direct-acting antivirals (DAA) and non-structural (NS)3/4A protease inhibitors have shown promising outcomes in combination with PEG-IFN/RBV in several clinical trials, with more than 70% of patients infected with HCV genotype 1 achieving SVR.[6–8]

Various viral and host factors have been identified as significant determinants of treatment outcome. Among patients with HCV genotype 1 infection, factors associated with a lower rate of SVR include a high baseline viral load, older age, African-American race, insulin resistance, advanced fibrosis, hepatic steatosis and administration of an insufficient dose of PEG-IFN or RBV.[9,10] Other viral factors include amino acid substitutions at positions 70 and 91 in the HCV core region,[11] and in the interferon sensitivity-determining region in NS5A.[12] In addition, recent genome-wide association studies, including our study of HCV infection,[13] have shown that a single nucleotide polymorphism (SNP) near the interleukin-28B (*IL28B*) gene is strongly associated with response to PEG-IFN/RBV therapy for chronic HCV genotype 1 infection.[13–18]

Chemokines and cytokines regulate immunity and inflammation in HCV infection. They also play critical roles in eradication of HCV during IFN-based treatment. Several studies have reported that interferon-γ-inducible protein 10 kDa (IP-10 or CXCL10) may be a prognostic maker for HCV treatment efficacy in HCV genotype 1 infection: elevated pretreatment serum IP-10 concentrations correlate with non-response to PEG-IFN/RBV therapy.[19–23] Darling *et al.* reported that pretreatment serum IP-10 of less than 600 pg/mL was an independent predictive factor for SVR in PEG-IFN/RBV therapy, especially among patients with the unfavorable *IL28B* genotype; SVR rates differed significantly according to pretreatment serum IP-10 concentrations.[24] Other studies have shown that *IL28B* genotype and pretreatment serum IP-10 concentrations were associated with early viral kinetics of HCV, the first phase decline or rapid virological response (RVR), as well as SVR in PEG-IFN/RBV therapy.[25,26] However, the impact of serum IP-10 concentrations on virological response to PEG-IFN/RBV/telaprevir therapy has not been elucidated yet.

Furthermore, it has been reported that pretreatment serum IP-10 concentrations tended to be higher in African-American patients infected with HCV than in white patients,[20,24] suggesting that serum IP-10 concentrations may vary according to race. Until now, there have been few reports concerning the association between pretreatment serum IP-10 concentrations and treatment efficacy in Asian populations infected with HCV.

In this study, we aimed to determine the impact of pretreatment serum IP-10 concentrations on virological responses to PEG-IFN/RBV or PEG-IFN/RBV/telaprevir therapy in a Japanese population infected with HCV genotype 1.

METHODS

Study population and treatment protocol

SERUM SAMPLES WERE obtained from 149 patients chronically infected with HCV genotype 1 who were treated at Nagoya City University Hospital and Nagoya Daini Red Cross Hospital. All patients had tested positive for HCV RNA for more than 6 months. Patients chronically infected with hepatitis B virus or HIV, or with other causes of liver disease such as autoimmune hepatitis and primary biliary cirrhosis, were excluded from this study. Written informed consent was obtained from each patient and the study protocol conformed to the ethics guidelines of the Declaration of Helsinki and was approved by the appropriate institutional ethics review committees.

One hundred and four patients were treated with PEG-IFN-α-2b (1.5 μg/kg bodyweight s.c. once a week) or PEG-IFN-α-2a (180 μg once a week) plus RBV (600–1000 mg daily according to bodyweight) for 48 weeks. The dose of PEG-IFN or RBV were reduced according to the recommendations on the package inserts or the clinical condition of individual patients. Forty-five patients received PEG-IFN/RBV/telaprevir therapy: PEG-IFN-α-2b (1.5 μg/kg bodyweight s.c. once a week), RBV (600–1000 mg daily according to bodyweight) and telaprevir (standard dose of 2250 mg daily three times a day every 8 h or a reduced dose of 1500 mg daily twice a day every 12 h) for 12 weeks, followed by an additional 12 weeks of PEG-IFN/RBV. In seven patients, the initial dose of telaprevir was reduced to 1500 mg daily according to age, bodyweight, sex or baseline hemoglobin level, according to the judgment of the physicians. When marked adverse effects (e.g. anorexia, anemia, neutropenia, thrombocytopenia, renal dysfunction, skin rash) developed, the dose of telaprevir was reduced to 1500 mg daily and that of PEG-IFN or RBV was reduced according to the recommendation on the package inserts or the clinical condition of individual patients.

Definition of virological response to treatment

The virological responses were defined as follows: SVR, HCV RNA undetectable at week 24 after the end of

therapy; transient virological response (TVR), HCV RNA became undetectable during therapy but reappeared after the end of treatment; non-virological response (NVR), HCV RNA remained detectable during therapy; RVR, HCV RNA undetectable at week 4 after the start of therapy; and very rapid virological response (vRVR), HCV RNA undetectable at week 2 after the start of therapy.

Laboratory tests

Blood samples were taken according to protocol from baseline until after the end of treatment and were analyzed by hematological tests and for blood chemistry. HCV RNA concentrations were measured at baseline, regularly during treatment and at follow-up visits after the end of treatment, using the COBAS TaqMan HCV test (Roche Diagnostics, Tokyo, Japan). The dynamic range of this assay is 1.2–7.8 log IU/mL.

Quantification of serum IP-10 concentrations

Serum IP-10 concentrations were measured in samples collected at baseline (prior to treatment) using the Quantikine human CXCL10/IP-10 immunoassay (R&D Systems, Minneapolis, MN, USA). All samples were diluted 1:2 and analyzed in duplicate. The linear dynamic range for IP-10 measurement by this assay is 8–500 pg/mL, with a detection limit of 7.8 pg/mL. Samples with IP-10 concentrations above 1000 pg/mL were diluted 1:5 and reanalyzed.

SNP genotyping

Genetic polymorphisms near the *IL28B* gene (rs8099917) were determined according to the manufacturer's recommendations using TaqMan SNP Genotyping Assays (Applied Biosystems, Carlsbad, CA, USA) or the DigiTag2 assay.[27]

Statistical analysis

Categorical variables were compared between groups by the χ^2-test or Fisher's exact test and non-categorical variables by the Mann–Whitney *U*-test. Pearson's correlation coefficient test was used to evaluate relationships between serum IP-10 concentrations and other variables. Multivariate logistic regression analysis with stepwise forward selection was performed with $P < 0.05$ in univariate analysis as the criteria for model inclusion. $P < 0.05$ was considered significant. To evaluate the discriminatory ability of IP-10 concentrations to predict SVR, receiver–operator curve (ROC) analysis was conducted and the cut-off value selected. These statistical analyses were carried out using SPSS software package version 18J (SPSS, Chicago, IL, USA).

RESULTS

Demographic characteristics

THE CLINICAL CHARACTERISTICS of the study population are described in Table 1. PEG-IFN/RBV/telaprevir therapy has been known to be poorly tolerated because of adverse effects. In addition, the treatment efficacy varied considerably according to *IL28B* genotype or past IFN-based treatment outcome. Therefore, we examined *IL28B* genotype in all cases before treatment and selected patients for treatment with PEG-IFN/RBV/telaprevir according to age, *IL28B* genotype, past IFN-based treatment response, among other criteria. As a result, the median age was younger and the rate of rs8099917:TT (*IL28B* favorable genotype) was higher in the patients treated with PEG-IFN/RBV/telaprevir. Of the 45 patients treated with PEG-IFN/RBV/telaprevir, 17 were naïve for IFN therapy, 22 were previously treated with PEG-IFN/RBV, three with IFN/RBV and three with IFN monotherapy. Of the 28 previously treated patients, 21 and seven resulted in TVR and NVR, respectively.

Table 1 Clinical characteristics of 149 patients infected with HCV genotype 1

	PEG-IFN/RBV ($n = 104$)	PEG-IFN/RBV/telaprevir ($n = 45$)
Sex, male	46	27
Age, years	59 (16–73)	55 (28–70)
Bodyweight, kg	57 (34–92)	61 (42–102)
Hemoglobin, g/dL	13.7 (9.9–17.7)	14.7 (12.0–16.7)
Platelet count, $\times 10^4/\mu L$	15.6 (6.3–28.1)	14.3 (9.8–31.9)
ALT, IU/L	45 (12–426)	37 (13–212)
γ-GT, IU/L	32 (10–222)	27 (12–258)
IP-10, pg/mL	325 (58–2053)	261 (57–1438)
HCV RNA, log IU/mL	6.5 (5.1–7.5)	6.7 (4.8–7.6)
rs8099917, TT/TG + GG	72/32	37/8
Treatment efficacy		
SVR/TVR/NVR	39/36/29	43/1/1

Data are expressed as number for categorical data or the median (range) for continuous data.
γ-GT, γ-glutamyltransferase; ALT, alanine aminotransferase; IP-10, interferon-gamma-inducible protein-10; NVR, non-virological response; PEG-IFN, pegylated interferon; RBV, ribavirin; SVR, sustained virological response; TVR, transient virological response.

