27. Urban TJ, Thompson AJ, Bradrick SS, *et al.* IL28B genotype is associated with differential expression of intrahepatic interferon-stimulated genes in patients with chronic hepatitis C. *Hepatology* 2010; 52:1888–1896.
28. Rossol S, Marinos G, Carucci P, Singer MV, Williams R, Naoumov NV. Interleukin-12 induction of Th1 cytokines is important for viral clearance in chronic hepatitis B. *J Clin Invest* 1997; 99:3025–3033.
29. Quiroga JA, Martin J, Navas S, Carreno V. Induction of interleukin-12 production in chronic hepatitis C virus infection correlates with the hepatocellular damage. *J Infect Dis* 1998; 178:247–251.
30. Marín-Serrano E, Rodriguez-Ramos C, Diaz F, Martin-Herrera L, Giron-Gonzalez JA. Modulation of the anti-inflammatory interleukin 10 and of proapoptotic IL-18 in patients with chronic hepatitis C treated with interferon alpha and ribavirin. *J Viral Hepat* 2006; 13:230–234.
31. Saraiva M, O'Garra A. The regulation of IL-10 production by immune cells. *Nat Rev Immunol* 2010; 10:170–181.
32. Trinchieri G. Interleukin-12: a proinflammatory cytokine with immunoregulatory functions that bridge innate resistance and antigen-specific adaptive immunity. *Annu Rev Immunol* 1995; 13:251–276.
33. Megjugorac NJ, Gallagher GE, Gallagher G. Modulation of human plasmacytoid DC function by IFN-lambda1 (IL-29). *J Leukoc Biol* 2009; 86:1359–1363.
34. Liu BS, Janssen HL, Boonstra A. IL-29 and IFNα differ in their ability to modulate IL-12 production by TLR-activated human macrophages, and exhibit differential regulation of the IFNγ receptor expression. *Blood* 2011; 117:2385–2395.

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Original Article

Serum chemokine levels are associated with the outcome of pegylated interferon and ribavirin therapy in patients with chronic hepatitis C

Suguru Yoneda,[1] Takeji Umemura,[1] Satoru Joshita,[1] Tetsuya Ichijo,[1] Akihiro Matsumoto,[1] Kaname Yoshizawa,[1] Yoshihiko Katsuyama,[3] Masao Ota,[2] Eiji Tanaka[1] and the Nagano Interferon Treatment Research Group*

[1]*Departments of Medicine, Division of Hepatology and Gastroenterology,* [2]*Department of Legal Medicine, Shinshu University School of Medicine, and* [3]*Department of Pharmacy, Shinshu University Hospital, Matsumoto, Japan*

Aim: Serum chemokine levels and amino acid substitutions in the interferon-sensitivity determining region (ISDR) and core region have been associated with treatment outcome of pegylated interferon and ribavirin therapy in genotype 1 hepatitis C virus (HCV)-infected patients. The present study was conducted to clarify the association between serum chemokines and treatment outcome in patients with chronic HCV-1 infection in a Japanese cohort.

Methods: A total of six serum chemokines were quantified before, during and after pegylated interferon and ribavirin treatment in 79 genotype 1 chronic HCV patients using a multiple bead array system. Viral ISDR and core region variants were determined by direct sequencing.

Results: The baseline serum levels of eotaxin, IP-10 and RANTES were significantly higher in chronic HCV patients than in controls. High levels of eotaxin and macrophage inflammatory protein (MIP)-1β before therapy and more than two mutations in the ISDR were associated with a sustained virological response, and patients with more than two mutations in the ISDR also had significantly higher MIP-1β levels. Receiver–operator curve analysis showed a 77% sensitivity and 73% specificity for predicting an SVR using MIP-1β values.

Conclusion: Serum MIP-1β levels may predict the response to HCV treatment with pegylated interferon and ribavirin and are associated with amino acid substitutions in the ISDR.

Key words: chemokines, core, interferon sensitivity determining region, MIP-1β, pegylated interferon, ribavirin

INTRODUCTION

HEPATITIS C VIRUS (HCV) infection is a major cause of chronic liver disease that leads to liver cirrhosis and/or hepatocellular carcinoma (HCC).[1] HCC is ranked fourth in men and fifth in women as a cause of death from malignant neoplasms in Japan.[2,3] Interferon (IFN)-based therapy can achieve HCV eradication and decrease the risk of HCC to improve prognosis; with pegylated (PEG) IFN and ribavirin therapy, approximately 50% of patients with genotype 1 HCV infection achieve a sustained virological response (SVR).[4,5]

Chemokines and their receptors play an important role in the pathogenesis of HCV infection.[6,7] Despite the growing amount of published research supporting the complex interactions of these inflammatory biomarkers in the outcome of antiviral therapy, the majority of recent studies have nearly exclusively concentrated on only one or a few markers. Thus, it is possible that a test evaluating several biomarkers may prove to be of greater value in predicting responses to therapy.

In the present study, we sought to determine the levels of six chemokines in patients with chronic HCV-1b

Correspondence: Dr Takeji Umemura, Department of Medicine, Shinshu University School of Medicine, 3-1-1 Asahi, Matsumoto 390-8621, Japan. Email: tumemura@shinshu-u.ac.jp

*The Nagano Interferon Treatment Research Group includes Drs Susumu Morita, Atsushi Kamijo, Michiharu Komatsu, Naoki Tanaka (Shinshu University Hospital), Dr Chiharu Miyabayashi (Chikuma Central Hospital, Chikuma) and Dr Yuriko Koike (Kawanakajima Clinic, Nagano).

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infection who underwent treatment with PEG IFN and ribavirin using a broad-spectrum bead-based multiplex immunoassay.

METHODS

Subjects

A TOTAL OF 79 treatment-naïve patients with chronic hepatitis C (40 men and 39 women; median age 60 years [range: 17–74]) were seen at Shinshu University Hospital or its affiliated hospitals in the Nagano Interferon Treatment Research Group. Alanine aminotransferase (ALT), aspartate aminotransferase (AST), and γ-glutamyl transpeptidase (γ-GTP) were tested using standard methods.[8] All patients, who were infected with genotype 1b HCV, received PEG IFN-α-2b (PegIntron; Schering-Plough, Tokyo, Japan; 1.5 μg/kg of bodyweight) and ribavirin (Rebetol; Schering-Plough; 600–1000 mg/day) adjusted to bodyweight for 48 weeks as described previously.[9] The pretreatment median value for ALT was 54 IU/L (range: 22–389), AST was 44 IU/L (range: 20–288) and HCV RNA was 1700 KIU/mL (range: 11–5100), as measured by COBAS AMPLICOR assays (Roche Diagnostic Systems, Tokyo, Japan). A group of 26 healthy individuals (13 men and 13 women; mean age 54 years [range: 28–60]) with hepatitis B and C negative serologies and normal transaminases were used as the control. All patients and controls were negative for the antibody to HIV. The protocol of this study was approved by the ethics committee of Shinshu University School of Medicine and all patients provided written informed consent. All serum samples were immediately stored at −70°C and remained in storage until testing.

Definition of treatment outcome

An SVR was concluded in those whose serum HCV RNA was undetectable 24 weeks after completing therapy. Post-treatment relapse was defined as the reappearance of HCV RNA in the serum after treatment in patients whose HCV RNA was undetectable during or at the completion of therapy. A non-response was defined as a decrease in HCV RNA to less than 2 log copies/mL at week 12 and detectable HCV RNA during the treatment course.

Detection of amino acid substitutions in core and interferon-sensitivity determining regions (ISDR)

The sequences of 1–191 amino acids (a.a.) in the core protein and 2209–2248 a.a. in the NS5A region of genotype 1b HCV were determined by direct sequencing using stored serum samples obtained before therapy, as reported previously. Nucleotide and a.a. sequences were compared with the nucleotide sequences of genotype 1b HCV-J.[10] Substitutions of a.a.70 arginine (Arg70) and glutamine (Gln70) or a.a.91 leucine (Leu91) and methionine (Met91)[11] and the number of a.a. substitutions in the ISDR were defined as wild-type (0), intermediate-type (1) and mutant-type (≥2).[12] Of the 79 patients, 75 were determined to have substitutions at a.a.70 and a.a.91, and 76 could be sequenced for their ISDR.

Detection of chemokines

Six chemokines (macrophage inflammatory protein [MIP]-1α, MIP-1β, eotaxin, IP-10, RANTES and interleukin [IL]-8) were quantified using Luminex Multiplex Cytokine Kits (Procarta Cytokine assay kit; Panomics, Fremont, CA, USA) from serum samples obtained before the start of treatment, 4 weeks after the start of treatment and 24 weeks after the completion of treatment, according to the manufacturer's instructions.[13]

Statistical analysis

The Mann–Whitney *U*-test and Kruskal–Wallis test were used to analyze continuous variables as appropriate. The Wilcoxon rank sum test and the Friedman test were used to evaluate changes in serum chemokine levels over time. Spearman's rank correlation coefficients were used to evaluate the relationship between each pair of markers. The χ^2-test with Yate's correction was used for the analysis of categorical data. In cases where the number of subjects was less than 5, Fisher's exact test was used. $P \leq 0.05$ was considered significant. To predict treatment outcome, each cut-off point for continuous variables was determined by receiver–operator curve (ROC) analysis. Statistical analyses were performed using SPSS ver. 18.0J.

RESULTS

OF THE 79 patients receiving PEG IFN and ribavirin therapy, 31 (39%) achieved an SVR, 23 (29%) relapsed, and 25 (32%) did not respond to treatment and were termed null viral responders (NVR). When stratified into three groups based on treatment outcome, patients with an NVR had a higher female ratio ($P = 0.030$) (Table 1). Before treatment, the median AST and γ-GTP levels in the SVR group were significantly lower than those in the relapsed and NVR groups. Substitutions of a.a.70 in the core region

Table 1 Clinical characteristics of patients with chronic hepatitis C

Characteristic	SVR ($n = 31$)	TR ($n = 23$)	NVR ($n = 25$)	P
Mean age, years (range)	55 (28–72)	57 (17–71)	59 (22–74)	0.20
Sex, male : female	23:8	9:14	8:17	0.030
Mean values (range)				
ALT (IU/L)	58 (24–172)	76 (24–389)	90 (22–357)	0.43
AST (IU/L)	41 (21–133)	57 (20–218)	78 (25–288)	0.042
γ-GTP (IU/L)	40 (13–147)	47 (12–167)	81 (17–439)	0.027
HCV RNA (10^3 IU/mL)	1962 (110–>5100)	2379 (360–>5100)	1934 (220–>5100)	0.23
Substitutions				
Core a.a. 70 (Arg70/Gln70)	22/6	14/8	11/14	0.034
Core a.a. 91 (Leu91/Met91)	20/8	17/5	17/8	0.78
ISDR of NS5A (0–1/≥ 2)	20/9	20/2	23/2	0.040

a.a., amino acid; ALT, alanine aminotransferase; AST, aspartate aminotransferase; HCV, hepatitis C virus; ISDR, interferon-sensitivity determining region; NVR, null virological response; SVR, sustained virological response; TR, transient response; γ-GTP, γ-glutamyl transpeptidase.

($P = 0.034$) and in the ISDR ($P = 0.040$) were both significantly associated with treatment outcome. Six serum chemokines were assessed before therapy in all patients and in 26 healthy controls, revealing that the median serum levels of eotaxin, IP-10 and RANTES were significantly higher in HCV-afflicted patients. The median serum IL-8 level in cases with chronic HCV infection was significantly lower compared with the control group (Table 2).

The median serum chemokines of our cohort are shown in Table 3. Before treatment, the median serum levels of three chemokines (eotaxin, MIP-1β and RANTES) were significantly higher in patients who achieved an SVR than in those who did not. Patients with a virological response had significantly higher MIP-1α (39.0 vs 25.9 pg/mL; $P = 0.001$) and MIP-1β (192.7 vs 110.0 pg/mL; $P < 0.001$) compared with non-responders.

