be selected, and therefore extended treatment may be needed.

With genotype 2, the SVR rate was relatively high (63%). The patients who could not attain SVR in re-treatment (two patients) showed NR in the previous treatment. Thus, the patients with genotype 2 and showing NR in previous treatment seemed to be difficult to treat and could be treated with other drugs. Among the patients with RVR in re-treatment, the SVR rates were similar among those with RVR in re-treatment between 24 weeks and 48 weeks of treatment. The effectiveness of extended treatment for the patients with genotype 2 in re-treatment could not be demonstrated because of their small number in this study. Further investigation is needed to clarify this.

In conclusion, this study shows that the efficacy of re-treatment for genotype 1 patients who failed to show SVR to previous treatment with PEG IFN plus ribavirin could be predicted from the previous treatment response and a low HCV RNA level at the start of re-treatment. Re-treatment for 72 weeks led to clinical improvement for genotype 1 patients with c-EVR and without RVR on re-treatment.

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Baseline Factors and Early Viral Response (Week 4) to Antiviral Therapy With Peginterferon and Ribavirin for Predicting Sustained Virologic Response in Patients Infected With Hepatitis C Virus Genotype 1: A Multicenter Study

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Both baseline predictive factors and viral response at week 4 of therapy are reported to have high predictive ability for sustained virologic response to peginterferon and ribavirin combination therapy in patients with hepatitis C virus (HCV) genotype 1. However, it is not clear how these baseline variables and week 4 response should be combined to predict sustained virologic response. In this multicenter study, the authors investigated the impact of baseline predictive factors on the predictive value of week 4 viral response. Receiver-operating characteristic curve analyses were performed to evaluate the ability of week 4 reduction in HCV RNA levels to predict sustained virologic response in 293 Japanese patients infected with HCV genotype 1b. Analyses were performed in all patients and in patient subgroups stratified according to baseline variables. Overall, week 4 viral reduction demonstrates a high predictive ability for sustained virologic response. The sensitivity, specificity, positive predictive value (PPV), negative predictive value, and accuracy were higher than those of viral reduction at week 12. However, the best cut-off levels differ depending on the baseline factors and they were lower in patients with unfavorable baseline predictors. When patients had the TG/GG rs8099917 genotype, the best cut-off was markedly low with low PPV. Week 4 viral response can be a predictor of sustained virologic response in patients with HCV genotype 1 and is better than week 12 viral response. However, the cut-off

levels should be modified based on the baseline predictive variables. **J. Med. Virol.**

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INTRODUCTION

Although the combination antiviral therapy with peginterferon (PEG-IFN) and ribavirin has increased markedly the rate of patients with a sustained virologic response, that is, the eradication of hepatitis C virus (HCV), only 50% of patients infected with HCV genotype 1 had achieved a sustained virologic response, approximately. Several studies reported that early HCV viral dynamics during therapy have a high

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predictive value for a sustained virologic response in HCV genotype 1-infected patients. Previous studies reported that the response of HCV during combination therapy, that is, the changes in serum HCV RNA levels after starting therapy, has been shown to be an important predictor of the treatment outcome [Zeuzem et al., 2001; Buti et al., 2002; Berg et al., 2006]. Several recent reports have emphasized the importance of evaluating the viral dynamics at 4 weeks after starting therapy to predict a sustained virologic response. A rapid virologic response, in which serum HCV RNA is undetectable at 4 weeks after starting therapy, has been a strong predictive factor of a sustained virologic response reportedly [Martinez-Bauer et al., 2006; Poordad et al., 2008; Martinot-Peignoux et al., 2009; de Segadas-Soares et al., 2009]. In addition to a rapid virologic response, reduced serum HCV RNA levels at 4 weeks after starting therapy has also been reported to have a strong predictive value for the likelihood of achieving sustained virologic response to PEG-IFN and ribavirin combination therapy in patients infected with HCV genotype 1 [Yu et al., 2007; Huang et al., 2010; Toyoda et al., 2011; Marcellin et al., 2012]. These studies suggested that a reduction in HCV RNA levels at week 4 is closely associated with the probability of achieving sustained virologic response.

Aside from early viral response to therapy, several baseline host and viral factors are associated with treatment outcome. Genetic polymorphism near the *IL28B* gene (rs12979860 or rs8099917) is the strongest baseline factor associated with treatment outcome in patients with HCV genotype 1 reportedly [Ge et al., 2009; Suppiah et al., 2009; Tanaka et al., 2009; McCarthy et al., 2010; Rauch et al., 2010]. In addition, studies from Japan have reported that amino acid substitutions at residue 70 of the HCV core region and amino acids 2,209–2,248 of the NS5A region of HCV (i.e., interferon sensitivity-determining region, ISDR) are viral factors associated with treatment outcome in patients with HCV genotype 1b [Enomoto et al., 1996; Akuta et al., 2005, 2007a; Donlin et al., 2007; Maekawa and Enomoto, 2009; Hayes et al., 2011]. Given these various predictors for a sustained virologic response, that is, week 4 viral response and baseline variables, how should they be combined to predict treatment outcome more precisely? In the present study, the authors investigated how to incorporate week 4 viral response to PEG-IFN and ribavirin combination therapy with baseline predictive factors to predict a sustained virologic response.

MATERIALS AND METHODS

Patients and Analyses

In this multicenter study, 682 patients who underwent PEG-IFN alpha-2b and ribavirin combination therapy in a standard treatment regimen at one of the participating institutions, (Musashino Red Cross Hospital, Kurume University Hospital, Shin-Matsudo

Central General Hospital, Kagawa Prefectural Central Hospital, and Ogaki Municipal Hospital) between December 2004 and January 2010 were initially included into the retrospective analyses. All patients were infected with HCV genotype 1b; patients with HCV genotype 1a are usually not found in the Japanese general population. Pretreatment HCV RNA levels were $\geq 5.0 \log_{10}$ IU/ml, based on a quantitative real-time PCR-based method (COBAS AmpliPrep/COBAS TaqMan HCV Test; Roche Molecular Systems, Pleasanton, CA; lower limit of quantification, $1.7 \log_{10}$ IU/ml; lower limit of detection, $1.0 \log_{10}$ IU/ml) [Colucci et al., 2007; Pittaluga et al., 2008], because the use of ribavirin along with PEG-IFN is not approved by Japanese National Medical Insurance System for patients with pretreatment HCV RNA levels $< 5.0 \log_{10}$ IU/ml. No patients had co-infection with hepatitis B virus or human immunodeficiency virus. All patients had 100% medication adherence for both PEG-IFN and ribavirin during the initial 4 weeks of therapy and 80% or more throughout the treatment period. Among these 682 patients, three baseline factors, genetic polymorphism near the *IL28B* gene, amino acid substitution at residue 70 of the HCV core region, and ISDR sequence had been measured prior to treatment in 405 patients. We excluded 112 of these 405 patients with extended treatment duration up to 72 weeks because the extension of treatment duration might influence outcomes, leaving 293 patients who underwent 48-week standard regimen included in the final sample (Fig. 1).

Receiver-operating characteristic (ROC) analyses were performed to evaluate the value of week 4 reduction in HCV RNA levels in predicting sustained virologic response and an area under the ROC curve (AUROC) was generated. Best cut-off levels were determined based on the sensitivity and specificity. Sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and accuracy were also calculated using these cut-off levels. Analyses were performed for all patients and subgroups according to baseline variables. The same analyses were performed on the reduction in HCV RNA levels at week 12 after starting therapy.

The study protocol was in compliance with the Helsinki Declaration and was approved by the ethics committee of each participating institution.