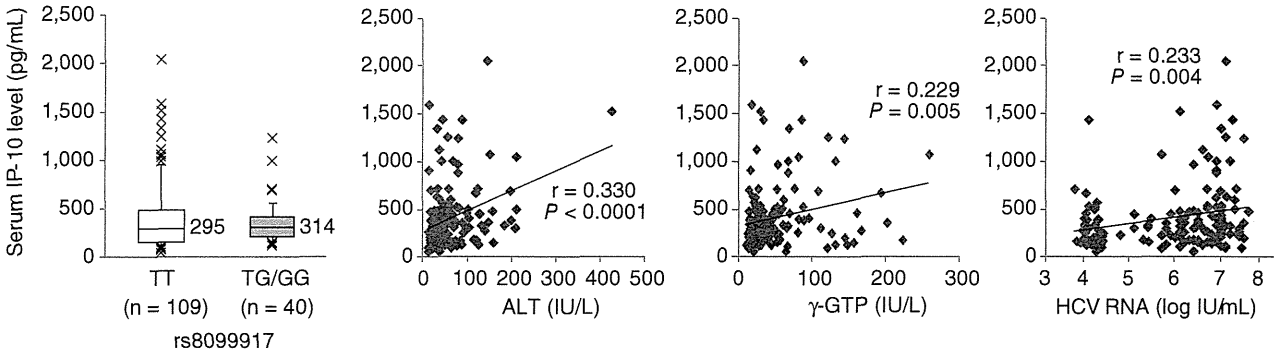

Figure 1 Correlations between pretreatment serum interferon-γ-inducible protein-10 (IP-10) concentrations and *IL28B* genotype or other variables. Boxes represent the interquartile range of the data. The lines across the boxes and the numbers indicate the median values. The hash marks above and below the boxes indicate the 90th and 10th percentiles for each group, respectively. The serum IP-10 concentrations were not correlated with the *IL28B* genotype but were weakly correlated with alanine aminotransferase (ALT), γ-glutamyltransferase (γ-GT) and hepatitis C virus (HCV) RNA concentrations.

Correlations between pretreatment serum IP-10 concentrations and other variables

The median pretreatment serum IP-10 concentration of the 149 patients was 301 pg/mL (range, 57–2053). The pretreatment serum IP-10 concentrations were not correlated with age, sex, *IL28B* genotype and platelet count, but alanine aminotransferase (ALT), γ-glutamyltransferase (γ-GT) and HCV RNA concentrations were weakly correlated ($r = 0.330$, 0.229 and 0.233, respectively) (Figs 1,S1).

Predictive factors for SVR in PEG-IFN/RBV therapy

We examined the factors associated with SVR in patients treated with PEG-IFN/RBV. By univariate analysis, age, platelet count, γ-GT, IP-10 and *IL28B* genotype were significantly associated with SVR (Table 2). Dividing the patients into three groups, SVR, TVR and NVR, the IP-10 concentrations were significantly lower in SVR than in TVR and NVR ($P = 0.017$ and 0.005, respectively) (Fig. 2). ROC analysis of the pretreatment serum IP-10 concentration for predicting SVR revealed that the area under the curve was 0.68 (Fig. S2). When we set the cut-off value at 300 pg/mL, the sensitivity was 0.67 and the specificity was 0.68. Then, we analyzed predictive factors for SVR in logistic regression models that included the following variables: age (≤58 vs ≥59 years), platelet count (≥15 vs $<15 \times 10^4$/μL), γ-GT (≤31 vs ≥32 IU/L), IP-10 concentration (<300 or ≥300 pg/mL) and rs8099917 genotype (TT vs TG/GG). The *IL28B* genotype and IP-10 concentration were independent

Table 2 Univariate analysis of factors associated with SVR in patients treated with PEG-IFN/RBV

	SVR ($n = 39$)	Non-SVR ($n = 65$)	*P*-value
Sex, male	20	26	n.s.
Age, years	57 (47–61)	60 (53–65)	0.035
Bodyweight, kg	58 (52–67)	56 (51–61)	n.s.
Hemoglobin, g/dL	13.9 (13.0–14.7)	13.6 (12.7–14.5)	n.s.
Platelet count, $\times 10^4$/μL	16.9 (12.5–20.5)	14.0 (10.9–18.1)	0.027
ALT, IU/L	49 (34–83)	44 (32–70)	n.s.
γ-GT, IU/L	26 (17–45)	33 (23–67)	0.024
IP-10, pg/mL	212 (144–384)	356 (239–509)	0.002
HCV RNA, log IU/mL	6.4 (5.9–6.9)	6.5 (6.2–6.8)	n.s.
rs8099917, TT/TG + GG	37/2	35/30	<0.0001

Data are expressed as number for categorical data or the median (first-third quartiles) for continuous data.
γ-GT, γ-glutamyltransferase; ALT, alanine aminotransferase; IP-10, interferon-gamma-inducible protein-10; n.s., not significant; NVR, non-virological response; PEG-IFN, pegylated interferon; RBV, ribavirin; SVR, sustained virological response.

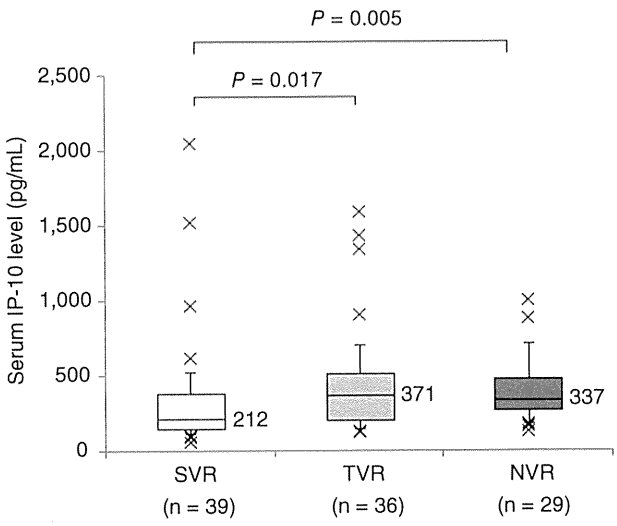

Figure 2 Pretreatment serum interferon-γ-inducible protein-10 (IP-10) concentrations according to treatment efficacy in patients treated with pegylated interferon (PEG-IFN)/ribavirin (RBV). Boxes represent the interquartile range of the data. The lines across the boxes and the numbers indicate the median values. The hash marks above and below the boxes indicate the 90th and 10th percentiles for each group, respectively. The serum IP-10 concentrations were lower in sustained virological response (SVR) than in transient virological response (TVR) and non-virological response (NVR) ($P = 0.017$ and 0.005, respectively). There was a significant difference in IP-10 concentrations between SVR and non-SVR (TVR + NVR) ($P = 0.002$).

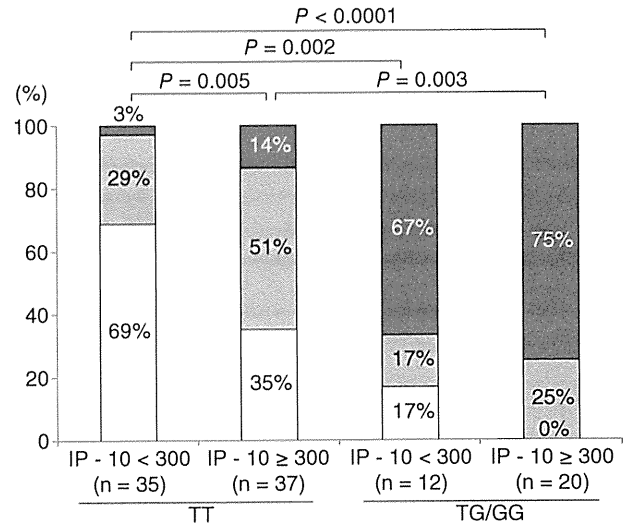

Figure 3 Treatment outcome according to *IL28B* genotype and pretreatment serum interferon-γ-inducible protein-10 (IP-10) concentrations in patients treated with pegylated interferon (PEG-IFN)/ribavirin (RBV). In the rs8099917:TT group, the sustained virological response (SVR) rate was higher in the patients with IP-10 of <300 pg/mL than in those with IP-10 of ≥300 pg/mL (69% vs 35%, $P = 0.005$), whereas no patient with rs8099917:TG/GG and IP-10 of ≥300 pg/mL achieved SVR. *P*-values were obtained by comparing the SVR rates among the groups. NVR, non-virological response; TVR, transient virological response. ▩, NVR; ▨, TVR; □, SVR.

predictive factors for SVR (odds ratio [OR] = 17.44 [$P < 0.001$] and OR = 3.86 [$P = 0.01$]) (Table 3). Therefore, we analyzed the associations between IP-10 concentrations and treatment outcome in subgroups according to *IL28B* genotype. In the rs8099917:TT group, the SVR rate was higher in the patients with IP-10 of less than 300 pg/mL than those with IP-10 of 300 pg/mL or more (69% vs 35%, $P = 0.005$), whereas no patient achieved SVR among those with rs8099917:TG/GG and IP-10 of 300 pg/mL or more (Fig. 3).