Table 2 Serum chemokines in patients with chronic hepatitis C and healthy controls

Chemokine	Chronic hepatitis C ($n = 79$)	Control ($n = 26$)	P
MIP-1α	36.4 (2.4–5021.3)	34.6 (10.6–92.8)	0.46
MIP-1β	160.2 (14.4–3341.6)	122.3 (21.1–1677.6)	0.18
Eotaxin	100.0 (2.4–1296.0)	19.8 (18.3–25.0)	<0.001
IP-10	1642.8 (57.7–11 487.0)	31.1 (21.3–80.6)	<0.001
RANTES	31 755.3 (17.9–83 248.0)	3460.0 (191.5–40 001.0)	<0.001
IL-8	12.9 (2.4–6324.3)	41.8 (2.4–327.6)	<0.001

Data are expressed as median (interquartile range) values (pg/mL).
IL, interleukin; MIP-1, macrophage inflammatory protein-1.

Table 3 Serum chemokines in treatment outcome to antiviral therapy

Chemokine	SVR ($n = 31$)	TR ($n = 23$)	NVR ($n = 25$)
MIP-1α	36.4 (32.3–99.3)	39.0 (33.7–52.9)	25.9 (17.7–40.8)
MIP-1β	264.4 (176.3–371.6)	155.0 (112.1–300.0)	110.2 (81.0–150.2)
Eotaxin	107.0 (66.9–180.4)	44.8 (28.1–87.6)	120.1 (50.7–234.5)
IP-10	1964.4 (956.4–5485.4)	1088.2 (818.6–2006.4)	1879.8 (653.4–2969.0)
RANTES	83 248.0 (31 755.3–83 248.0)	8633.4 (3469.1–22 498.6)	30 970.8 (3638.7–83 248.0)
IL-8	12.5 (8.7–24.2)	10.6 (2.4–17.8)	13.6 (12.1–15.2)

Data are expressed as median (interquartile range) values (pg/mL).
NVR, null virological response; SVR, sustained virological response; TR, transient response.

Table 4 Serum chemokine level changes before, during and after treatment in patients with chronic hepatitis C

Chemokine	Treatment outcome	Baseline	Week 4	Week 72	*P*
MIP-1α	SVR	36.4 (32.3–99.3)	34.4 (20.3–60.5)	17.4 (5.6–27.9)	<0.001
	Non-SVR	36.1 (25.2–49.2)	28.8 (22.2–45.0)	29.3 (23.2–46.1)	0.331
MIP-1β	SVR	264.4 (176.3–371.6)	161.7 (112.0–223.3)	158.7 (78.8–249.6)	<0.001
	Non-SVR	131.2 (97.0–187.8)	83.6 (59.2–108.9)	105.8 (79.9–148.0)	<0.001
Eotaxin	SVR	107.0 (66.9–180.4)	190.3 (115.4–274.7)	161.8 (101.5–221.2)	0.044
	Non-SVR	78.7 (30.4–141.2)	142.7 (76.3–226.4)	103.6 (30.6–228.5)	0.030
IP-10	SVR	1964.4 (956.4–5485.4)	2322.6 (1222.1–3411.2)	1085.2 (718.5–2314.4)	<0.001
	Non-SVR	1422.7 (766.8–2645.8)	1168.9 (654.3–1713.5)	1458.5 (525.0–3045.6)	0.047
RANTES	SVR	83 248.0 (57 501.7–83 248.0)	83 248.0 (31 037.0–83 248.0)	83 248.0 (17 542.9–83 248.0)	0.091
	Non-SVR	14 670.7 (3730.4–55 199.4)	25 377.2 (11 272.6–83 248.0)	21 707.6 (8746.5–83 248.0)	0.057
IL-8	SVR	12.5 (9.3–22.2)	11.4 (8.9–16.1)	8.2 (6.6–12.0)	<0.001
	Non-SVR	13.1 (10.0–16.3)	12.7 (10.3–14.2)	12.5 (9.3–14.7)	0.418

Data are expressed as median (interquartile range) values (pg/mL).
IL, interleukin; MIP-1, macrophage inflammatory protein-1; SVR, sustained virological response.

We also measured chemokine levels 4 weeks after the initiation of therapy and 6 months after its completion (Table 4). The serum levels of MIP-1α ($P < 0.001$, Friedman test), MIP-1β ($P < 0.001$), eotaxin ($P = 0.044$), IL-8 ($P < 0.001$) and IP-10 ($P < 0.001$) were significantly decreased in samples collected from patients who achieved an SVR from baseline to 6 months after completion. The levels of MIP-1β ($P < 0.001$), eotaxin ($P = 0.03$) and IP-10 ($P = 0.047$) were lower in patients with a non-SVR as well. In addition, MIP-1α ($P = 0.004$, Wilcoxon rank sum test), MIP-1β ($P < 0.001$) and IL-8 ($P = 0.045$) levels were significantly decreased in samples collected from patients who achieved an SVR from pretreatment to 4 weeks after the start of therapy. MIP-1β ($P < 0.001$) was similarly decreased in patients with a non-SVR.

Several demographic (age and sex) and clinical (ALT, AST, viral load and histology) findings were examined for their correlation with serum chemokines in patients with HCV infection. Serum IP-10 levels significantly correlated with ALT ($P = 0.038$, $r = 0.234$), AST ($P = 0.015$, $r = 0.284$) and fibrosis ($P = 0.045$, $r = 0.257$). Serum MIP-1β was significantly correlated with MIP-1α ($P < 0.001$, $r = 0.451$) and RANTES ($P < 0.001$, $r = 0.443$).

The frequency of Gln70 in the core region was significantly higher in patients with a non-SVR than in those with an SVR (22/47 vs 6/28; $P = 0.028$). Mutant ISDR was significantly prevalent in patients with an SVR (9/29 vs 4/47; $P = 0.026$). We next analyzed whether substitutions in the ISDR and core region were associated with serum chemokine levels because substitutions in these regions have been linked with treatment outcome in patients with chronic hepatitis C. The median baseline serum level of MIP-1β was significantly higher in patients with a mutant-type than in those with intermediate- or wild-type (249.2 vs 155.0 pg/mL; $P = 0.039$) (Table 5). Other chemokines

Table 5 Serum chemokine levels according to substitutions in the ISDR

Chemokine	Mutant-type ($n = 63$)	Intermediate- and wild-type ($n = 13$)	*P*
MIP-1α	67.3 (29.2–247.2)	36.4 (25.9–47.4)	0.57
MIP-1β	249.2 (185.1–371.0)	155.0 (106.9–275.5)	0.039
Eotaxin	100.0 (70.0–188.8)	101.1 (41.9–157.7)	0.18
IP-10	1809.4 (1166.7–6437.8)	1576.2 (818.6–3138.4)	0.12
RANTES	83 248.0 (6309.0–83 248.0)	29 705.6 (6713.2–83 248.0)	0.07
IL-8	20.3 (10.4–46.3)	12.9 (8.7–15.7)	0.38

Data are expressed as median (interquartile range) values (pg/mL).
IL, interleukin; MIP-1, macrophage inflammatory protein-1.

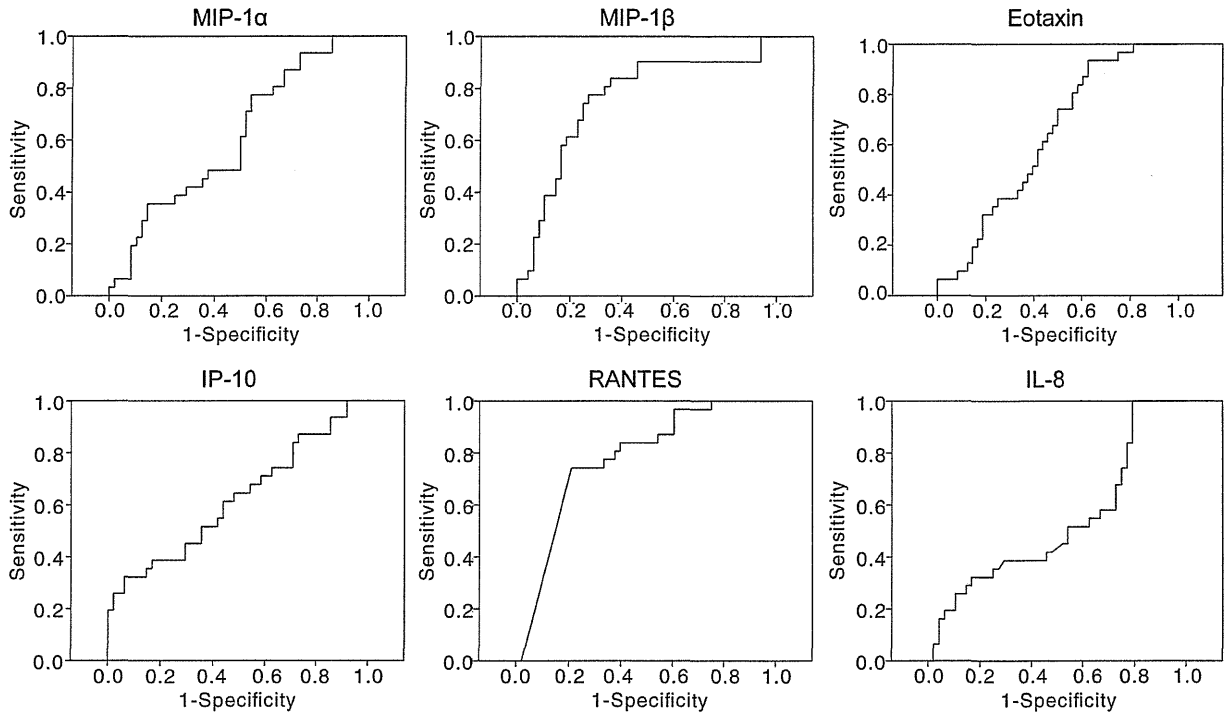

Figure 1 Receiver–operator curves for serum chemokine levels on treatment outcome. The areas under the curve for macrophage inflammatory protein (MIP)-1α, MIP-1β, eotaxin, IP-10, RANTES and interleukin (IL)-8 were 0.612, 0.756, 0.629, 0.623, 0.739 and 0.530, respectively.

were not significantly correlated with substitutions in the ISDR or core region.

Lastly, ROC curve analyses were performed to determine whether serum chemokines could predict an SVR (Fig. 1). MIP-1β only had a significant area under the curve, with values of sensitivity and specificity being 77.4% and 72.9%, respectively. The positive and negative predictive values for MIP-1β were 64.9% and 83.3%, respectively. The area under the curve (AUC) value was high at 0.76 (95% confidence interval = 0.64–0.87), indicating a strong predictive association.

DISCUSSION

IN THIS STUDY, we measured the levels of six chemokines in patients with genotype 1 chronic hepatitis C and analyzed their association with the outcome of PEG IFN and ribavirin therapy. Our data showed that baseline serum levels of eotaxin, IP-10 and RANTES were higher in HCV patients compared to healthy controls. Furthermore, elevated levels of eotaxin and MIP-1β before therapy were associated with an SVR. Serum cytokines have also been associated with pathogenesis in HCV infection. Because an association between serum cytokines and treatment outcome in HCV patients has already been reported in a prior study, only chemokines were assessed in this report.