Measurements of Serum HCV RNA Levels, Amino Acid Substitution at Residue 70 in the HCV Core, Amino Acid Sequence of ISDR, and Genetic Polymorphism Near the *IL28B* Gene

After each patient gave informed consent, serum samples were obtained during the patient's regular hospital visits just prior to beginning treatment, every 4 weeks during the treatment period, and during the 24-week follow-up period after treatment. Serum samples were stored at -80°C until they were analyzed. HCV RNA levels were measured using a quantitative

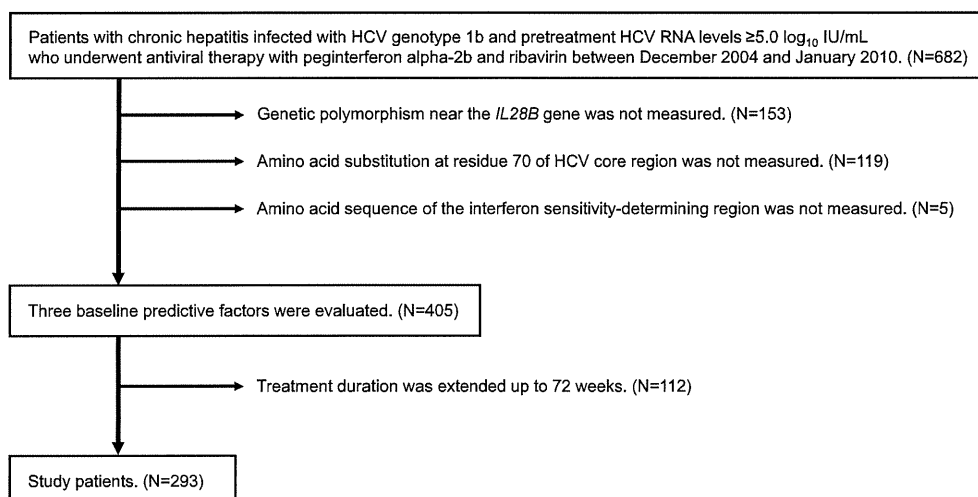


Fig. 1. Schematic representation of the study patients.

real-time PCR-based method (COBAS AmpliPrep/COBAS TaqMan HCV Test). The reductions in HCV RNA 4 and 12 weeks after starting therapy were calculated based on the pretreatment HCV RNA levels. When calculating the reduction in HCV RNA levels, HCV RNA concentration was defined as zero when HCV RNA was undetectable (i.e., rapid virologic response at week 4 and complete early virologic response at week 12).

Amino acid 70 of the HCV core region and the amino acid sequence of the ISDR were analyzed by direct nucleotide sequencing of each region as described previously [Enomoto et al., 1996; Akuta et al., 2007b]. The following PCR primer pairs were used for direct sequencing of the HCV core region:

5'-GCCATAGTGGTCTGCGGAAC-3' (outer, sense primer), 5'-GGAGCAGTCCTTCGTGACATG-3' (outer, antisense primer), 5'-GCTAGCCGAGTAGTGT-3' (inner, sense primer), and 5'-GGAGCAGTCCTTCGTGACATG-3' (inner, antisense primer). The following PCR primers were used for direct sequencing of ISDR: 5'-TTCCACTACGTGACGGGCAT-3' (outer, sense primer), 5'-CCCCTCCATGTGTAGGACAT-3' (outer, antisense primer), 5'-GGGTCACAGCTCCCTGTGAGCC-3' (inner, sense primer), and 5'-GAGGGTTGTAATCCGGCGTGC-3' (inner, antisense primer). When evaluating the ISDR, HCV was defined as wild-type when there were zero or one amino acid substitutions in residues 2,209–2,248 as compared with the HCV-J strain [Kato et al., 1990], and as non-wild-type when there was more than one substitution.

Genotyping of rs8099917 polymorphisms near the *IL28B* gene was performed using the TaqMan SNP assay (Applied Biosystems, Carlsbad, CA) according to the manufacturer's guidelines. A pre-designed and functionally tested probe was used for rs8099917 (C_11710096_10, Applied Biosystems). Genetic polymorphism of rs8099917 reportedly corresponds to

rs12979860 in more than 99% of individuals of Japanese ethnicity [Tanaka et al., 2010]. The TT genotype of rs8099917 corresponds to the CC genotype of rs12979860, the GG genotype of rs8099917 corresponds to the TT genotype of rs12979860, and the TG heterozygous genotype of rs8099917 corresponds to the CT of rs12979860.

RESULTS

Patients Characteristics and Baseline Variables

Table I summarizes patient characteristics. The polymorphism of rs8099917 was TT genotype in 204 patients (69.6%). Amino acid substitution at residue 70 was arginine in 200 patients (68.3%). HCV-ISDR was non-wild-type in 78 patients (26.6%). All these variables (TT genotype of rs8099917, arginine at residue 70, and non-wild-type ISDR) were reportedly associated with favorable response to therapy.

As a final outcome, 113 patients (38.6%) achieved sustained virologic response. Sensitivity, specificity, PPV, NPV, and accuracy were 97%, 48%, 54%, 97%, and 67%, respectively, according to genotypes of rs8099917 near the *IL28B* gene. They were 85%, 42%, 48%, 82%, and 59%, respectively, according to amino acid substitutions at residue 70 in the HCV core region, and 43%, 84%, 63%, 70%, and 78%, respectively, according to ISDR of HCV NS5A region.

Association Between Week 4 Viral Reduction and Treatment Outcome Based on Baseline Predictive Factors

Table II shows the predictive value of a reduction in serum HCV RNA levels at week 4 of therapy in all patients and based on each baseline predictive variable. Week 4 viral reduction demonstrates a high predictive ability for a sustained virologic response with

TABLE I. Characteristics of Study Patients

Age (years), median (range)	60 (20–80)
Sex (male/female) (%)	150 (51.2)/143 (48.8)
BMI, median (range)	22.6 (15.8–33.3)
Prior treatment for HCV (no/yes) (%)	201 (68.6)/92 (31.4)
Initial dose of PEG-IFN (μg), median (range)	80.0 (40.0–150.0)
Initial dose of ribavirin (mg), median (range)	600 (200–1,000)
Pretreatment HCV RNA levels (\log_{10} IU/ml), median (range)	6.1 (5.0–7.4)
Platelet count ($\times 10^3/\mu\text{l}$)	159 (43–373)
Hemoglobin (g/dl)	13.9 (8.6–18.1)
Neutrophil count (μl^{-1})	2,430 (4,670–7,480)
Alanine aminotransferase (IU/L)	49 (10–485)
Genetic polymorphisms of rs8099917 (TT/TG or GG) (%)	204 (69.6)/89 (30.4)
Amino acid at residue 70 of HCV core (arginine/glutamine or histidine) (%)	200 (68.3)/93 (31.7)
Amino acid sequence of ISDR (non-wild-type/wild-type) (%)	78 (26.6)/215 (73.4)

(N = 293).

BMI, body mass index; HCV, hepatitis C virus; PEG-IFN, peginterferon; ISDR, interferon sensitivity-determining region.

a high AUROC in all patients, in which sensitivity, specificity, PPV, NPV, and accuracy were more than 80%. The best cut-off for the prediction was 3.1- \log_{10} reduction. When patients were stratified according to baseline predictive factors, AUROC remained above 0.85, indicating retention of high predictive ability. However, the best cut-off levels differ depending on baseline factors, and they were lower in patients with unfavorable baseline predictors (TG/GG genotype of rs8099917 near the *IL28B* gene, glutamine/histidine at residue 70 of the HCV core region, and wild-type of ISDR). Especially, when patients had the TG/GG rs8099917 genotype, the calculated best cut-off level was markedly lower than that of patients with the TT genotype. Sensitivity, specificity, PPV, NPV, and accuracy were more than 70% in all patient subgroups, except for patients with the TG/GG genotype in whom PPV was only 10%.

Association Between Week 12 Viral Reduction and Treatment Outcome Based on Baseline Predictive Factors

Table III shows the predictive value of a reduction in serum HCV RNA levels at week 12 of therapy in all patients and based on each baseline predictive variable. The predictive ability of week 12 viral reduction

for sustained virologic response was decreased in comparison to that of week 4 with a low AUROC in all patients. The specificity, PPV, and accuracy of the prediction at week 12 were also lower than those at week 4. The best cut-off levels increased to 5.0- \log_{10} reduction. When patients were stratified according to the genetic polymorphisms of rs8099917 near the *IL28B* gene and according to amino acid substitutions at residue 70 of the HCV core region, the differences of the best cut-off levels based on these baseline factors were less marked than those at week 4, although the best cut-off levels remained lower in patients with unfavorable baseline predictors. The difference of best cut-off levels between patients with TT genotype and with TG/GG genotype of rs8099917 also decreased, but PPV in patients with TG/GG genotype remained low (21%). In contrast, the difference in the best cut-off levels increased when patients were stratified according to amino acid sequences in ISDR. The best cut-off level of the reduction in HCV RNA levels at week 12 for predicting sustained virologic response was higher in patients with HCV of wild-type ISDR, an unfavorable baseline variable, than in patients with HCV of favorable non-wild-type ISDR, which was inverse to the evaluation with week 4 viral reduction in which the cut-off level was higher in patients with HCV of non-wild-type ISDR.