Table 3 Logistic regression analysis of factors associated with sustained virological response in patients treated with PEG-IFN/RBV

	Odds ratio (95% CI)	*P*-value
Age, ≤58 years	1.46 (0.53–4.03)	0.467
Platelet count, ≥15 × 10^4/μL	1.84 (0.68–4.95)	0.228
γ-GT, ≤31 IU/L	1.09 (0.40–3.00)	0.861
IP-10, <300 pg/mL	3.86 (1.39–10.75)	0.010
rs8099917, TT	17.44 (3.62–83.94)	<0.001

γ-GT, γ-glutamyltransferase; CI, confidence interval; IP-10, interferon-γ-inducible protein-10; PEG-IFN, pegylated interferon; RBV, ribavirin.

Virological response to PEG-IFN/RBV/telaprevir therapy

The percentages of patients in whom HCV RNA became undetectable in each period of treatment with PEG-IFN/RBV/telaprevir are shown in Figure 4. The RVR rate was high regardless of *IL28B* genotype: 82% (37/45) of all patients. All the patients with the *IL28B* favorable genotype achieved SVR but, of the eight patients with the unfavorable genotype, two failed to achieve SVR. Therefore, it was difficult to evaluate the association between pretreatment serum IP-10 concentrations and RVR or SVR. Then, we examined the factors associated with vRVR, defined as HCV RNA undetectable at week 2 after the start of therapy. The vRVR rate was 45% (18/45) of

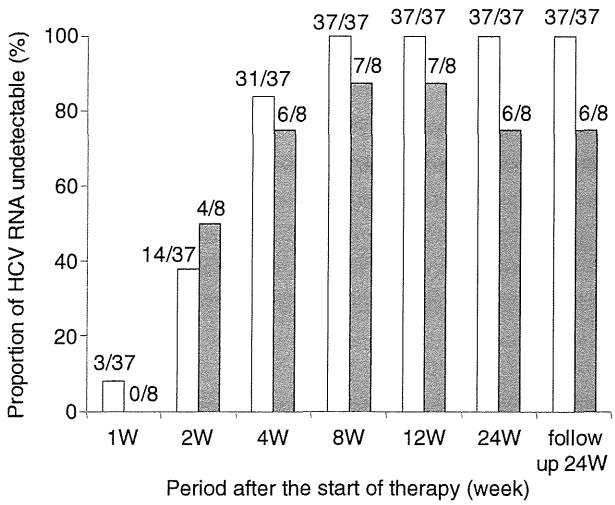

Figure 4 Percentages of patients in whom hepatitis C virus (HCV) RNA became undetectable in each period of treatment with pegylated interferon (PEG-IFN)/ribavirin (RBV)/telaprevir, according to *IL28B* genotype. The percentages with very rapid virological response (vRVR), defined as HCV RNA undetectable at week 2 after the start of therapy, and RVR among all patients were 45% (18/45) and 82% (37/45), respectively. All patients with rs8099917:TT (*IL28B* favorable genotype) achieved a sustained virological response (SVR) but two of the eight patients with rs8099917:TG/GG did not achieve SVR. □, TT ($n = 37$); ▨, TG/GG ($n = 8$).

all patients. By univariate analysis, low concentrations of IP-10 and HCV RNA at baseline were significantly associated with vRVR (Table 4). There was a weak correlation between pretreatment serum IP-10 concentrations and HCV RNA levels in patients treated with PEG-IFN/RBV/telaprevir, but it was not significant ($r = 0.256$, $P = 0.090$) (Fig. S3), therefore, they were considered almost independent predictors of vRVR. In addition, in the subgroup with the *IL28B* favorable genotype, IP-10 concentrations were also significantly lower in vRVR ($P = 0.009$) (Fig. 5).

DISCUSSION

IN THIS STUDY, we have identified an association between pretreatment serum IP-10 concentrations and treatment efficacy in patients infected with HCV genotype 1 and treated with PEG-IFN/RBV. Considering a previous report,[24] the pretreatment serum IP-10 concentrations tended to be lower in this study than in Caucasian and African-American populations. Some previous reports have mentioned differences in serum IP-10 concentrations between in African-American and white patients,[20,24] and our findings suggest that serum IP-10 concentrations may be lower in Asian patients infected with HCV. Therefore, there is the likelihood that the appropriate cut-off values of pretreatment serum IP-10 concentrations for predicting SVR vary among different races. To assess the potential predictive value of IP-10 measurements, other studies stratified the patients according to a 600 pg/mL cut-off of IP-10 concentrations,[22,24] while we set the cut-off value as 300 pg/mL on the basis of the ROC analysis. In multivariate analysis, the *IL28B* favorable genotype and pretreatment serum IP-10 of less than 300 pg/mL were independent factors for predicting SVR. Therefore, the ability to predict treatment efficacy was improved by

Table 4 Univariate analysis of factors associated with very rapid virological response in patients treated with PEG-IFN/RBV/telaprevir

	vRVR ($n = 18$)	Non-vRVR ($n = 27$)	*P*-value
Sex, male	12	14	n.s.
Age, years	57 (41–63)	55 (48–62)	n.s.
Bodyweight, kg	65 (58–76)	57 (50–70)	n.s.
Hemoglobin, g/dL	15.1 (13.6–15.6)	14.2 (13.1–15.3)	n.s.
Platelet count, $\times 10^4/\mu L$	15.9 (13.6–19.4)	13.6 (11.8–17.4)	n.s.
ALT, IU/L	34 (25–60)	45 (30–73)	n.s.
γ-GT, IU/L	28 (20–51)	26 (19–58)	n.s.
IP-10, pg/mL	190 (126–336)	300 (223–616)	0.007
HCV RNA, log IU/mL	6.3 (5.8–6.9)	6.8 (6.6–7.2)	0.006
rs8099917, TT/TG + GG	14/4	23/4	n.s.

γ-GT, γ-glutamyltransferase; IP-10, interferon-γ-inducible protein-10; n.s., not significant; PEG-IFN, pegylated interferon; RBV, ribavirin; vRVR, very rapid virological response.
Data are expressed as number for categorical data or the median (first-third quartiles) for continuous data.

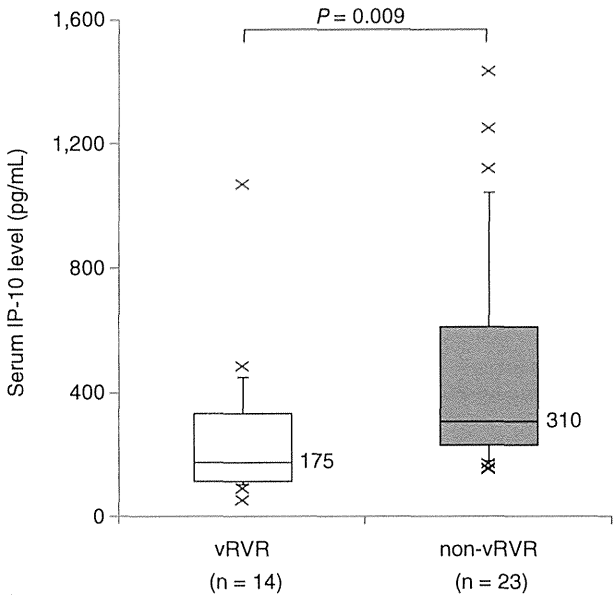

Figure 5 Pretreatment serum interferon-γ-inducible protein-10 (IP-10) concentrations in *IL28B* favorable patients with or without a very rapid virological response (vRVR) to treatment with pegylated interferon (PEG-IFN)/ribavirin (RBV)/telaprevir. Boxes represent the interquartile range of the data. The lines across the boxes and the numbers indicate the median values. The hash marks above and below the boxes indicate the 90th and 10th percentiles for each group, respectively. The serum IP-10 concentrations were significantly lower in vRVR than in non-vRVR ($P = 0.009$).

considering the *IL28B* genotype and pretreatment serum IP-10 concentration together in PEG-IFN/RBV therapy: the positive predictive value of *IL28B* favorable genotype and IP-10 of less than 300 pg/mL for predicting SVR was 69% and the negative predictive value of *IL28B* favorable genotype or IP-10 of less than 300 pg/mL was 100% (Fig. 3, Table S1).

It has been reported that serum IP-10 concentrations correlated with *IL28B* genotype, activity of hepatitis, progressive liver fibrosis and HCV RNA concentrations.[23,25,28] In this study, the serum IP-10 concentrations were not correlated with the *IL28B* genotype but were weakly correlated with ALT, γ-GT and HCV RNA concentrations.

