

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-IFN α -2b (PegIntron, Schering-Plough K.K.; \leq 45 kg, 60 μ g/dose; 46–60 kg, 80 μ g/dose; 61–75 kg, 100 μ g/dose; 76–90 kg, 120 μ g/dose; \geq 91 kg, 150 μ g/dose), and ribavirin (Rebetol, Schering-Plough K.K.; \leq 60 kg, 600 mg/day; 61 kg–80 kg, 800 mg/day; \geq 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 re-appearance 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 (<i>n</i> = 79)	SVR (<i>n</i> = 31)	Non-SVR (<i>n</i> = 48)	<i>P</i>
Median age, y (range)	60 (17–74)	56 (28–72)	61 (17–74)	0.08
Male, <i>n</i> (%)	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 $\leq .05$ 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

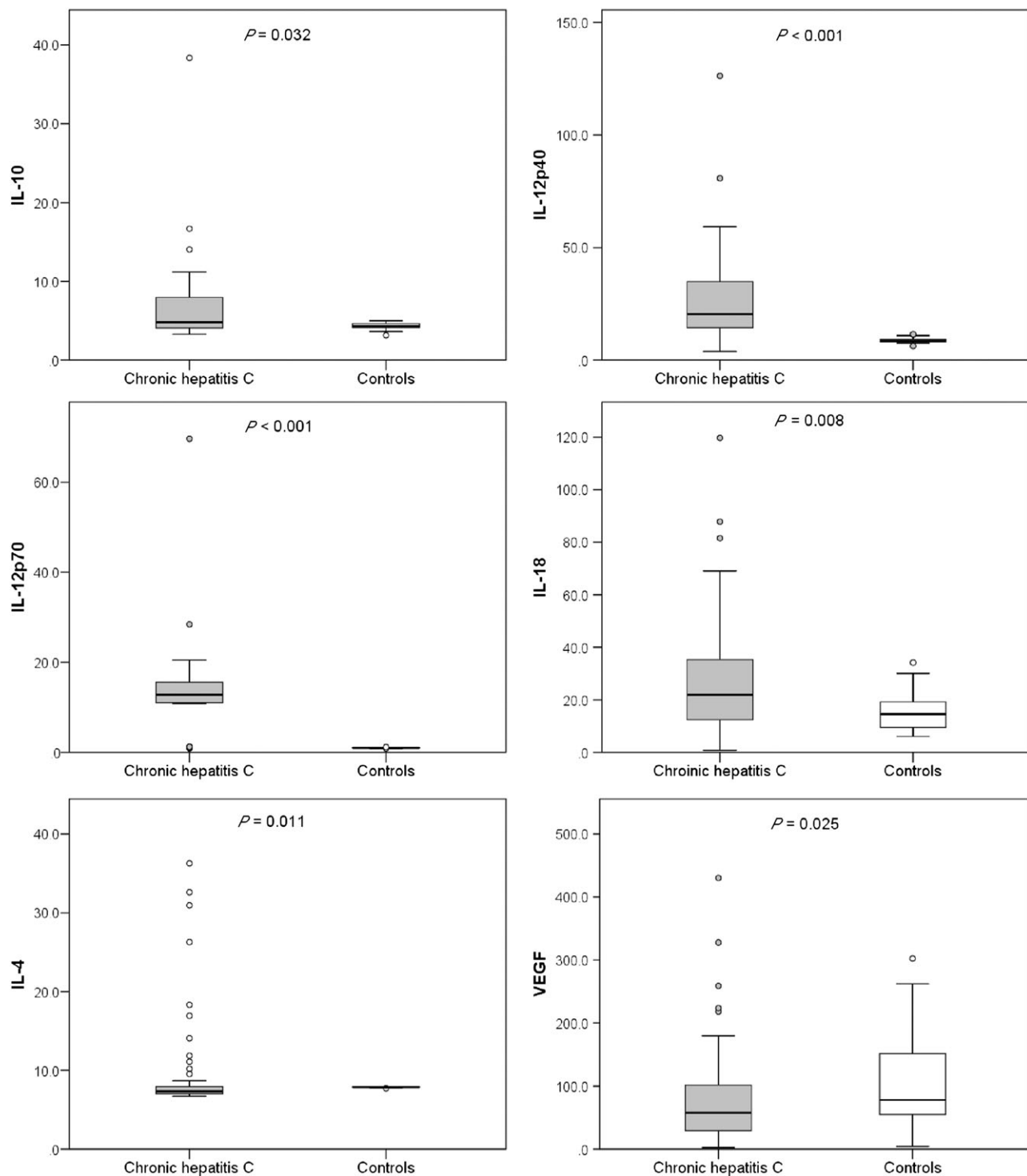


Figure 1. Detection of Serum Cytokines in Patients with HCV Infection and Healthy Subjects. Boxes represent the interquartile range of the data. The lines across the boxes indicate the median values. The hash marks above and below the boxes indicate the 90th and 10th percentiles for each group, respectively. 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

