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.

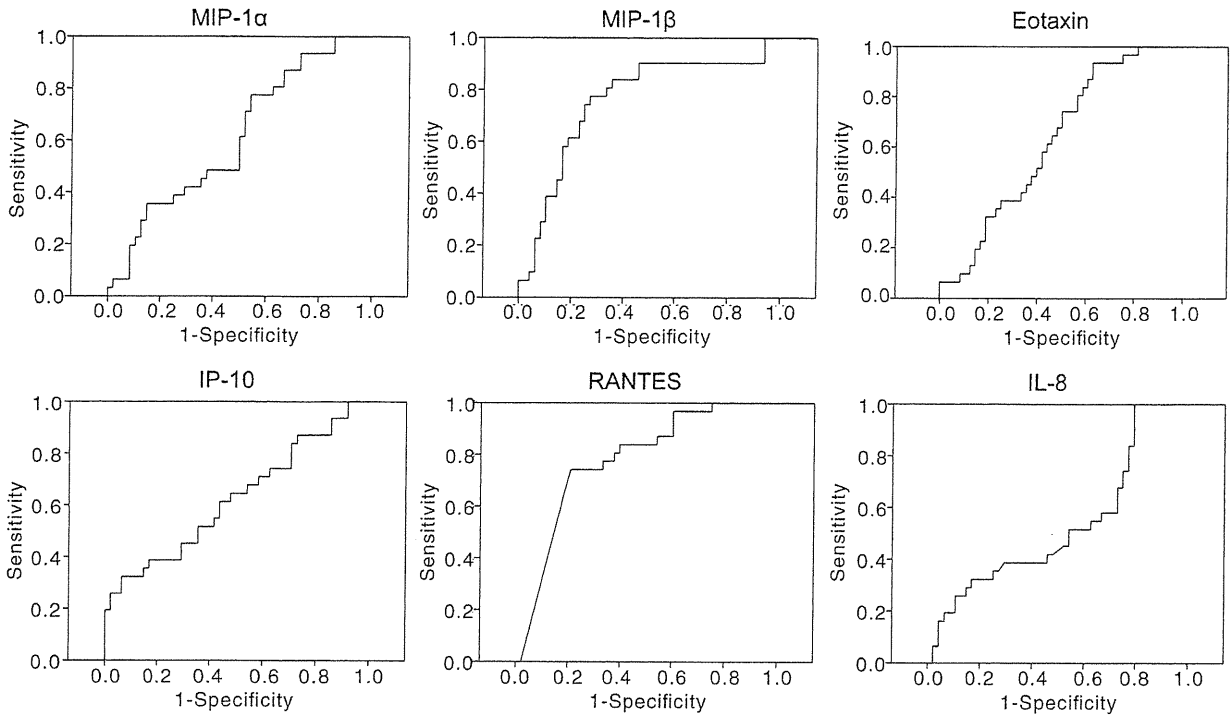

Figure 1 Receiver–operator curves for serum chemokine levels on treatment outcome. The areas under the curve for macrophage inflammatory protein (MIP)-1α, MIP-1β, eotaxin, IP-10, RANTES and interleukin (IL)-8 were 0.612, 0.756, 0.629, 0.623, 0.739 and 0.530, respectively.

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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Genome-wide association study identified *ITPA/DDRGK1* variants reflecting thrombocytopenia in pegylated interferon and ribavirin therapy for chronic hepatitis C

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Hematologic abnormalities during current therapy with pegylated interferon and ribavirin (PEG-IFN/RBV) for chronic hepatitis C (CHC) often necessitate dose reduction and premature withdrawal from therapy. The aim of this study was to identify host factors associated with IFN-induced thrombocytopenia by genome-wide association study (GWAS). In the GWAS stage using 900K single-nucleotide polymorphism (SNP) microarrays, 303 Japanese CHC patients treated with PEG-IFN/RBV therapy were genotyped. One SNP (rs11697186) located on *DDRGK1* gene on chromosome 20 showed strong associations in the minor-allele-dominant model with the decrease of platelet counts in response to PEG-IFN/RBV therapy [$P = 8.17 \times 10^{-9}$; odds ratio (OR) = 4.6]. These associations were replicated in another sample set ($n = 391$) and the combined *P*-values reached 5.29×10^{-17} (OR = 4.5). Fine mapping with 22 SNPs around *DDRGK1* and *ITPA* genes showed that rs11697186 at the GWAS stage had a strong linkage disequilibrium with rs1127354, known as a functional variant in the *ITPA* gene. The

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ITPA-AA/CA genotype was independently associated with a higher degree of reduction in platelet counts at week 4 ($P < 0.0001$), as well as protection against the reduction in hemoglobin, whereas the CC genotype had significantly less reduction in the mean platelet counts compared with the AA/CA genotype ($P < 0.0001$ for weeks 2, 4, 8, 12), due to a reactive increase of the platelet count through weeks 1–4. Our present results may provide a valuable pharmacogenetic diagnostic tool for tailoring PEG-IFN/RBV dosing to minimize drug-induced adverse events.

INTRODUCTION

Chronic infection with hepatitis C virus (HCV) presents a significant health problem worldwide, with ~2.3% of the world population, i.e. more than 120–130 million people, being infected (1). Only 20–30% of HCV-infected individuals recover spontaneously. The remaining 70–80% go on to develop chronic infection, being at significant risk for progressive liver fibrosis and subsequent liver cirrhosis (LC) and hepatocellular carcinomas (HCC). Successful treatment of chronic hepatitis C (CHC) leads to a reduction of liver fibrosis stage of patients, and also prevents HCC development (2).

Antiviral treatment has been shown to improve liver histology and decrease incidence of hepatocellular carcinoma in CHC (3,4). Current therapy for CHC consists of treatment with pegylated interferon (IFN), which acts both as an antiviral and as an immunoregulatory cytokine, and ribavirin (RBV), an antiviral pro-drug that interferes with RNA metabolism (5,6). However, <50% of patients infected with HCV genotype 1 treated in this way achieve a sustained viral response (SVR) or cure of the infection (5,7). Older patients with liver fibrosis showed a significantly lower SVR rate due to poor adherence resulting from adverse events and laboratory abnormalities (8–10). In particular, hematologic abnormalities often necessitate dose reduction, and premature withdrawal from therapy in 10–14% of patients (5,11–14). New drugs and therapeutic approaches for CHC are actively developed and several candidates are in early trial phase (15,16). Given this background, effective pre-treatment screening for predictive biomarkers with the aim of evaluating possible risks over benefits of currently available treatment will avoid these side effects in patients who will not be helped by treatment, as well as reduce the substantial cost of treatment.

The completion of the Human Genome Project has led to the advent of a new era of scientific research, including a revolutionary approach: the genome-wide association study (GWAS). Several recent studies, including our study, have demonstrated marked associations between single-nucleotide polymorphisms (SNPs) within and around *IL28B* gene, which codes for IFN–λ3 (16–21). Another recent study indicated that genetic variants of *ITPA* gene leading to inosine triphosphatase (ITPA) deficiency could protect against hemolytic anemia (HA) in CHC patients receiving RBV (22).

In Japan, HCV-infected patients are relatively old and some of them have had severe fibrosis (9). Thrombocytopenia is one of the critical adverse events by IFN-based therapy among liver cirrhotic patients (23), because low platelet count (PLT), i.e. <30.0 (10^9/l), would be a risk factor for any bleeding, as well as it would lead to poor treatment efficiency due to the initial or early dose reduction of PEG-IFN. Based on its pathogenesis, drug-induced thrombocytopenia is usually due to bone marrow suppression, immune-mediated destruction and platelet aggregation (24). In this study, we firstly found that genetic variants in the *ITPA/DDRGK1* genes were associated with IFN-induced thrombocytopenia, and then examined the correlation between IFN-induced thrombocytopenia and RBV-induced HA in Japanese CHC patients under PEG-IFN/RBV treatment.

RESULTS

Genetic variants associated with IFN-induced thrombocytopenia

In this study, we conducted a GWAS to identify host genes associated with the decrease of platelets in response to PEG-IFN/RBV treatment in 303 Japanese HCV patients (107 patients with the decrease of PLT versus 196 patients without the decrease of PLT based on the criteria described in Materials and Methods), using a genome-wide SNP typing array (Affymetrix SNP 6.0 for 900K SNPs). The characteristics of patients for each GWAS stage and replication stage are summarized in Table 1. Figure 1 shows a genome-wide view of the single-point association data based on allele frequencies. One SNP (rs11697186) located on *DDRGK1* gene on chromosome 20 showed strong associations in the allele frequency model ($P = 8.17 \times 10^{-9}$) with the decrease of PLT in response to PEG-IFN plus RBV treatment. The association reached genome-wide level of significance [Bonferroni criterion $P < 8.40 \times 10^{-8}$ (0.05/595052)], and another SNP (rs6139030) near *ITPA* gene had a marginal significance ($P = 4.30 \times 10^{-7}$, in Table 2).

To validate the results of the GWAS stage, 22 SNPs were selected for the replication in a set of 391 Japanese HCV patients with and without platelet reduction (Supplementary Material, Table S1). The associations of the original significant SNP (rs11697186) and the marginal SNP (rs6139030) at the GWAS stage were replicated in the second set of 391 patients in the minor-allele-dominant model [$P = 5.88 \times 10^{-10}$, odds ratio (OR) = 4.6 for rs11697186; $P = 3.83 \times 10^{-10}$, OR = 4.3 for rs6139030, Table 2]. The combined P-values for both stages reached 5.29×10^{-17} (OR = 4.5; 95% CI = 3.1–6.5) and 1.33×10^{-15} (OR = 3.9; 95% CI = 2.8–5.5), respectively (Table 2).