In addition, we identified that pretreatment serum IP-10 concentrations and HCV RNA levels almost independently affected the early viral kinetics of HCV in patients treated with PEG-IFN/RBV/telaprevir. IP-10 concentrations were significantly lower in patients with vRVR, defined as HCV RNA undetectable at week 2 after the start of therapy, than in those with non-vRVR. Moreover, that result was similar in the subgroup of patients with the *IL28B* favorable genotype, suggesting that pretreatment serum IP-10 concentrations are associated with early viral kinetics of HCV, independent of the *IL28B* genotype. We selected patients for treatment with PEG-IFN/RBV/telaprevir according to age, *IL28B* genotype and past IFN-based treatment response. As a result, the virological responses were excellent regardless of *IL28B* genotype and it was difficult to evaluate the association between pretreatment serum IP-10 concentrations or *IL28B* genotype and RVR or SVR because of the strong antiviral effect of treatment with telaprevir on the selected patients in this study. However, patients with vRVR during triple therapy may benefit from a reduction of the duration of treatment, namely, 12 weeks to achieve SVR; *IL28B* genotype, pretreatment serum IP-10 concentrations, and HCV RNA levels that predict vRVR may be useful for shortening the triple therapy. Further studies are necessary to reveal the impact of pretreatment IP-10 concentrations on treatment efficacy, especially in refractory patients treated with regimens including DAA.

Interferon-γ-inducible protein-10 is a CXC chemokine that lacks chemotactic activity for neutrophils but rather targets T lymphocytes, natural killer cells and monocytes.[29–31] Through its receptor, CXCR3,[32,33] IP-10 is produced by a variety of cells, including hepatocytes,[34,35] and IP-10 concentrations in plasma are mirrored by intrahepatic IP-10 mRNA and strongly predict the first phase of HCV RNA decline during PEG-IFN/RBV therapy.[36] On the other hand, it has been reported that high levels of expression of intrahepatic IFN-stimulated genes (ISG) were associated with a poor response to PEG-IFN/RBV therapy.[37,38] This is consistent with data, including our results, showing that elevated pretreatment serum IP-10 concentrations correlate with non-response to PEG-IFN/RBV therapy.[19–23] In addition, two recent studies have revealed an association between *IL28B* genotype and the expression levels of intrahepatic ISG,[39,40] and others have reported that the serum IP-10 concentration, as well as *IL28B* genetic variants, are predictive factors for spontaneous clearance of acute HCV infection.[41] However, the mechanisms through which ISG, IP-10 and *IL28B* genetic variants affect the elimination of HCV RNA are not known yet and further studies are warranted.

In conclusion, pretreatment serum IP-10 concentrations are associated with treatment efficacy in PEG-IFN/RBV and with early viral kinetics in PEG-IFN/RBV/telaprevir therapy.

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SUPPORTING INFORMATION

ADDITIONAL SUPPORTING INFORMATION may be found in the online version of this article at the publisher's website.

Figure S1 Correlations between pretreatment serum interferon-γ-inducible protein 10 kDa (IP-10) concentrations and sex, age or platelet count.
Figure S2 Receiver–operator curve (ROC) analysis for pretreatment serum interferon-γ-inducible protein 10 kDa (IP-10) concentration for prediction of a sustained virological response (SVR) to pegylated interferon (PEG-IFN)/ribavirin (RBV).
Figure S3 Correlations between pretreatment serum IP-10 concentrations and hepatitis C virus (HCV) RNA levels in patients treated with pegylated interferon (PEG-IFN)/ribavirin (RBV)/telaprevir.
Table S1 Sensitivity, specificity and positive and negative predictive values of the likelihood of patients treated with pegylated interferon (PEG-IFN)/ribavirin (RBV) achieving sustained virological response.

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Impact of a Single Nucleotide Polymorphism Upstream of the IL28B Gene in Patients Positive for Anti-HCV Antibody in an HCV Hyperendemic Area in Japan

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The influence of genetic variation at the interleukin-28B (IL28B) locus on the natural course of hepatitis C virus (HCV) infection has not been fully investigated. The goal of this study was to examine whether an IL28B polymorphism (rs8099917) is associated with natural clearance of HCV and with disease parameters of HCV infection in an HCV hyperendemic area of Japan. The patients were 502 anti-HCV antibody-positive residents who participated in liver disease screening program from 2002 to 2004. Patients who underwent interferon-based therapy or had hepatocellular carcinoma were excluded. Of these patients, 149 were negative for HCV RNA (prior infection) and 353 were positive for HCV RNA or HCV core antigen (HCV carriers). In multivariate analysis, the IL28B TT genotype was a predictor for prior HCV infection. In addition, nine of the patients with prior HCV infection were positive for anti-HCV antibody with positive for HCV core antigen or HCV RNA before 2001, and these nine patients all had the IL28B TT genotype. Furthermore, the IL28B TT genotype was associated independently with higher HCV core antigen levels in HCV carriers. In contrast, the IL28B genotype did not affect the biochemical markers, such as alanine aminotransferase, hepatic fibrosis markers, and α-fetoprotein, and the degree of hepatic fibrosis assessed by transient elastography in HCV carriers. We concluded that IL28B polymorphism (TT genotype) is associated with spontaneous clearance of HCV and conversely with high viral loads in HCV carriers. In contrast, the IL28B genotype does not affect disease progression such as hepatic fibrosis. ***J. Med. Virol. 86:1877–1885, 2014.*** © 2014 Wiley Periodicals, Inc.

KEY WORDS: interleukin 28B; hepatitis C virus; SNP; natural course

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INTRODUCTION

Around 25% of patients with hepatitis C virus (HCV) infection achieve spontaneous clearance (self-limiting cases), but the remaining 75% develop chronic infection [Hajarizadeh et al., 2013]. The HCV carrier rate in Japan is 0.2–2.1% and the rate worldwide is approximately 3.0%, while the numbers of HCV carriers in Japan and worldwide are approximately two million and 150 million, respectively. HCV carriers have a higher risk of development of chronic hepatitis C, which may progress to liver cirrhosis and hepatocellular carcinoma (HCC), and the annual number of deaths of patients with HCV-related liver diseases is 0.35 million globally [World Health Organization, 2013]. Therefore, elucidation of the mechanism of persistent HCV infection may contribute to establishment of new methods for diagnosis, prevention, and treatment of advanced HCV-related liver diseases, and lead to an improved prognosis.

HCV infection is mainly treated by interferon (IFN)-based therapy and the rate of sustained viral response has increased as new concomitant drugs, such as NS3/4A protease inhibitor have been developed [Jacobson et al., 2011; Zeuzem et al., 2011; Marcellin et al., 2011; Chayama et al., 2013]. Recent studies have shown that a single nucleotide polymorphism (SNP) near the interleukin 28B (IL28B) region is related to the efficacy of IFN-based therapy in patients with chronic hepatitis C [Ge et al., 2009; Suppiah et al., 2009; Tanaka et al., 2009]. Moreover, IL28B is effective even in IFN-free therapy, such as combination therapy with direct acting anti-hepatitis C virus agents [Zeuzem et al., 2013]. IL28B, one of three subtypes of IFNλ, is located on the long arm of chromosome 19 and is also referred to as IFNλ3. IFNλ activates the JAK/STAT signaling pathway and induces downstream IFN-stimulated genes (ISGs) to achieve antiviral effects [Sirén et al., 2005; Onoguchi et al., 2007]. Several studies have also shown an association of IL28B SNPs with spontaneous clearance of acute HCV infection [van den Berg et al., 2011; Beinhardt et al., 2012; Liu et al., 2012], but the effect of SNPs on the natural course of HCV-infected Japanese patients has not been determined.

In this study, data were used from 2002 to 2004 taken from a community-based cohort study we performed from 1995 to 2005 in an HCV hyperendemic area in Japan [Uto et al., 2009]. These data are used to evaluate the effect of an IL28B SNP on clinical features, such as spontaneous clearance of HCV and liver biochemistry in patients with anti-HCV antibody.

METHODS

Patients

Since 1995, we have offered a general health examination for residents over 20-years-old who live in an HCV hyperendemic area in Japan and we have found 1,321 anti-HCV-positive residents in this area. A cohort study in these residents was continued until 2005 [Uto et al., 2009]. The HCV antibody-positive rate in the area was around 20%, which is greater than in other areas, and about 70% of the residents had persistent HCV infection [Uto et al., 2006]. Of these residents, 502 anti-HCV-seropositive patients who participated in at least one ultrasonography-based liver disease-screening program between 2002 and 2004 and agreed to serve as patients in SNP analysis were enrolled in the current study. Lymphocytes and serum used in this study were collected from 25 patients in 2002, 107 patients in 2003, and 370 patients in 2004. Patients who underwent IFN-based therapy or were diagnosed with hepatocellular carcinoma were excluded from the study. This study was approved by the ethical committees of Kagoshima University Graduate School of Medical and Dental Sciences, the Faculty of Medicine, University of Miyazaki, and Nagoya City University Graduate School of Medical Sciences.

Patients who were positive for HCV core antigen (HCVcAg) or HCV RNA based on screening performed between 2002 and 2004 were considered to have hepatitis C viremia and were classified as HCV carriers. Patients who were negative for both HCVcAg and HCV RNA (i.e., viremia-negative) were considered to have a history of HCV infection and were classified as patients with prior HCV infection.