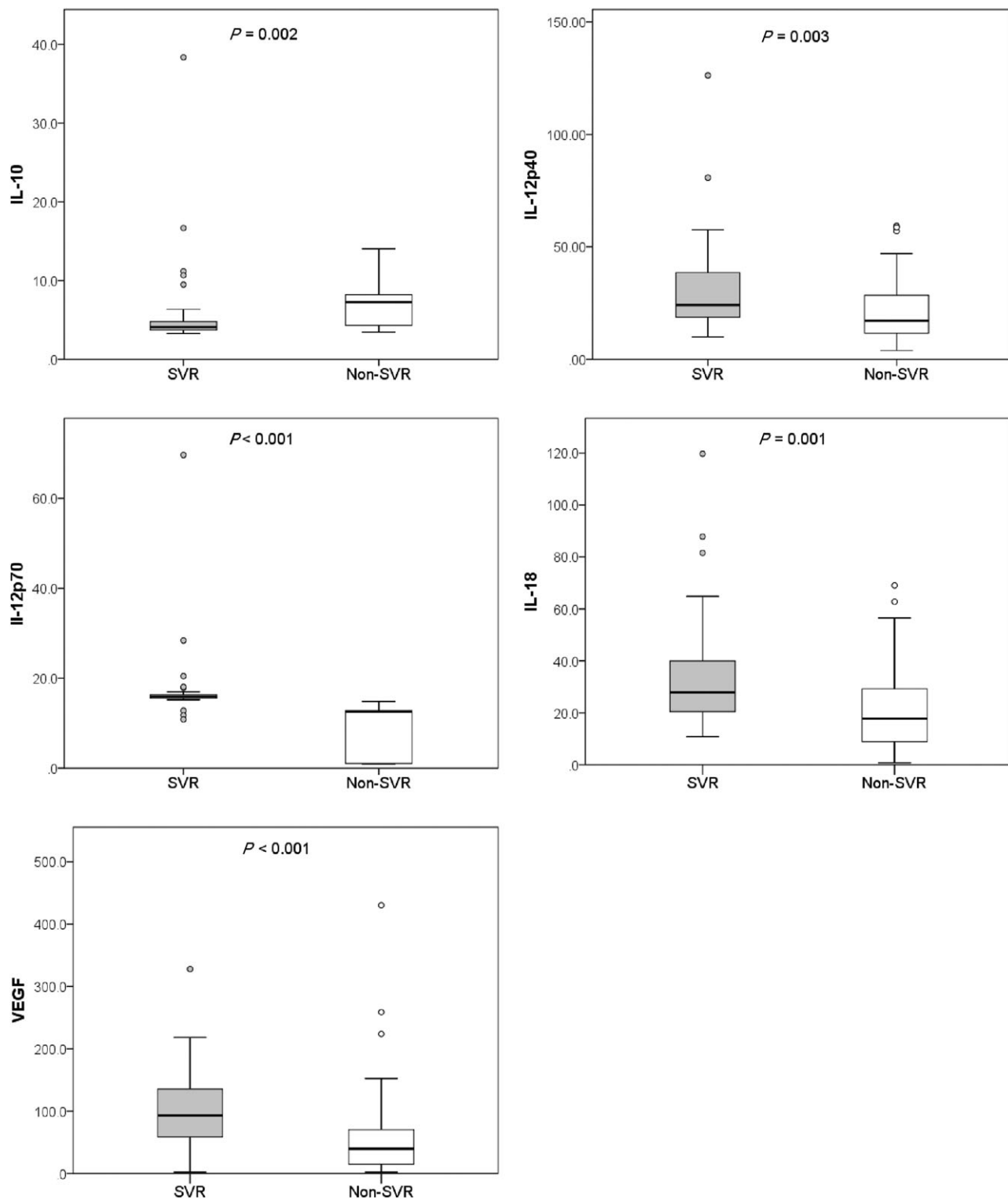


Figure 2. Serum Cytokines Related to Antiviral Therapy Outcome. Boxes represent the interquartile range of the data. The lines across the boxes indicate the median values. The hash marks above and below the boxes indicate the 90th and 10th percentiles for each group, respectively. (A) Serum IL-10, IL-12p40, 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.