Genetic variants associated with RBV-induced anemia

We also conducted a GWAS to identify host genes associated with a quantitative change in hemoglobin (Hb) levels from baseline to week 4 of PEG-IFN/RBV treatment in the above 303 Japanese HCV patients (94 patients with an Hb reduction of ≥3 g/dl at week 4 and 209 patients without Hb reduction), using a genome-wide SNP typing array (Affymetrix SNP 6.0 for 900K SNPs). Two SNPs (rs11697186 and rs6139030)

Table 1. Clinical characteristics of patients in this study

	GWAS ($n = 303$)	Replication ($n = 391$)
Age	57.4 (9.7)	56.8 (9.9)
Sex (M/F)	151/152	209/182
Weight (kg)	60.6 (10.4)	61.3 (10.7)
Body mass index	23.5 (3.1)	23.7 (4.1)
Baseline Hb (g/dl)	14.1 (1.4)	14.1 (1.4)
Baseline platelet count (10^9/l)	151.3 (54.3)	159.7 (55.0)
Baseline ALT (IU/l)	83.5 (79.4)	86.8 (71.9)
Baseline creatinine (mg/dl)	0.70 (0.15)	0.72 (0.16)
Baseline liver fibrosis (F0–2/F3–4/ND)	153/77/73	175/59/43
rs8099917: TT/non-TT	165/138	296/95
rs1127354: AA/CA/CC	4/79/220	6/101/284
Week 4 Hb (g/dl)	11.8 (1.7)	11.9 (1.5)
Week 4 platelet count (10^9/l)	127.6 (48.2)	132.4 (51.0)
Hb reduction at week 4	−2.3 (1.4)	−2.2 (1.4)
Platelet reduction at week 4	−22.2 (38.4)	−24.7 (30.4)

located on *DDRGK1* gene and *ITPA* gene on chromosome 20 showed strong associations in the allele frequency model ($P = 3.29 \times 10^{-10}$ and $P = 2.56 \times 10^{-9}$) with Hb reduction in response to PEG-IFN plus RBV treatment (Table 3).

The above 22 SNPs were selected for the replication study and fine mapping, including rs1127354, which was reported by the US group (22) to be strongly associated with Hb reduction (Supplementary Material, Table S2). All SNPs were genotyped using the DigiTag2 assay in an independent set of 391 Japanese HCV patients with quantitative change in Hb in response to PEG-IFN/RBV treatment [137 patients with Hb reduction versus 254 patients without Hb reduction (Table 3)]. The associations of the original SNPs were replicated in the second set of 391 patients in the minor-allele-dominant model ($P = 3.86 \times 10^{-16}$, OR = 0.02 for rs11697186; $P = 6.90 \times 10^{-18}$, OR = 0.03 for rs6139030, Table 3). The combined *P*-values for both stages reached 9.43×10^{-25} (OR = 0.03; 95% CI = 0.01–0.08) and 2.12×10^{-25} (OR = 0.04; 95% CI = 0.02–0.09), respectively (Table 3). The rs1127354 was also strongly associated with a quantitative change in Hb in response to PEG-IFN/RBV treatment in a set of 694 Japanese HCV patients (303 patients from the GWAS stage plus the second set of 391 patients) with and without Hb reduction ($P = 4.58 \times 10^{-26}$, OR = 0.03; 95% CI = 0.01–0.08).

Fine mapping with 22 SNPs around *DDRGK1* and *ITPA* genes showed that four significant SNPs (rs11697186, rs6139030, rs1127354 and rs13830) at the GWAS stage had a strong linkage disequilibrium (LD) ($r^2 > 0.86$) within the 22.7 kb region (Fig. 2). As the rs1127354 is known as a functional variant in the *ITPA* gene that caused ITPase deficiency and protected against RBV-induced HA (22,25), the representative SNP was applied for the following detailed studies.

ITPA/DDRGK1 variants reflect anemia and reactive increase of the platelet count

The mean quantitative reduction of blood cells from the baseline according to the *ITPA* rs1127354 genotypes is shown in Figure 3. Patients with the rs1127354 genotypes AA and CA showed lower degree of Hb reduction at weeks 2, 4, 8 and 12 during therapy compared with those with the CC genotype ($P < 0.0001$ for weeks 2, 4, 8 and 12 in Fig. 3A). The most difference of mean Hb reduction was found at week 4 (AA/CA −1.14 versus CC −2.72). These results show that the AA and CA genotypes are significantly associated with less absolute reduction in Hb levels, especially during the early weeks of therapy, and protect against the development of severe anemia. Interestingly, the CC genotype had significantly less reduction in the mean platelet count compared with the AA/CA genotype ($P < 0.0001$ for weeks 2, 4, 8; $P = 0.019$ for week 12 in Fig. 3B), due to a reactive increase of platelet count through weeks 1–4. The most difference of mean platelet reduction was found at week 4 [AA/CA −41.2 versus CC −18.0 (10^9/l)]. There was no difference in the neutrophil leukocyte count between genotypes (Fig. 3C). We then compared the percentage of patients with platelet count reduction in the *ITPA* rs1127354 genotypes at week 4 of PEG-IFN/RBV therapy (Fig. 4). The percentage of patients with a platelet count reduction of <30 (10^9/l) at week 4 was significantly higher in the rs1127354 genotypes CC ($P < 0.0001$), indicating that the degree of platelet count reduction was less in patients with the rs1127354 genotype CC. A multivariate analysis for factors associated with a platelet reduction >30 (10^9/l) at week 4 showed that lower platelet count at the baseline and the rs1127354 genotypes AA/CA were independently associated with platelet reduction (OR = 1.15; 95% CI = 1.11–1.20; $P < 0.0001$, OR = 5.92; 95% CI = 3.82–9.17; $P < 0.0001$, respectively).

Figure 5 showed reactive increase of the platelet count through weeks 1–4 of PEG-IFN/RBV therapy. Patients with anemia (Hb reduction ≥3.0 g/dl) at week 4 had a significantly higher degree of the reactive increase of the platelet count than those without anemia ($P < 0.0001$ in Fig. 5A). Within a subgroup of patients with the rs1127354 genotypes CC, patients with anemia still had a significantly higher degree of reactive increase of the platelet count than those without anemia ($P = 0.004$ in Fig. 5B). On the other hand, patients with the rs1127354 genotypes CC had a significantly higher degree of the reactive increase of the platelet count than those with genotypes AA/CA ($P < 0.0001$ in Fig. 5C), and a similar result was obtained in a subgroup of patients without anemia (Fig. 5D). To elucidate the significant factors associated with the rs1127354 genotypes by multivariate analysis, the rs1127354 genotypes AA/CA were independently associated with protection against the reduction in Hb and more reduction in platelet counts at week 4 due to a lower degree of the reactive increase of the platelet count (OR = 0.029; 95% CI = 0.009–0.092; $P < 0.0001$, OR = 4.73; 95% CI = 3.04–7.37; $P < 0.0001$, respectively). Indeed, the reactive increase of the platelet count through weeks 1–4 was positively correlated with a high platelet count at the baseline and anemia (Hb reduction ≥3.0 g/dl) at week 4, but was negatively correlated with rs1127354 genotypes AA/CA and a platelet count reduction of ≥30 (10^9/l) at week 4 (Table 4).

Relationship between *ITPA rs1127354* genotypes and treatment outcome due to dose reduction of PEG-IFN or RBV

In this population, a multivariate analysis showed that SVR was significantly associated with *IL28B* TT-genotype [OR

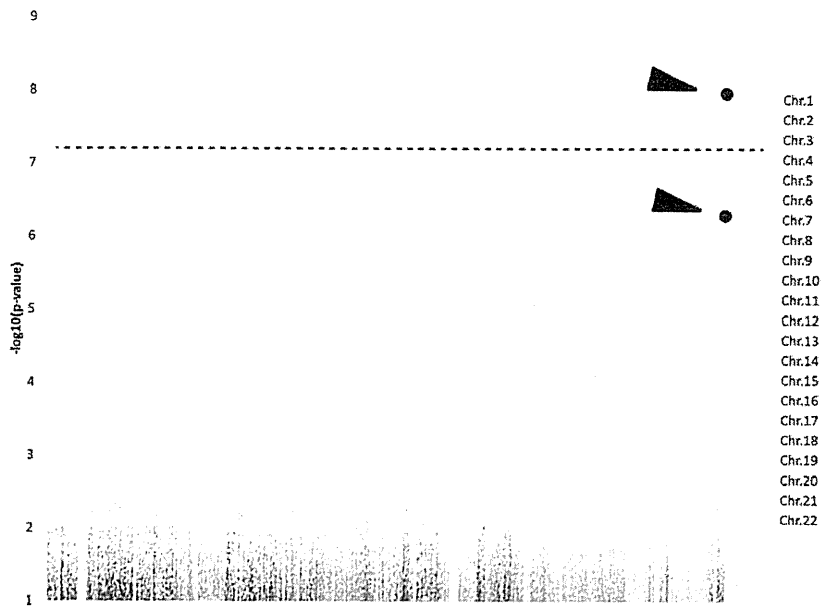

Figure 1. Genome-wide association results in 303 Japanese HCV patients with the decrease of platelets in response to PEG-IFN plus RBV treatment (107 patients with the decrease of PLT and 196 patients without the decrease of PLT). *P*-values were calculated using the χ^2 test for allele frequencies. Dots with arrow on chromosome 20 showed a significant SNP ($P = 8.17 \times 10^{-9}$ for rs11697186) and a candidate SNP with a marginal significance ($P = 4.30 \times 10^{-7}$ for rs6139030) associated with the decrease of PLT with response to PEG-IFN/RBV treatment. The dotted line indicates a genome-wide significance ($P < 8.40 \times 10^{-8}$).

Table 2. Two SNPs (rs11697186 and rs6139030) significantly associated with the decrease of PLT in response to PEG-IFN/RBV treatment

dbSNP rsID	Nearest gene	MAF[a] (allele)	Allele (1/2)	Stage	Patients with the decrease of PLT			Patients without the decrease of PLT			OR (95% CI)[b]	*P*-value[c]
					11	12	22	11	12	22		
rs11697186	*DDRGK1*	0.15 (T)	T/A	GWAS	3 (2.8)	48 (44.9)	56 (52.3)	0 (0.0)	32 (16.6)	161 (83.4)	4.6 (2.7–7.8)	8.17×10^{-9}
				Replication	3 (1.8)	65 (39.9)	95 (58.3)	3 (1.4)	25 (12.0)	181 (86.6)	4.6 (2.8–7.7)	5.88×10^{-10}
				Combined	6 (2.2)	113 (41.9)	151 (55.9)	3 (0.7)	57 (14.2)	342 (85.1)	4.5 (3.1–6.5)	5.29×10^{-17}
rs6139030	*ITPA*	0.17 (C)	T/C	GWAS	56 (52.3)	48 (44.9)	3 (2.8)	157 (80.1)	38 (19.4)	1 (0.5)	3.7 (2.2–6.1)	4.30×10^{-7}
				Replication	96 (54.9)	74 (42.3)	5 (2.9)	181 (83.8)	32 (14.8)	3 (1.4)	4.3 (2.7–6.8)	3.83×10^{-10}
				Combined	152 (53.9)	122 (43.3)	8 (2.8)	338 (82.0)	70 (17.0)	4 (1.0)	3.9 (2.8–5.5)	1.33×10^{-15}

[a]Minor allele frequency and minor allele in 184 healthy Japanese individuals.
[b]OR for the minor allele in a dominant model.
[c]*P*-value by χ^2 test for the minor allele dominant model.