Serological Markers

Serum anti-HCV antibodies were detected using a second-generation enzyme immunoassay (Immunocheck F-HCV Antibody; International Reagents Co., Kobe, Japan) or a third-generation chemiluminescence enzyme immunoassay (Lumipulse Ortho II; Ortho-Clinical Diagnostics, Tokyo, Japan). In the anti-HCV-positive patients, HCV core antigen levels were also measured with an immunoradiometric assay (Ortho HCV Ag IRMA test; Ortho-Clinical Diagnostic), using a cutoff value for a positive result of 20 fmol/L from 2002 to 2004. The presence of HCV RNA was determined by reverse transcription polymerase chain reaction (Amplicor HCV Monitor, ver. 1.0 or ver. 2.0 [Nippon Roche or Roche Diagnostics K.K., Tokyo, Japan]) in patients with HCVcAg levels below the detection threshold. Before 2001, serum levels of HCVcAg had been tested with a fluorescence enzyme immunoassay (Immunocheck F-HCV Ag Core; International Reagents Co., Kobe, Japan) with a detection threshold of 8 pg/mL. The HCV serotype was determined with a serological genotyping assay kit (Immunocheck F-HCV Grouping; International Reagents Co., Tokyo, Japan). If the HCV serotype was not determined, the HCV genotype was determined (HCV Core Genotype; SRL, Tokyo, Japan). Serotype 1 included HCV genotype 1b, and serotype 2 included genotypes 2a and 2b. No other HCV

genotype was detected in this study population [Uto et al., 2009].

Determination of the IL28B SNP (rs8099917)

The rs8099917 polymorphism was determined using a TaqMan predesigned SNP genotyping assay [Ito et al., 2011].

Hepatic Fibrosis

The degree of hepatic fibrosis was evaluated using transient elastography (Fibroscan; Echosens, Paris, France) [Fraquelli et al., 2007; Friedrich-Rust et al., 2009; Berzigotti et al., 2010].

Statistical Analysis

Data were analyzed by chi-square test, Mann–Whitney U test, or multivariate stepwise logistic regression analysis using SPSS ver.17, GraphPad Prism 5 (SPSS Inc., Chicago, IL). The significance level was set at $P < 0.05$.

RESULTS

Background of HCV Carriers and Patients with Prior HCV Infection

The background characteristics of the 502 patients are shown in Table I. The median age was 73.0-years-old and 66.7% were female. The IL28B SNPs in the 502 patients were TT (82.9%, 416/502), TG (15.9%, 80/502), and GG (1.2%, 6/502). Of the patients, 353 were HCV core antigen- or HCV RNA-positive carriers (HCV carriers) and 149 were classified as patients with prior HCV infection. There were no significant differences in age, sex, and history of alcohol consumption and blood transfusion between the two groups. In contrast, genotype distribution of the IL28B rs8099917 polymorphism in HCV carriers and patients with prior HCV infection was significantly different. Furthermore, serum levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT), ferritin, hyaluronic acid, type IV collagen 7S, α-fetoprotein (AFP) and des-γ-carboxy prothrombin (DCP) were significantly higher and platelet count and serum levels of cholesterol and albumin were significantly lower in HCV carriers compared to patients with prior HCV infection.

IL28B SNP is Involved in Spontaneous Clearance of HCV Infection

Homozygous for the IL28B SNPs (rs8099917) major allele (TT genotype) was significantly more frequent in patients with prior HCV infection compared to HCV carriers (92.0%, 137/149 vs. 79.0%, 279/353, $P < 0.001$) (Fig. 1). Multivariate analysis using host factors such as age, sex, body mass index, IL28B SNPs, and history of alcohol consumption and blood transfusion, showed that the IL28B SNPs was associated independently

TABLE I. Characteristics of the 502 Patients, Including 353 HCV Carriers and 149 Patients With Prior HCV Infection

	All patients	HCV carrier*	Prior HCV infection*	
Characteristics	(n = 502)	(n = 353)	(n = 149)	*P* value
Age (years)	73.0 (37–97)	73.0 (42–97)	72.0 (37–89)	0.495
Sex [Male/Female]	167/335	121/232	46/103	0.460
Body mass index (kg/m^2)**	23.1 (14.6–32.5) (n = 420)	23.0 (14.6–32.5) (n = 291)	23.1 (16.8–32.4) (n = 129)	0.443
IL28B SNPs (rs8099917) [TT/TG/GG]	416/80/6	279/69/5	137/11/1	0.002
Alcohol consumption [Yes/No]**	159/333	111/235	48/98	0.863
Blood transfusion [Yes/No]**	63/429	39/307	24/122	0.117
HCV RNA serotype [1/2]**		232/120		
HCV core antigen (fmol/L) [< 20/≥ 20–< 1000/≥ 1000]		3290.0# (< 20–≥ 20000) (17/77/259)		
AST (IU/L)	34 (10–738)	40.0 (16–738)	24.0 (10–67.0)	< 0.001
ALT (IU/L)	27.5 (7–575)	34.0 (7–575)	18.0 (7–62)	< 0.001
Total cholesterol (mg/dL)	187.5 (84–323)	179.0 (84–323)	207.0 (132–308)	< 0.001
Albumin (g/dL)	4.4 (2.5–5.3)	4.4 (2.5–5.3)	4.5 (3.7–5.1)	0.004
Ferritin (ng/mL)	85 (3–2100)	96.0 (3–2100)	74.0 (3–797)	0.041
Platelet count (× 10^4/μL)	19.9 (4.4–44.5)	18.8 (4.4–44.5)	22.8 (10.7–39.3)	< 0.001
Hyaluronic acid (ng/mL)	102.5 (9–4560)	126.0 (9–4560)	60.0 (9–880)	< 0.001
Type IV collagen 7S (ng/mL)	4.7 (1.7–19)	5.0 (1.7–19)	4.2 (2.3–6.2)	< 0.001
AFP (ng/mL)	4.4 (1.4–1059)	4.9 (1.6–1059)	3.6 (1.4–16)	< 0.001
DCP (mAU/mL)	22.0 (6–554)	23.0 (6–554)	21.0 (11–36)	0.006

Continuous variables are expressed as median (range) and compared using the Mann–Whitney U test.
Categorical variables are expressed as number and compared using the chi-square test.
HCV, hepatitis C virus; IL, interleukin; SNP, single nucleotide polymorphism; AST, aspartate aminotransferase; ALT, alanine aminotransferase; γ-GTP, γ-glutamyl transpeptidase; AFP, α-fetoprotein; DCP, des-γ-carboxy prothrombin.
*Definition is stated in the Method.
**Patients with missing data were excluded.
#Data was calculated in subject whose HCV core antigen levels were ≥ 20.

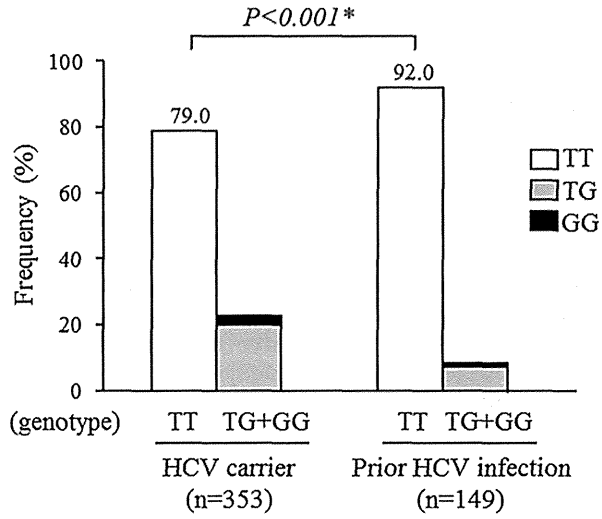

Fig. 1. Genotype distribution of the IL28B rs8099917 polymorphism in HCV carriers and patients with prior HCV infection. The frequency of the TT genotype was significantly higher in patients with prior HCV infection. *Chi-square test.

with persistence of HCV infection (odds ratio [OR] of TT genotype = 0.323, 95% confidence interval [CI]: 0.154–0.678, $P = 0.003$) (Table II). In addition, among the 149 patients with prior HCV infection confirmed between 2002 and 2004, nine were positive for HCV core antigen or HCV RNA with positive for anti-HCV antibody before 2001 and all nine of these patients had the TT genotype.

Association between IL28B SNPs and Clinical Features in HCV Carriers

HCV carriers were divided into those with TT and non-TT (TG + GG) IL28B SNPs (rs8099917). There were no significant differences in age when lymphocytes used in this study were collected, sex, history of alcohol consumption and blood transfusion, platelet count, transaminase and ferritin between these groups (Table III). In addition, the IL28B SNP was not associated with hepatic fibrosis assessed by hepatic fibrosis markers (hyaluronic acid, type IV collagen 7S) and ultrasonic elastography (Fig. 2). Furthermore, the distribution of liver fibrosis stages (mild/advanced fibrosis) based on previously established cutoffs for liver stiffness assessed by ultrasonic elastography [Friedrich-Rust et al., 2010] and the aspartate aminotransferase-to-platelet ratio index (APRI) [Ahmad et al., 2011] was not associated with IL28B SNP (Table IV). In contrast, HCV core antigen levels were significantly higher in the TT group of HCV carriers (Table III, Fig. 3).