TABLE II. AUROC, Best Cut-Off Level, Sensitivity, Specificity, PPV, NPV, and Accuracy of the Reduction in Serum HCV RNA Levels 4 Weeks After Starting PEG-IFN and Ribavirin Combination Therapy From Pretreatment Levels for Predicting Sustained Virologic Response

	N	AUROC	Best cut-off	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Accuracy (%)
Overall	293	0.92746	3.1 \log_{10}	88	87	81	92	87
<i>IL28B</i> -TT	204	0.88353	3.2 \log_{10}	87	78	82	84	83
<i>IL28B</i> -TG or GG	89	0.84302	1.1 \log_{10}	100	69	10	100	70
Core 70-R	200	0.91023	3.2 \log_{10}	86	83	82	87	85
Core 70-Q or H	93	0.94350	2.8 \log_{10}	88	93	75	97	92
ISDR-non-wild type	78	0.93455	3.0 \log_{10}	90	90	94	84	90
ISDR-wild type	215	0.92654	2.9 \log_{10}	92	84	71	96	87

AUROC, area under the receiver-operating characteristics curve; PPV, positive predictive value; NPV, negative predictive value; HCV, hepatitis C virus; PEG-IFN, peginterferon; R, arginine; Q, glutamine; H, histidine; ISDR, interferon sensitivity-determining region.

TABLE III. AUROC, Best Cut-Off Level, Sensitivity, Specificity, PPV, NPV, and Accuracy of the Reduction in Serum HCV RNA Levels 12 Weeks After Starting PEG-IFN and Ribavirin Combination Therapy From Pretreatment Levels for Predicting Sustained Virologic Response

	N	AUROC	Best cut-off	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Accuracy (%)
Overall	293	0.86907	5.0 log ₁₀	88	73	67	91	79
<i>IL28B</i> -TT	204	0.79216	5.11 log ₁₀	81	61	70	73	71
<i>IL28B</i> -TG or GG	89	0.92829	4.6 log ₁₀	100	87	21	100	88
Core 70-R	200	0.81791	5.0 log ₁₀	88	63	69	86	75
Core 70-Q or H	93	0.94272	4.9 log ₁₀	100	84	59	100	87
ISDR-non-wild type	78	0.87298	5.0 log ₁₀	88	79	88	79	85
ISDR-wild type	215	0.89572	5.4 log ₁₀	84	79	63	92	81

AUROC, area under the receiver-operating characteristics curve; PPV, positive predictive value; NPV, negative predictive value; HCV, hepatitis C virus; PEG-IFN, peginterferon; R, arginine; Q, glutamine; H, histidine; ISDR, interferon sensitivity-determining region.

DISCUSSION

This study was conducted to confirm the predictive value of week 4 viral dynamics of HCV for predicting sustained virologic response to the combination therapy with PEG-IFN and ribavirin in patients infected with HCV genotype 1 and with pretreatment HCV RNA levels of ≥ 5.0 log₁₀ IU/ml in a large multicenter study of Japan. The comparison of the predictability for sustained virologic response between week 4 and week 12 viral reductions revealed the higher predictive ability of week 4 viral response. In a recent study, Marcellin et al., [2012] suggested that a ≥ 3 log₁₀ reduction in HCV RNA levels at week 4 of PEG-IFN and ribavirin combination therapy is a reliable factor for predicting sustained virologic response in patients with HCV genotype 1. Our current results are consistent with their analysis for patients with HCV genotype 1b and those with pretreatment HCV RNA levels ≥ 5.0 log₁₀ IU/ml overall. The reduction in HCV RNA levels at week 4 appears to be a good and reliable predictor for a sustained virologic response. Although week 12 viral response (i.e., early virologic response) has been used as a pivotal decision criterion to extend treatment duration or to discontinue treatment, the predictive value is lower when the reduction in HCV RNA levels is compared to week 4 viral response.

When patients were stratified based on baseline predictive factors, however, the best cut-off levels for sustained virologic response were not constant. The cut-off levels decreased in patients with unfavorable baseline factors, that is, TG/GG genotype of rs8099917, glutamine/histidine at residue 70 of the HCV core region, and wild-type sequence of ISDR, indicating that the reduction in HCV RNA occurs slowly in patients with these unfavorable baseline variables. Conversely and paradoxically, the results may indicate that one can expect sustained virologic response in patients with a smaller reduction in HCV RNA levels at week 4 if they have unfavorable baseline variables.

When predictive value was evaluated using week 12 viral reduction, the best cut-off levels remained lower in patients with unfavorable TG/GG rs8099917 genotype and patients with HCV of unfavorable

glutamine/histidine at residue 70 of the HCV core region. In contrast, the best cut-off level was higher in patients with HCV of unfavorable wild-type ISDR. Previous studies reported the association between the genetic polymorphisms near the *IL28B* gene (rs12979860 and rs8099917) and amino acid substitution at residue 70 of HCV core region [Abe et al., 2010; Kobayashi et al., 2010], whereas no associations were reported between these two variables and ISDR mutation. This might explain the difference in the relationship of early viral response during therapy between with two baseline predictive factors, *IL28B* genetic polymorphisms and amino acid substitution of HCV core region and with ISDR mutation.

The calculated PPV was markedly low in patients with the unfavorable TG/GG genotype of rs8099917 (CT/TT genotype of rs12979860) both by the evaluations at weeks 4 and 12 viral responses. Therefore, it appears to be difficult to identify patients in this subgroup who are likely to achieve a sustained virologic response by their week 4 viral response, although week 4 viral response can be a factor used to identify patients with a high likelihood of achieving sustained virologic response in other subgroups.

In conclusion, week 4 viral response can be a predictor of sustained virologic response in patients with HCV genotype 1. However, the cut-off levels should be modified based on baseline host and viral predictive variables. In addition, week 4 viral response is not predictive in patients with unfavorable genotype of genetic polymorphism near the *IL28B* gene.

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Noninvasive estimation of fibrosis progression overtime using the FIB-4 index in chronic hepatitis C

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SUMMARY. The FIB-4 index is a simple formula to predict liver fibrosis based on the standard biochemical values (AST, ALT and platelet count) and age. We here investigated the utility of the index for noninvasive prediction of progression in liver fibrosis. The time-course alteration in the liver fibrosis stage between paired liver biopsies and the FIB-4 index was examined in 314 patients with chronic hepatitis C. The average interval between liver biopsies was 4.9 years. The cases that showed a time-course improvement in the fibrosis stage exhibited a decrease in the FIB-4 index, and those that showed deterioration in the fibrosis stage exhibited an increase in the FIB-4 index with a significant correlation ($P < 0.001$). Increase in the Δ FIB-4 index per year was an independent predictive factor for the progression in

liver fibrosis with an odds ratio of 3.90 ($P = 0.03$). The area under the receiver operating characteristic curve of the Δ FIB-4 index/year for the prediction of advancement to cirrhosis was 0.910. Using a cut-off value of the Δ FIB-4 index/year < 0.4 or ≥ 0.4 , the cumulative incidence of fibrosis progression to cirrhosis at 5 and 10 years was 34% and 59%, respectively in patients with the Δ FIB-4 index/year ≥ 0.4 , whereas it was 0% and 3% in those with the Δ FIB-4 index/year < 0.4 ($P < 0.001$). In conclusion, measurement of the time-course changes in the FIB-4 index is useful for the noninvasive and real-time estimation of the progression in liver fibrosis.

Keywords: FIB-4, fibrosis, HCV, noninvasive.