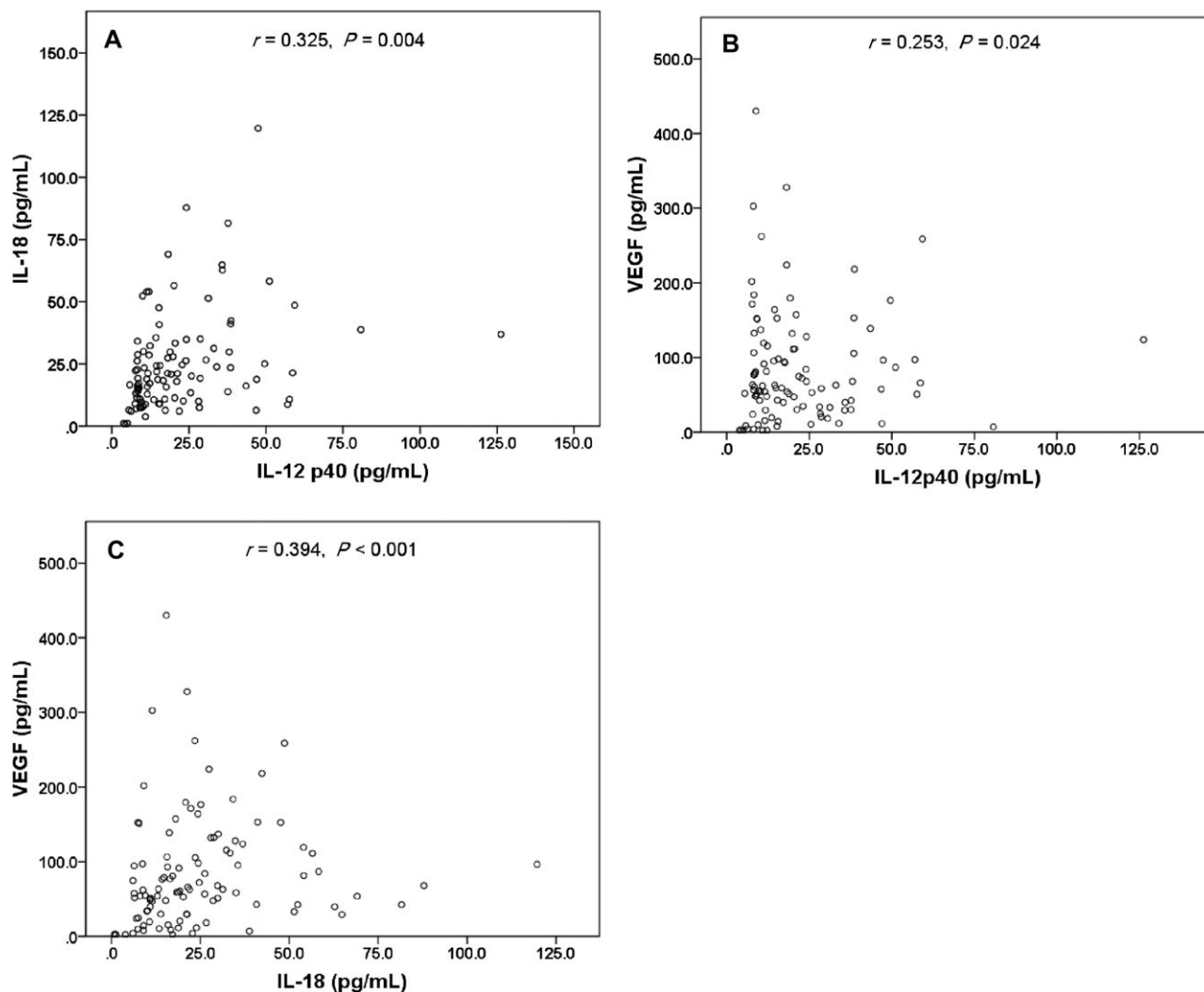


Figure 3. Correlation Between Serum Cytokines in 79 Patients with HCV Infection. (A–B) Serum IL-12p40 was significantly correlated with the level of (A) IL-18 ($r = .325$; $P = .004$) and (B) VEGF ($r = .253$; $P = .024$). (C) Serum IL-18 was correlated with the level of VEGF ($r = .394$; $P < .001$). **NOTE.** HCV, hepatitis C virus; IL, interleukin; VEGF, vascular endothelial growth factor.

Table 2. Optimal Cutoff Value, Sensitivity, Specificity, Area Under The Curve, and Predictive Values of Serum IL-10, IL-12p40, IL-18, and VEGF at Baseline and After 4 Weeks of Treatment in 79 Patients with Chronic Hepatitis C

Cytokine	Collection Time	Cutoff Value	Sensitivity (%) (95% CI)	Specificity (%) (95% CI)	AUC (95% CI)	PPV (%)	NPV (%)
IL-10	baseline	5.0	100 (86–100)	80 (67–89)	.86 (.84–.98)	69	100
	4 wk	6.8	82 (69–91)	100 (86–100)	.86 (.78–.95)	100	71
IL-12p40	baseline	17.4	81 (63–93)	52 (37–67)	.70 (.59–.82)	52	81
	4 wk	21.3	81 (63–93)	60 (45–74)	.69 (.57–.81)	57	83
IL-18	baseline	15.4	97 (83–100)	46 (31–61)	.72 (.61–.83)	54	96
	4 wk	24.6	87 (70–96)	42 (28–57)	.62 (.50–.75)	49	83
VEGF	baseline	57.6	77 (59–90)	69 (54–81)	.74 (.63–.86)	62	83
	4 wk	62.6	74 (55–88)	67 (52–80)	.70 (.58–.82)	59	80

NOTE. CI, confidence interval; AUC, area under the curve; PPV, positive predictive value; NPV, negative predictive value; IL, interleukin