IL28B SNP is Associated with HCV Core Antigen Levels in HCV Carriers

Univariate analysis showed that IL28B SNP, HCV serotype and serum levels of ALT, and total cholesterol were significant factors contributing to HCV core antigen levels (Table V). In multivariate analysis using these significant factors, the major (TT) allele of the IL28B SNP, HCV serotype 1 and ALT ≤ 30 (IU/L) emerged as independent predictors for higher HCV core antigen levels (≥ 1000 fmol/L, Table V).

DISCUSSION

This study shows that the frequency of the major (TT) allele of IL28B SNP rs8099917 was significantly higher in patients with prior HCV infection than in HCV carriers. The TT allele was also present in all nine patients with anti-HCV antibody who achieved spontaneous HCV clearance during the observation period between 1995 and 2005. These findings suggest that IL28B SNP rs8099917 might play a role in both HCV clearance in the acute stage of HCV infection and in spontaneous clearance in the chronic stage of infection. The IL28B SNP has previously

TABLE II. Factors Associated With Persistence of HCV Infection

Variable	Status	Odds ratio	95% CI	*P* value
Univariate analysis				
Age (years)	< 65	1		
	≥ 65	1.185	0.828–1.694	0.401
Sex	Female	1		
	Male	1.052	0.923–1.199	0.470
Body mass index (kg/m^2)	< 22	1		
	≥ 22	0.898	0.675–1.196	0.509
IL28B SNPs (rs8099917)	TG + GG	1		
	TT	0.384	0.215–0.686	<0.001
Alcohol consumption	No	1		
	Yes	0.988	0.864–1.131	0.916
Blood transfusion	No	1		
	Yes	0.942	0.868–1.021	0.139
*Multivariate analysis**				
IL28B SNPs (rs8099917)	TG + GG	1		
	TT	0.323	0.154–0.678	0.003

HCV, hepatitis C virus; CI, confidence interval; IL, interleukin; SNP, single nucleotide polymorphism.
*Multiple stepwise logistic regression analysis was carried out using variables in this table as co-variables.

TABLE III. Characteristics of HCV Carriers Based on the IL28B SNPs (rs8099917)

Characteristics	IL28B SNP (rs8099917) TT (n = 279)	TG + GG (n = 74)	*P* value
Age (years)	73.0 (44–97)	74.0 (42–95)	0.900
Sex [Male/Female]	98/181	23/51	0.515
Body mass index (kg/m^2)*	23.1 (14.6–32.5) (235)	22.8 (16.2–29.4) (56)	0.433
Alcohol consumption[Yes/No]*	88/186	23/49	0.404
Blood transfusion [Yes/No]*	33/240	6/67	0.353
HCV-RNA serotype [1/2]*	177/101	55/19	0.086
HCV core antigen (fmol/L)	4020.0(< 20–≥ 20000)	1820.0(< 20–≥ 20000)	0.003
[< 20/≥ 20–< 1000/≥ 1000]	13/54/212	4/23/47	0.082
AST (IU/L)	41.0 (16–738)	40.0 (22–278)	0.694
ALT (IU/L)	35.0 (7–575)	31.0 (7–209)	0.206
Total cholesterol (mg/dL)	178.0 (112–323)	183.0 (84–274)	0.518
Albumin (g/dL)	4.4 (3.1–5.3)	4.5 (2.5–5.1)	0.315
Ferritin (ng/mL)	105.0 (3–2100)	71.5 (3–658)	0.063
Platelet count ($\times 10^4/\mu L$)	18.5 (4.4–44.5)	19.2 (5.2–31.5)	0.317
AFP (ng/mL)	4.8 (1.6–1059)	5.4 (2.2–85.5)	0.109
DCP (mAU/mL)	23.0 (6–554)	24.0 (13–75)	0.411

Continuous variables are expressed as median (range) and compared using the Mann-Whitney U test.
Categorical variables are expressed as number and compared using the chi-square test.
HCV, hepatitis C virus; SNP, single nucleotide polymorphism; IL, interleukin; AST, aspartate aminotransferase; ALT, alanine aminotransferase; γ-GTP, γ-glutamyl transpeptidase; AFP, α-fetoprotein; DCP, des-γ-carboxy prothrombin.
*Patients with missing data were excluded.
#Data was calculated in patients whose HCV core antigen levels were ≥ 20.

been reported to influence the efficacy of IFN-based treatment and spontaneous HCV clearance in acute hepatitis C [Thomas et al., 2009; Montes-Cano et al., 2010; Rauch et al., 2010; Tillmann et al., 2010], but this study may suggest that this SNP could be associated with spontaneous clearance of chronic HCV infection.

A cross-sectional study in 353 patients showed that the frequency of the major allele of IL28B rs12979860 (genotype CC) was significantly higher in patients with prior HCV infection (72.5%) compared to HCV carriers (45.6%) [Montes-Cano et al., 2010]. In a review of several cohort studies, the CC allele of IL28B rs12979860 contributed to spontaneous clearance of HCV [Thomas et al., 2009]. In a cross-sectional study in 136 females with HCV acute infection, the rate of spontaneous HCV clearance was higher (64%) in patients with the IL28B rs12979860 CC genotype compared to those with CT (24%) and TT (6%) [Tillmann et al., 2010]. A multicenter cohort study showed that the minor allele of IL28B rs8099917 (TG + GG) was likely to induce persistence of HCV infection (OR = 2.31; 95% CI: 1.74–3.06; $P = 6.07 \times 10^{-9}$) [Rauch et al., 2010]. Therefore, there are many reports indicating a relationship between IL28B SNPs and spontaneous HCV clearance, but these are mainly hospital-based studies and none examined spontaneous clearance in patients with chronic HCV infection. Although the estimated age at HCV infection was unclear, this community-based

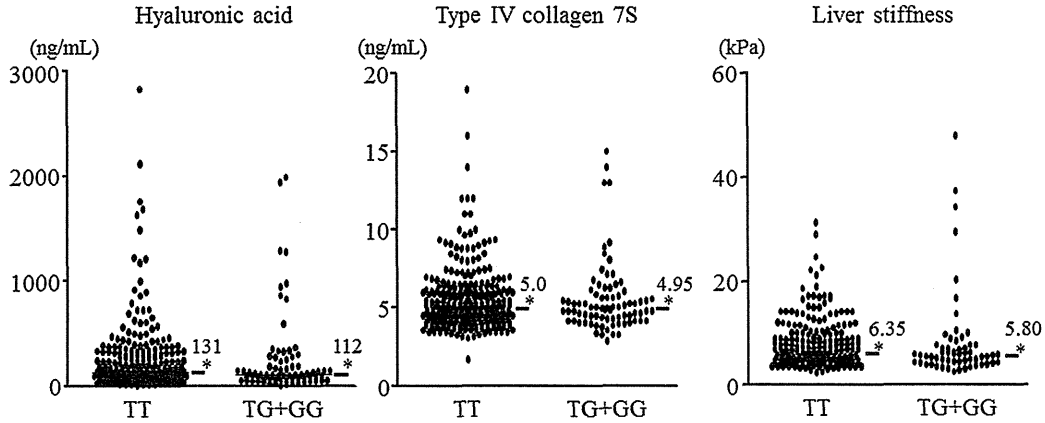

Fig. 2. Association between the IL28B rs8099917 polymorphism and hepatic fibrosis in HCV carriers. Hepatic fibrosis was evaluated using serum markers (n = 353), such as hyaluronic acid and type IV collagen 7S and by ultrasonic elastography (n = 268). *Lines indicate the median in each group.

TABLE IV. Liver Fibrosis Assessed by Transient Elastography and APRI in HCV Carriers According to IL28B Polymorphism

Characteristics	IL28B SNP (rs8099917) TT	TG + GG	*P* value
Liver stiffness (kPa) [mild fibrosis/ advanced fibrosis]*	154/56**	48/10**	0.140
APRI [mild fibrosis/ advanced fibrosis]*	215/64	64/10	0.077

APRI, aspartate aminotransferase-to-platelet ratio index [Ahmad et al., 2011]; HCV, hepatitis C virus.
*The distribution of liver fibrosis stages (mild/advanced fibrosis) based on previously established cutoffs [Friedrich-Rust et al., 2010; Ahmad et al., 2011.].
**Patients with missing data were excluded.

cohort study may have less bias in the clinical course compared to the past studies and may provide direct evidence that an IL28B SNP can influence the natural course in patients with chronic HCV infection.