INTRODUCTION

Advanced stage of liver fibrosis in chronic hepatitis C is associated with failure of interferon therapy or development of major concomitant disease such as variceal bleeding, liver failure and hepatocellular carcinoma [1–3]. Therefore, evaluation of the stage of liver fibrosis is essential in clinical practice. Liver biopsy is the gold standard for diagnosis of liver fibrosis [4,5], but inaccuracy in evaluation of fibrosis because of sampling errors [6–8] or by the inter-observer variation has been reported [9]. Real-time assessment of liver fibrosis may be clinically useful, but the invasiveness of liver biopsy precludes repeated examinations.

A variety of noninvasive methods to diagnose liver fibrosis have been proposed. Recently, transient elastography [10–13] and real-time tissue elastography [14] using ultrasonography

have been developed, but these modalities are not widely available. For blood tests, the aspartate aminotransferase (AST)/alanine aminotransferase (ALT) ratio [15], the AST/platelet ratio index (APRI) [16,17] and the Fibrotest [18,19] have been reported to be useful. The FIB-4 index is another prediction value of liver fibrosis in chronic hepatitis C based on the standard biochemical values and age. The FIB-4 index has been reported to be markedly useful for the prediction of advanced liver fibrosis [20,21]. Given its noninvasiveness and simplicity, the FIB-4 index has the advantage of an easy follow-up of the time-course changes by repeated measurements.

In the present study, we investigated the utility of the real-time assessment of the FIB-4 index for the prediction of time-course progression in liver fibrosis.

PATIENTS AND METHODS

Patients

A total of 421 patients with chronic hepatitis C who had repeated liver biopsies between 1991 and 2010 at the Musashino Red Cross hospital were consecutively investigated. All patients received interferon therapy after the first biopsy and had nonsustained virological response. A second

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; HBV, hepatitis B virus; HCV, hepatitis C virus; HIV, human immunodeficiency virus.

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biopsy was performed at least 6 months after the completion of interferon therapy. Exclusion criteria were as follows: (i) co-infection with HBV or HIV ($n = 1$), (ii) alcohol abuse (intake of alcohol equivalent to pure alcohol 40 g/day or more) ($n = 8$), (iii) the presence of nonalcoholic steatohepatitis ($n = 14$), (iv) the presence of hepatocellular carcinoma ($n = 15$), (v) interval between paired biopsies was <1.5 years ($n = 41$) and (vi) length of biopsy sample <15 mm ($n = 28$). The demographic characteristics of the 314 patients enrolled are shown in Table 1.

Assessment of liver fibrosis stage

Liver biopsy was carried out under laparoscopic or ultrasonographic guidance. A sample 15 mm or larger was collected and evaluated. The fibrosis stage was categorized according to the METAVIR score: F0, no fibrosis; F1, portal fibrosis without septa; F2, portal fibrosis with few septa; F3, numerous septa without cirrhosis; and F4, cirrhosis. Two pathologists examined all samples and determined the fibrosis stage. When staging was inconsistent between the two pathologists, an appropriate stage was determined by discussion between the two.

Calculation of FIB-4 index

The FIB-4 index at the time of each liver biopsy was calculated based on the blood test results within 1 month before

liver biopsy according to the following formula: The FIB-4 index = (age [years] \times AST [IU/L]) / (platelet count [10^9 /L] \times (ALT [IU/L])^{1/2}). Change in the FIB-4 index per year (Δ FIB-4 index/year) was calculated by the following formula: Δ FIB-4 index/year = (the FIB-4 index at the second liver biopsy – the FIB-4 index at the first liver biopsy) / interval between paired biopsies (years). Change in AST, ALT, platelet counts per year (Δ AST/year, Δ ALT/year, Δ Platelet counts/year) and the degree of changes in the fibrosis stage per year were calculated similarly.

Statistical analysis

The SPSS software package 15.0 (SPSS Inc, Chicago, IL, USA) was used for statistical analysis. Categorical data were analysed using Fisher's exact test. Continuous variables were compared with Student's *t*-test. Factors associated with the progression in liver fibrosis were analysed by multivariate logistic regression analysis. Association between progression in fibrosis stage and changes in the FIB-4 was analysed by Spearman's rank correlation test. Kaplan–Meier method and log-rank test were used to analyse time to occurrence of fibrosis progression to cirrhosis. A *P*-value of < 0.05 was considered statistically significant.

RESULTS

Changes in liver fibrosis stage overtime

The clinical backgrounds of patients at the first and second biopsies are shown in Table 1. The average interval was 4.9 years between the two liver biopsies. The fibrosis stage progressed over time in 23%, regressed in 17% and remained unchanged in 60%. Changes of fibrosis stage stratified by the fibrosis stage at the first liver biopsy are shown in Table 2.

Comparison of FIB-4 index and liver fibrosis stage

For the prediction of advanced liver fibrosis (F3–4), a FIB-4 index <1.45 had a negative predictive value of 97%, whereas a FIB-4 > 3.25 had a positive predictive value of 49% at first biopsy. Similarly, a FIB-4 < 1.45 had a negative predictive value of 98%, and a FIB-4 > 3.25 had a positive predictive value of 54% at second biopsy (Fig. 1).

Table 1 Clinical background of patients

	First biopsy	Second biopsy
Age (years)	53.7 \pm 9.8	58.7 \pm 9.4
Gender (male/female)	149/165	
AST (IU/L)	64.5 \pm 36.7	58.5 \pm 37.7
ALT (IU/L)	87.7 \pm 58.9	69.9 \pm 53.9
Platelet counts ($\times 10^9$ /L)	165 \pm 48	159 \pm 48
Histological findings		
Activity: 0/1/2/3	38/143/117/16	10/147/131/26
Fibrosis: 0–1/2/3/4	139/107/61/7	134/101/63/16
Interval of between biopsies (years)	4.9 \pm 2.9	–

AST, aspartate aminotransferase; ALT, alanine aminotransferase.

Table 2 Changes of fibrosis stage over time

Fibrosis stage at first biopsy	Fibrosis stage at second biopsy				Total
	F0–1 (%)	F2 (%)	F3 (%)	F4 (%)	
F0–1	98 (71)	33 (24)	8 (5)	–	139
F2	33 (31)	50 (47)	21 (20)	3 (2)	107
F3	3 (5)	18 (29)	33 (55)	7 (11)	61
F4	–	–	1 (14)	6 (86)	7

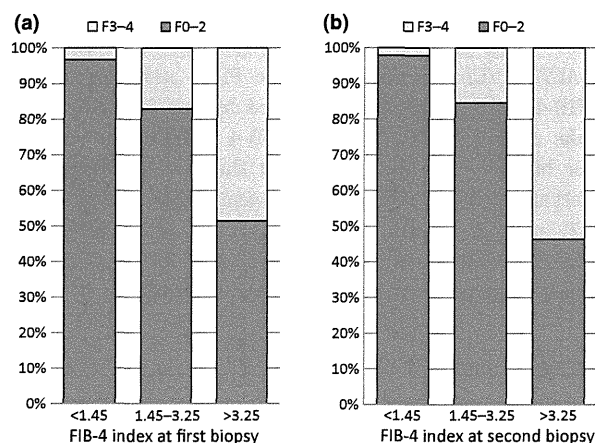


Fig. 1 Comparison of the FIB-4 index and liver fibrosis stage. Patients were categorized into three groups according to the FIB-4 index using cut-off values of < 1.45, 1.45–3.25, > 3.25 at liver biopsy. The lower bar chart (dark grey) indicates patients with F0–2, while the upper bar chart (light grey) indicates patients with F3–4. (a) comparison of the FIB-4 index and liver fibrosis stage at first biopsy and (b) at second biopsy.

Predictive factors for the progression of fibrosis

Higher level of Δ AST/year, lower level of Δ ALT/year, lower level of Δ Platelet counts/year and higher level of the Δ FIB-4/year were significantly associated with the progression of fibrosis overtime (Table 3). Multivariate analysis demonstrated that only the Δ FIB-4 index/year was an independent

predictive factor for the progression of fibrosis stage ($P = 0.03$) with an odds ratio of 3.70 (95% CI:1.07–12.5).