All AUC values were significantly higher than a 0.50 nonpredictive value ($P < .01$ for all comparisons). Cutoff values were determined by constructing receiver operating characteristic curves and are expressed as pg/mL. IL-10 is predictive of a nonresponse. IL-12p40, IL-18, and VEGF are predictive of a sustained virological response.

Table 3. Multivariate Analysis of Factors Independently Associated with a Sustained Virological Response to Pegylated Interferon and Ribavirin Therapy in Patients Infected with Hepatitis C Virus Genotype 1

Factors	OR	95% CI	P
Gender: male	10.932	2.178–54.780	.004
AST \geq 40 IU/L	.946	.906–.989	.013
IL-10 \geq 5.0 pg/mL	.823	.704–.962	.014
IL-12p40 \geq 17.4 pg/mL	1.071	1.009–1.137	.024
IL-18 \geq 15.4 pg/mL	1.085	1.024–1.150	.006

NOTE. OR, odds ratio; CI, confidence interval; AST, aspartate amino-transferase; IL, interleukin.

Only variables that achieved statistical significance ($P < .05$) in multivariate logistic regression analysis are shown.

patients (6%), respectively, which was statistically significant ($P < .001$).

Serum levels of IL-12p70 were significantly correlated with the number of substitutions in the ISDR (Kruskal–Wallis; $P = .027$). In addition, median baseline serum IL-12p70 levels were significantly higher in patients with mutant-type ISDR than in those with wild or intermediate types (15.6 vs 12.7 pg/mL; $P = .009$).