HCV-RNA levels are greater in HCV-infected patients with the major (CC or CT) allele of IL28B SNP rs12979860 than in those with the minor (TT) allele [Ge et al., 2009; Labarga et al., 2011]. HCV core antigen levels in the current study were also significantly higher in patients with the major (TT) allele of IL28B rs8099917 compared to the minor (TG + GG) allele group. Based on the correlation of HCV core antigen levels with HCV RNA levels, the HCV RNA or core antigen level was likely to have been greater

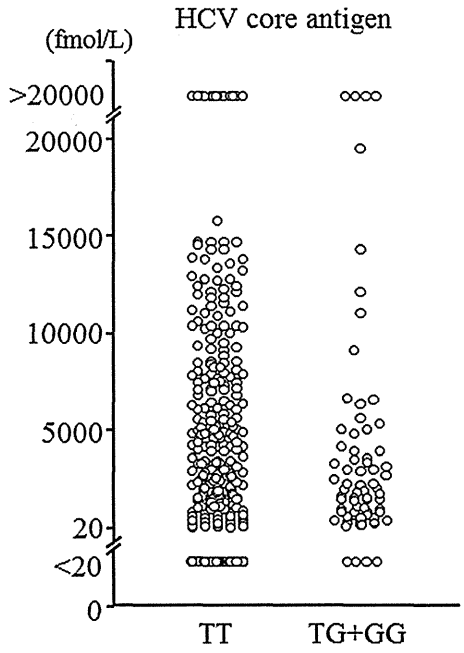

Fig. 3. HCV core antigen levels based on the IL28B rs8099917 polymorphism in HCV carriers (n = 353).

in patients with the TT allele of IL28B rs8099917. This effect might be associated with lower ISG expression in the liver of patients with IL28B rs8099917 TT compared with TG + GG patients [Honda et al., 2010; Abe et al., 2011; Chayama et al., 2012]. On the other hand, interferon-stimulated gene (ISG) expression after IFN administration was induced more in patients with the TT allele and this made the antiviral effect stronger, which may influence the efficacy of IFN therapy [Chayama et al., 2012]. Similarly, in terms of host immunological responsiveness during HCV acute infection, greater ISG expression was induced in patients with the TT allele, which suggests that ISG expression can be induced by natural immunity during acute infection and that this might promote HCV clearance. Thus, expression levels of ISGs regulated by the IL28B SNP might be lower in TT patients compared to TG + GG patients, but induction of ISG expression might be higher in the TT group.

Around 25% of patients with HCV infection undergo spontaneous clearance during the early stage of HCV infection, but such clearance is less likely to occur after the infection becomes persistent [Maasoumy and Wedemeyer, 2012; Hajarizadeh et al., 2013]. Of the patients in the current study, the majority of those with a history of HCV infection, including HCV antibody-positive patients and those who were HCV core antigen-negative and HCV RNA-negative, were thought to achieve spontaneous HCV clearance early after the onset of acute infection. In contrast, there were nine HCV carriers who appeared to achieve spontaneous clearance at a later stage because these patients were positive for anti-HCV antibody with viremia in observation period before 2001. The IL28B gene may have been involved in clearance in these patients, but the underlying mechanism is unclear. HCV levels were lower in these nine HCV carriers (data not shown) and continuous high expression of ISGs, unlike during IFN therapy and acute HCV infection, might result in a lower viral load and spontaneous HCV clearance. Rs8099917 has been reported to be the most significant IL28B SNP for a complete response to IFN-based therapy [Suppiah et al., 2009; Tanaka et al., 2009], while another group suggested that IL28B SNP rs12979860 was more important [Ge et al., 2009]. IL28B SNP rs8099917 and rs12979860 are located close together on the genome. According to HapMap data, linkage disequilibrium may be present and rs8099917 and rs12979860 are 99% matched in the Japanese population [Kobayashi et al., 2012]. However, the concordance rate varies with race [Thio and Thomas, 2010] and a further study of the effects on spontaneous HCV clearance of the major and minor alleles of rs8099917, rs12979860 or other undetermined SNP is required.

Persistent HCV infection induces hepatic inflammation and fibrosis, which lead to liver cirrhosis and hepatocarcinogenesis. It has been reported that the

TABLE V. Factors Contributing to Higher HCV Core Antigen Levels (≥ 1000 fmol/L) in HCV Carriers Based on Univariate Analysis and Multivariate Analysis

Variable	Status	Odds ratio	95% CI	*P* value
Univariate analysis				
Age (years)	<65	1		
	≥65	1.181	0.750–1.858	0.546
Sex	Female	1		
	Male	0.877	0.728–1.056	0.163
Body mass index (kg/m^2)	<22	1		
	≥22	1.111	0.804–1.534	0.587
IL28B SNPs (rs8099917)	TG+GG	1		
	TT	1.583	1.050–2.386	0.038
Alcohol consumption	No	1		
	Yes	0.997	0.845–1.177	>0.999
Blood transfusion	No	1		
	Yes	0.954	0.869–1.048	0.334
HCV RNA serotype	2	1		
	1	1.960	1.488–2.583	<0.001
AST (IU/L)	≤30	1		
	≥31	0.689	0.435–1.091	0.128
ALT (IU/L)	≤30	1		
	≥31	0.705	0.518–0.960	0.021
Total cholesterol (mg/dL)	<180	1		
	≥180	1.265	1.024–1.561	0.041
Albumin (g/dL)	<3.8	1		
	≥3.8	1.286	0.541–3.056	0.619
Ferritin (ng/mL)	<138	1		
	≥138	0.972	0.816–1.159	0.801
Platelet count (×10^4/μL)	<18	1		
	≥18	1.154	0.912–1.461	0.278
Hyaluronic acid (ng/mL)	<130	1		
	≥130	0.825	0.638–1.066	0.148
Type IV collagen 7S (ng/mL)	<5.5	1		
	≥5.5	0.924	0.764–1.117	0.454
AFP (ng/mL)	<10	1		
	≥10	0.991	0.888–1.107	0.875
DCP (mAU/mL)	<40	1		
	≥40	0.944	0.880–1.013	0.069
*Multivariate analysis**				
IL28B SNPs (rs8099917)	TG+GG	1		
	TT	2.347	1.302–4.232	0.005
HCV RNA serotype	2	1		
	1	3.766	2.238–6.337	<0.001
ALT (IU/L)	≤30	1		
	≥31	0.448	0.265–0.758	0.003

HCV, hepatitis C virus; IL, interleukin; SNP, single nucleotide polymorphism; AST, aspartate aminotransferase; ALT, alanine aminotransferase; γ-GTP, γ-glutamyl transpeptidase; AFP, α-fetoprotein; DCP, des-γ-carboxy prothrombin.
*Multiple stepwise logistic regression analysis was carried out using significant variables in univariate analysis.

major (TT) allele of IL28B did not affect serum ALT in HCV carriers, but was involved in hepatic inflammation and hepatic fibrosis [Abe et al., 2010]. Another report indicated that the TT allele of IL28B SNP rs12979860 was a factor in progression of hepatic fibrosis in patients with chronic hepatitis C [Fabris et al., 2012]. In contrast, Marabita et al. reported that IL28B SNP was not involved in hepatic fibrosis [Marabita et al., 2011]. Thus, there is no consensus on the involvement of the IL28B SNP in progression of hepatic fibrosis. In this study, we found no significant relationship between the IL28B SNP and hepatic fibrosis based on serum markers, such as platelet count, hyaluronic acid, type IV collagen 7S and APRI, and on ultrasonic elastography. Hepatic fibrosis in our patients was relatively mild because we studied a community-based population. Therefore, differences in the characteristics of the patients between this study and previous studies may have influenced the relationship between the IL28B SNP and hepatic fibrosis. Factors such as obesity are also involved in progression of hepatic fibrosis besides persistence of HCV infection [Cross et al., 2010; Kobayashi et al., 2011] and the IL28B SNP might only make a small contribution to hepatic inflammation and fibrosis.

The CT allele of IL28B SNP rs12979860 was related to risks of onset of liver cirrhosis and hepatocellular carcinoma [Fabris et al., 2011], but there is a conflicting report showing that the IL28B SNP has no effect on prognosis of liver diseases [Joshita et al., 2012]. At least six patients in the current study (four with IL28B SNP rs8099917 TT vs. two with non-TT) died

of liver diseases during the observation period from 2002 to 2005 (data not shown). Statistical analysis indicated that the IL28B SNP had no effect on prognosis, but this conclusion is limited by the small number of patients in the study.

There were several limitations in this study. Firstly, patients on IFN therapy were excluded based on an interview before the start of the study, but it is possible that those with a sustained viral response to IFN might have been included in the patients with prior HCV infection. However, the interview was repeated several times from 1995 and so this possibility is very low. In contrast, detailed information on alcohol intake was not available, and there may be underestimation of the effect of alcohol abuse on liver disease. Secondly, details of the clinical course in nine viremia patients who experienced spontaneous HCV clearance could not be fully evaluated, although several data in three of these case were previously reported [Uto et al., 2006]. Furthermore, in eight of these cases, viremia was confirmed only once. Therefore, it is possible that the HCV viremia-positive status was caused by contamination, but this is not likely. Confirmation of this finding requires analysis in more patients. Thirdly, the patients were relatively old and some patients in the previous cohort study could not be followed up due to disease progression or death [Uto et al., 2009]. Furthermore, patients with HCC were excluded because many such patients were referred to hospital and did not participate in the study. The source of HCV infection was also unclear. These situations may have caused a selection bias. Thus, it is desirable to conduct a long-term longitudinal study in younger patients, including patients with HCC. Fourthly, recent studies have shown that interferon lambda-4 (IFNL4) rs469415590 is strongly associated with IL28B SNP rs12979860 [Prokunina-Olsson et al., 2013; Stättermayer et al., 2014]. The impact of IFNL4 rs469415590 in the natural clearance of HCV and prognosis in a community-based population is of particular interest. In this cohort study, however, the rs469415590 polymorphism was not examined. Further studies are required to evaluate this issue.