As CC chemokines, MIP-1α, MIP-1β and RANTES are important in hepatic immunity because they are expressed on the portal vessel endothelium to provide a mechanism for the recruitment of CCR5 memory T cells in portal areas during immune surveillance and against inflammatory liver diseases.[14] Therefore, in the present study, lower MIP-1α and MIP-1β serum levels following treatment suggests that a decrease in the trans-endothelial migration of leukocytes occurs in responsive patients, which may preclude the retention and survival of lymphocytes in the liver and, thereby, ameliorate tissue damage and fibrosis. In particular, patients with an SVR had significantly higher MIP-1β compared to those without, in agreement with a previous study.[15]

The association between substitutions in the NS5A region of the ISDR and elevated MIP-1β levels that

was seen in our study is intriguing. Ahlenstiel *et al.*[16] reported that only HCV proteins, such as HCV core and NS5A, can modify RANTES secretion by altering RANTES promoter activity. To explain the observed association between MIP-1β and substitutions in the NS5A region of ISDR, one could hypothesize that IFN induces high levels of chemokines or other antiviral mediators that preferentially kill HCV; however, such a notion is highly speculative and would require additional studies to establish its validity. MIP-1β-mediated T-cell infiltration is essential for the delivery of IFN-γ to mediate protective downstream responses against HCV infection in the liver. It has been shown from the intrahepatic gene expression profiles of chimpanzees that MIP-1β was upregulated during acute infection at the time of viral clearance, but not in those who failed to eradicate the virus,[17] and previous studies have shown that HCV-infected individuals have a diminished response to MIP-1β in the liver.[18] As ROC analysis showed that MIP-1β could predict an SVR in our cohort, our data support that elevated serum levels of MIP-1β at baseline might be a favorable indicator of treatment outcome in patients with chronic hepatitis C.

Eotaxin is a chemokine that is thought to selectively attract eosinophils by activating CCR3 receptors. Several studies have shown that eotaxin is involved in the pathogenesis of inflammatory processes during liver diseases as well.[19,20] Vargas *et al.* recently analyzed the association between chemokines and virological response to IFN and ribavirin in HIV and HCV co-infected patients;[21] in patients achieving an SVR, plasma eotaxin levels before therapy were statistically higher than in non-responders. Thus, both our and their studies suggest that eotaxin may also be a useful marker in predicting an SVR to HCV treatment with PEG IFN and ribavirin.

There have been reports of increased serum and intrahepatic levels of IP-10 in HCV genotype 1-infected individuals.[22,23] Related studies have found elevated IP-10 to be associated with increased liver damage, and it has also been shown that serum IP-10 concentrations are higher in non-responders to HCV therapy than in those who achieve an SVR.[24–29] The serum level of IP-10 was not significantly associated with treatment outcome in our study, but the degree of fibrosis was well correlated with IP-10, as in a previous study.[30] These conflicting findings may reflect patient selection, sample size or racial differences.

Overall, the serum levels of eotaxin, IL-8, IP-10, MIP-1α and MIP-1β decreased during treatment and remained low in patients with an SVR. Because no direct correlation between chemokine levels and HCV RNA viral load was noticed, it is possible that chemokines may in fact compromise host immune responses to the virus.

One limitation of this study is a small sample size. Because we could not perform multivariate statistical analysis, it was difficult to draw a definitive conclusion on the most relevant chemokine. Hence, ROC analysis only was performed in our study. Larger studies are needed in the future. Another limitation of our findings is that we could not confirm if the stored serum chemokine levels were consistent with the original fresh serum samples. However, we can presume that this effect was minimal because all samples were stored immediately at −70 °C until use. Furthermore, our prior study with the same samples showed data consistent with those of other published work for the Luminex bead assay.

In conclusion, our data show that chemokines, especially MIP-1β, eotaxin and IP-10, have the potential to be effective and non-invasive markers of an SVR and potential prognostic surrogates for therapeutic outcome. Assessing chemokines may help elucidate the pathogenic processes of this disease on an individual basis, thereby assisting with prognostication and treatment decisions.

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REFERENCES

1 Kiyosawa K, Sodeyama T, Tanaka E *et al.* Interrelationship of blood transfusion, non-A, non-B hepatitis and hepatocellular carcinoma: analysis by detection of antibody to hepatitis C virus. *Hepatology* 1990; **12**: 671–5.
2 Umemura T, Kiyosawa K. Epidemiology of hepatocellular carcinoma in Japan. *Hepatol Res* 2007; **37** (Suppl 2): S95–S100.
3 Umemura T, Ichijo T, Yoshizawa K *et al.* Epidemiology of hepatocellular carcinoma in Japan. *J Gastroenterol* 2009; 44 (Suppl 19): 102–7.
4 Manns MP, McHutchison JG, Gordon SC *et al.* Peginterferon alfa-2b plus ribavirin compared with interferon alfa-2b plus ribavirin for initial treatment of chronic hepatitis C: a randomised trial. *Lancet* 2001; **358**: 958–65.

5 Fried MW, Shiffman ML, Reddy KR *et al.* Peginterferon alfa-2a plus ribavirin for chronic hepatitis C virus infection. *N Engl J Med* 2002; **347**: 975–82.
6 Zeremski M, Petrovic LM, Talal AH. The role of chemokines as inflammatory mediators in chronic hepatitis C virus infection. *J Viral Hepat* 2007; **14**: 675–87.
7 Heydtmann M, Adams DH. Chemokines in the immunopathogenesis of hepatitis C infection. *Hepatology* 2009; **49**: 676–88.
8 Umemura T, Zen Y, Hamano H *et al.* Immunoglobin G4-hepatopathy: association of immunoglobin G4-bearing plasma cells in liver with autoimmune pancreatitis. *Hepatology* 2007; **46**: 463–71.
9 Shirakawa H, Matsumoto A, Joshita S *et al.* Pretreatment prediction of virological response to peginterferon plus ribavirin therapy in chronic hepatitis C patients using viral and host factors. *Hepatology* 2008; **48**: 1753–60.
10 Kato N, Hijikata M, Ootsuyama Y *et al.* Molecular cloning of the human hepatitis C virus genome from Japanese patients with non-A, non-B hepatitis. *Proc Natl Acad Sci USA* 1990; **87**: 9524–8.
11 Akuta N, Suzuki F, Sezaki H *et al.* Association of amino acid substitution pattern in core protein of hepatitis C virus genotype 1b high viral load and non-virological response to interferon-ribavirin combination therapy. *Intervirology* 2005; **48**: 372–80.
12 Enomoto N, Sakuma I, Asahina Y *et al.* Mutations in the nonstructural protein 5A gene and response to interferon in patients with chronic hepatitis C virus 1b infection. *N Engl J Med* 1996; **334**: 77–81.
13 Yoneda S, Umemura T, Katsuyama Y *et al.* Association of serum cytokine levels with treatment response to pegylated interferon and ribavirin therapy in genotype 1 chronic hepatitis C patients. *J Infect Dis* 2011; **203**: 1087–95.
14 Shields PL, Morland CM, Salmon M *et al.* Chemokine and chemokine receptor interactions provide a mechanism for selective T cell recruitment to specific liver compartments within hepatitis C-infected liver. *J Immunol* 1999; **163**: 6236–43.
15 Wright H, Alex P, Nguyen T *et al.* Multiplex cytokine profiling of initial therapeutic response in patients with chronic hepatitis C virus infection. *Dig Dis Sci* 2005; **50**: 1793–803.
16 Ahlenstiel G, Woitas RP, Rockstroh J *et al.* CC-chemokine receptor 5 (CCR5) in hepatitis C–at the crossroads of the antiviral immune response? *J Antimicrob Chemother* 2004; **53**: 895–8.
17 Bigger CB, Guerra B, Brasky KM *et al.* Intrahepatic gene expression during chronic hepatitis C virus infection in chimpanzees. *J Virol* 2004; **78**: 13779–92.
18 Lichterfeld M, Leifeld L, Nischalke HD *et al.* Reduced CC chemokine receptor (CCR) 1 and CCR5 surface expression on peripheral blood T lymphocytes from patients with chronic hepatitis C infection. *J Infect Dis* 2002; **185**: 1803–7.
19 Pham BN, Bemuau J, Durand F *et al.* Eotaxin expression and eosinophil infiltrate in the liver of patients with drug-induced liver disease. *J Hepatol* 2001; **34**: 537–47.
20 Tacke F, Trautwein C, Yagmur E *et al.* Up-regulated eotaxin plasma levels in chronic liver disease patients indicate hepatic inflammation, advanced fibrosis and adverse clinical course. *J Gastroenterol Hepatol* 2007; **22**: 1256–64.
21 Vargas A, Berenguer J, Catalan P *et al.* Association between plasma levels of eotaxin (CCL-11) and treatment response to interferon-alpha and ribavirin in HIV/HCV co-infected patients. *J Antimicrob Chemother* 2010; **65**: 303–6.
22 Harvey CE, Post JJ, Palladinetti P *et al.* Expression of the chemokine IP-10 (CXCL10) by hepatocytes in chronic hepatitis C virus infection correlates with histological severity and lobular inflammation. *J Leukoc Biol* 2003; **74**: 360–9.
23 Zeremski M, Petrovic LM, Chiriboga L *et al.* Intrahepatic levels of CXCR3-associated chemokines correlate with liver inflammation and fibrosis in chronic hepatitis C. *Hepatology* 2008; **48**: 1440–50.
24 Butera D, Marukian S, Iwamaye AE *et al.* Plasma chemokine levels correlate with the outcome of antiviral therapy in patients with hepatitis C. *Blood* 2005; **106**: 1175–82.
25 Lagging M, Romero AI, Westin J *et al.* IP-10 predicts viral response and therapeutic outcome in difficult-to-treat patients with HCV genotype 1 infection. *Hepatology* 2006; **44**: 1617–25.
26 Romero AI, Lagging M, Westin J *et al.* Interferon (IFN)-gamma-inducible protein-10: association with histological results, viral kinetics, and outcome during treatment with pegylated IFN-alpha 2a and ribavirin for chronic hepatitis C virus infection. *J Infect Dis* 2006; **194**: 895–903.
27 Diago M, Castellano G, Garcia-Samaniego J *et al.* Association of pretreatment serum interferon gamma inducible protein 10 levels with sustained virological response to peginterferon plus ribavirin therapy in genotype 1 infected patients with chronic hepatitis C. *Gut* 2006; **55**: 374–9.
28 Reiberger T, Aberle JH, Kundi M *et al.* IP-10 correlates with hepatitis C viral load, hepatic inflammation and fibrosis and predicts hepatitis C virus relapse or non-response in HIV-HCV coinfection. *Antivir Ther* 2008; **13**: 969–76.
29 Askarieh G, Alsio A, Pugnale P *et al.* Systemic and intrahepatic interferon-gamma-inducible protein 10 kDa predicts the first-phase decline in hepatitis C virus RNA and overall viral response to therapy in chronic hepatitis C. *Hepatology* 2010; **51**: 1523–30.
30 Zeremski M, Dimova R, Brown Q *et al.* Peripheral CXCR3-associated chemokines as biomarkers of fibrosis in chronic hepatitis C virus infection. *J Infect Dis* 2009; **200**: 1774–80.