6.12 (2.78–13.46), $P < 0.0001$] as well as platelet counts [OR 1.18 (1.11–1.26), $P < 0.00001$]. We analyzed whether the rs1127354 genotype could influence the treatment outcome by PEG-IFN/RBV therapy. When analyzed in the patients available for treatment outcome (172 with *ITPA*-AA/CA and 450 with *ITPA*-CC), the percentage of patients receiving >80% of the expected PEG-IFN and RBV dose at baseline and week 4 was not significantly different among the rs1127354 genotypes. However, the rate of SVR tended to be higher in patients with *ITPA*-AA/CA genotype than those with *ITPA*-CC (48.8 versus 37.3%), because the relapse rate was lower in patients with *ITPA*-AA/CA. To investigate the influence on treatment outcome by dose reduction of PEG-IFN, in a subgroup of patients with low platelet counts (<10) at baseline (19 with *ITPA*-AA/CA and 53 with *ITPA*-CC) we analyzed the treatment outcome according to rs1127354 genotypes. The SVR rate was very low in each group (21.1% in *ITPA*-AA/CA and 17.0% in *ITPA*-CC), because many patients had the initial dose reduction of PEG-IFN (<80% of standard dose)—36.8% of patients with *ITPA*-AA/CA and 44.6% of patients with *ITPA*-CC genotype. Further prospective studies are required among the pre-cirrhotic or cirrhotic patients with low platelet counts.

DISCUSSION

Recent genome-wide association studies, including our study on HCV infection, have identified two important host genetic variants: the SNP in *IL28B* gene, which is strongly associated with response to therapy for chronic genotype 1 HCV infection (16–21), and the SNP in *ITPA* gene, which precisely predicts RBV-induced anemia in

Table 3. Two SNPs (rs11697186 and rs6139030) significantly associated with quantitative change in Hb levels from baseline to week 4 of PEG-IFN/RBV treatment

dbSNP rsID	Nearest gene	MAF[a] (allele)	Allele (1/2)	Stage	Patients with quantitative change in Hb			Patients without quantitative change in Hb			OR (95% CI)[b]	*P*-value[c]
					11	12	22	11	12	22		
rs11697186	*DDRGK1*	0.15 (T)	T/A	GWAS	0 (0.0)	3 (3.3)	89 (96.7)	3 (1.5)	77 (37.0)	128 (61.5)	0.06 (0.02–0.16)	3.29×10^{-10}
				Replication	0 (0.0)	2 (1.5)	134 (98.5)	6 (2.5)	88 (37.3)	142 (60.2)	0.02 (0.01–0.09)	3.86×10^{-16}
				Combined	0 (0.0)	5 (2.2)	223 (97.8)	9 (2.0)	165 (37.2)	270 (60.8)	0.03 (0.01–0.08)	9.43×10^{-25}
rs6139030	ITPA	0.17 (C)	T/C	GWAS	88 (93.6)	6 (6.4)	0 (0.0)	125 (59.8)	80 (38.3)	4 (1.9)	0.08 (0.03–0.22)	2.56×10^{-9}
				Replication	134 (97.8)	3 (2.2)	0 (0.0)	143 (56.3)	103 (40.6)	8 (3.1)	0.03 (0.01–0.08)	6.90×10^{-18}
				Combined	222 (96.1)	9 (3.9)	0 (0.0)	268 (57.9)	183 (39.5)	12 (2.6)	0.04 (0.02–0.09)	2.12×10^{-25}

[a]Minor allele frequency and minor allele in 184 healthy Japanese individuals.
[b]OR for the minor allele in a dominant model.
[c]*P*-value by χ^2 square test for the minor allele dominant model.

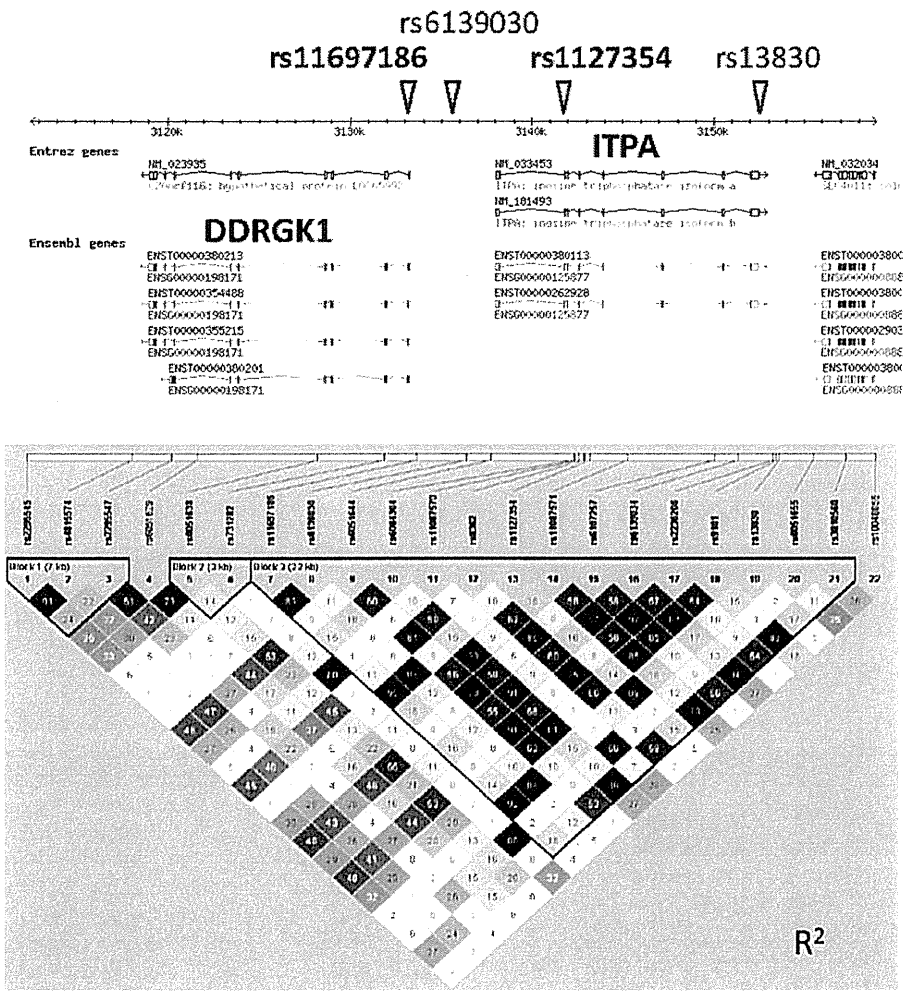

Figure 2. Pairwise LD (r^2) diagrams for *DDRGK1* and *ITPA*. Lower panel shows estimates of pairwise r^2 for 22 SNPs selected in the replication study using the second set of 391 Japanese HCV patients with and without quantitative change in PLT levels from baseline to week 4 of PEG-IFN/RBV treatment.

European-American population (22) and Japanese population (26). The genetic variation of *ITPA* causing an accumulation of inosine triphosphate (ITP) has been shown to protect patients against RBV-induced anemia during treatment for CHC infection. A recent report showed the biologic mechanism that ITP confers protection against RBV-induced ATP reduction by substituting for erythrocyte GTP, which is depleted by RBV, in the biosynthesis of ATP (25).

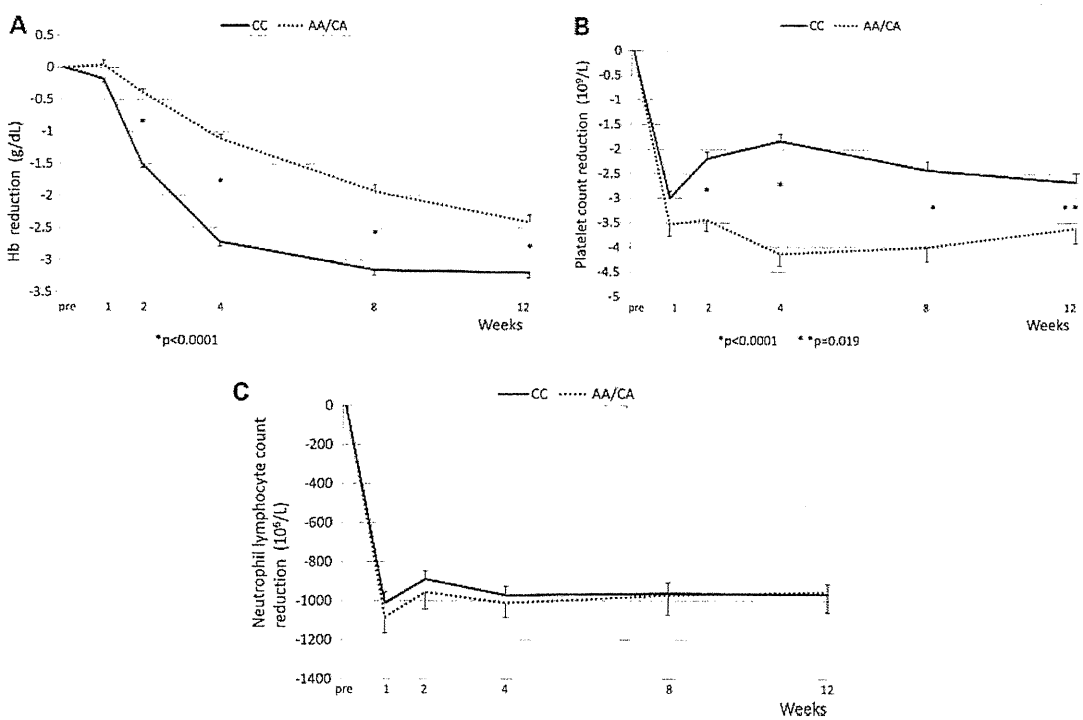

Figure 3. *ITPA* rs1127354 genotypes and the quantitative reduction of blood cells from baseline. Mean reduction of (**A**) Hb levels, (**B**) platelet counts and (**C**) neutrophil leukocyte counts during treatment according to rs1127354 genotype is shown. Solid and dotted lines indicate patients with CC and AA/CA genotypes, respectively. Error bars indicate standard error. CC genotype had more reduction in mean Hb levels during therapy compared with the AA/CA genotype ($^{*}P < 0.0001$ for weeks 2, 4, 8, 12). CC genotype had less of a reduction in mean platelet counts ($^{*}P < 0.0001$ for weeks 2, 4, 8, and $^{**}P = 0.019$ for week 12), and showed a reactive increase of platelet counts through weeks 1–4.