CONCLUSION

This study suggests that the IL28B SNP is associated with spontaneous HCV clearance and HCV core antigen levels in patients in HCV hyperendemic areas and that this SNP is unlikely to be related to histopathological findings such as hepatic fibrosis.

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RESEARCH ARTICLE

Influence of Genes Suppressing Interferon Effects in Peripheral Blood Mononuclear Cells during Triple Antiviral Therapy for Chronic Hepatitis C

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Abstract

The levels of expression of interferon-stimulated genes (ISGs) in liver are associated with response to treatment with pegylated interferon (PEG-IFN) plus ribavirin (RBV). However, associations between the responses of ISGs to IFN-based therapy and treatment efficacy or interleukin-28B (*IL28B*) genotype have not yet been determined. Therefore, we investigated the early responses of ISGs and interferon-lambdas (IFN-λs) in peripheral blood mononuclear cells (PBMCs) during PEG-IFN/RBV plus NS3/4 protease inhibitor (PI) therapy. We prospectively enrolled 50 chronic hepatitis C patients with HCV genotype 1, and collected PBMCs at baseline, 8 and 24 h after the initial administration of PEG-IFN/RBV/PI. Levels of mRNAs for selected ISGs and IFN-λs were evaluated by real-time PCR. All 31 patients with a favorable *IL28B* genotype and 13 of 19 with an unfavorable genotype achieved sustained virological responses (SVR). Levels of mRNA for *A20, SOCS1*, and *SOCS3*, known to suppress antiviral activity by interfering with the IFN signaling pathway, as well as *IRF1* were significantly higher at 8 h in patients with an unfavorable *IL28B* genotype than in those with a favorable one ($P = 0.007$, 0.026, 0.0004, 0.0006, respectively), especially in the non-SVR group. Particularly, the fold-change of *IRF1* at 8 h relative to baseline was significantly higher in non-SVR than in SVR cases with an unfavorable *IL28B* genotype ($P = 0.035$). In conclusion, levels of several mRNAs of genes suppressing antiviral activity in PBMCs during PEG-IFN/RBV/PI differed according to *IL28B* genotypes, paralleling treatment efficacy.

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Introduction

Chronic hepatitis C virus (HCV) infection is a significant risk factor for progressive liver fibrosis and hepatocellular carcinoma (HCC). Antiviral treatment improves the natural course in chronic hepatitis C (CHC) [1, 2]. Newly-developed treatments involving direct-acting antivirals (DAAs), including nonstructural (NS) 3/4A protease inhibitors have shown promising outcomes in combination with pegylated interferon (PEG-IFN) plus ribavirin (RBV) in several clinical trials. Thus >70% of patients infected with HCV genotype 1 are reported to achieve sustained virological responses (SVR) [3–5].

Recent genome-wide association studies (GWAS), including our own study on HCV infection [6], have identified a single nucleotide polymorphism (SNP) near the interleukin-28B (*IL28B*) gene encoding type III IFN-λ3 that was strongly associated with the response to PEG-IFN/RBV therapy for chronic HCV genotype 1 infection [6–9]. Furthermore, a recent meta-analysis showed that the *IL28B* genotype was also associated with efficacy of PEG-IFN/RBV plus NS3/4A protease inhibitor (PI) treatment, including telaprevir or boceprevir [10]. However, it is not known how the *IL28B* gene influences the elimination of HCV.

IFNs mediate their potent antiviral effects through the regulation of hundreds of IFN-stimulated genes (ISGs). Type I and III IFNs induce the transcription of ISGs by activating the Janus kinase-signal transducer and activator of transcription (Jak-STAT) pathway through different cell surface receptors [11–14]. Because it has been reported that a high level of expression of intrahepatic ISGs at baseline affects responses to PEG-IFN/RBV therapy [15, 16], several groups have investigated an association between *IL28B* genotype and the expression of intrahepatic ISGs [17, 18]. In addition, intrahepatic expression of genes involved in innate immunity, Toll-like receptor 3 (TLR3) and retinoic acid-inducible gene I (RIG-I) which are important in signaling pathways for IFN-β induction, were also associated with the *IL28B* genotype and response to PEG-IFN/RBV [19]. Nevertheless, we cannot fully explain treatment outcome by evaluating *IL28B* genotypes and measuring intrahepatic gene expressions at baseline. Changes of intrahepatic gene expressions cannot easily be evaluated due to the risk of complications caused by taking a liver biopsy. For this reason, several groups have assessed the response of ISGs to PEG-IFN/RBV using peripheral blood mononuclear cells (PBMCs) as a surrogate. However, most of these earlier studies found less marked correlations between the expression of ISGs in PBMCs and treatment efficacy or *IL28B* genotype, relative to what was seen in the liver of the same patients [15, 20, 21]. We also analyzed the expression of ISGs, which included previously reported genes [17, 19–21], in PBMCs during PEG-IFN/RBV therapy, indicating that several ISGs that suppressed the antiviral state by interfering with the IFN signaling pathway were associated with the *IL28B* genotype or response to PEG-IFN/RBV therapy. These included *A20*, suppressor of cytokine signaling 1 (*SOCS1*), *SOCS3*. In PEG-IFN/RBV/PI therapy, the expression of ISGs, IFN-λs, and molecules related to the innate immune system is expected to be changed greatly soon after the start of therapy, due to the effects of the PI. Hence, we prospectively collected PBMCs of patients treated with PEG-IFN/RBV/PI, and then evaluated associations between the levels of mRNAs for the selected ISGs or IFN-λs and the *IL28B* genotype or patient's response to treatment.

Patients and Methods

Patients and treatment protocol

We prospectively enrolled a total of 50 CHC individuals infected with HCV genotype 1 who were treated with PEG-IFN/RBV/PI at Nagoya City University Hospital; 32 patients received telaprevir and 18 faldaprevir. All patients had tested positive for HCV RNA for more than 6

months. Patients chronically infected with hepatitis B virus or human immunodeficiency virus, or with other liver diseases such as autoimmune hepatitis and primary biliary cirrhosis, were excluded from this study.

The regimen of PEG-IFN/RBV/telaprevir therapy was as follows: PEG-IFN-α2b (1.5 μg/kg body weight subcutaneously once a week), RBV (600–1000 mg daily according to body weight), and telaprevir (standard dose of 2250 mg daily given three times a day every 8 hours or reduced dose of 1500 mg daily given twice a day every 12 hours) for 12 weeks, followed by an additional 12 weeks of PEG-IFN/RBV. In several patients, the initial dose of telaprevir was reduced to 1500 mg daily according to age, body weight, gender, or baseline hemoglobin level, at the discretion of the attending physicians. When marked adverse effects such as anorexia, anemia, neutropenia, thrombocytopenia, renal dysfunction or skin rash, developed, the dose of telaprevir was reduced to 1500 mg daily, and that of PEG-IFN or RBV was reduced according to the recommendation on the package inserts or the clinical condition of individual patients. The regimen of PEG-IFN/RBV/faldaprevir was as follows: PEG-IFN-α2a (180 μg subcutaneously once a week), RBV (600–1000 mg daily according to body weight), and faldaprevir (120 or 240 mg once-daily) for 12 or 24 weeks, followed by an additional PEG-IFN/RBV, making a total of 24 or 48 weeks. When marked adverse effects developed, the dose of PEG-IFN or RBV was reduced as mentioned above.

Written informed consent was obtained from each patient and the study protocol conformed to the ethics guidelines of the Declaration of Helsinki and was approved by the ethics review committees of Nagoya City University Hospital.

Definition of virological response to treatment

Treatment outcomes were defined as SVR (undetectable HCV RNA levels 24 weeks after cessation of treatment), transient virological response (TVR; HCV RNA levels became undetectable during treatment but reappeared after the end of treatment), and non-virological response (NVR; HCV RNA levels never became undetectable).

Detection of HCV RNA

Blood samples were obtained before treatment, and at week 1, 2, 4, 8, 12, and every 4 weeks up to treatment completion, and hematologic tests, blood chemistry and HCV RNA assays were performed. Follow-up measurements were obtained at week 4, 12 and 24 weeks after the end of treatment. HCV RNA levels were measured throughout the course of therapy using the COBAS TaqMan HCV test (Roche Diagnostics K.K., Tokyo, Japan). The measurement range of this assay is 1.2–7.8 log IU/mL.

SNP genotyping

Genetic polymorphisms in SNPs of the *IL28B* gene (rs8099917) were determined according to the manufacturer's instructions using TaqMan SNP Genotyping Assays and an ABI PRISM 7900HT Fast Real-Time PCR System (Applied Biosystems, Carlsbad, CA).

Measurement of gene expression in PBMCs

Blood samples were collected from the patients at baseline, 8, and 24 hours (h) after the initial administration of PEG-IFN/RBV/PI. PBMCs were isolated from blood by Ficoll gradient centrifugation. Total RNA was extracted from PBMCs using the RNeasy Mini Kit (Qiagen, Valencia, CA). Complementary DNA (cDNA) synthesis was performed using 1.0 μg of total RNA isolated from PBMCs using the High Capacity RNA-to-cDNA kit (Applied Biosystems,