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Association of IL28B Variants With Response to Pegylated-Interferon Alpha Plus Ribavirin Combination Therapy Reveals Intersubgenotypic Differences Between Genotypes 2a and 2b

Naoya Sakamoto, MD, PhD,[1,2]* Mina Nakagawa,[1] Yasuhito Tanaka,[3] Yuko Sekine-Osajima,[1] Mayumi Ueyama,[1] Masayuki Kurosaki,[4] Nao Nishida,[5] Akihiro Tamori,[6] Nishimura-Sakurai Yuki,[1] Yasuhiro Itsui,[1,7] Seishin Azuma,[1] Sei Kakinuma,[1,2] Shuhei Hige,[8] Yoshito Itoh,[9] Eiji Tanaka,[10] Yoichi Hiasa,[11] Namiki Izumi,[4] Katsushi Tokunaga,[5] Masashi Mizokami,[12] Mamoru Watanabe[1] and the Ochanomizu-Liver Conference Study Group

[1]*Department of Gastroenterology and Hepatology, Tokyo Medical and Dental University, Tokyo, Japan*
[2]*Department for Hepatitis Control, Tokyo Medical and Dental University, Tokyo, Japan*
[3]*Department of Virology & Liver Unit, Nagoya City University Graduate School of Medical Sciences, Mizuho-ku Nagoya, Japan*
[4]*Division of Gastroenterology and Hepatology, Musashino Red Cross Hospital, Tokyo, Japan*
[5]*Department of Human Genetics, Graduate School of Medicine, The University of Tokyo, Tokyo, Japan*
[6]*Department of Hepatology, Osaka City University Graduate School of Medicine, Osaka, Japan*
[7]*Department of Internal Medicine, Soka Municipal Hospital, Saitama, Japan*
[8]*Department of Internal Medicine, Hokkaido University Graduate School of Medicine, Sapporo, Japan*
[9]*Molecular Gastroenterology and Hepatology, Kyoto Prefectural University of Medicine, Kyoto, Japan*
[10]*Department of Medicine, Shinshu University School of Medicine, Matsumoto, Japan*
[11]*Department of Gastroenterology and Metabology, Ehime University Graduate School of Medicine, Ehime, Japan*
[12]*Research Center for Hepatitis and Immunology, International Medical Center of Japan Konodai Hospital, Ichikawa, Japan*

Genetic polymorphisms of the interleukin 28B (IL28B) locus are associated closely with outcomes of pegylated-interferon (PEG-IFN) plus ribavirin (RBV) combination therapy. The aim of this study was to investigate the relationship between IL28B polymorphism and responses to therapy in patients infected with genotype 2. One hundred twenty-nine chronic hepatitis C patients infected with genotype 2, 77 patients with genotype 2a and 52 patients with genotype 2b, were analyzed. Clinical and laboratory parameters, including genetic variation near the IL28B gene (rs8099917), were assessed. Drug adherence was monitored in each patient. Univariate and multivariate statistical analyses of these parameters and clinical responses were carried out. Univariate analyses showed that a sustained virological response was correlated significantly with IL28B polymorphism, as well as age, white blood cell and neutrophil counts, adherence to RBV, and rapid virological response. Subgroup analysis revealed that patients infected with genotype 2b achieved significantly lower rapid virological response rates than those with genotype 2a. Patients with the IL28B-major allele showed higher virus clearance rates at each time point than those with the IL28B-minor allele, and the differences were more profound in patients infected with genotype 2b than those with genotype 2a. Furthermore, both rapid and sustained virological responses were associated significantly with IL28B alleles in patients with genotype

Abbreviations: HCV, hepatitis C virus; HCC, hepatocellular carcinoma; IFN, interferon; PEG-IFN, pegylated-interferon; RBV, ribavirin; IL28B, interleukin 28B; SNPs, single nucleotide polymorphisms; BMI, body mass index; ALT, alanine transaminase; ISDR, the interferon sensitivity determining region; ITPA, inosine triphosphatase

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Naoya Sakamoto and Mina Nakagawa contributed equally to this work.

*Correspondence to: Naoya Sakamoto, MD, PhD, Department of Gastroenterology and Hepatology, Tokyo Medical and Dental University, 1-5-45 Yushima, Bunkyo-ku, Tokyo 113-8519, Japan. E-mail: nsakamoto.gast@tmd.ac.jp

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2b. IL28B polymorphism was predictive of PEG-IFN plus RBV combination treatment outcomes in patients infected with genotype 2 and, especially, with genotype 2b. In conclusion, IL-28B polymorphism affects responses to PEG-IFN-based treatment in difficult-to-treat HCV patients. ***J. Med. Virol. 83:871–878, 2011.*** © 2011 Wiley-Liss, Inc.

KEY WORDS: hepatitis C virus (HCV); chronic hepatitis C; genotype 2; PEG-IFN plus RBV therapy; combination therapy; IL28B; interferon-λ3

INTRODUCTION

Hepatitis C virus (HCV) infects around 170 million people worldwide and is characterized by a high probability of developing chronic inflammation and fibrosis of the liver, leading to end-stage liver failure and hepatocellular carcinoma (HCC) [Alter, 1997; Sakamoto and Watanabe, 2009]. Since the first report in 1986, type I interferons have been the mainstay of HCV therapy [Hoofnagle, 1994]. Current standards of care consist of a combination of ribavirin (RBV) plus pegylated interferon (PEG-IFN)-alpha for 48 weeks for infection with genotypes 1 and 4, and for 24 weeks for the other genotypes [Zeuzem et al., 2000; Fried et al., 2002]. Although this treatment improved substantially sustained virological response rates, it may result also in serious adverse effects and a considerable proportion of patients require early discontinuation of treatment. Patients of African origin have even poorer treatment outcomes [Rosen and Gretch, 1999]. Given this situation, a precise assessment of the likely treatment outcomes before the initiation of treatment may improve substantially the quality of antiviral treatment.

Recently, several studies have reported that genetic polymorphisms of the IL28B locus, which encodes interferon-λ3 (interleukin 28B), are associated with response to interferon-based treatment of chronic HCV infections with genotype 1 [Ge et al., 2009; Suppiah et al., 2009; Tanaka et al., 2009] and also spontaneous clearance of HCV [Thomas et al., 2009].

While chronic HCV infections with genotype 2 are associated with good treatment outcome, there are some refractory cases among patients infected with genotype 2, similar to genotype 1. The aims of this study were to analyze retrospectively clinical and virological factors associated with treatment response in patients with chronic HCV infection with genotype 2 who were treated with PEG-IFN plus RBV combination therapy and to clarify the relationship between IL28B polymorphism and the response to combination therapy.

PATIENTS AND METHODS

The authors analyzed retrospectively 129 patients with chronic HCV infection with genotype 2 who received combination therapy with PEG-IFN plus RBV between December 2004 and December 2009 at 10 multicenter hospitals (liver units with hepatologists) throughout Japan. All patients had chronic active hepatitis confirmed histologically or clinically and were positive for anti-HCV antibodies and serum HCV RNA by quantitative or qualitative assays. Patients with a positive test for serum hepatitis B surface antigen, coinfection with other HCV genotypes, coinfection with human immunodeficiency virus, other causes of hepatocellular injury (such as alcoholism, autoimmune hepatitis, primary biliary cirrhosis, or a history of treatment with hepatotoxic drugs), and a need for hemodialysis were excluded.

Study Design

Each patient was treated with combination therapy with PEG-IFN-α2b (Peg-Intron, Schering-Plough Nordic Biotech, Stockholm, Sweden, at a dose of 1.2–1.5 μg/kg subcutaneously once a week) or PEG-IFN-α2a (Pegasys; Roche, Basel, Switzerland, at a dose of 180 μg subcutaneously once a week) plus RBV (Rebetol, Schering-Plough Nordic Biotech or Copegus; Roche) 600–1,000 mg daily depending on the body weight (b.w.) (b.w. <60 kg: 600 mg po daily; b.w: 60–80 kg: 800 mg po daily; b.w. >80 kg: 1,000 mg po daily; in two divided doses). The duration of the combination therapy was set at a standard 24 weeks, but treatment reduction or discontinuation was permitted by doctor's decision. The rates of PEG-IFN and RBV administration achieved were calculated as percentages of actual total dose administered of a standard total dose of 24 weeks, according to body weight before therapy. During treatment, patients were assessed as outpatients at weeks 2, 4, 6, 8, and then every 4 weeks for the duration of treatment and at every 4 weeks after the end of treatment. Biochemical and hematological testing was carried out in a central laboratory. Serum HCV RNA was measured before treatment, during treatment at 4 weekly intervals, and after therapy at 4 weekly intervals for 24 weeks, by quantitative or qualitative assays.

Patient Evaluation

The following factors were analyzed to determine whether they were related to the efficacy of combination therapy: age, gender, body mass index (BMI), previous IFN therapy, grade of inflammation and stage of fibrosis on liver biopsy, pretreatment biochemical parameters, such as white blood cells, neutrophils, hemoglobin, platelet count, alanine transaminase (ALT) level, serum HCV RNA level (log IU/ml), and single nucleotide polymorphism (SNPs) in the *IL28B* locus (rs8099917). Liver biopsy specimens were evaluated blindly, to determine the grade of inflammation and stage of fibrosis, by an independent interpreter who was not aware of the clinical data. Activity of inflammation was graded on a scale of 0–3: A0 shows no activity, A1 shows mild activity, A2 shows moderate activity and A3 shows severe activity. Fibrosis was staged on a scale of 0–4:

F0 shows no fibrosis, F1 shows moderate fibrosis, F2 shows moderate fibrosis with few septa, F3 shows severe fibrosis with numerous septa without cirrhosis and F4 shows cirrhosis.

Informed written consent was obtained from each patient who participated in the study. The study protocol conformed to the ethical guidelines of the Declaration of Helsinki and to the relevant ethical guidelines as reflected in a priori approval by the ethics committees of all the participating universities and hospitals.

SNP Genotyping

Human genomic DNA was extracted from whole blood of each patient. Genetic polymorphism of IL28B was determined by DigiTag2 assay by typing one tag SNP located within the IL28B locus, rs8099917 (22). Heterozygotes (T/G) or homozygotes (G/G) of the minor allele (G) were defined as having the IL28B minor allele, whereas homozygotes for the major allele (T/T) were defined as having the IL28B major allele.

Outcomes

The primary end point was a sustained biochemical and virological response. A sustained virological response was defined as serum HCV RNA undetectable at 24 weeks after the end of treatment. Secondary end points were a rapid virological response (HCV RNA undetectable in serum at week 4) and end-of-treatment virological response. In addition, tolerability (adverse events) and drug adherence were recorded and factors potentially associated with virological response explored.

Statistical Analysis

SPSS software package (SPSS 18J, SPSS, Chicago, IL) was used for statistical analysis. Discrete variables were evaluated by Fisher's exact probability test and distributions of continuous variables were analyzed by the Mann–Whitney *U*-test. Independent factors possibly affecting response to combination therapy were examined by stepwise multiple logistic-regression analysis. All *P*-values were calculated by two-tailed tests, and those of less than 0.05 were considered statistically significant.

RESULTS

Clinical Characteristics and Response to Therapy

The clinical characteristics and response rates to therapy of 129 patients are summarized in Tables I and II. Sixty-eight patients achieved a rapid virological response, whereas 44 patients remained HCV-RNA positive at week 4. Treatment reduction or cessation was permitted also to avoid side effects, and one patient stopped treatment at week 12 because he was anticipated to be a non-responder. On an intention-to-treat analysis, serum HCV-RNA levels were negative at the end of treatment in 125 of the 129 patients (97%) treated and, among them, 98 (76%) achieved a sustained virological response. The rapid virological response rate of patients infected with genotype 2b was lower significantly than that of patients infected with genotype 2a ($P = 0.036$) (Table II). The sustained virological response rate decreased with RBV drug discontinuation and dose reduction (84% and 66% with ≧80% and <80% of RBV dose, $P = 0.021$, Table III). Adherences to PEG-IFN did not influence a sustained virological response or end of treatment response significantly, while RBV adherence was associated significantly with a sustained virological response (Table III).