Correlation between the degree of changes in the fibrosis stage and the Δ FIB-4 index per year

When the patients were categorized into five groups according to the degree of changes in the fibrosis stage per year (< -0.2, -0.2 – < 0, 0, > 0 – 0.2 and > 0.2), median value of the Δ FIB-4 index/year was -0.29, -0.02, 0.04, 0.16 and 0.47, respectively. The FIB-4 index reduced along the regression of the fibrosis stage, while the FIB-4 index increased along the progression of the fibrosis stage, which showed a significant correlation ($P < 0.001$) (Fig. 2).

Prediction of progression to cirrhosis by the changes in the FIB-4 index per year

The area under the receiver operating characteristic curve of the Δ FIB-4 index/year for the prediction of advancement to cirrhosis was 0.910. By the Δ FIB-4 index/year of 0.4, the sensitivity and specificity for the prediction of advancement to cirrhosis was 80% and 91%. The cumulative incidence of fibrosis progression to cirrhosis, at 5 and 10 years, was 34% and 59%, respectively, in patients with the Δ FIB-4 index/year ≥ 0.4 , whereas it was 0% and 3% in those with the Δ FIB-4 index/year < 0.4 ($P < 0.001$) (Fig. 3).

DISCUSSION

Recently, noninvasive markers of liver fibrosis have been used as a predictive factor of liver-related outcome such as

Table 3 Factors associated with the progression of liver fibrosis

	Progression of	Nonprogression of	P-value
	Liver fibrosis	Liver fibrosis	
Gender (male/female)	31/42	118/123	0.33
Age at first biopsy (years)	54.4 \pm 8.7	53.5 \pm 10.2	0.50
AST at first biopsy (IU/L)	63.9 \pm 35.0	64.8 \pm 37.3	0.85
ALT at first biopsy (IU/L)	86.5 \pm 58.4	88.1 \pm 59.2	0.84
Platelet counts at first biopsy ($10^9/L$)	15.8 \pm 4.6	16.7 \pm 4.8	0.16
Change between biopsies			
Δ AST (IU/L)/year	3.8 \pm 19.5	-4.1 \pm 14.8	<0.001
Δ ALT (IU/L)/year	-1.9 \pm 28.4	7.2 \pm 22.6	0.005
Δ Platelet counts ($10^9/L$)/year	-4.1 \pm 9.5	-0.002 \pm 9.5	0.001
Δ FIB-4 index/year	0.31 \pm 0.52	-0.005 \pm 0.37	<0.001

Δ AST/year: (AST at the second liver biopsy – AST at the first liver biopsy) /interval between paired biopsies (years); Δ ALT/year: (ALT at the second liver biopsy – ALT at the first liver biopsy) /interval between paired biopsies (years); Δ Platelet counts/year: (platelet counts at the second liver biopsy – platelet counts at the first liver biopsy) /interval between paired biopsies (years); Δ FIB-4 index /year: (the FIB-4 index at the second liver biopsy – the FIB-4 index at the first liver biopsy) /interval between paired biopsies (years).

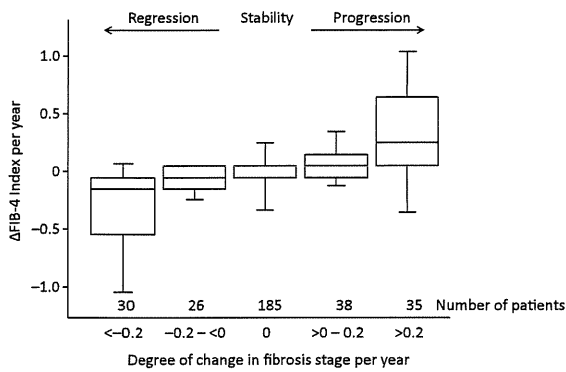


Fig. 2 Correlation between the degree of changes in the fibrosis stage and the Δ FIB-4 index per year. Boxplot of the Δ FIB-4 index/year is shown according to the degree of changes in the fibrosis stage per year. The bottom and top of each box represent the 25 and 75th percentiles, giving the interquartile range. The line through the box indicates the median value, and the error bar indicates the 5 and 95th percentiles.

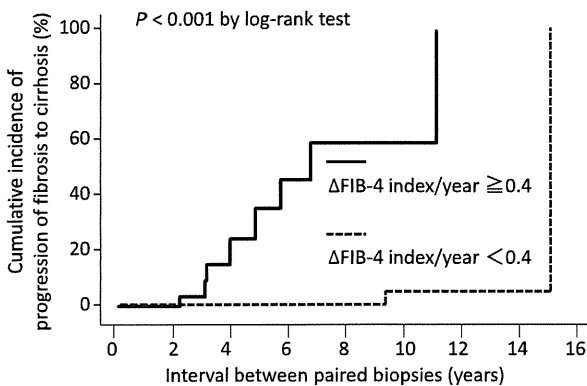


Fig. 3 Cumulative incidence of fibrosis progression to cirrhosis. Patients were categorized into two groups according to the Δ FIB-4 index/year using cut-off value of < 0.4 or ≥ 0.4 .

mortality [22–24] or HCC development [24–26] in patients with chronic liver disease. There have been few studies that investigated the association between changes of noninvasive markers and liver-related outcome [27–29]. However, it is still unclear whether there is a relation between the time-course changes in the value of noninvasive markers and progression of liver fibrosis.

The aim of the study was to evaluate the utility of the real-time assessment of the FIB-4 index for the prediction of time-course progression in liver fibrosis. We have shown that the FIB-4 index reduced along the regression of the fibrosis stage, while the FIB-4 index increased along the progression of the fibrosis stage. These results indicate that the measurement of the time-course changes in the FIB-4 index may

be useful for the noninvasive and real-time estimation of the progression in liver fibrosis overtime.

Although the gold standard for diagnosis of liver fibrosis is liver biopsy, there are a variety of problems including invasiveness and sampling errors [6]. Diagnostic methods of liver fibrosis by measurement of elasticity of the liver by ultrasonography [10–14] have been developed, but these modalities are not widely available.

The FIB-4 index has an advantage among these noninvasive liver fibrosis diagnostic methods. Firstly, it is quite easily calculated. The parameters required for calculation are only age, AST, ALT and platelet counts, which are measured at the routine examination of patients with liver disease. Therefore, additional blood collection is unnecessary, and the index can be calculated at no cost. Secondly, because of its simple calculation, it is possible to evaluate the clinical conditions in a real-time manner. Repeated measurements of the FIB-4 index make it possible to predict deterioration in liver fibrosis continuously over time. Because no special equipment or system is necessary, and objective data on the clinical conditions are provided in a real-time manner, the FIB-4 index is simple and convenient compared with other noninvasive liver fibrosis diagnostic methods.

It is widely known that a decrease in platelet counts is useful for the prediction of the progression of fibrosis stage [30]. We have reported that elevated AST or ALT is also associated with the progression of liver fibrosis [31]. However, the results of this study showed that a change in the FIB-4 index over time was a more useful factor for the prediction of the progression of fibrosis stage than AST, ALT and changes in platelet counts.

Liver biopsy is still an important examination as the gold standard for diagnosis of liver fibrosis, but time-course changes cannot be readily observed by repeated biopsies because of its invasiveness. On the other hand, it is possible to estimate the progression of liver fibrosis by repeated measurement of the FIB-4 index. Therefore, two examinations should be combined: liver biopsy may be utilized to determine the baseline of fibrosis stage, and the serial measurement of the FIB-4 index may be utilized to predict changes of fibrosis stages overtime in a real-time manner.

In conclusion, we believe that measurement of the time-course changes in the FIB-4 index is useful for the noninvasive and real-time estimation of the progression in liver fibrosis.

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CONFLICT OF INTEREST

No conflicts of interest exist for all authors.

