

immune escape mutants, it is possible that HBVs with pre-S substitutions are advantageous for viral survival and replication in hepatocytes, despite that virion production is reduced. In addition, those infected cells are themselves protected from apoptosis. In those circumstances of persistent viral replication, the chances of acquiring the essential substitutions in the polymerase gene conferring lamivudine resistance might increase.

Before these findings can be applied confidently in clinical settings, some caveats must be considered. First, the number of patients analyzed in the study was quite small, and therefore the potential role of the substitutions detected in drug susceptibility must be evaluated by studies of larger populations. Second, because HBV sequences were determined directly, as opposed to first cloning multiple genomes, the dynamics of minor HBV populations and their contribution to the appearance of resistance are not known. Subcloning analysis or deep sequencing might help further to establish the clinical importance and role of these substitutions in drug resistance. The utility of these viral substitutions for designing HBV therapies with the second-generation nucleoside analogs requires additional research. As for the stability of these predictive viral regions during the treatment period, five patients were available for the analysis of the complete HBV genome sequence after the acquisition of lamivudine resistance. Interestingly, the predictive positions of Pre-S1 84 and Pre-S2 1 changed after the acquisition of lamivudine resistance in some patients. However, the role of those changes needs to be further clarified by larger sample sizes.

In conclusion, it was demonstrated that the presence of pre-S1 and pre-S2 substitutions in the HBV genome prior to treatment might play an important role in the subsequent evolution of lamivudine resistance.

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Original Article

# Serum RANTES level influences the response to pegylated interferon and ribavirin therapy in chronic hepatitis C

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**Aim:** Prediction of treatment responses to pegylated interferon (PEG IFN) plus ribavirin (RBV) therapy is uncertain for genotype 1b chronic hepatitis C.

**Methods:** In this study, 96 patients were investigated for the correlation between 36 pretreatment serum chemokine/cytokine levels and PEG IFN/RBV treatment efficacy by a sandwich enzyme-linked immunoassay (ELISA) and a bead array.

**Results:** First, chemokines/cytokines were measured semi-quantitatively by sandwich ELISA in 31 randomly-selected patients and the serum regulated on activation normal T-cell expressed and secreted (RANTES) level was found to be significantly higher in the sustained virological response (SVR) group than the non-SVR group ( $P = 0.048$ ). Precise RANTES

measurement in all 96 patients using a bead array confirmed this correlation ( $P = 0.002$ ). However, the genetic RANTES haplotype was not significantly related to the serum level. The serum RANTES level was extracted by multivariate analysis (odds ratio = 4.09, 95% confidence interval = 1.02–16.5,  $P = 0.048$ ) as an independent variable contributing to SVR.

**Conclusion:** The serum RANTES level is an important determinant influencing the virological response to PEG IFN/RBV therapy in chronic hepatitis C.

**Key words:** hepatitis C virus, pegylated interferon plus ribavirin therapy, RANTES

## INTRODUCTION

HEPATITIS C VIRUS (HCV) is a major cause of chronic liver disease worldwide and persistent infection may lead to liver cirrhosis and hepatocellular carcinoma.<sup>1</sup> Therapy leading to HCV eradication is the only treatment with proven efficacy in decreasing the occurrence of hepatocellular carcinoma.<sup>2</sup> Recently, treatment with telaprevir, a non-structural (NS)3/4A protease inhibitor, combined with pegylated interferon

(PEG IFN) and ribavirin (RBV), increased the rates of sustained viral response (SVR) up to 64–75%<sup>3,4</sup> compared to the SVR rate of approximately 50% for the previous PEG IFN/RBV therapy. However, it has become evident that genotype 1-infected patients with a null response to previous PEG IFN/RBV therapy have poor responses to PEG IFN/RBV/telaprevir,<sup>5</sup> with an SVR rate as low as approximately 30%, illustrating the difficulty in treating patients infected with genotype 1 HCV. Therefore, precise and accurate prediction of the viral response to PEG IFN/RBV therapy remains an important issue.