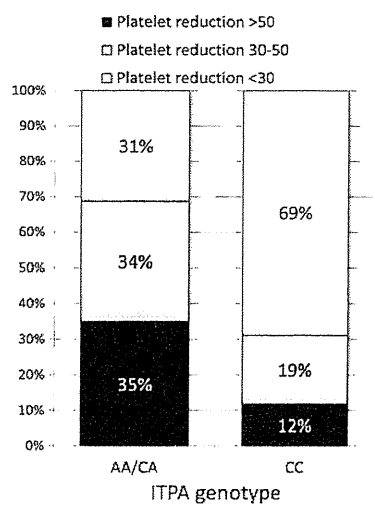

Figure 4. *ITPA* rs1127354 genotypes and reduction of platelet counts at week 4 of PEG-IFN/RBV therapy. The percentage of patients with platelet count reduction of >50 (10^9/l) (black bar), 30–50 (10^9/l) (gray bar) and <30 (10^9/l) (white bar) at week 4 is shown for rs1127354 genotypes. The incidence of platelet count reduction of >50 and <30 was significantly lower in patients with the rs1127354 genotypes CC compared with AA/CA genotypes: 12 versus 35%, $P < 0.0001$, and 69 versus 31%, $P < 0.0001$, respectively.

In this study, two SNPs, rs11697186 and rs6139030, which were within and around *DDRGK1* gene on chromosome 20, were strongly associated with thrombocytopenia as well as with Hb reduction at week 4. In clinical practice, the positive predictive value and negative predictive value by rs11697186 genotypes were 66.5 and 69.4% for thrombocytopenia, as well as 97.2 and 45% for RBV-induced anemia at week 4. As previously reported (22,26), a functional SNP (rs1127354) in the *ITPA* locus, which is in strong LD with rs11697186, was the most significant SNP associated with RBV-induced anemia and, in this study, IFN-induced thrombocytopenia in Japanese genetic populations. Note that severe Hb decline, which is mainly found in ITPA-CC patients, was inversely correlated with platelet reduction. This would contribute to an association between severe anemia and relative reactive increase of platelet count in this population, which attenuated the IFN effect on the platelet count. Our data supported a previous report which described that the current use of RBV, inducing severe anemia, might blunt the thrombocytopenic effect of IFNs as a result of reactive increase of platelet counts (27).

A previous paper showed hematological and bone marrow effects of RBV in rhesus monkeys (28). Hb values decreased significantly during RBV administration due to dose-related erythroid hypoplasia in bone marrow and returned to normal following withdrawal. On the other hand, increase of the platelet count occurred in both low- and high-dose treatment groups during RBV administration, with a fall of the platelet count to normal after drug withdrawal. The effect on platelet count was clearly dose related, with maximum counts rising to twice and three times above baseline levels in the low- and high-dose groups, respectively. This caused a significant increase of

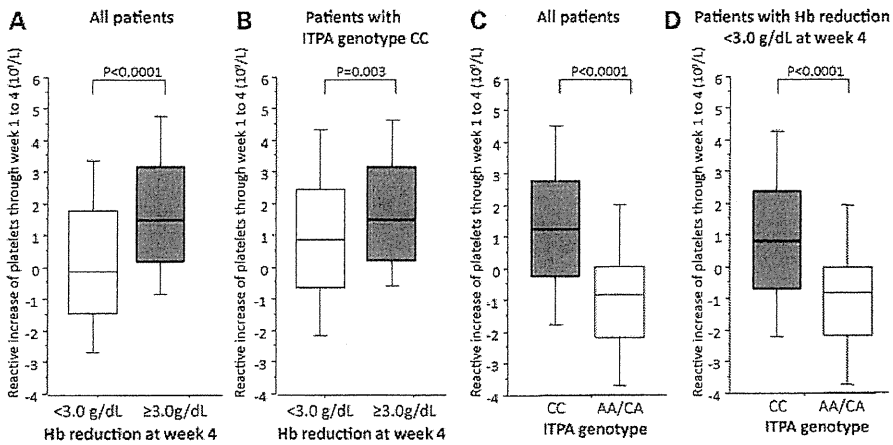

Figure 5. Reactive increase of platelet counts through weeks 1–4. Box plots of reactive increase of platelet count through weeks 1–4 according to the degree of anemia at week 4 are shown for all patients (**A**) and a subgroup of patients with the rs1127354 genotypes CC (**B**). Patients with anemia (Hb reduction ≥3.0 g/dl) at week 4 had a significantly higher degree of reactive increase of platelet count than those without anemia ($P < 0.0001$). Box plots of reactive increase of platelet counts according to the rs1127354 genotype CC are shown for all patients (**C**) and a subgroup of patients without anemia (**D**) (Hb reduction <3.0 g/dl) at week 4. Patients with the rs1127354 genotypes CC had a significantly high degree of reactive increase of platelet counts compared with those with genotypes AA/CA ($P < 0.0001$).

Table 4. Multivariate analysis of factors associated with reactive increase of platelets ≥20 (10^9/l) through weeks 1–4

	OR	95% CI	*P*-value
Baseline platelet counts	1.168	1.101–1.239	<0.0001
ITPA AA/CA	0.379	0.168–0.856	0.0196
Platelet reduction ≥30 (10^9/l) at week 4	0.051	0.021–0.120	<0.0001
Hb reduction ≥3.0 g/dl at week 4	1.602	0.914–2.809	0.0996

the platelet count associated with increased numbers of megakaryocytes. Additionally, the sequence homology of thrombopoietin (TPO) and erythropoietin (EPO) may explain the synergy of the physiologic role of TPO and EPO in platelet production. When EPO is elevated, as in iron deficiency anemia, an amino acid sequence similar to TPO may increase the platelet count (29).

Another possibility is a direct association between *ITPA* SNPs or the related SNPs with a strong LD and IFN-induced thrombocytopenia. *DDRGK1* (DDRGK domain-containing protein 1) is a novel C53/LZAP-interacting protein. C53/LZAP (also named as Cdk5rap3) is a putative tumor suppressor that plays important roles in multiple cell signaling pathways, including DNA damage response and NF-kappaB signaling (30); however, it remains largely unknown how the function of *DDRGK1* variants is regulated. Further studies are required to elucidate the possible association between *DDRGK1* variants and thrombocytopenia.

Multivariate analysis demonstrated that rs1127354 in the *ITPA* gene was independently associated with RBV-induced severe anemia and IFN-induced thrombocytopenia. This finding suggests that rs1127354 would be a useful marker to predict these hematological side effects by PEG-IFN/RBV therapy, indicating that genetic testing of *ITPA* variant might be applied to establish personalized dosages of PEG-IFN/RBV therapy. The rate of SVR tended to be higher in patients with *ITPA*-AA/CA genotype than those with *ITPA*-CC in this population. This might reflect decreased treatment efficacy (higher relapse rate) due to dose reduction of RBV in patients with *ITPA*-CC genotype. Our recent paper also demonstrated that the incidence of early dose reduction was significantly higher in *ITPA*-major (CC) patients as expected and, more importantly, that a significantly higher SVR rate was achieved in *ITPA*-hetero/minor (CA/AA) patients with HCV non-1b or low viral load strains (31) and in a subset of Japanese patients with the favorable TT genotype at rs8099917 of *IL28B* (32). Taken together, our results indicate that the *ITPA* minor variant A is not only a protective allele against PEG-IFN and RBV treatment-associated anemia in Japanese population, but also a significant predictor of SVR in certain HCV strains that show good response to IFN. The possible mechanism of protection against RBV-induced hemolysis is that ITP deficiency or low-activity variants (*ITPA* minor variant A) in turn lead to the accumulation of ITP in red blood cells (33,34), and the ITP confers protection against RBV-induced ATP reduction by substituting for erythrocyte GTP (25). On the other hand, half of the *ITPA*-major (CC) patients did not develop a significant Hb decline. This finding suggests other low-frequency *ITPA* variants or SNPs in other enzymes that are involved in erythrocyte purine nucleoside metabolism.

In Japan, the older HCV-infected patients developing liver fibrosis have been prevalent (mean age 62 years) (9). Thrombocytopenia by PEG-IFN/RBV therapy could lead to poor treatment efficiency among such Japanese patients with LC due to the initial or early dose reduction of PEG-IFN. In fact, ~40% of such population in this study had the initial dose reduction of PEG-IFN, resulting in a low SVR rate. Splenectomy or embolization of the splenic artery might be one of the options to increase the SVR rate, but a sufficient treatment outcome had not been obtained at present (35). Based on the recently accumulated SNP data, if patients had favorable *IL28B* genotype and *ITPA*-CC (lower reduction of platelet counts), a standard dose of PEG-IFN might be available for

the patients with lower platelet counts and the SVR rate might be increased due to sufficient dose of PEG-IFN.

Several STAT-C agents (specifically targeted antiviral therapies for hepatitis C) are being tested for clinical efficacy against hepatitis C (12,13,15,16). Most experts believe that when new drugs are approved to treat hepatitis C, they will be used in combination with PEG-IFN and RBV. Moreover, recent clinical trials, including NS3 protease inhibitors, have shown that PEG-IFN plus RBV would be necessary to achieve optimal treatment responses (12,13). Our present results may provide a valuable pharmacogenetic diagnostic tool for tailoring PEG-IFN and RBV dosing to minimize drug-induced adverse events and for further optimization of clinical anti-HCV chemotherapeutics.