Factors Independently Associated with a Sustained Virological Response

We evaluated several factors found in association with an SVR from PEG-IFN and ribavirin therapy for their independence by multivariate analysis (Table 3). Male (odds ratio 10.93 [95% confidence interval 2.18–54.87], $P = .004$), AST \geq 40 IU/L (.95 [.91–.99], $P = .013$), IL-10 \geq 5.0 pg/mL (.82 [.70–.96], $P = .014$), IL-12p40 \geq 17.4 pg/mL (1.07 [1.01–1.14], $P = .024$), and IL-18 \geq 15.4 pg/mL (1.09 [1.02–1.15], $P = .006$) were independent risk factors related to an SVR. Conversely, core region or ISDR substitutions were not significant independent associations in this study.

Serum Cytokine Changes During and After Treatment

We next measured cytokine levels 4 weeks after the initiation of therapy and 6 months after its completion (Table 4). The levels of IL-10 ($P < .001$, Friedman test), IL-12p40 ($P = .008$), and

IL-18 ($P < .001$) were significantly decreased in samples collected from patients who achieved an SVR. The reduction in serum cytokine levels from baseline to 4 weeks of treatment was determined and compared between SVR and non-SVR groups, and showed that the ratio of IL-10 had a significant negative association with both an EVR ($P = .024$) and an SVR ($P = .001$).

DISCUSSION

In this study, we measured the levels of 8 cytokines in patients with genotype 1 chronic hepatitis C and analyzed their association with the outcome of PEG-IFN and ribavirin therapy using a newly developed bead-array multiplex system. Serum IL-10, IL-12p40, IL-12p70, and IL-18 were higher in patients with HCV infection than in healthy participants. In addition, cytokines IL-10, IL-12p40, and IL-18 all decreased during treatment and remained low in patients with an SVR. These findings suggest that cytokines may in fact compromise host immune responses to the virus.

A strong association between high baseline serum IL-10 and a nonresponse to PEG - IFN and ribavirin therapy was found in our cohort, which is consistent with previous studies [7, 14, 15]. We found achievement of an EVR or SVR to be diminished in patients who had a lower IL-10 ratio between baseline and 4 weeks of treatment. In addition, using ROC curve analysis, we found sensitivity, specificity, and AUC were all high for IL-10, suggesting that serum IL-10 values at baseline and 4 weeks of treatment are predictive markers for treatment nonresponse (Table 2). Although humoral immunity is said to play a minor role in recovery from HCV infection and B-cell immunity is strongest in those with persistent infection [8, 16], a strong natural killer cell-mediated and Th1 cell-mediated immune response seems to be a key factor in protection from HCV infection. IL-10 was originally described as a cytokine synthesis inhibitory factor [17, 18], but recent studies have demonstrated that IL-10 produced by Th17 cells restrains the pathologic effects of Th17 [19, 20]. Furthermore, there is strong evidence of a substantial genetic component to IL-10 production [21, 22]; the -1082 G/G genotype is known to be related to increased IL-

Table 4. Serum Cytokine Levels Changes During and After Treatment of Pegylated Interferon Plus Ribavirin

Cytokines	Treatment Outcome	Baseline	Week 4	Week 72	P
IL-10	SVR	4.1 (3.3–25.4)	3.7 (3.1–19.9)	3.5 (2.9–9.0)	< .001
	Non-SVR	7.3 (3.7–10.8)	7.5 (3.9–8.8)	7.4 (3.9–10.9)	0.962
IL-12p40	SVR	24.1 (11.3–99.0)	22.1 (11.6–75.2)	18.4 (7.8–76.5)	0.008
	Non-SVR	17.2 (4.6–57.9)	19.2 (8.1–50.1)	21.6 (5.8–77.0)	0.281
IL-18	SVR	27.9 (13.8–100.6)	25.1 (13.2–95.2)	23.3 (6.6–48.5)	< .001
	Non-SVR	17.7 (1.1–59.9)	31.3 (10.3–90.6)	17.4 (5.4–52.0)	< .001

Data are median (5th–95th percentile) values.

Abbreviations: IL, interleukin; SVR, sustained virological response.

10 production and is associated with a high risk of inefficient HCV clearance [23, 24] and resistance to IFN treatment [25–28].