Treatment resistance is attributed to various factors associated with the virus and host. Viral factors, such as amino acid (a.a.) sequence variation in the core and NS5A regions, have been investigated extensively for their contribution to the outcome of IFN-based therapy,<sup>6,7</sup> including PEG IFN/RBV therapy. On the other hand, host factors such as African-American race, older age, being obese, the presence of cirrhosis and

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steatosis, and insulin resistance have been reported to be associated with treatment resistance.<sup>8-11</sup> Especially, single nucleotide polymorphisms (SNP) near the interleukin (*IL*)-28B gene, including rs12979860 and rs8099917, have been reported to have a significant correlation with the response to IFN-based therapy.<sup>12,13</sup> However, even with inclusion of these factors, prediction of the treatment response in chronic HCV infection remains uncertain at present.

Chemokines are a group of small, exogenously secreted cytokines that modulate the migration of leukocytes to sites of tissue damage and inflammation in a variety of infectious and autoimmune diseases.<sup>14</sup> In chronic HCV infection, chemokines such as *RANTES* (regulated on activation normal T-cell expressed and secreted), macrophage inflammatory protein (*MIP*)-1 $\alpha$ , *MIP*-1 $\beta$  and interferon- $\gamma$  inducible protein 10 kDa (*IP*-10) are elevated and considered to play crucial roles in inflammatory processes and viral elimination, as well as the transition from innate to adaptive immunity.<sup>14,15</sup> Upregulation of several serum chemokines, such as eotaxin, *IP*-10 and *RANTES* also has been reported in HCV infection, possibly reflecting hepatic inflammation.<sup>16</sup> Considering the roles of chemokines/cytokines in establishing chronic hepatitis, it is possible that these chemokines also affect the response to antiviral therapy, and actually several chemokines as interleukin (*IL*)-8, *IL*-10, *MIP*-1 $\beta$ , *RANTES* or *IP*-10 have been investigated previously for their association with the treatment response.<sup>16-20</sup> However, the importance of those chemokines has not been established yet and, moreover, these studies did not characterize in detail these chemokines in association with other factors, including *IL*-28B influencing the response to therapy.

In this study, we explored extensively the association of 36 serum cytokines/chemokines and the treatment response, with detailed information of host and virus, to predict better the treatment response to PEG IFN and RBV therapy in genotype 1b HCV infection. Because the pretreatment serum *RANTES* level was found to be correlated significantly with the response, we analyzed further the association between the serum level of *RANTES* and the genomic SNP.

## METHODS

### Patients

NINETY-SIX CONSECUTIVE PATIENTS with genotype 1b HCV and receiving PEG IFN/RBV therapy between 2004 and 2010 at Yamanashi University Hospital were recruited retrospectively into the study. All

patients received the standard therapy according to the treatment protocol of PEG IFN/RBV therapy for Japanese patients, established by a hepatitis study group of the Ministry of Health, Labor and Welfare, Japan (PEG IFN- $\alpha$ -2b 1.5  $\mu$ g/kg bodyweight, once weekly s.c., and RBV 600–800 mg daily p.o. for 48 weeks).<sup>21</sup> All patients enrolled fulfilled the following criteria: (i) negative for hepatitis B surface antigen; (ii) no other forms of hepatitis, such as primary biliary cirrhosis, autoimmune liver disease or alcoholic liver disease; (iii) not co-infected with HIV; and (iv) a signed consent was obtained for the study protocol that had been approved by the Human Ethics Review Committee of Yamanashi University Hospital. The study was approved by the ethics committees of University of Yamanashi, and the study protocol conformed to the ethical guidelines of the 2008 Declaration of Helsinki.

### Definition of treatment outcome

An SVR was defined as undetectable serum HCV RNA at 24 weeks after the end of treatment. Relapse was defined as reappearance of detectable HCV RNA levels following discontinuation of treatment. Null response was defined as less than 2 log decrease of the baseline HCV RNA levels after 12 weeks of treatment. Based on this definition, when patients were classified according to the achievement of SVR, patients with relapse or null response were classified as non-SVR.