MATERIALS AND METHODS

Patients

From April 2007 to April 2010, samples were obtained from 303 patients with chronic HCV (genotype 1) infection who were treated at 14 multi-center hospitals (liver units with hepatologists) throughout Japan. Each patient was treated with PEG-IFN-α2b (1.5 μg/kg body weight, subcutaneously once a week) or PEG-IFN-α2a (180 μg once a week) plus RBV (600–1000 mg daily according to body weight) for 48 weeks. Treatment duration was extended in some patients up to 72 weeks, according to the physicians' preferences. The dose of PEG-IFN or RBV was reduced according to the recommendations on the package inserts or the clinical conditions of the individual patients. EPO or other growth factors were not given. 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 institutional ethics review committees. HBsAg-positive and/or anti-HIV-positive patients were excluded from this study.

In the following stage of replication study, SNP genotyping in an independent set of 391 Japanese HCV patients treated with PEG-IFN plus RBV treatment was completed using the DigiTag2 or TaqMan assay (ABI) following the manufacturer's protocol. The characteristics of patients for each GWAS stage and replication stage are summarized in Table 1.

SNP genotyping and data cleaning

In the GWAS stage, we genotyped 303 Japanese HCV patients with and without the decrease of platelet counts from baseline to week 4 of PEG-IFN/RBV treatment [107 patients with a decrease of >30 (10^9/l) in platelet counts and 196 patients without a decrease of >30 (10^9/l) in platelet counts], using the Affymetrix Genome-Wide Human SNP Array 6.0 according to the manufacturer's instructions. The cut-off value was calculated to maximize the difference, which was also close to the median change. The average overall call rate of patients with and without the decrease of PLT reached 98.69 and 98.72%, respectively. We then applied the following thresholds for SNP QC in data cleaning: SNP call rate $\geq$95% for all samples, MAF $\geq$1% for all samples. A total of 595 052 SNPs on autosomal chromosomes passed the QC filters and were used for association analysis. All cluster plots of SNPs showing $P < 0.0001$ in association analyses by comparing allele frequencies in both groups with and without the decrease of PLT were checked by visual inspection, and SNPs with ambiguous genotype calls were excluded.

In the following stage of the replication study and high-density association mapping, we selected 23 tag SNPs from the 44.7 kb region, including *DDRGK1* gene and *ITPA* gene by analyzing LD and haplotype structure based on the HapMap data of Japanese, using the Haploview software. Of these tag SNPs, rs1127354 within the *ITPA* gene, which was associated with RBV-induced anemia (22), was included; however, rs7270101 was excluded because recent papers studying Japanese patients showed no variants in rs7270101 (26,31,32). The SNP genotyping in an independent set of 391 Japanese HCV patients with and without quantitative change in PLT levels from baseline to week 4 of PEG-IFN/RBV treatment (175 patients with quantitative change in PLT and 216 patients without quantitative change in PLT) was completed using the DigiTag2 assay (36). Twenty-two of the 23 SNPs were successfully analyzed and were used for SNP genotyping and data cleaning. All 22 SNPs in the replication study cleared HWE P-value >0.001.

Based on the above SNPs data obtained from 303 Japanese HCV patients, using the Affymetrix Genome-Wide Human SNP Array 6.0, we also performed GWAS between 94 patients with a quantitative change of >3 g of reduction in Hb and 209 patients without quantitative change in Hb levels from baseline to week 4 of PEG-IFN/RBV treatment. SNP genotyping in an independent set of 391 Japanese HCV patients with and without quantitative change in Hb levels from baseline to week 4 of PEG-IFN/RBV treatment (137 patients with quantitative change in Hb and 254 patients without quantitative change in Hb) was also completed using the DigiTag2 assay (36). Twenty-two of the 23 SNPs were successfully analyzed and were used for SNP genotyping and data cleaning.

An application of the Cochrane–Armitage test on all the SNPs showed the genetic inflation factor $\lambda = 1.000$ for thrombocytopenia and $\lambda = 1.006$ for anemia in the GWAS stage (Supplementary Material, Figs S1 and S2). In addition, principal component analysis was performed in 303 samples for the GWAS stage together with the HapMap samples (CEU, YRI, CHB and JPT) (Supplementary Material, Fig. S3). These results implied that the effect of population stratification was negligible, except one sample, which was excluded from further analysis.

Laboratory and histological tests

Blood samples were obtained at baseline, 1, 2, 4, 8 and 12 weeks after the start of therapy and for hematologic tests after the start of therapy and for hematologic tests, blood chemistry and HCV-RNA. Genetic polymorphism in the *IL28B* gene (rs8099917) was determined using the ABI TaqMan assay (Applied Biosystems, Carlsbad, CA, USA). Fibrosis was evaluated on a scale of 0–4 according to the METAVIR scoring system. The SVR was defined as an undetectable HCV-RNA level by qualitative PCR with a lower detection limit of 50 IU/ml (Amplicor, Roche Diagnostic Systems, CA, USA) or by Cobas Ampliprep/Cobas TaqMan assay (CAP/CTM) with a lower detection limit of

15 IU/ml (Roche Diagnostic Systems) 24 weeks after the completion of therapy.

Statistical analysis

The observed association between an SNP and the decrease of platelets/quantitative change in Hb levels with response to PEG-IFN plus RBV treatment was assessed by χ^2 test with a two-by-two contingency table in three genetic models: allele frequency model, dominant-effect model and recessive-effect model. SNPs on chromosome X were removed because gender was not matched between groups with and without the decrease of PLT and quantitative change in Hb levels. A total of 595 052 SNPs passed the quality control filters in the GWAS stage; therefore, significance levels after Bonferroni correction for multiple testing were $P = 8.40 \times 10^{-8}$ (0.05/595052) in the GWAS stage and $P = 2.27 \times 10^{-3}$ (0.05/22) in the replication stage.

The association between an SNP of the *ITPA* gene (rs1127354) and the incidence of platelet reduction at week 4 was analyzed by Fisher's exact test. The association between *ITPA* polymorphisms and the degree of reduction in platelet counts and Hb levels at each time point during therapy were analyzed by Mann–Whitney *U* test. Multivariable regression analysis was used to analyze the factors associated with *ITPA*, the rs1127354 genotype, factors associated with platelet count reductions and factors associated with the reactive increase in platelet counts. IBM-SPSS software v.15.0 (SPSS, Inc., Chicago, IL, USA) was used for these analyses.

Possible heterogeneity in allele frequencies at rs1127354 was assessed by Tarone's test. The association between the SNP and thrombocytopenia/anemia were analyzed by the Cochran–Mantel–Haenszel test. Both analyses were performed using the R (version 2.9.0) software (Supplementary Material, Table S3).

AUTHORS' CONTRIBUTIONS

Drafting of the paper, statistical analysis and approval of the final draft submitted: M.M.; drafting of the paper, statistical analysis, collecting samples and clinical data and approval of the final draft submitted: Y.T. and M.K.; statistical analysis and approval of the final draft submitted: N.N., M.S. and K.T.; collecting samples and clinical data and approval of the final draft submitted: K.M., N.S., N.E., H.Y., S.N., K.H., S.H., Y.I., E.T., S.M., M.H., Y.H., F.S., S.K. and N.I.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at *HMG* online.

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REFERENCES

1. Global Burden of Hepatitis C Working Group (2004) Global burden of disease (GBD) for hepatitis C. *J. Clin. Phamacol.*, **44**, 20–29.
2. Shiratori, Y., Shiina, S., Imamura, M., Kato, N., Kanai, F., Okudaira, T., Teratani, T., Tohgo, G., Toda, N., Ohashi, M. *et al.* (1995) Characteristic difference of hepatocellular carcinoma between hepatitis B- and C-viral infection in Japan. *Hepatology*, **22**, 1027–1033.
3. Yoshida, H., Tateishi, R., Arakawa, Y., Sata, M., Fujiyama, S., Nishiguchi, S., Ishibashi, H., Yamada, G., Yokosuka, O., Shiratori, Y. *et al.* (2004) Benefit of interferon therapy in hepatocellular carcinoma prevention for individual patients with chronic hepatitis C. *Gut*, **53**, 425–430.
4. George, S.L., Bacon, B.R., Brunt, E.M., Mihindukulasuriya, K.L., Hoffmann, J. and Di Bisceglie, A.M. (2009) Clinical, virologic, histologic, and biochemical outcomes after successful HCV therapy: a 5-year follow-up of 150 patients. *Hepatology*, **49**, 729–738.
5. Fried, M.W., Shiffman, M.L., Reddy, K.R., Smith, C., Marinos, G., Goncales, F.L. Jr, Haussinger, D., Diago, M., Carosi, G., Dhumeaux, D. *et al.* (2002) Peginterferon alfa-2a plus ribavirin for chronic hepatitis C virus infection. *N. Engl. J. Med.*, **347**, 975–982.
6. Manns, M.P., McHutchison, J.G., Gordon, S.C., Rustgi, V.K., Shiffman, M., Reindollar, R., Goodman, Z.D., Koury, K., Ling, M. and Albrecht, J.K. (2001) Peginterferon alfa-2b plus ribavirin compared with interferon alfa-2b plus ribavirin for initial treatment of chronic hepatitis C: a randomised trial. *Lancet*, **358**, 958–965.
7. Hadziyannis, S.J., Sette, H. Jr, Morgan, T.R., Balan, V., Diago, M., Marcellin, P., Ramadori, G., Bodenheimer, H. Jr, Bernstein, D., Rizzetto, M. *et al.* (2004) Peginterferon-alpha2a and ribavirin combination therapy in chronic hepatitis C: a randomized study of treatment duration and ribavirin dose. *Ann. Intern. Med.*, **140**, 346–355.
8. Hiramatsu, N., Oze, T., Tsuda, N., Kurashige, N., Koga, K., Toyama, T., Yasumaru, M., Kanto, T., Takehara, T., Kasahara, A. *et al.* (2006) Should aged patients with chronic hepatitis C be treated with interferon and ribavirin combination therapy? *Hepatol. Res.*, **35**, 185–189.
9. Iwasaki, Y., Ikeda, H., Araki, Y., Osawa, T., Kita, K., Ando, M., Shimoe, T., Takaguchi, K., Hashimoto, N., Kobatake, T. *et al.* (2006) Limitation of