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Original Article

Pegylated interferon monotherapy in patients with chronic hepatitis C with low viremia and its relationship to mutations in the NS5A region and the single nucleotide polymorphism of interleukin-28B

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Aim: Previous studies have suggested that patients with chronic hepatitis C with a low pretreatment hepatitis C virus (HCV) level have a high sustained virological response (SVR) rate, and that there would be a subpopulation of patients in which HCV can be eradicated with pegylated interferon (PEG IFN) alone without a decrease in SVR. However, the efficacy of PEG IFN monotherapy in patients with low HCV RNA levels is unclear. Several studies have reported that interferon sensitivity-determining region (ISDR) and the single-nucleotide polymorphism (SNP) of interleukin-28B (IL-28B) contribute to IFN response, but these relationships are controversial. The aim of this study was to determine whether the SNP of IL-28B (rs8099917) and amino acid substitutions in the ISDR among patients with low HCV levels affect the response to PEG IFN monotherapy.

Methods: One hundred and four patients with low-level HCV infection were studied. Low HCV level was defined as 100 KIU/mL or less.

Results: SVR was achieved in 94 patients (92.2%). HCV levels (≤ 50 KIU/mL) and ISDR (≥ 2 mutations) were associated with SVR on univariate analysis. The rates of SVR in the patients with IL-28B genotypes TT, TG and GG were 94.5%, 77.8% and 100%, respectively. The G allele tended to be associated with poor response to IFN therapy ($P = 0.0623$). On multivariate analysis, the ISDR was the factor predictive of SVR ($P = 0.004$).

Conclusion: The ISDR is significantly associated with a good response to PEG IFN monotherapy in patients with low HCV levels.

Key words: hepatitis C virus, interferon sensitivity-determining region, interferon, interleukin-28B, rapid virological response

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INTRODUCTION

HEPATITIS C VIRUS (HCV) is a member of the Flaviviridae family and causes chronic hepatitis that can develop into cirrhosis and hepatocellular carcinoma (HCC) that easily progresses to end-stage liver disease.¹ Because 170 000 000 persons are infected with HCV worldwide, HCV infection is a significant global health problem.

The current recommended therapy for patients with chronic hepatitis C is a combination of pegylated interferon (PEG IFN) and ribavirin and/or telaprevir or boceprevir.²⁻⁶ HCV RNA levels, as well as genotypes, are an important factor associated with sustained virological response (SVR) to IFN therapy.^{3,4} Patients with low HCV RNA levels have a high SVR rate, and even standard IFN monotherapy is useful for eradication of HCV in patients with low viral loads.⁷⁻⁹ Several studies have succeeded in reducing the duration of treatment without risk of relapse.^{10,11} Although patients with low HCV RNA have higher response rates to IFN treatment, not all patients achieve SVR. Other factors for improving the prediction of SVR in patients with low HCV RNA levels are needed. The predictive factors for SVR in patients with genotype 1b and high HCV RNA levels have been investigated, and several studies have shown that the single nucleotide polymorphism of interleukin-28B (IL-28B) and amino acid substitutions in the core and NS5A region affect the response to IFN therapy.¹²⁻¹⁶ However, the predictive factors for SVR among patients with low HCV RNA levels treated with PEG IFN monotherapy have been unclear.

Hepatitis C virus consists of three structural proteins (core, envelope 1 and envelope 2) and six non-structural proteins (NS2 to NS5). HCV NS5A protein was reported to have a domain associated with IFN response. This domain in the region of HCV genotype 1b is closely associated with response to IFN therapy and is known as the IFN sensitivity-determining region (ISDR).^{12,15-21} IFN acts to control replication of the virus by inducing the dsRNA-dependent protein kinase (PKR). The ISDR is located in the PKR-binding domain, is inhibited by PKR *in vitro*,²² and is useful for prediction in patients with genotypes 2a, 2b and 3a.²³⁻²⁸ Therefore, ISDR heterogeneity is an important factor that may affect response to IFN in patients with low HCV RNA levels. We hypothesized that ISDR heterogeneity could be predicted in patients with low HCV RNA levels in which HCV can be eradicated with PEG IFN- α alone without a decrease in SVR.

Not only genetic heterogeneity in the HCV genome but also host genetics contribute to IFN treatment outcomes. Therefore, several studies were performed to understand the host factors associated with IFN responsiveness; these showed that IL-28B polymorphisms are strongly associated with response to PEG IFN and ribavirin combination therapy in patients with genotype 1b and high viral load.^{13,14,16,29} However, the associations between ISDR and IL-28B and the effects of PEG IFN- α

monotherapy in patients with low HCV RNA levels are not well known.

The aim of the present study was to determine whether genomic heterogeneity of the ISDR and the SNP of IL-28B among patients with low HCV RNA levels affects the response to PEG IFN- α -2a monotherapy.

METHODS

A TOTAL OF 295 patients with chronic hepatitis C were treated by PEG IFN- α -2a monotherapy at Nagoya University Hospital and Affiliated Hospitals; 104 patients with low HCV RNA levels were selected for this study. The patients consisted of 62 men and 42 women with a mean age of 55.1 years (range, 19-78). All patients were positive for serum anti-HCV antibody by a commercial enzyme-linked immunosorbent assay (Dinabot, Tokyo, Japan) and for HCV RNA by a commercial polymerase chain reaction (PCR) (Roche Diagnostic Systems, Tokyo, Japan).

A low HCV level was defined as 100 KIU/mL or less, as previously reported.^{4,7,9,11} No patient had hepatitis B surface antigen, co-infection with HIV, autoimmune disease or chronic alcohol abuse.

Schedule of IFN therapy

Patients received PEG IFN- α -2a (Pegasys Chugai-Roche, Tokyo, Japan) at a dose of 180 μ g injected s.c. once per week for 24 or 48 weeks. The patients were allocated, at the discretion of the physician in charge, to a protocol lasting either 24 or 48 weeks. Laboratory tests and evaluations of adverse events were performed once per week during treatment.

The dose of PEG IFN- α -2a was reduced to 90 μ g when clinically significant adverse events or laboratory abnormalities such as neutropenia (<750 cells/mm³) or thrombocytopenia (<50 000 cells/mm³) occurred. PEG IFN- α -2a was discontinued when neutropenia of less than 250 cells/mm³ or a platelet count of less than 25 000 cells/mm³ was seen.

Hepatitis C virus RNA in serum samples was examined at 4 weeks, at the end of IFN therapy, and at 6 months after the end of treatment (ETR). Serum was stored at -80°C for virological examination at pretreatment.

Patients who were persistently negative for serum HCV RNA and who had a normal serum alanine aminotransferase (ALT) level at 24 weeks after withdrawal of IFN treatment were considered to have SVR. Patients who were HCV negative at the ETR but returned to HCV

positive status after withdrawal of IFN were defined as virological relapsers. Patients who did not become HCV negative with IFN therapy were defined as non-virological responders.

This study was approved by the ethics committee of each institution involved. Informed consent was obtained from each patient, and the study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki.

Virological tests

Hepatitis C virus was genotyped by direct sequencing of the 5'-untranslated region and/or E1 regions, as described previously.^{30,31} Genotypes were classified according to the nomenclature proposed by Simmonds *et al.*³²

Nested PCR analysis and direct sequencing of the NS5A-ISDR were performed as previously reported for each genotype.^{15,16,27,28} In brief, RNA was extracted from 140 μ L serum using a QIAamp Viral RNA Kit (Qiagen, Valencia, CA, USA) and dissolved in 50 μ L diethylpyrocarbonate-treated water. RNA (10 ng) was used for reverse transcription with oligo and random hexamer primers with an iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA). NS5A-ISDR was sequenced after amplification by nested PCR as previously described.^{15,16,27,28}

The primers used were as follows: NS5A-ISDR of genotype 1b, sense 5'-TGGATGGAGTGC GGTTGCACA GGTA-3' and antisense 5'-TCTTTCTCCGTGGAGGTGGT ATTG-3'; NS5A-ISDR of genotype 2a, sense 5'-ACGTCC ATGCTAACAGACCC-3' and antisense 5'-GGGAATCT CTTCTTGGGGAG-3'; and NS5A-ISDR of genotype 2b, sense 5'-TCTCAGCTCCCTTGCATCCTGA-3' and antisense 5'-GATGGTATCGAAGGCTC-3'. Amplification conditions consisted of 10 min at 94°C, followed by 40 cycles of 94°C for 10 s, 55°C for 30 s and 72°C for 30 s in a thermal cycler (GeneAmp PCR System 9700; Applied Biosystems, Foster City, CA, USA). The second PCR was done using the following sets of primers: NS5A-ISDR of genotype 1b, sense 5'-CAGGTACGC TCCGGCGTGCA-3' and antisense 5'-GGGGCCTTGGT AGGTGGCAA-3'; NS5A-ISDR of genotype 2a, sense from the first-round PCR and a new antisense primer 5'-CGAGAGAGTCCAGAACGACC-3'; and NS5A-ISDR of genotype 2b, sense 5'-AGCTCCTCAGCGAGCCA GCT-3' and antisense 5'-GATGGTATCGAAGGCTC-3'. PCR products were separated by electrophoresis on 2% agarose gels, stained with ethidium bromide and visualized under ultraviolet light. PCR products were then purified and sequenced with the second-round

PCR primers with a dye terminator sequencing kit (BigDye Terminator v1.1 Cycle Sequencing Kit; Applied Biosystems) and an ABI 310 DNA Sequencer (Applied Biosystems).