TABLE I. Baseline Characteristics of Participating Patients Infected With HCV Genotype 2

Total number	129
Genotype (2a/2b)	77/52
IL28B SNPs (rs8099917)	
TT/TG/GG	100/28/1
Age (years)[a]	64 (20–73)
Gender (male/female)	64/65
Body mass index (kg/m^2)[a] (N = 80)	23.7 (16.9–33.5)
Previous interferon therapy (no/yes)	102/21 (unknown 6)
Histology at biopsy (N = 96)	
Grade of inflammation	
A0/1/2/3	10/53/29/4
Stage of fibrosis	
F0/1/2/3	7/59/19/11
White blood cells (/μl)[b] (N = 94)	5,115 ± 1,630
Neutrophils (/μl)[b] (N = 94)	2,765 ± 1,131
Hemoglobin (g/dl)[b] (N = 95)	14.2 ± 1.3
Platelet count (×10^{-3}/μl)[b] (N = 98)	187 ± 95
ALT (IU/L)[b] (N = 95)	82 ± 78
Serum HCV-RNA level (log(IU/ml))[a,c]	6.2 (3.6–7.4)
Treatment duration (>16, ≦24)	19/110

SNPs, single nucleotide polymorphisms; ALT, alanine transaminase.
[a]Data are shown as median (range) values.
[b]Data are expressed as mean ± SD.
[c]Data are shown as log(IU/ml)).

Factors Associated With a Sustained Virological Response

Next the host clinical and viral factors associated with a sustained virological response were analyzed. Univariate statistical analysis showed that six parameters were associated significantly with the sustained virological response rates, including age, white blood cells, neutrophils, adherence to RBV, rapid virological response and an IL28B SNP (rs8099917) (Table IV). There was no significant association of sustained virological response with gender, previous interferon therapy, stage of fibrosis, pretreatment HCV titer or adherence to PEG-IFN. Further multivariate analyses were conducted using significant factors identified by the univariate analysis (Table V). The multiple logistic-regression analysis showed that only a rapid virological response was associated with a sustained virological response (OR = 0.170, $P = 0.019$).

TABLE II. Response Rates to Therapy

Character			Number/total number (%)
Overall			
RVR			68/112 (61)
ETR			125/129 (97)
SVR			98/129 (76)
Genotype	2a	2b	*P*-value
RVR	46/67 (69)	22/45 (49)	**0.036**
ETR	74/77 (96)	51/52 (98)	NS
SVR	56/77 (73)	42/52 (81)	NS

RVR, rapid virological response; ETR, end of treatment response; SVR, sustained virological response.
Bold indicated *P*-value of less than 0.05.

TABLE III. Response Rates to Treatment According to Drug Adherence

	≧80%	<80%	*P*-value
PEG-IFN adherence			
ETR	94/96 (98)	31/33 (94)	NS
SVR	75/96 (78)	23/33 (70)	NS
RBV adherence			
ETR	72/73 (99)	53/56 (95)	NS
SVR	61/73 (84)	37/56 (66)	**0.021**

ETR, end of treatment response; SVR, sustained virological response; PEG-IFN, pegylated interferon; RBV, ribavirin.
The rates of PEG-IFN and RBV administration achieved were calculated as percentages of actual total dose administered of a standard total dose of 24 weeks, according to body weight before therapy.
Bold indicated *P*-value of less than 0.05.

Comparison of Sustained Virological Response Rates According to IL28B SNPs

The PEG-IFN plus RBV treatment efficacy was compared after dividing the study subjects into two groups based on IL28B alleles (Table VI). Patients homozygous for the IL28B major allele (TT allele) achieved significantly higher rapid and sustained virological response rates than those heterozygous or homozygous for the IL28B minor allele (TG/GG alleles) ($P < 0.05$). In addition, responses to PEG-IFN plus RBV treatment were analyzed after dividing the study subjects into those with genotype 2a and with genotype 2b. The rapid and sustained virological response rates tended to be higher in patients homozygous for the IL28B major allele than those heterozygous or homozygous for the

TABLE IV. Clinical and Virological Characteristics of Patients Based on Therapeutic Response

	SVR (n = 98)	Non-SVR (n = 31)	*P*-value
Genotype (2a/2b)		56/42	21/10
IL28B SNPs (rs8099917)			
TT/TG + GG	81/17	19/12	**0.024**
Age (years)[a]	56 (20–73)	61 (40–72)	**0.002**
Gender (male/female)	51/47	13/18	NS
Body mass index (kg/m^2)[a]	22.8 (16.9–33.5)	24.1 (20.3–27.6)	NS
Previous Interferon therapy (no/yes)	80/14	22/7	NS
Grade of inflammation (A0-1/2-3)	46/28	15/7	NS
Stage of fibrosis (F0-2/3-4)	64/10	21/1	NS
White blood cells (/μl)[b]	5,318 ± 1,617	4,489 ± 1,540	**0.032**
Neutrophils (/μl)[b]	2,913 ± 1,139	2,278 ± 983	**0.021**
Hemoglobin (g/dl)[b]	14.2 ± 1.4	14.1 ± 1.1	NS
Platelet count (×10^{-3}/μl)[b]	193 ± 105	171 ± 54	NS
ALT (IU/ml)[b]	79 ± 73	94 ± 92	NS
Pretreatment Serum HCV-RNA level (log(IU/ml))[a,c]	6.1 (3.6–7.4)	6.3 (4.0–6.7)	NS
PEG-IFN adherence (≧80%/<80%)	75/23	21/10	NS
RBV adherence (≧80%/<80%)	61/37	12/19	**0.024**
RVR/non-RVR	57/24	11/20	**0.001**

SNPs, single nucleotide polymorphisms; ALT, alanine transaminase; RVR, rapid virological response.
[a]Data are show as median (range) values.
[b]Data are expressed as mean ± SD.
[c]Data are shown as log (IU/ml)).
Bold indicated *P*-value of less than 0.05.

TABLE V. Multivariate Analysis for the Clinical and Virological Factors Related to Sustained Response With Peg-IFN Plus RBV Therapy in 63 Patients

Factor	Category	Odds ratio (95% CI)	*P*-value
Regression analysis			
RVR	RVR	1	**0.019**
	Non-RVR	0.170 (0.039–0.744)	
RBV adherence	≧80%	1	0.061
	<80%	0.250 (0.059–1.064)	
IL28B SNPs (rs8099917)	TT	1	0.104
	TG + GG	0.252 (0.048–1.330)	
Age		1.087 (0.976–1.211)	0.128
Neutrophils		0.999 (0.997–1.001)	0.209
White blood cells		1.000 (0.999–1.002)	0.504

CI, confidence interval; SNPs, single nucleotide polymorphisms; RVR, rapid virological response, RBV, ribavirin.
Bold indicated *P*-value of less than 0.05.

IL28B minor allele infected with both genotype 2a and 2b, and these differences were more profound in patients infected with genotype 2b than with genotype 2a. The rapid and sustained virological response rates of patients with the major IL28B allele were higher significantly than those of patients with the minor IL28B allele infected only with genotype 2b (rapid virological response: 58% and 0% with IL28B major and hetero/minor, $P = 0.002$, sustained virological response: 88% and 44% with IL28B major and hetero/minor, $P = 0.009$).

Although the rapid virological response rate of patients infected with genotype 2b was lower significantly than that of patients infected with genotype 2a, the sustained virological response rate was higher in patients infected with genotype 2b than with genotype 2a (Table II), In order to investigate that discrepancy, sustained virological response rates in patients with or without rapid virological response were analyzed according to IL28B SNPs. In patients infected with genotype 2b and a non-rapid virological response, the sustained virological response rates differed significantly between IL28B major and hetero/minor groups (sustained virological response with non-rapid virological response: 75% and 29% with IL28B major and hetero/minor, $P = 0.044$), and no one achieved a rapid virological response among the patients infected with genotype 2b and with the IL28B hetero/minor allele. In patients infected with genotype 2a, on the contrary, there was no significant correlation of rapid and sustained virological response rates between IL28B SNPs (sustained virological response with rapid virological response: 78% and 70% with IL28B major and hetero/minor, $P = 0.630$, sustained virological response with non-rapid virological response: 57% and 43% with IL28B major and hetero/minor, $P = 0.552$).

Next, changes in virological response rates over time were investigated in patients treated with PEG-IFN plus RBV and the time course was analyzed after separating the patients infected with genotype 2a and 2b (Fig. 1). Patients with IL28B-TG and -GG showed significantly lower rates of rapid and sustained virological response, compared to patients with IL28B-TT, and greater differences were observed according to IL28B SNPs among patients infected with genotype 2b than with 2a.

Side Effects

Side effects leading to Peg-IFN plus RBV discontinuation occurred in eight patients (6.2%) and discontinuation of RBV alone occurred in four patients (3.1%).

TABLE VI. Rapid and Sustained Virological Response Rates to Treatment According to IL28B SNPs

Character	IL28B major	IL28B hetero/minor	*P*-value
Number/total number (%)			
Overall			
RVR	58/88 (66)	10/24 (42)	0.031
SVR	81/100 (81)	17/29 (59)	0.013
Genotype 2a			
RVR	36/50 (72)	10/17 (59)	NS
SVR	43/57 (75)	13/20 (65)	NS
Genotype 2b			
RVR	22/38 (58)	0/7 (0)	0.002
SVR	38/43 (88)	4/9 (44)	0.009

RVR, rapid virological response; ETR, end of treatment response; SVR, sustained virological response.

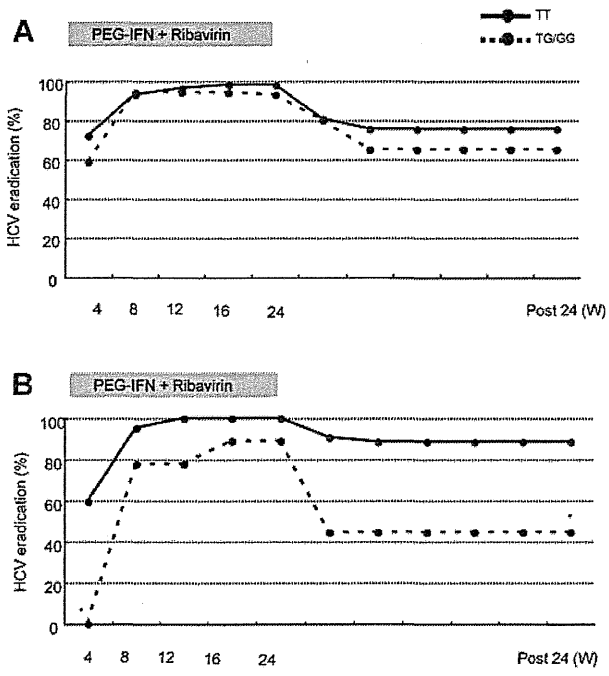

Fig. 1. Changes over time in virological response rates were confirmed in patients treated with PEG-IFN plus RBV, and the time courses were analyzed after separating the patients infected with genotypes 2a and 2b. Patients with the IL28B major (TT allele) are indicated in the figure by a continuous line and those with IL28B hetero or minor (TG or GG), by a dotted line. IL28B-TG and -GG patients showed significantly lower rates of rapid and sustained virological response, compared to IL28B-TT patients. *P*-values were two-tailed and those of less than 0.05 were considered to be statistically significant. $^{*}P < 0.01$.

Among the eight patients who withdrew from both drugs, four, including one who stopped at week 7, had achieved a sustained virological response. Among four patients who withdrew from RBV alone, three had achieved a sustained virological response. The events leading to drug withdrawal were HCC treatment (n = 2), general fatigue (n = 2), retinopathy, neuro-psychiatric event, severe dermatological symptoms suggestive of the drug-induced hypersensitivity syndrome, and arrhythmia.