combination therapy of interferon and ribavirin for older patients with chronic hepatitis C. *Hepatology*, **43**, 54–63.
10. Sezaki, H., Suzuki, F., Akuta, N., Yatsuji, H., Hosaka, T., Kobayashi, M., Suzuki, Y., Arase, Y., Ikeda, K., Miyakawa, Y. *et al.* (2009) An open pilot study exploring the efficacy of fluvastatin, pegylated interferon and ribavirin in patients with hepatitis C virus genotype 1b in high viral loads. *Intervirology*, **52**, 43–48.
11. Bruno, R., Sacchi, P., Maiocchi, L., Patruno, S. and Filice, G. (2006) Hepatotoxicity and antiretroviral therapy with protease inhibitors: a review. *Dig. Liver Dis.*, **38**, 363–373.
12. Hezode, C., Forestier, N., Dusheiko, G., Ferenci, P., Pol, S., Goeser, T., Bronowicki, J.P., Bourliere, M., Gharakhanian, S., Bengtsson, L. *et al.* (2009) Telaprevir and peginterferon with or without ribavirin for chronic HCV infection. *N. Engl. J. Med.*, **360**, 1839–1850.
13. McHutchison, J.G., Everson, G.T., Gordon, S.C., Jacobson, I.M., Sulkowski, M., Kauffman, R., McNair, L., Alam, J. and Muir, A.J. (2009) Telaprevir with peginterferon and ribavirin for chronic HCV genotype 1 infection. *N. Engl. J. Med.*, **360**, 1827–1838.
14. Suzuki, F., Akuta, N., Suzuki, Y., Sezaki, H., Yatsuji, H., Kawamura, Y., Hosaka, T., Kobayashi, M., Arase, Y., Ikeda, K. *et al.* (2009) Rapid loss of hepatitis C virus genotype 1b from serum in patients receiving a triple treatment with telaprevir (MP-424), pegylated interferon and ribavirin for 12 weeks. *Hepatol. Res.*, **39**, 1056–1063.
15. Sakamoto, N. and Watanabe, M. (2009) New therapeutic approaches to hepatitis C virus. *J. Gastroenterol.*, **44**, 643–649.
16. Afdhal, N.H., McHutchison, J.G., Zeuzem, S., Mangia, A., Pawlotsky, J.M., Murray, J.S., Shianna, K.V., Tanaka, Y., Thomas, D.L., Booth, D.R. *et al.* (2010) Hepatitis C pharmacogenetics: state of the art in 2010. *Hepatology*, **53**, 336–345.
17. Tanaka, Y., Nishida, N., Sugiyama, M., Kurosaki, M., Matsuura, K., Sakamoto, N., Nakagawa, M., Korenaga, M., Hino, K., Hige, S. *et al.* (2009) Genome-wide association of IL28B with response to pegylated interferon-alpha and ribavirin therapy for chronic hepatitis C. *Nat. Genet.*, **41**, 1105–1109.
18. Ge, D., Fellay, J., Thompson, A.J., Simon, J.S., Shianna, K.V., Urban, T.J., Heinzen, E.L., Qiu, P., Bertelsen, A.H., Muir, A.J. *et al.* (2009) Genetic variation in IL28B predicts hepatitis C treatment-induced viral clearance. *Nature*, **461**, 399–401.
19. Suppiah, V., Moldovan, M., Ahlenstiel, G., Berg, T., Weltman, M., Abate, M.L., Bassendine, M., Spengler, U., Dore, G.J., Powell, E. *et al.* (2009) IL28B is associated with response to chronic hepatitis C interferon-alpha and ribavirin therapy. *Nat. Genet.*, **41**, 1100–1104.
20. Thomas, D.L., Thio, C.L., Martin, M.P., Qi, Y., Ge, D., O'Huigin, C., Kidd, J., Kidd, K., Khakoo, S.I., Alexander, G. *et al.* (2009) Genetic variation in IL28B and spontaneous clearance of hepatitis C virus. *Nature*, **461**, 798–801.
21. Tanaka, Y., Nishida, N., Sugiyama, M., Tokunaga, K. and 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.
22. Fellay, J., Thompson, A.J., Ge, D., Gumbs, C.E., Urban, T.J., Shianna, K.V., Little, L.D., Qiu, P., Bertelsen, A.H., Watson, M. *et al.* (2010) ITPA gene variants protect against anaemia in patients treated for chronic hepatitis C. *Nature*, **464**, 405–408.
23. Afdhal, N., McHutchison, J., Brown, R., Jacobson, I., Manns, M., Poordad, F., Weksler, B. and Esteban, R. (2008) Thrombocytopenia associated with chronic liver disease. *J. Hepatol.*, **48**, 1000–1007.
24. Wazny, L.D. and Ariano, R.E. (2000) Evaluation and management of drug-induced thrombocytopenia in the acutely ill patient. *Pharmacotherapy*, **20**, 292–307.
25. Hitomi, Y., Cirulli, E.T., Fellay, J., McHutchison, J.G., Thompson, A.J., Gumbs, C.E., Shianna, K.V., Urban, T.J. and Goldstein, D.B. (2011) Inosine triphosphate protects against ribavirin-induced adenosine triphosphate loss by adenylosuccinate synthase function. *Gastroenterology*, **140**, 1314–1321.
26. Ochi, H., Maekawa, T., Abe, H., Hayashida, Y., Nakano, R., Kubo, M., Tsunoda, T., Hayes, C.N., Kumada, H., Nakamura, Y. *et al.* (2010) ITPA polymorphism affects ribavirin-induced anemia and outcomes of therapy—a genome-wide study of Japanese HCV virus patients. *Gastroenterology*, **139**, 1190–1197.
27. Ong, J.P. and Younossi, Z.M. (2004) Managing the hematologic side effects of antiviral therapy for chronic hepatitis C: anemia, neutropenia, and thrombocytopenia. *Cleve. Clin. J. Med.*, **71** (Suppl. 3), S17–S21.
28. Canonico, P.G., Kastello, M.D., Cosgriff, T.M., Donovan, J.C., Ross, P.E., Spears, C.T. and Stephen, E.L. (1984) Hematological and bone marrow effects of ribavirin in rhesus monkeys. *Toxicol. Appl. Pharmacol.*, **74**, 163–172.
29. Akan, H., Guven, N., Aydogdu, I., Arat, M., Beksac, M. and Dalva, K. (2000) Thrombopoietic cytokines in patients with iron deficiency anemia with or without thrombocytosis. *Acta Haematol.*, **103**, 152–156.
30. Wu, J., Lei, G., Mei, M., Tang, Y. and Li, H. (2010) A novel C53/LZAP-interacting protein regulates stability of C53/LZAP and DDRGK domain-containing protein 1 (DDRGK1) and modulates NF-kappaB signaling. *J. Biol. Chem.*, **285**, 15126–15136.
31. Sakamoto, N., Tanaka, Y., Nakagawa, M., Yatsuhashi, H., Nishiguchi, S., Enomoto, N., Azuma, S., Nishimura-Sakurai, Y., Kakinuma, S., Nishida, N. *et al.* (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.
32. Kurosaki, M., Tanaka, Y., Tanaka, K., Suzuki, Y., Hoshioka, Y., Tamaki, N., Kato, T. and Yasui, Y. (2011) Analysis of the correlations between genetic polymorphisms of the ITPA gene and hemolytic anemia or outcome after treatment with pegylated-interferon and ribavirin in genotype 1b chronic hepatitis C. *Antivir. Ther.*, in press.
33. Shipkova, M., Lorenz, K., Oellerich, M., Wieland, E. and von Ahsen, N. (2006) Measurement of erythrocyte inosine triphosphate pyrophosphohydrolase (ITPA) activity by HPLC and correlation of ITPA genotype-phenotype in a Caucasian population. *Clin. Chem.*, **52**, 240–247.
34. Fraser, J.H., Meyers, H., Henderson, J.F., Brox, L.W. and McCoy, E.E. (1975) Individual variation in inosine triphosphate accumulation in human erythrocytes. *Clin. Biochem.*, **8**, 353–364.
35. Kumada, H., Okanoue, T., Onji, M., Moriwaki, H., Izumi, N., Tanaka, E., Chayama, K., Sakisaka, S., Takehara, T., Oketani, M. *et al.* (2010) Guidelines for the treatment of chronic hepatitis and cirrhosis due to hepatitis C virus infection for the fiscal year 2008 in Japan. *Hepatol. Res.*, **40**, 8–13.
36. Nishida, N., Tanabe, T., Takasu, M., Suyama, A. and Tokunaga, K. (2007) Further development of multiplex single nucleotide polymorphism typing method, the DigiTag2 assay. *Anal. Biochem.*, **364**, 78–85.

MAJOR ARTICLE

Association of Serum Cytokine Levels With Treatment Response to Pegylated Interferon and Ribavirin Therapy in Genotype 1 Chronic Hepatitis C Patients

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Background. We sought to clarify the associations among serum cytokines, amino acid substitutions in the interferon sensitivity–determining region (ISDR) and core region, and treatment outcome of pegylated interferon and ribavirin therapy in genotype 1 hepatitis C virus (HCV)-infected patients.

Methods. We quantified a total of 8 serum cytokines before, during, and after treatment in 79 genotype 1 chronic HCV patients. Viral ISDR and core region variants were determined by direct sequencing.

Results. High levels of interleukin (IL)-12 and IL-18 and more than 2 mutations in the ISDR were associated with a sustained virological response (SVR). Conversely, high baseline IL-10 levels and glutamine at amino acid 70 of the HCV core protein (Gln70) were significantly associated with a nonresponse to treatment, and patients with Gln70 had significantly higher IL-10 levels. In multivariate analysis, low IL-10, high IL-12, and high IL-18 levels were independently associated with an SVR. These 3 cytokine levels were decreased from baseline levels 4 weeks into treatment and remained low in patients with an SVR.

Conclusion. Serum IL-10, IL-12, and IL-18 levels are predictive of the response to HCV treatment with pegylated interferon and ribavirin and are associated with amino acid substitutions in the ISDR and core region.