Genomic analysis

Detection of the SNP of IL-28B (rs8099917) was done by a real-time PCR system, as previously reported.¹⁶ In brief, genomic DNA was extracted from 15 μ L of whole blood using a commercial kit (QIAamp DNA Blood mini Kit; Qiagen) and dissolved in 50 μ L diethylpyrocarbonate-treated water. DNA (1 ng) was used for PCR with primers and probes of commercial kit (Taqman SNP Genotyping Assays; Applied Biosystems). The SNP of IL-28B (rs8099917) was amplified, and the results were analyzed by real-time PCR in a thermal cycler (7300 Real time PCR System; Applied Biosystems).

Statistical analysis

Data are expressed as mean \pm standard deviation. A paired Student's *t*-test or Fisher's exact test were used to analyze differences in variables. $P < 0.05$ was considered significant. Multiple logistic regression models were used to identify factors predictive of SVR. Statview ver. 5.0 software (SAS Institute, Cary, NC, USA) was used for all analyses.

RESULTS

Background

PATIENTS' CLINICAL CHARACTERISTICS are summarized in Table 1. HCV genotypes 1b ($n = 34$), 2a ($n = 58$), 2b ($n = 9$) and unknown ($n = 3$) were detected.

Table 1 Clinical characteristics at pretreatment

Clinical characteristics	$n = 104$
Age (years)	55.1 \pm 12.5
Sex: male/female	62/42
AST (IU/L)	50.0 \pm 28.2
ALT (IU/L)	62.7 \pm 47.3
Platelet count (10^4 /uL)	18.4 \pm 5.7
HCV RNA level (KIU/mL)	36 (1.6–100)
HCV genotype (1b/2a/2b/unknown)	34/58/9/3
IFN length (weeks) (24/48/<17)	49/45/10
Body mass index	22.7 \pm 3.2

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

Table 2 Virological response in each group

(a) Virological response according to durations of IFN therapy				
	Overall (<i>n</i> = 102)	24W (<i>n</i> = 48)	48W (<i>n</i> = 45)	<17W (<i>n</i> = 9)
RVR	81.4% (<i>n</i> = 83)	87.5% (<i>n</i> = 42)	73.3% (<i>n</i> = 33)	88.9% (<i>n</i> = 8)
ETR	100% (<i>n</i> = 102)	100% (<i>n</i> = 48)	100% (<i>n</i> = 45)	100% (<i>n</i> = 9)
SVR	92.2% (<i>n</i> = 94)	93.8% (<i>n</i> = 45)	91.1% (<i>n</i> = 41)	88.9% (<i>n</i> = 8)
(b) Virological response according to HCV genotypes				
	Overall (<i>n</i> = 102)	1b (<i>n</i> = 32)	2a (<i>n</i> = 58)	2b (<i>n</i> = 9)
RVR	81.4% (<i>n</i> = 83)	81.3% (<i>n</i> = 26)	81.0% (<i>n</i> = 47)	88.9% (<i>n</i> = 8)
SVR	92.2% (<i>n</i> = 94)	87.5% (<i>n</i> = 28)	93.1% (<i>n</i> = 54)	100% (<i>n</i> = 9)

ETR, end of treatment response; HCV, hepatitis C virus; IFN, interferon; RVR, rapid virological response; SVR, sustained virological response; W, weeks.

All patients had serum HCV RNA levels of 100 KIU/mL or less, and the median HCV RNA level was 36 KIU/mL.

One hundred and four patients were initially included in this study; 49 patients were treated with PEG IFN- α -2a for 24 weeks, and 45 patients were treated for 48 weeks. Ten patients withdrew from IFN therapy within 17 weeks, and two of these 10 patients could not be followed. The reasons for discontinuing therapy were fatigue (*n* = 3), depression (*n* = 1), rash (*n* = 1), appetite loss (*n* = 1), liver failure (*n* = 1) and unknown (*n* = 3). The two patients who withdrew from follow up were excluded from the analysis, and the remaining 102 patients were followed for 6 months after the ETR.

Virological response

Virological response is shown in Table 2. Rapid virological response (RVR), which was defined as negativity for HCV after 4 weeks of treatment, for the overall group, the 48 weeks' group, the 24 weeks' group and the under 17 weeks' group was 81.4% (83/102), 73.3% (33/45), 87.5% (42/48) and 88.9% (8/9), respectively. Virological response at the ETR was 100% among all patients. Finally, 94 (92.2%) of 102 patients achieved SVR.

There was no significant difference in virological response between patients treated for 24 weeks and those treated for 48 weeks. The virological response according to HCV genotype is shown in Table 2(b). Patients with genotype 1b had a lower SVR rate than genotypes 2a and 2b, but no significant differences in genotype were noted.

Genetic heterogeneity in NS5A-ISDR and response to IFN therapy

The prevalences of the number of amino acid substitutions in ISDR according to HCV genotypes are summa-

rized in Figure 1. The ISDR were examined by direct sequencing, and classification involved counting the number of amino acid substitutions compared to consensus strains of each genotype, as previously reported.^{15,24,27,28}

Interferon sensitivity-determining region sequences were obtained in 81 patients. Five patients did not have serum at pretreatment, and 16 patients could not be amplified by PCR. Sixty-one patients (84.7%) had one mutation or more. SVR according to the ISDR is shown in Figure 2. All patients with three or more mutations in the ISDR achieved SVR, but 18 (69.2%) of 26 patients with two or less mutations in the ISDR achieved SVR. Patients with two or less mutations in the ISDR were poor responders to IFN therapy.

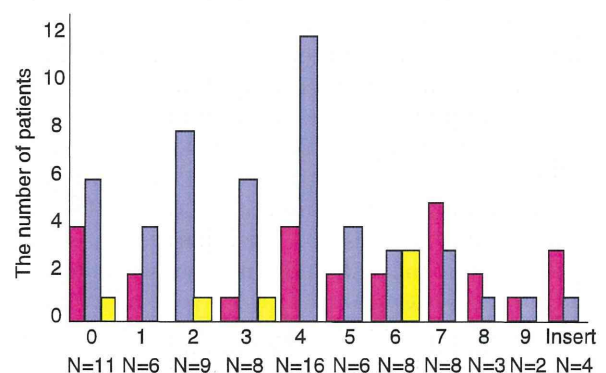


Figure 1 Number of amino acid substitutions in interferon sensitivity-determining region (ISDR) according to hepatitis C virus (HCV) genotypes. ■, HCV genotypes 1b; ■, HCV genotypes 2a; ■, HCV genotypes 2b.

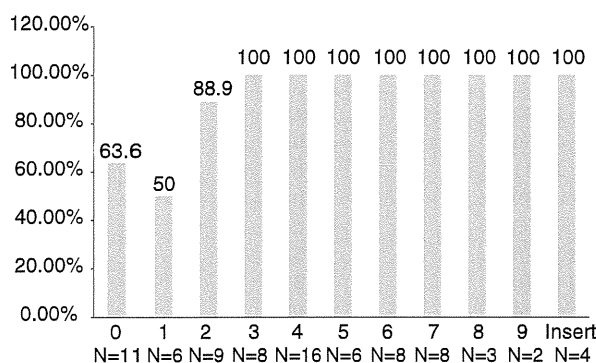


Figure 2 Sustained virological response (SVR) according to the number of amino acid substitutions in interferon sensitivity-determining region (ISDR).