DISCUSSION

Recent studies suggest that genetic variations in IL28B are strongly associated with response to therapy of chronic HCV infection with genotype 1 [Ge et al., 2009; Suppiah et al., 2009; Tanaka et al., 2009] and with spontaneous HCV clearance [Thomas et al., 2009]. In this study, univariate analyses showed that the sustained virological response was correlated significantly with IL28B polymorphism (rs8099917) as well as age, adherence to RBV and rapid virological response, and multiple logistic-regression analysis showed that only a rapid virological response was associated with a sustained virological response in all patients infected with genotype 2 (Table V). Although the IL28B polymorphisms are not so useful for predicting the clinical outcomes of PEG-IFN plus RBV combination therapy among patients with genotype 2, compared to genotype 1, IL28B polymorphism was predictive of PEG-IFN plus RBV treatment outcomes among patients with genotype 2 and, more remarkably, among patients with genotype 2b in this study. Indeed, both rapid and sustained virological response rates according to the rs8099917 genotypes were different significantly in patients with genotype 2b but not in patients with genotype 2a. Furthermore, in the plot of virological response (Fig. 1), a stronger effect of the IL28B allele was observed in patients with genotype 2b than with genotype 2a.

It has been reported that there was no significant association between genetic variation in IL28B and response to therapy of HCV patients infected with genotype 2 or 3, indicating that the prognostic value of the risk allele for treatment response might be limited to individuals with difficult-to-treat HCV genotypes [Rauch et al., 2010]. This report lacks details of the distribution of the various genotypes. The present study agrees with a more recent report that the IL28B polymorphism was associated with a sustained virological response in patients with chronic HCV infection with genotype 2 or 3 who did not achieve a rapid virological response [Mangia et al., 2010]. In Japan, the percentage of HCV infection with genotype 1b is 70%, genotype 2a is 20% and genotype 2b is 10%, whilst other genotypes are observed only rarely. In this study, the association of IL28B polymorphism with response to therapy was analyzed in more detail, considering the subtypes 2a and 2b, and IL28B polymorphism (rs8099917) found to be linked more closely to the virological response of patients infected with genotype 2b than those with genotype 2a. A recent in vitro study, which constructed several chimeric virus clones between HCV-2b and HCV-JFH1 (2a), also supported subgenotypic differences between genotype 2a and 2b [Suda et al., 2010]. The authors speculated that the prognostic value of the risk allele for treatment response might be more pronounced in individuals with difficult-to-treat HCV subgenotypes, such as patients infected with genotype 2b, compared with 2a. In addition, the prevalence of the IL28B minor allele is much higher in Caucasians and African Americans than in eastern Asian populations [Thomas et al., 2009], which suggest that the effects of IL28B polymorphism could be more pronounced in non-Asian populations. In the present results, however, the sustained virological response rate of patients infected with genotype 2b was higher than that of patients with genotype 2a overall. We speculate that, among patients infected with genotype 2b, only those with the IL28B minor variant might be treatment-refractory. That possibility might be validated further by a larger cohort study with genotype 2b.

The sustained virological response rates decreased significantly with failure of adherence to RBV (Table III), which was extracted as a factor associated with sustained virological response by univariate

analysis (Table IV). Regardless of the drug adherence, end of treatment response rates of patients infected with genotype 2 were around 94–99%, but the sustained virological response rates of the patients who received a total cumulative treatment dose of RBV of <80% was reduced significantly. As reported previously, increased RBV exposure during the treatment phase was associated with an increased likelihood of a sustained virological response [McHutchison et al., 2009] and these results confirm the importance of RBV in order to prevent relapse. Furthermore, host genetic variation leading to inosine triphosphatase (ITPA) deficiency protects against hemolytic anemia in chronic hepatitis C patients receiving RBV as revealed recently [Fellay et al., 2010]. We have reported also that the *ITPA* SNP, rs1127354, is confirmed to be a useful predictor of RBV-induced anemia in Japanese patients and that the incidence of early dose reduction was significantly higher in patients with ITPA-major (CC) variant as expected and, more importantly, that a significant higher sustained virological response rate was achieved in patients with the *ITPA-hetero*/minor (CA/AA) variant with non-genotype 1 or low viral loads [Sakamoto et al., 2010].

A rapid virological response was extracted in this study as a factor associated with sustained virological response only by multivariate analysis. It has been reported recently that a rapid virological response is an important treatment predictor and that drug adherence, which is reported to affect the therapeutic efficacy in patients infected with genotype 1, had no impact on the both sustained and rapid virological responses in combination therapy for patients infected with genotype 2 [Inoue et al., 2010]. The reasons why several host factors useful for predicting the response to therapy in patients with genotype 1, such as gender, age, progression of liver fibrosis and IL28B polymorphism had no influence on the efficacy in patients with genotype 2, can be attributed to IFN-sensitive genotypes. Similarly, the other viral factors useful for predicting the response to therapy, such as viral load and amino acid substitutions in the Core and NS5A regions had no influence on treatment outcomes. In this study, patients who achieved a rapid virological response had a high sustained virological response rate, regardless of IL28B polymorphism in patients with genotype 2a but, interestingly, none of the IL28B-TG and -GG patients with genotype 2b achieved a sustained virological response (although there were nine IL28B-TG and -GG patients with genotype 2b, two could not be determined as rapid virological response because the times at which they became HCV-negative were not recorded clearly, being described as 4–8 weeks.) These results also suggest that patients with both genotype 2b and IL28B minor allele are refractory cases.

IL28B encodes a protein also known as IFN-λ3 [O'Brien, 2009]. *IL28A* (IFN-λ2) and *IL29* (IFN-λ1) are found adjacent to *IL28B* on chromosome 19. These three IFN-λ cytokines, discovered in 2003 by two independent groups [Kotenko et al., 2003; Sheppard et al., 2003] have been suggested to be involved in the suppression of replication of a number of viruses, including HCV [Robek et al., 2005; Marcello et al., 2006; Tanaka et al., 2010]. Humans have these three genes for IFN-λ, and this group of cytokines is now collectively referred to as type III IFN [Zhou et al., 2007]. IFN-λ functionally resembles type I IFN, inducing antiviral protection in vitro [Kotenko et al., 2003; Sheppard et al., 2003] as well as in vivo [Ank et al., 2006]. Type III IFN utilizes a receptor complex different from that of type I IFN, but both types of IFN induce STAT1, STAT2, and STAT3 activation by activation of a highly overlapping set of transcription factors, and the two types of IFN seem to have similar biological effects at a cellular level. Some in vitro studies have suggested that IFN-α induces expression of IFN-λ genes [Siren et al., 2005]. Other in vitro studies also suggest that IFN-λ inhibits hepatitis C virus replication through a pattern of signal transduction and regulation of interferon-stimulated genes that is distinct from IFN-α and that the anti-HCV activity of either IFN-α or IFN-λ is enhanced by a low dose of the other [Marcello et al., 2006]. A novel mechanism of the interaction between IFN-α and IFN-λ may play a key role in the suppression of HCV [O'Brien, 2009].

In conclusion, IL28B polymorphism is predictive of PEG-IFN plus RBV treatment outcomes in patients infected with genotype 2, and more remarkably with genotype 2b. These results suggest that IL-28B polymorphism affects responses to IFN-based treatment in more difficult-to-treat subpopulations of HCV patients, and that intersubgenotypic differences between genotype 2a and 2b are revealed by responses to PEG-IFN plus RBV treatment according to IL28B variants.

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REFERENCES

Alter MJ. 1997. Epidemiology of hepatitis C. Hepatology 26:62S–65S.

Ank N, West H, Bartholdy C, Eriksson K, Thomsen AR, Paludan SR. 2006. Lambda interferon (IFN-lambda), a type III IFN, is induced by viruses and IFNs and displays potent antiviral activity against select virus infections in vivo. J Virol 80:4501–4509.

Fellay J, Thompson AJ, Ge DL, Gumbs CE, Urban TJ, Shianna KV, Little LD, Qiu P, Bertelsen AH, Watson M, Warner A, Muir AJ, Brass C, Albrecht J, Sulkowski M, McHutchison JG, Goldstein DB. 2010. ITPA gene variants protect against anaemia in patients treated for chronic hepatitis C. Nature 464:405–408.

Fried MW, Shiffman ML, Reddy KR, Smith C, Marinos G, Goncales FL, Haussinger D, Diago M, Carosi G, Dhumeaux D, Craxi A, Lin A, Hoffman J, Yu J. 2002. Peginterferon alfa-2a plus ribavirin for chronic hepatitis C virus infection. N Engl J Med 347:975–982.

Ge D, Fellay J, Thompson AJ, Simon JS, Shianna KV, Urban TJ, Heinzen EL, Qiu P, Bertelsen AH, Muir AJ, Sulkowski M, McHutchison JG, Goldstein DB. 2009. Genetic variation in IL28B predicts hepatitis C treatment-induced viral clearance. Nature 461:399–401.

Hoofnagle JH. 1994. Therapy of acute and chronic viral hepatitis. Adv Intern Med 39:241–275.

Inoue Y, Hiramatsu N, Oze T, Yakushijin T, Mochizuki K, Hagiwara H, Oshita M, Mita E, Fukui H, Inada M, Tamura S, Yoshihara H, Hayashi E, Inoue A, Imai Y, Kato M, Miyagi T, Hohsui A, Ishida H, Kiso S, Kanto T, Kasahara A, Takehara T, Hayashi N. 2010. Factors affecting efficacy in patients with genotype 2 chronic hepatitis C treated by pegylated interferon alpha-2b and ribavirin: Reducing drug doses has no impact on rapid and sustained virological responses. J Viral Hepat 17:336–344.

Kotenko SV, Gallagher G, Baurin VV, Lewis-Antes A, Shen M, Shah NK, Langer JA, Sheikh F, Dickensheets H, Donnelly RP. 2003. IFN-lambdas mediate antiviral protection through a distinct class II cytokine receptor complex. Nat Immunol 4:69–77.

Mangia A, Thompson AJ, Santoro R, Piazzolla V, Tillmann HL, Patel K, Shianna KV, Mottola L, Petruzzellis D, Bacca D, Carretta V, Minerva N, Goldstein DB, McHutchison JG. 2010. An IL28B polymorphism determines treatment response of hepatitis C virus genotype 2 or 3 patients who do not achieve a rapid virologic response. Gastroenterology 139:821–827.

Marcello T, Grakoui A, Barba-Spaeth G, Machlin ES, Kotenko SV, MacDonald MR, Rice CM. 2006. Interferons alpha and lambda inhibit hepatitis C virus replication with distinct signal transduction and gene regulation kinetics. Gastroenterology 131:1887–1898.

McHutchison JG, Lawitz EJ, Shiffman ML, Muir AJ, Galler GW, McCone J, Nyberg LM, Lee WM, Ghalib RH, Schiff ER, Galati JS, Bacon BR, Davis MN, Mukhopadhyay P, Koury K, Noviello S, Pedicone LD, Brass CA, Albrecht JK, Sulkowski MS. 2009. Peginterferon alfa-2b or alfa-2a with ribavirin for treatment of hepatitis C infection. N Engl J Med 361:580–593.

O'Brien TR. 2009. Interferon-alfa, interferon-lambda and hepatitis C. Nat Genet 41:1048–1050.

Rauch A, Kutalik Z, Descombes P, Cai T, di Iulio J, Mueller T, Bochud M, Battegay M, Bernasconi E, Borovicka J, Colombo S, Cerny A, Dufour JF, Furrer H, Gunthard HF, Heim M, Hirschel B, Malinverni R, Moradpour D, Mullhaupt B, Witteck A, Beckmann JS, Berg T, Bergmann S, Negro F, Telenti A, Bochud PY. 2010. Genetic variation in IL28B is associated with chronic hepatitis C and treatment failure—A genome-wide association study. Gastroenterology 138:1240–1243.

Robek MD, Boyd BS, Chisari FV. 2005. Lambda interferon inhibits hepatitis B and C virus replication. J Virol 79:3851–3854.

Rosen HR, Gretch DR. 1999. Hepatitis C virus: Current understanding and prospects for future therapies. Mol Med Today 5:393–399.