In agreement with our findings, recent studies have indicated that Gln70 substitutions in the HCV core region are associated with treatment failure [11, 29–32]. Additionally, patients with Gln70 had higher IL-10 levels compared with those with Arg70. Among the 28 HCV patients who had Gln70, all 14 non-responders had higher IL-10 (≥ 5.0 pg/mL), whereas 11 of 14 responders had lower IL-10 levels ($P < .001$). This association between Gln70 and elevated IL-10 levels is intriguing. Dolganiuc et al reported that HCV core and NS3 proteins in monocytes and dendritic cells induce IL-10 [33], so further studies are needed to clarify the relationship between IL-10 and core region amino acid substitutions.

This report demonstrates the beneficial role of IL-12 in achieving an SVR during PEG-IFN and ribavirin therapy. IL-12 is a proinflammatory cytokine that promotes the differentiation of Th1 cells, suppresses Th2 function, and amplifies the cytotoxicity of cytotoxic T lymphocytes and natural killer cells [34]. Thus, production of IL-12 is directed toward the elimination of intracellular pathogens and viruses. Elevated serum IL-12 has been noted in patients with chronic HBV or HCV infection, and is even more prominent among responders to IFN- α treatment [35, 36]. In our study, we noted significantly higher serum IL-12p70 in participants carrying mutant-type ISDR than in those with intermediate- or wild-type ISDR. This correlation between IL-12 and ISDR substitutions is striking and requires further study to verify its favorable effect during PEG-IFN and ribavirin therapy.

It is believed that the dynamics of the Th1/Th2 response determine the outcome of antiviral therapy to chronic hepatitis C [10] and that IL-18 is an important mediator of the Th1/Th2 balance. IL-18 plays a critical role in host defense against infection by intracellular microbes but also induces autoimmune diseases and propagates inflammation [37]. IL-18 is significantly upregulated in patients with chronic HCV infection and is correlated with hepatic injury [38, 39], indicating a key role in disease pathogenesis. However, the effect of IL-18 on antiviral therapy for chronic hepatitis C is still unclear. We found that IL-18 levels were significantly higher in patients with chronic HCV infection compared with healthy controls, but they were also higher at baseline in patients who achieved an SVR than in those who did not. In addition, there was a significant correlation between IL-18 and IL-12; in the presence of IL-12, IL-18 stimulates *IFNG* expression, thus promoting the Th1-mediated immune response. Without IL-12, IL-18 stimulates Th2 responses [37]. In this study, because serum IFN- γ levels were below detection thresholds, we could not assess the association of such cytokines.

Lastly, we observed that pretreatment serum VEGF levels were associated with an SVR. A previous study showed no association between baseline VEGF and treatment outcome, but only 36

patients, including 19 with genotype 1, were studied [40]. Hence, it is still unclear if this angiogenesis marker plays a critical role in response to antiviral therapy in chronic HCV infection. Furthermore, we correlated VEGF with IL-12 and IL-18 in our study. In particular, IL-18 enhances the production of VEGF in rheumatoid arthritis synovial fibroblasts, suggesting that IL-18 could be an angiogenic mediator with triggering effects on VEGF production [41]. Although the preoperative serum VEGF level was found to be a significant predictor of tumor recurrence and overall survival in patients with HCC [42], there have been no reports regarding treatment response in patients with chronic hepatitis C during antiviral therapy.

In multivariate analysis of our cohort, low IL-10, high IL-12p40, and high IL-18 were independent factors related to an SVR in patients treated with PEG-IFN and ribavirin. Our results indicate that such 3-cytokine profiling may offer clinicians another tool in predicting treatment outcome of HCV infection. Further investigation must be done in vitro and using many samples to validate the significance of our findings.

In conclusion, several cytokines were seen to be elevated in patients with chronic hepatitis C using the multiplex bead assay. Serum IL-10 levels and amino acid substitutions at the 70 aa core region of HCV are useful for predicting a nonresponse to PEG-IFN and ribavirin therapy in patients with chronic hepatitis C genotype 1. A higher level of serum IL-12 is considered to be favorable for response to antiviral therapy, and is correlated with substitutions in the ISDR. Lastly, IL-18 is notably high in patients with chronic HCV infection, and is correlated with IL-12.