Prevalence of the SNP of IL-28B (rs8099917) T (major allele) and G (minor allele) and response to IFN therapy

The frequencies of the IL-28B genotypes were: major homozygotes (TT), 73; heterozygotes (TG), 18; and minor homozygotes (GG), two. The rates of SVR in the patients with TT, TG and GG were 94.5% (69/73), 77.8% (14/18) and 100% (2/2), respectively. The SVR rate of patients with G allele of the IL-28B genotype was 80.0% (16/20), and that with T allele was 94.5% (69/73). Patients with T allele of the IL-28B genotype had a slightly higher SVR rate than did those with G allele, but there were no significant differences ($P = 0.0623$).

Analysis for factors predictive of SVR

The results of univariate analysis for factors predictive of SVR are shown in Table 3. HCV RNA levels were lower

in patients with SVR than in those without SVR ($P = 0.0154$). SVR was achieved in 41.2% of patients with less than two mutations in the ISDR and 98.4% of patients with two or more mutations in the ISDR ($P = 0.0001$). HCV RNA levels and ISDR were associated with SVR on univariate analyses.

Results of multivariate analyses of factors predictive of SVR are shown in Table 4. Variables were recorded categorically as ordinal data. Background factors were age (<60 vs ≥ 60 years), sex (male vs female), platelet count (< $15 \times 10^4/\text{mm}^3$ vs $\geq 15 \times 10^4/\text{mm}^3$), HCV RNA level (<50 vs ≥ 50 KIU/mL), ALT levels (<70 vs ≥ 70 IU/L), aspartate aminotransferase (AST) levels (<60 vs ≥ 60 IU/L), HCV genotype (1 vs 2), ISDR (<2 vs ≥ 2 mutations), IL-28B (TT vs TG and GG) and RVR (yes vs no). As can be seen in Table 4, factors such as age, sex, platelet count, HCV RNA level, ALT levels, AST levels, HCV genotype, IL-28B and RVR did not have any effect on SVR. In contrast, the ISDR was the most influential factor.

DISCUSSION

THE HCV RNA level is one of the most important factors affecting response to IFN therapy. Patients with high HCV RNA levels respond poorly to IFN therapy, whereas patients with low HCV RNA levels have a high SVR rate to IFN therapy. Thus, most patients with low HCV RNA levels have achieved SVR, but other therapeutic options for patients who fail IFN therapy are needed. Several studies have attempted to reduce the duration of treatment, reduce the dose of IFN and/or ribavirin, or use standard IFN without risk of relapse.⁸⁻¹⁰ The present study confirmed the high SVR rate (92.2%) in patients with low HCV RNA levels (≤ 100 KIU/mL)

Table 3 Univariate analysis: factors predictive of SVR

Factors	SVR (n = 94)	Non-SVR (n = 8)	P-value
Age (years)	54.6 \pm 12.6	57.4 \pm 8.8	0.5528
Sex: male/female	58/36	2/6	0.0619
ALT (IU/L)	63.2 \pm 48.3	56.3 \pm 32.5	0.7126
AST (IU/L)	50.7 \pm 28.6	41.4 \pm 21.6	0.4043
PLT ($\times 10^4/\text{mm}^3$)	18.5 \pm 5.8	18.0 \pm 5.0	0.8292
HCV RNA level (KIU/mL)	42.5 \pm 34.8	75.0 \pm 45.7	0.0154
HCV genotype: 1/2	29/63	4/3	0.4337
ISDR: <2/ ≥ 2	10/63	7/1	0.0001
IL-28B: TT/TG, GG	69/16	4/4	0.0623
RVR: yes/no	78/16	5/3	0.1661

ALT, alanine aminotransferase; AST, aspartate aminotransferase; HCV, hepatitis C virus; IL-28B, interleukin-28B; ISDR, interferon sensitivity-determining region; PLT, platelets; RVR, rapid virological response; SVR, sustained virological response.

Table 4 Multivariate analysis: factors predictive of SVR

Factors	P-value	Risk ratio	95% CI	
Age: <60 years	0.4556	2.837	0.183	43.891
Sex: male	0.8712	0.756	0.026	22.166
AST: <60 IU/L	0.7806	2.131	0.010	438.334
ALT: <70 IU/L	0.6063	0.239	0.001	55.563
Platelet count: <15 × 10 ⁴ /uL	0.6873	0.463	0.011	19.680
HCV RNA: <50 KIU/mL	0.1046	13.170	0.585	296.318
Genotype: 2	0.1693	14.110	0.324	614.872
ISDR: <2	0.0074	0.004	0.001	0.235
IL-28B: TT	0.2684	5.978	0.252	141.852
RVR: yes	0.7495	1.756	0.055	55.696

95% CI, 95% confidence interval; ALT, alanine aminotransferase; AST, aspartate aminotransferase; HCV, hepatitis C virus; IL-28B, interleukin 28B; ISDR, interferon sensitivity-determining region; RVR, rapid virological response; SVR, sustained virological response.

treated by PEG IFN- α -2a monotherapy. Although the effects of shortened treatment duration of PEG IFN- α with ribavirin for patients with low HCV RNA levels are unclear, PEG IFN- α -2a monotherapy could reduce the cost and adverse events of ribavirin while maintaining a high SVR rate. This treatment would be a good therapeutic option for patients with low HCV RNA levels. However, selection by HCV RNA level alone was insufficient to predict IFN responsiveness completely, and other factors would be necessary to improve the positive predictive values for SVR in patients infected with low HCV RNA levels.

Hepatitis C virus genotype is another major factor, in addition to HCV RNA levels, that is associated with response to IFN therapy. In the present study, the SVR rates of genotypes 1 and 2 were 87.5% and 94.0%, respectively. Patients infected with genotypes 2 had a slightly higher SVR rate than did those with genotype 1, but there were no significant differences in our small study. The difference in SVR according to genotype may exist, but HCV genotype did not have enough power to be a determinant of IFN response completely among patients with low HCV RNA levels because of the bias for HCV RNA levels. However, patients infected with low HCV RNA levels respond differently to IFN therapy, suggesting that an additional factor associated with resistance to IFN exists.

The heterogeneity of the HCV NS5A region is an important factor that may affect response to IFN in patients with HCV genotype 1b and was named the ISDR.¹⁷ Mutations in the ISDR affect the interaction with PKR and may inhibit viral replication. Therefore, ISDR of other HCV genotypes, in addition to 1b, could be used as predictors of IFN responsiveness.²³⁻²⁸ In the

present study, it was hypothesized that the amino acid substitutions in the ISDR would explain differences in IFN resistance in patients infected with low HCV RNA levels. Therefore, the utility of substitutions of amino acids in the ISDR for predicting IFN responsiveness was investigated. The ISDR was the most influential factor for SVR on multivariate analyses. All patients with three or more mutations in the ISDR achieved SVR, and 18 of 26 patients with less than three mutations in the ISDR achieved SVR. Thus, patients with less than three mutations in the ISDR would be resistant to PEG IFN- α -2a monotherapy and may need to receive much more powerful treatment, even if they have low HCV RNA levels. The ISDR system could be used as a diagnostic tool to predict SVR in patients infected with low HCV RNA levels. An individualized treatment strategy based on HCV RNA levels and the ISDR in patients with chronic hepatitis C would be an important consideration to achieve optimal therapy and avoid unnecessary treatment.

Some studies of SVR to PEG IFN- α -2b and ribavirin and/or telaprevir combination therapy for chronic hepatitis C patients with genotype 1 and high viral load identified genetic variation near the IL-28B gene associated with IFN responsiveness.^{13,14,16} However, the effects of genetic variation near the IL-28B gene on SVR in patients with low HCV RNA levels treated with PEG IFN monotherapy are unknown. Therefore, the utility of the SNP of IL-28B for predicting IFN responsiveness was investigated. Patients with IL-28B (rs8099917) genotypes TG and GG had a lower SVR rate than genotype TT, but no significant differences in genotype were found in this study. The SNP of IL-28B would be associated with the response to IFN, especially for poor responders, and