Sakamoto N, Watanabe M. 2009. New therapeutic approaches to hepatitis C virus. J Gastroenterol 44:643–649.

Sakamoto N, Tanaka Y, Nakagawa M, Yatsuhashi H, Nishiguchi S, Enomoto N, Azuma S, Nishimura-Sakurai Y, Kakinuma S, Nishida N, Tokunaga K, Honda M, Ito K, Mizokami M, Watanabe M. 2010. ITPA gene variant protects against anemia induced by pegylated interferon-alpha and ribavirin therapy for Japanese patients with chronic hepatitis C. Hepatol Res 40:1063–1071.

Sheppard P, Kindsvogel W, Xu W, Henderson K, Schlutsmeyer S, Whitmore TE, Kuestner R, Garrigues U, Birks C, Roraback J, Ostrander C, Dong D, Shin J, Presnell S, Fox B, Haldeman B, Cooper E, Taft D, Gilbert T, Grant FJ, Tackett M, Krivan W, McKnight G, Clegg C, Foster D, Klucher KM. 2003. IL-28, IL-29 and their class II cytokine receptor IL-28R. Nat Immunol 4:63–68.

Siren J, Pirhonen J, Julkunen I, Matikainen S. 2005. IFN-alpha regulates TLR-dependent gene expression of IFN-alpha, IFN-beta, IL-28, and IL-29. J Immunol 174:1932–1937.

Suda G, Sakamoto N, Itsui Y, Nakagawa M, Mishima K, Onuki-Karakama Y, Yamamoto M, Funaoka Y, Watanabe T, Kiyohashi K, Nitta S, Azuma S, Kakinuma S, Tsuchiya K, Imamura M, Hiraga N, Chayama K, Watanabe M. 2010. IL-6-mediated intersubgenotypic variation of interferon sensitivity in hepatitis C virus genotype 2a/2b chimeric clones. Virology 407:80–90.

Suppiah V, Moldovan M, Ahlenstiel G, Berg T, Weltman M, Abate ML, Bassendine M, Spengler U, Dore GJ, Powell E, Riordan S, Sheridan D, Smedile A, Fragomeli V, Muller T, Bahlo M, Stewart GJ, Booth DR, George J. 2009. IL28B is associated with response to chronic hepatitis C interferon-alpha and ribavirin therapy. Nat Genet 41:1100–1104.

Tanaka Y, Nishida N, Sugiyama M, Kurosaki M, Matsuura K, Sakamoto N, Nakagawa M, Korenaga M, Hino K, Hige S, Ito Y, Mita E, Tanaka E, Mochida S, Murawaki Y, Honda M, Sakai A, Hiasa Y, Nishiguchi S, Koike A, Sakaida I, Imamura M, Ito K, Yano K, Masaki N, Sugauchi F, Izumi N, Tokunaga K, Mizokami M. 2009. Genome-wide association of IL28B with response to pegylated interferon-alpha and ribavirin therapy for chronic hepatitis C. Nat Genet 41:1105–1109.

Tanaka Y, Nishida N, Sugiyama M, Tokunaga K, Mizokami M. 2010. Lambda-Interferons and the single nucleotide polymorphisms: A milestone to tailor-made therapy for chronic hepatitis C. Hepatol Res 40:449–460.

Thomas DL, Thio CL, Martin MP, Qi Y, Ge D, O'Huigin C, Kidd J, Kidd K, Khakoo SI, Alexander G, Goedert JJ, Kirk GD, Donfield SM, Rosen HR, Tobler LH, Busch MP, McHutchison JG, Goldstein DB, Carrington M. 2009. Genetic variation in IL28B and spontaneous clearance of hepatitis C virus. Nature 461:798–801.

Zeuzem S, Feinman SV, Rasenack J, Heathcote EJ, Lai MY, Gane E, O'Grady J, Reichen J, Diago M, Lin A, Hoffman J, Brunda MJ. 2000. Peginterferon alfa-2a in patients with chronic hepatitis C. N Engl J Med 343:1666–1672.

Zhou Z, Hamming OJ, Ank N, Paludan SR, Nielsen AL, Hartmann R. 2007. Type III interferon (IFN) induces a type I IFN-like response in a restricted subset of cells through signaling pathways involving both the Jak-STAT pathway and the mitogen-activated protein kinases. J Virol 81:7749–7758.

Impact of Viral Amino Acid Substitutions and Host Interleukin-28B Polymorphism on Replication and Susceptibility to Interferon of Hepatitis C Virus

Nobuhiko Hiraga,[1,2] Hiromi Abe,[1,2] Michio Imamura,[1,2] Masataka Tsuge,[1,2] Shoichi Takahashi,[1,2] C. Nelson Hayes,[1,2] Hidenori Ochi,[2,3] Chise Tateno,[2,4] Katsutoshi Yoshizato,[2,4] Yusuke Nakamura,[5] Naoyuki Kamatani,[6] and Kazuaki Chayama[1,2,3]

Amino acid (aa) substitutions of core 70 and 91 and in the NS5A (nonstructural protein 5A) interferon sensitivity determining region (ISDR) as well as genetic polymorphisms in the host interleukin-28B (IL28B) locus affect the outcome of interferon (IFN)-based therapies for patients with chronic hepatitis C. The combination of these factors and the quasispecies nature of the virus complicate understanding of the underlying mechanism. Using infectious hepatitis C virus (HCV) genotype 1b clone HCV-KT9, we introduced substitutions at both core aa70 (Arg to Gln) and aa91 (Leu to Met). We also introduced four and nine ISDR aa substitutions into core mutant HCV-KT9. Using human hepatocyte chimeric mice with different IL28B genotypes, we examined the infectivity, replication ability, and susceptibility to IFN of these clones. Although aa substitutions in the ISDR significantly impaired infectivity and replication ability of the virus, core aa70 and 91 substitutions did not. The effect of IFN treatment was similar in core wild-type and mutant viruses. Interestingly, virus titer was significantly higher in mice with the favorable IL28B allele (rs8099917 TT and rs12979860 CC) in the transplanted hepatocytes than in mice with hepatocytes from rs8099917 TG and rs12979860 TT donors ($P < 0.001$). However, the effect of IFN was significantly greater, and intrahepatic expression levels of IFN-stimulated genes were significantly higher in mice with the favorable IL28B allele. *Conclusion*: Our data suggest that HCV replication levels and response to IFN are affected by human hepatocyte IL28B single-nucleotide polymorphism genotype and mutations in the ISDR. The mechanism underlying the clinically observed association of wild-type core protein in eradication-favorable host cells should be investigated further. (HEPATOLOGY 2011;54:764-771)

Chronic hepatitis C virus (HCV) infection is the leading cause of cirrhosis, liver failure, and hepatocellular carcinoma.[1,2] Interferon (IFN) is an essential component of therapy for patients with chronic HCV infection, and the most effective currently available therapy is combination therapy with pegylated (PEG)-IFN and ribavirin (RBV).[3-5] Among HCV genotypes, genotype 1 is the most resistant to

Abbreviations: aa, amino acid; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HCV, hepatitis C virus; HSA, human serum albumin; IFN, interferon; IL28B, interleukin-28B; ISDR, interferon-sensitivity–determining region; ISG, interferon-stimulated gene; MxA, myxovirus resistance protein A; NVR, nonvirological response; OAS, oligoadenylate synthetase; PBS, phosphate-buffered saline; PEG, pegylated; PKR, RNA-dependent protein kinase; RBV, ribavirin; RT-PCR, reverse-transcription polymerase chain reaction; SCID, severe combined immunodeficiency; SNP, single-nucleotide polymorphism; SVR, sustained virological response; uPA, urokinase-type plasminogen activator.

From the [1]Department of Medicine and Molecular Science, Division of Frontier Medical Science, Programs for Biomedical Research, Graduate School of Biomedical Sciences, Hiroshima University, Hiroshima, Japan; [2]Liver Research Project Center, Hiroshima University, Hiroshima, Japan; [3]Laboratory for Digestive Diseases, RIKEN Center for Genomic Medicine, Hiroshima, Japan; [4]PhoenixBio Co., Ltd., Higashi-Hiroshima, Japan; [5]Laboratory of Molecular Medicine, Human Genome Center, The Institute of Medical Science, University of Tokyo, Tokyo, Japan; and [6]Laboratory for Statistics, RIKEN Center for Genomic Medicine, Yokohama, Japan.

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Address reprint requests to: Kazuaki Chayama, M.D., Ph.D., Department of Medical and Molecular Science, Division of Frontier Medical Science, Programs for Biomedical Research, Graduate school of Biomedical Science, Hiroshima University, 1-2-3 Kasumi, Minami-ku, Hiroshima 734-8551, Japan. E-mail: chayama@hiroshima-u.ac.jp; fax: +81-82-255-6220.

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IFN therapy.[6] The limited success of combination therapy for genotype 1 HCV infection is because of the low response rate during therapy and high relapse rate after therapy.[7]

Recent studies have identified both viral and host factors predictive of IFN therapy. Among the viral factors, amino acid (aa) substitutions in the IFN-sensitivity–determining region (ISDR) (nucleotides 2209-2248 or aa positions 237-276 within the NS5A region) are associated with sustained virological response (SVR) after IFN treatment in HCV genotype 1b patients.[8,9] Akuta et al. reported that substitution of aa70 or 91 in the HCV core region are independent predictors of SVR and nonvirological response (NVR).[10-12] Recently, we[13] and another group[14] also reported that wild-type HCV core aa70 and two or more aa substitutions in the ISDR are effective predictors of SVR in patients with HCV genotype 1b.

Among host factors associated with SVR, many common genetic polymorphisms in the human genome have been identified, including single-nucleotide polymorphisms (SNPs).[15-19] More recently, an association between several linked SNPs in the interleukin-28B (IL28B) locus and the effect of combination therapy has been reported.[20-22]

We recently reported that the core aa wild type is significantly more likely to be found in patients with the eradication-favorable IL28B SNP genotype.[23-25] The underlying mechanism of this association as well as the reason for the differential response to therapy by viruses with core aa substitutions are unknown. This is partly because of the presence of HCV quasispecies in human serum samples and the difficulty of performing infection experiments in a small animal model.

The severe combined immunodeficient (SCID) urokinase-type plasminogen activator (uPA) mouse permits repopulation of the liver with human hepatocytes, resulting in human hepatocyte chimeric mice able to develop HCV viremia after injection of serum samples positive for the virus.[26] We and other groups have reported that the human hepatocyte chimeric mouse is useful for evaluating anti-HCV drugs, such as IFN-alpha and NS3-4A protease inhibitor.[27,28] We have further improved the replacement levels of the human hepatocytes in this mouse model,[29] which enabled us to perform infection experiments more easily because highly repopulated mice (defined as human serum albumin [HSA] levels well above 1 mg/mL) successfully develop viremia more often than poorly repopulated mice.[30] Using this mouse model, we developed a reverse genetics system for HCV.[31,32] This system is useful for studying characteristics of HCV strains with various substitutions of interest, because the effects of quasispecies can be minimized. Furthermore, as there is no adaptive immune system in this mouse model, we are able to examine the replication of HCV and the effect of therapy while avoiding the influence of the immunological response. In the present study, we investigated effects of viral and host factors on HCV infectivity, replication ability, and IFN susceptibility using genetically engineered genotype 1b HCV-infected mice that underwent transplantation with hepatocytes having eradication-favorable or eradication-unfavorable IL28B SNP genotypes.