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References

- Alter HJ, Purcell RH, Shih JW, et al. Detection of antibody to hepatitis C virus in prospectively followed transfusion recipients with acute and chronic non-A, non-B hepatitis. *N Engl J Med* **1989**; *321*:1494–500.
- 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.
- Umemura T, Kiyosawa K. Epidemiology of hepatocellular carcinoma in Japan. *Hepatol Res* **2007**; *37*:S95–S100.
- Umemura T, Ichijo T, Yoshizawa K, Tanaka E, Kiyosawa K. Epidemiology of hepatocellular carcinoma in Japan. *J Gastroenterol* **2009**; *44*:102–7.

5. 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.
6. 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.
7. Wan L, Kung YJ, Lin YJ, et al. Th1 and Th2 cytokines are elevated in HCV-infected SVR(-) patients treated with interferon- α . *Biochem Biophys Res Commun* **2009**; 379:855–60.
8. Umemura T, Wang RY, Schechterly C, Shih JW, Kiyosawa K, Alter HJ. Quantitative analysis of anti-hepatitis C virus antibody-secreting B cells in patients with chronic hepatitis C. *Hepatology* **2006**; 43:91–9.
9. Umemura T, Zen Y, Hamano H, Kawa S, Nakanuma Y, Kiyosawa K. Immunoglobulin G4-hepatopathy: Association of immunoglobulin G4-bearing plasma cells in liver with autoimmune pancreatitis. *Hepatology* **2007**; 46:463–71.
10. Shirakawa H, Matsumoto A, Yoshita 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.
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. 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.
13. 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.
14. Kuzushita N, Hayashi N, Katayama K, et al. High levels of serum interleukin-10 are associated with a poor response to interferon treatment in patients with chronic hepatitis C. *Scand J Gastroenterol* **1997**; 32:169–74.
15. Marín-Serrano E, Rodríguez-Ramos C, Díaz F, Martín-Herrera L, Girón-González 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**; 13:230–4.
16. Takaki A, Wiese M, Maertens G, et al. Cellular immune responses persist and humoral responses decrease two decades after recovery from a single-source outbreak of hepatitis C. *Nat Med* **2000**; 6:578–82.
17. Fiorentino DF, Bond MW, Mosmann TR. Two types of mouse T helper cell. IV. Th2 clones secrete a factor that inhibits cytokine production by Th1 clones. *J Exp Med* **1989**; 170:2081–95.
18. Fiorentino DF, Zlotnik A, Vieira P, et al. IL-10 acts on the antigen-presenting cell to inhibit cytokine production by Th1 cells. *J Immunol* **1991**; 146:3444–51.
19. McGeachy MJ, Bak-Jensen KS, Chen Y, et al. TGF- β and IL-6 drive the production of IL-17 and IL-10 by T cells and restrain T(H)-17 cell-mediated pathology. *Nat Immunol* **2007**; 8:1390–7.
20. Stumhofer JS, Silver JS, Laurence A, et al. Interleukins 27 and 6 induce STAT3-mediated T cell production of interleukin 10. *Nat Immunol* **2007**; 8:1363–71.
21. Crawley E, Kon S, Woo P. Hereditary predisposition to low interleukin-10 production in children with extended oligoarticular juvenile idiopathic arthritis. *Rheumatology (Oxford)* **2001**; 40:574–8.
22. Reuss E, Fimmers R, Kruger A, Becker C, Rittner C, Höhler T. Differential regulation of interleukin-10 production by genetic and environmental factors—a twin study. *Genes Immun* **2002**; 3:407–13.
23. Oleksyk TK, Thio CL, Truelove AL, et al. Single nucleotide polymorphisms and haplotypes in the IL10 region associated with HCV clearance. *Genes Immun* **2005**; 6:347–57.
24. Paladino N, Fainboim H, Theiler G, et al. Gender susceptibility to chronic hepatitis C virus infection associated with interleukin 10 promoter polymorphism. *J Virol* **2006**; 80:9144–50.