Hepatitis C virus (HCV) infection is a major cause of chronic liver disease worldwide. More than half of patients with acute HCV infections develop chronic hepatitis, which leads to liver cirrhosis or hepatocellular carcinoma (HCC) in at least 20% of cases [1, 2]. HCC is ranked fourth in men and fifth in women as a cause of death from malignant neoplasms in Japan [3, 4]. Because approximately 70%–80% of Japanese HCC patients are infected with HCV, viral eradication is important to decrease the incidence of HCC. Interferon-based therapy can reduce HCV to undetectable levels and improve prognosis. The primary aim of antiviral therapy in HCV patients is a sustained virological response (SVR), which is defined as undetectable serum HCV RNA 24 weeks after completion of therapy. Despite recent advances, however, approximately 50% of patients with genotype 1 HCV infection do not achieve an SVR by antiviral therapy [5, 6].

Cytokines play an important role in the pathogenesis, progression, and treatment outcome of HCV infection. Because the control of cytokine production is highly complex and the effects of cytokines are widespread throughout multiple regulatory networks, it would seem that screening for multiple biomarkers could best clarify the immunopathogenesis of the disease and predict responses to antiviral therapy. However, such analysis is difficult using enzyme-linked immunosorbent assay, which requires each biomarker be tested individually. In

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this study, we used a new broad-spectrum bead-based multiplex immunoassay to simultaneously test multiple factors in the sera of patients with chronic hepatitis C. Wan et al recently reported that some cytokines are elevated in non-SVR HCV patients using this bead system, but only 17 patients with genotype 1 were evaluated [7]. Thus, the association between multiple cytokines and treatment outcome are largely unknown.

The objective of this study was to determine which cytokines in patients with genotype 1 chronic hepatitis C relate to the clinical and virologic characteristics of hepatitis and how they affect the HCV response to pegylated interferon (PEG-IFN) and ribavirin therapy.

PATIENTS AND METHODS

Participants

We included 79 consecutive patients with genotype 1 chronic hepatitis C in this study. We based diagnosis of chronic hepatitis C on the following criteria, as reported previously [8]: 1) presence of serum HCV antibodies and detectable viral RNA; 2) absence of detectable hepatitis B surface antigen; and 3) exclusion of other causes of chronic liver disease. No patient had a history of or developed decompensated cirrhosis or hepatocellular carcinoma. The baseline characteristics of patients are shown in Table 1. We used a group of 26 healthy individuals with normal transaminase levels and negative serologic results for hepatitis B and hepatitis C as the control. All participants were negative for the antibody to the human immunodeficiency virus. The protocol of this study was approved by the ethics committee of the Shinshu University School of Medicine, and all patients provided written informed consent.

Laboratory Testing

We measured antibodies to HCV in serum samples via third-generation enzyme-linked immunosorbent assays (EIA-3; Abbott Laboratories). We determined serum levels of HCV RNA using the COBAS AMPLICOR assays (Roche Diagnostic Systems), which amplify HCV RNA by reverse transcriptase–polymerase chain reaction. The lower limit of the assay was 50 IU/mL. We determined HCV genotypes using INNO-LiPA HCV II (Innogenetics). We found that all patients in our test cohort were infected with genotype 1b. We performed alanine aminotransferase (ALT), aspartate aminotransferase (AST), and other relevant biochemical tests using standard methods [9].

Antiviral Therapy

All patients received body weight–adjusted PEG-IFNα-2b (PegIntron, Schering-Plough K.K.; ≤45 kg, 60 μg/dose; 46–60 kg, 80 μg/dose; 61–75 kg, 100 μg/dose; 76–90 kg, 120 μg/dose; ≥ 91 kg, 150 μg/dose), and ribavirin (Rebetol, Schering-Plough K.K.; ≤60 kg, 600 mg/day; 61 kg–80 kg, 800 mg/day; ≥81 kg, 1000 mg/day) for 48 weeks, as reported previously [10].

Definition of Viral Kinetic Response and Treatment Outcome

An early virological response (EVR) was defined as undetectable serum HCV RNA by 12 weeks of therapy. An SVR was classified as serum HCV RNA that was undetectable 24 weeks after completing therapy. Post-treatment relapse was defined as a reappearance of serum HCV RNA after treatment in patients whose HCV RNA level was undetectable during or at the completion of therapy. A nonresponse was defined as a decrease in HCV RNA of <2 log copies/mL at week 12 and detectable HCV RNA during the treatment course.

Detection of Amino Acid Substitutions in the Core and NS5A Regions

We determined the sequence of 1–191 amino acids (aa) in the core protein of genotype 1b HCV, and we evaluated substitutions at aa70 of arginine (Arg70) or glutamine (Gln70) [11] with the use of HCV-J as a reference [12]. We also determined the sequence of 2209–2248 aa in the NS5A region of genotype 1b HCV containing the interferon sensitivity–determining region (ISDR), and the number of aa substitutions in the ISDR was defined as wild-type (0), intermediate-type (1), or mutant-type

Table 1. Demographic and Clinical Characteristics of Patients with Hepatitis C Virus Infection

Characteristics	All (n = 79)	SVR (n = 31)	Non-SVR (n = 48)	P
Median age, y (range)	60 (17–74)	56 (28–72)	61 (17–74)	0.08
Male, n (%)	40 (51)	23 (74)	17 (35)	0.001
Median values (range)				
ALT, IU/L (range)	54 (22–389)	53 (24–172)	61 (22–389)	0.25
AST, IU/L (range)	44 (20–288)	36 (21–133)	48 (20–288)	0.012
HCV RNA, 10^5 IU/mL(range)	17 (1.1–51)	15 (1.1–50)	19 (2.2–51)	0.13
Substitutions				
Core aa 70(Arg70/Gln70)	47/28	22/6	25/22	0.028
ISDR of NS5A(wild/intermediate/mutant)	46/17/13	13/7/9	33/10/4	0.026

NOTE. HCV, hepatitis C virus; SVR, sustained virological response; AST, aspartate aminotransferase; ALT, alanine aminotransferase; aa, amino acid; ISDR, interferon sensitivity–determining region.

(≥2) [13]. We determined all aa substitutions in the core region and ISDR by direct sequencing.

Detection of Cytokines

We quantified 8 cytokines (interleukin [IL]-2, IL-4, IL-6, IL-10, IL-12p40, IL-12p70, IL-18, and vascular endothelial growth factor [VEGF]) using Luminex Multiplex Cytokine Kits (Procarta Cytokine assay kit) for serum samples obtained before the start of treatment, 4 weeks after the start of treatment, and 24 weeks after treatment completion. All collected samples were immediately stored at −70°C and remained in storage until testing.

Statistical Analysis

We used the Mann–Whitney *U* test and Kruskal–Wallis test to analyze continuous variables where appropriate. We used the Friedman test to evaluate changes in serum cytokine levels over time. We used the Spearman rank correlations to evaluate the relationship between pairs of markers. We used the χ^2 test with the Yates correction for the analysis of categorical data. In cases where the number of participants was <5, we used the Fisher exact test. We considered a *P* value of ≤.05 statistically significant. To predict treatment outcome, cutoff points for continuous variables were decided by receiver-operating characteristic (ROC) curve analysis. Multivariate analysis was performed using a stepwise logistic regression model. Statistical analyses were performed using SPSS software version 18.0J.

RESULTS

Detection and Quantification of Serum Markers in Patients with Chronic Hepatitis C and Controls

Of the 79 patients receiving PEG-IFN and ribavirin therapy, 31 (39%) were sustained responders with accompanying normalization of ALT levels. Of the 48 patients without an SVR, 23 had a relapse and 25 did not respond to treatment. Patients with an SVR had a higher male ratio compared with patients without ($P = .001$) (Table 1). Before treatment, the median AST level in the SVR group was significantly lower than that in the non-SVR group (36 vs 48 IU/L; $P = .012$). Substitutions of aa 70 in the core region ($P = .028$) and in the ISDR ($P = .026$) were both significantly associated with treatment outcome.

Serum samples obtained prior to antiviral therapy were examined for the presence of 8 cytokines by multiplex assays. Of these, 6 could be reliably quantified in a large majority of samples. As shown in Figure 1, the median baseline serum concentrations of 4 cytokines [IL-10 (4.8 vs 4.3 pg/mL; $P = .032$), IL-12p40 (20.4 vs 8.5 pg/mL; $P < .001$), IL-12p70 (12.8 vs 1.0 pg/mL; $P < .001$), and IL-18 (21.9 vs 14.5 pg/mL; $P = .008$)] were significantly higher in patients with HCV infection than in healthy controls. Conversely, serum levels of IL-4 (7.3 vs 7.9 pg/mL; $P = .011$) and VEGF (57.5 vs 78.0 pg/mL; $P = .025$) were significantly lower in patients with HCV infection compared with those in controls.

Effects of Antiviral Therapy on Serum Cytokine Levels

The median baseline serum levels of 4 cytokines (IL-12p40 [24.1 vs 17.2 pg/mL; $P = .003$], IL-12p70 [15.9 vs 12.6 pg/mL; $P < .001$], IL-18 [27.9 vs 17.7 pg/mL; $P = .001$], and VEGF [93.0 vs 39.7 pg/mL; $P < .001$]) were significantly higher in patients who achieved an SVR than in those who did not (Figure 2). In contrast, SVR patients showed significantly lower baseline IL-10 concentrations (4.1 pg/mL) than non-SVR patients (7.3 pg/mL; $P = .002$).

Significantly higher baseline levels of 3 cytokines (IL-4 [7.8 vs 7.0 pg/mL; $P = .001$], IL-12p40 [24.1 vs 14.6 pg/mL; $P < .001$], and VEGF [65.5 vs 43.0 pg/mL; $P = .025$]) were observed in patients with a virological response compared with levels in those without. Conversely, IL-10 levels (4.3 vs 7.9 pg/mL; $P < .001$) were significantly lower in virological responders compared with that in nonresponders.

Several demographic (age and sex) and clinical (ALT level, AST level, and viral load) findings were examined for their correlation with serum cytokines in patients with HCV infection, but no significant associations were observed. However, serum IL-12p40 levels were significantly correlated with serum IL-18 ($P = .004$, $r = 0.325$) (Figure 3A) and VEGF ($P = .024$, $r = 0.253$) (Figure 3B). There was also a significant correlation between IL-18 and VEGF ($P < .001$, $r = 0.394$) (Figure 3C).