Table 1. Characteristics of Donors for Transplanted Human Hepatocytes

Donor	A	B	C	D
Sex	Female	Male	Female	Male
Age	10	2	5	2
Ethnic group	Caucasian	Caucasian	African American	Hispanic
rs8099917	TG	TT	TG	TT
rs8109886	AA	CC	AA	CC
rs12979860	TT	CC	TT	CC
rs11882871	GG	AA	GG	AA
rs73930703	TT	CC	TT	CC
rs8107030	AG	AA	AG	AA
rs28416813	GG	CC	GG	CC
rs8103142	CC	TT	CC	TT
rs11881222	GG	AA	GG	AA
rs4803217	AA	CC	AA	CC

Materials and Methods

Animal Treatment. Generation of the $uPA^{+/+}$/$SCID^{+/+}$ mice and transplantation of human hepatocytes were performed as described previously.[29] All animal protocols described in this study were performed in accord with the guidelines of the local committee for animal experiments, and all animals received humane care. Infection, extraction of serum samples, and sacrifice were performed under ether anesthesia. Mouse serum concentrations of HSA, which serve as useful markers of the extent of repopulation, were measured as previously described.[29] Mice underwent transplantation with frozen human hepatocytes obtained from four different human donors (Table 1). Genotyping of IL28B SNPs of human hepatocytes was performed using the Invader assay as described previously.[33,34] We used 1000 IU/g/day of IFN-alpha (Dainippon Sumitomo Pharma Co., Tokyo, Japan) for 2 weeks. This dosage was selected based on a previous report showing that this regimen reduced mouse serum

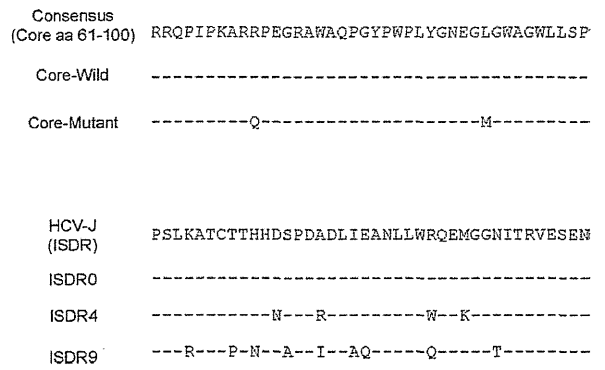

Fig. 1. The aa sequences of infectious genotype 1b HCV clones, Core-Wild, Core-Mutant (substitutions at aa70 and aa91), and ISDR variants (with 0, 4, and 9 substitutions).

HCV RNA levels by 0.5-2 log copies/mL during therapy.[31]

HCV RNA Transcription and Inoculation into Mice. We previously established an infectious genotype 1b HCV clone, HCV-KT9, that was obtained from a Japanese patient with severe acute hepatitis (GenBank accession no. AB435162).[32] Ten micrograms of plasmid DNA, linearized by digestion with *Xba*I (Promega, Madison, WI), was transcribed in a 100-μL reaction volume with T7 RNA polymerase (Promega) at 37°C for 2 hours and then analyzed by agarose gel electrophoresis. Each transcription mixture was diluted with 400 μL of phosphate-buffered saline (PBS) and injected into the livers of chimeric mice.[32] The HCV-KT9 clone has aa substitutions at aa70 and 91 (arginine to glutamine and leucine to methionine, respectively) in the core region (Core-Mutant), compared to the consensus sequence,[10-12] and no aa substitutions in the ISDR (ISDR0),[8] relative to the prototype sequence (HCV-J).[35] Using the original HCV-KT9 clone, we created two additional HCV clones having wild-type core aa70 and 91 (Core-Wild) and four (ISDR4) and nine (ISDR9) aa substitutions in the ISDR, respectively (Fig. 1). To introduce the aa substitutions, site-directed mutagenesis was performed with a QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA).

Human Serum Samples. Human serum samples containing a high titer of genotype 1b HCV (2.2×10^6 copies/mL) were obtained from a patient with chronic hepatitis after obtaining written informed consent. Aliquots of serum were stored in liquid nitrogen until use. Core 70 and 91 aas were Gln and Leu, respectively, and only one aa substitution was present in the ISDR. The study protocol involving human subjects conformed to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the institutional review committee.

Quantitation of HCV RNA and IFN-stimulated gene-expression levels. RNA was extracted from mice serum and liver samples by Sepa Gene RV-R (Sankojunyaku, Tokyo, Japan), dissolved in 8.8 μL of ribonuclease-free H_2O, and reverse transcribed using random primer (Takara Bio Inc., Shiga, Japan) and M-MLV reverse transcriptase (ReverTra Ace, TOYOBO Co., Osaka, Japan) in 20 μL of reaction mixture according to the instructions provided by the manufacturer. Nested polymerase chain reaction (PCR) and quantitation of HCV by Light Cycler (Roche Diagnostics, Tokyo, Japan) were performed as previously described.[32] Quantitation of IFN-stimulated genes (ISGs) (myxovirus resistance protein A [MxA], oligoadenylate synthetase [OAS], and RNA-dependent protein kinase [PKR]) was performed using real-time PCR Master Mix (Toyobo, Kyoto, Japan) and TaqMan Gene Expression Assay primer and probe sets (PE Applied Biosystems, Foster City, CA). Thermal cycling conditions were as follows: a precycling period of 1 minute at 95°C, followed by 40 cycles of denaturation at 95°C for 15 seconds and annealing/extension at 60°C for 1 minute. ISG messenger RNA expression levels were expressed relative to the endogenous RNA levels of the housekeeping reference gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

Statistical Analysis. The HCV infectious ratio of chimeric mice was assessed using the chi-square test. Mice serum HCV RNA titers, HSA concentrations, and ISG expression levels were compared using the Mann-Whitney U test. A P value less than 0.05 was considered statistically significant.

Results

Influence of aa Substitutions in the HCV Core Region and ISDR on HCV Infectivity and Replication Ability. We investigated the influence of aa substitutions in the core region and ISDR on HCV infectivity and replication ability in mice that underwent transplantation with human hepatocytes obtained from donor A (Table 1). Each 30 μg of *in vitro*–transcribed RNA was inoculated into the livers of mice. Six weeks after inoculation, serum HCV RNA titers increased above the detectable limit (1000 copies/mL) in 11 of 12 (92%) mice infected with Core-Wild-ISDR0 and in 14 of 16 (88%) mice with Core-Mutant-ISDR0 (Fig. 2A). HCV RNA titers in Core-Wild-ISDR0– and Core-Mutant-ISDR0–infected mice increased to the same levels (Fig. 2B). In contrast, serum HCV

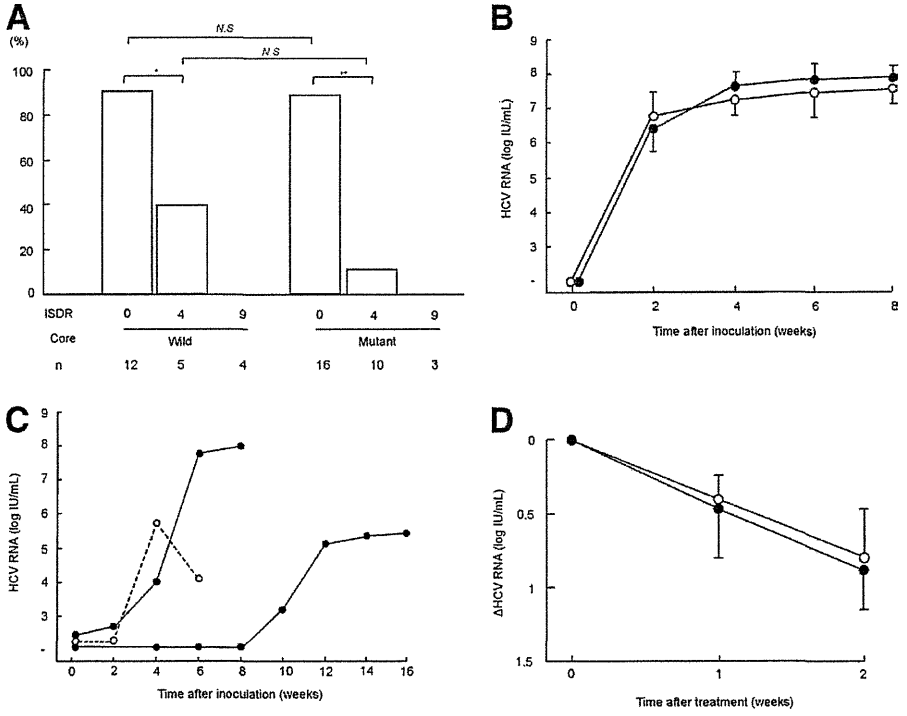

Fig. 2. Infectivity and replication ability of HCV clones. Mice that underwent transplantation with hepatocytes obtained from donor A were inoculated with 30 μg of *in vitro*-transcribed RNAs of indicated clones. (A) Proportion of HCV-infected mice. Infection was defined as serum HCV RNA titer above the detection limit (1000 copies/mL) 6 weeks after inoculation. aa sequences of the core (Wild or Mutant) and number of substitutions in the ISDR are noted below the graph. (B) Time course of serum HCV RNA levels in mice inoculated with either Core-Wild-ISDR0 (closed circles, n = 11) or Core-Mutant-ISDR0 (open circles, n = 14) HCV clones. Data are represented as mean ± standard deviation. (C) Time course of serum HCV RNA levels in two Core-Wild-ISDR4-infected mice (closed circles) and a Core-Mutant-ISDR4-infected mouse (open circles). Serum HCV RNA levels were measured until the mice died. (D) Core-Wild-ISDR0- (closed circles, n = 8) and Core-Mutant-ISDR0 (open circles, n = 4)-infected mice were treated daily with 1000 IU/g/day of IFN-alpha for 2 weeks. Mice serum HCV RNA titers were measured at the indicated times. $*P < 0.05$, $**P < 0.01$; NS, not significant.

RNA titer increased above the detection limit in only two of five (40%) Core-Wild-ISDR4 mice and in only 1 of 10 (10%) Core-Mutant-ISDR4 mice, and the titers in these mice were lower than in mice with ISDR0 (Fig. 2C). HCV RNA titers failed to increase above the detection limit in mice with Core-Wild-ISDR9 and Core-Mutant-ISDR9 (Fig. 2A).

Influence of Core aa Substitutions on the Effect of IFN. To investigate the influence of aa substitutions in the core region on the effect of IFN, Core-Wild-ISDR0– and Core-Mutant-ISDR0–infected mice were treated with 1000 IU/g of human IFN-alpha daily for 2 weeks. The treatment resulted in a 0.84 ± 0.3 log IU/mL reduction of HCV RNA titer in Core-Wild-ISDR0–infected mice and a 0.79 ± 0.34 log IU/mL reduction in Core-Mutant-ISDR0–infected mice (Fig. 2D).

We also investigated the influence of aa substitutions in the core region on the effect of IFN plus RBV combination therapy. Core-Wild-ISDR0– and Core-Mutant-ISDR0–infected mice were treated with 1000 IU/g of human IFN-alpha and 20 mg/kg of RBV daily for 2 weeks. The treatment resulted in similar HCV RNA reductions in all treated mice. However, as with IFN monotherapy, there were no significant differences in HCV reductions among mice with different aa substitutions in the core region (data not shown). The dose of ribavirin used was relatively small, however, because of the drug's toxicity in mice.

HCV Infectivity, Replication Levels, and IFN Susceptibility by Core aa Substitutions and Genetic Variation in the IL28B Locus. We investigated the influence of IL28B genotypes on HCV infectivity, replication ability, and IFN susceptibility. *In vitro*–transcribed RNA (30 μg) was inoculated into the livers of mice with hepatocytes from donor A (rs8099917 TG and rs12979860 TT) or donor B (rs8099917 TT and rs12979860 CC). Eight weeks after inoculation, serum HCV RNA titers increased above the detection limit in 22 of 25 (88%) mice with hepatocytes from donor A and in 20 of 23 (87%) mice with hepatocytes from donor B (Fig. 3A). Serum HCV RNA levels were