25. Edwards-Smith CJ, Jonsson JR, Purdie DM, Bansal A, Shorthouse C, Powell EE. Interleukin-10 promoter polymorphism predicts initial response of chronic hepatitis C to interferon alfa. *Hepatology* **1999**; 30:526–30.
26. Yee LJ, Tang J, Gibson AW, Kimberly R, Van Leeuwen DJ, Kaslow RA. Interleukin 10 polymorphisms as predictors of sustained response in antiviral therapy for chronic hepatitis C infection. *Hepatology* **2001**; 33:708–12.
27. Knapp S, Hennig BJ, Frodsham AJ, et al. Interleukin-10 promoter polymorphisms and the outcome of hepatitis C virus infection. *Immunogenetics* **2003**; 55:362–9.
28. Morgan TR, Lambrecht RW, Bonkovsky HL, et al. DNA polymorphisms and response to treatment in patients with chronic hepatitis C: Results from the HALT-C trial. *J Hepatol* **2008**; 49:548–56.
29. Mori N, Imamura M, Kawakami Y, et al. Randomized trial of high-dose interferon-alpha-2b combined with ribavirin in patients with chronic hepatitis C: Correlation between amino acid substitutions in the core/NS5A region and virological response to interferon therapy. *J Med Virol* **2009**; 81:640–9.
30. Kurbanov F, Tanaka Y, Matsuura K, et al. Positive selection of core 70Q variant genotype 1b hepatitis C virus strains induced by pegylated interferon and ribavirin. *J Infect Dis* **2010**; 201:1663–71.
31. Nakagawa M, Sakamoto N, Ueyama M, et al. Mutations in the interferon sensitivity determining region and virological response to combination therapy with pegylated-interferon alpha 2b plus ribavirin in patients with chronic hepatitis C-1b infection. *J Gastroenterol* **2010**; 45:656–65.
32. Hayashi K, Katano Y, Ishigami M, et al. Mutations in the core and NS5A region of hepatitis C virus genotype 1b and correlation with response to pegylated-interferon-alpha 2b and ribavirin combination therapy [published online ahead of print 28 March 2010]. *J Viral Hepat*. doi: 10.1111/j.1365-2893.2010.01305.x.
33. Dolganiuc A, Kodys K, Kopasz A, et al. Hepatitis C virus core and nonstructural protein 3 proteins induce pro- and anti-inflammatory cytokines and inhibit dendritic cell differentiation. *J Immunol* **2003**; 170:5615–24.
34. Trinchieri G. Interleukin-12: A proinflammatory cytokine with immunoregulatory functions that bridge innate resistance and antigen-specific adaptive immunity. *Annu Rev Immunol* **1995**; 13:251–76.
35. 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**; 99:3025–33.
36. Quiroga JA, Martín J, Navas S, Carreño V. Induction of interleukin-12 production in chronic hepatitis C virus infection correlates with the hepatocellular damage. *J Infect Dis* **1998**; 178:247–51.
37. Nakanishi K, Yoshimoto T, Tsutsui H, Okamura H. Interleukin-18 regulates both Th1 and Th2 responses. *Annu Rev Immunol* **2001**; 19:423–74.
38. Loffreda S, Muratori P, Muratori L, Mele L, Bianchi FB, Lenzi M. Enhanced monocyte Th1 cytokine production in HCV-infected cryoglobulinemic patients. *J Hepatol* **2003**; 38:230–6.
39. Schvoerer E, Navas MC, Thumann C, et al. Production of interleukin-18 and interleukin-12 in patients suffering from chronic hepatitis C virus infection before antiviral therapy. *J Med Virol* **2003**; 70:588–93.
40. Salcedo X, Medina J, Sanz-Cameno P, et al. The potential of angiogenesis soluble markers in chronic hepatitis C. *Hepatology* **2005**; 42:696–701.
41. Cho ML, Jung YO, Moon YM, et al. Interleukin-18 induces the production of vascular endothelial growth factor (VEGF) in rheumatoid arthritis synovial fibroblasts via AP-1-dependent pathways. *Immunol Lett* **2006**; 103:159–66.
42. Chao Y, Li CP, Chau GY, et al. Prognostic significance of vascular endothelial growth factor, basic fibroblast growth factor, and angiogenin in patients with resectable hepatocellular carcinoma after surgery. *Ann Surg Oncol* **2003**; 10:355–62.