Prediction of Treatment Outcome in Patients with Chronic Hepatitis C

We performed ROC curve analyses to determine the optimal cutoff values for serum cytokines in predicting treatment outcome for genotype 1 HCV–infected patients. We obtained the ROC curve for serum IL-10 via calculations using the values obtained from 25 nonresponders and 54 patients with a virological response. The ROC curves for serum IL-12p40, IL-18, and VEGF were obtained from 31 patients who achieved an SVR and 48 non-SVR patients. We selected optimal cutoff point values based on the cytokine level at which accuracy was maximal. The optimal cutoff value, sensitivity, specificity, positive predictive value, negative predictive value, and calculated area under the curve (AUC) for the 4 cytokines are listed in Table 2. The AUC values were consistently high and ranged between.70 (IL-12p40) and.86 (IL-10).

In addition, ROC curves for serum IL-10, IL-12p40, IL-18, and VEGF at 4 weeks after the start of treatment were obtained (Table 2). The AUCs for these 4 cytokines (.62–.86) were also high, but lower than those at baseline.

Correlation Between Core Region and Interferon Sensitivity–Determining Region Amino Acid Substitutions and Cytokine Production.

Because core region and ISDR substitutions have been associated with treatment outcome both in this study and elsewhere, we analyzed whether substitutions in these regions were correlated with baseline serum cytokine concentrations as

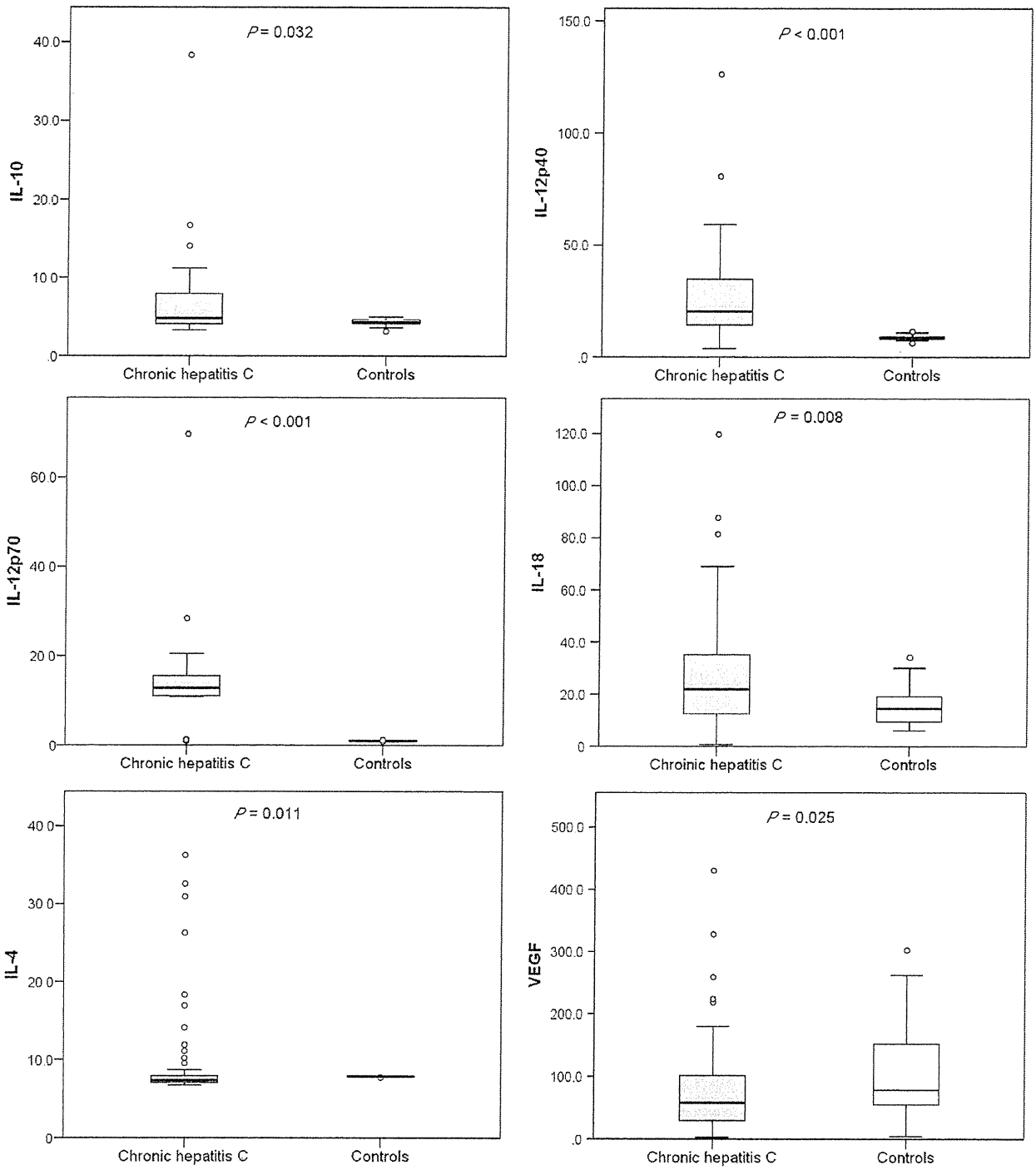

Figure 1. Detection of Serum Cytokines in Patients with HCV Infection and Healthy Subjects. Boxes represent the interquartile range of the data. The lines across the boxes indicate the median values. The hash marks above and below the boxes indicate the 90th and 10th percentiles for each group, respectively. Serum IL-10, IL-12p40, IL-12p70, IL-18, IL-4, and VEGF levels were detected in 79 patients with HCV infection and 26 controls. **NOTE.** HCV, hepatitis C virus; IL, interleukin; VEGF, vascular endothelial growth factor.

well. Before treatment, median IL-10 levels in patients with Gln70 (7.5 pg/mL) were significantly higher than those in patients with Arg70 (4.3 pg/mL; $P = .045$). The prevalence of higher serum IL-10 (≥5.0 pg/mL at baseline) was significantly greater in the nonresponse group than in the response group (25 of 25 patients [100%] vs 11 of 50 [22%]; $P < .001$). The frequencies of the combination of higher IL-10 and HCV with and without core Gln70 were 14 of 25 patients (56%) and 3 of 50

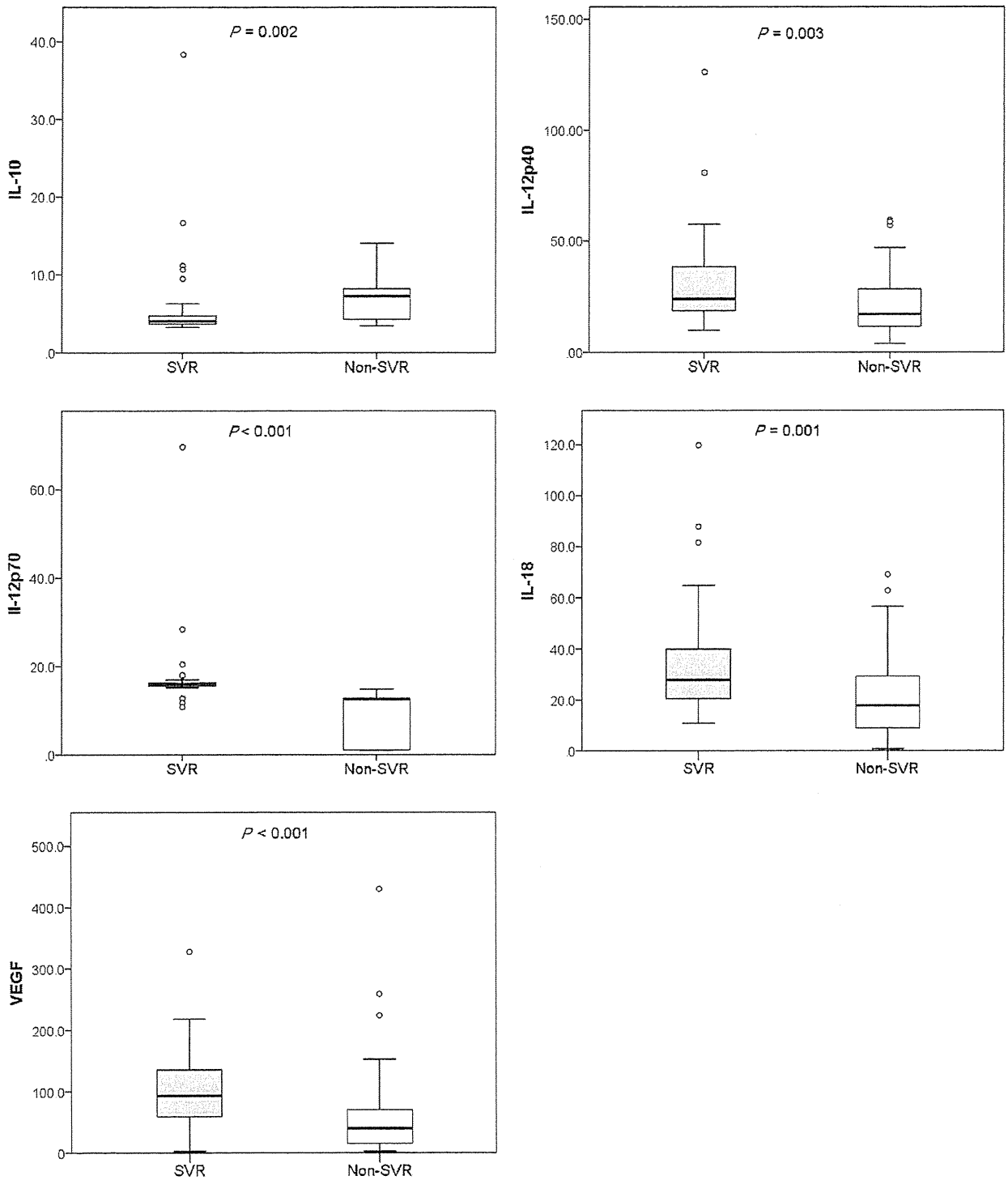

Figure 2. Serum Cytokines Related to Antiviral Therapy Outcome. Boxes represent the interquartile range of the data. The lines across the boxes indicate the median values. The hash marks above and below the boxes indicate the 90th and 10th percentiles for each group, respectively. (A) Serum IL-10, IL-12p40, IL-12p70, IL-18, and VEGF were detected in 31 patients who achieved a sustained virological response and 48 patients who did not. **NOTE.** SVR, sustained virological response; IL, interleukin; VEGF, vascular endothelial growth factor.