### Serum cytokine measurement

#### Sandwich enzyme-linked immunosorbent assay (ELISA)

Blood samples were collected before initiation of treatment and were stored at  $-80^{\circ}\text{C}$  until use. Semiquantitation of serum cytokines was performed using the Proteome Profiler Human Cytokine Array Kit Panel A (R&D Systems, Minneapolis, CA, USA) according to the manufacturer's instructions. The kit consists of a nitrocellulose membrane containing 36 different anti-cytokine antibodies (anti-C5a, anti-CD154, anti-G-CSF, anti-GM-CSF, anti-CXCL1, anti-CCL1, anti-sICAM-1, anti-IFN- $\gamma$ , anti-IL-1 $\alpha$ , anti-IL-1 $\beta$ , anti-IL-1ra, anti-IL-2, anti-IL-4, anti-IL-5, anti-IL-6, anti-IL-8, anti-IL-10, anti-IL-12p70, anti-IL-13, anti-IL-16, anti-IL-17, anti-IL-17E, anti-IL-23, anti-IL-27, anti-IL-32 $\alpha$ , anti-IP-10, anti-CXCL11, anti-CCL2, anti-MIF, anti-CCL3, anti-CCL4, anti-PAI-1, anti-*RANTES*, anti-CXCL12, anti-TNF- $\alpha$ , anti-sTREM-1), spotted in duplicate. Serum samples were diluted and mixed with a cocktail of biotinylated detection antibodies. The sample/antibody mixture



was then incubated with the membrane. Any cytokine/detection antibody complex present was bound to the membrane by its cognate immobilized capture antibody. Following washing to remove unbound material, streptavidin-horseradish peroxidase and chemiluminescent detection reagents (ECL Western Blotting Analysis System; GE Healthcare, Buckinghamshire, UK) were added sequentially. Arrays were scanned using a LAS-3000 mini-luminescent image analyzer (Fujifilm, Tokyo, Japan) and were quantified for the densities using Multi Gauge ver. 3.0 software (Fujifilm). Concentrations of cytokines and chemokines were expressed as their signal intensity ratios relative to that of the positive control spotted on the same membrane.

#### Bead array

Precise serum concentrations of regulated on *RANTES* were measured using the Luminex Bio-Plex system (Bio-Rad, Hercules, CA, USA) and the Procarta Cytokine Assay Kit (Panomics, Fremont, CA, USA) in a 96-well plate ELISA-based format according to the manufacturers' recommendations. The sensitivity of the assays is greater than 10 pg/mL cytokine. Serum and standards were incubated with a mixture of the Luminex antibody-conjugated beads for 30 min with constant shaking. After washing, the detection antibodies and substrates were added and incubated for another 30 min. Fluorescent signals were collected and data expressed, using internal standards, in pg/mL as the mean of two individual experiments carried out in duplicate.

#### Viral core and interferon sensitivity-determining region (ISDR) sequence determination by direct sequencing

Hepatitis C virus RNA extraction from serum samples, complementary DNA synthesis and amplification by two-step nested polymerase chain reaction (PCR) were carried out using specific primers for the HCV core and ISDR. PCR amplicons were sequenced directly by Big Dye Terminator ver. 3.1 (ABI, Tokyo, Japan) with universal M13 forward and reverse primers using an ABI prism 3130 sequencer (ABI). The sequence files generated were assembled using Vector NTI software (Invitrogen, Tokyo, Japan) and base-calling errors were corrected following inspection of the chromatogram.

#### SNP typing of the *RANTES* and *IL-28B* genes

Genomic DNA of the patients was extracted from peripheral blood using a blood DNA extraction kit

(QIAGEN, Tokyo, Japan) according to the manufacturer's protocol. The allele typing of each DNA sample was performed by real-time PCR with a model 7500 (ABI) using FAM-labeled SNP primers for the loci rs2107538, rs2280788, rs2280789, rs4796120 and rs3817655 (ABI) for *RANTES* and the locus rs8099917 (ABI) for *IL-28B*.

#### Statistical analysis

Student's *t*-test and Mann-Whitney *U*-test were used to analyze continuous variables, as appropriate. Fisher's exact test was used for the analysis of categorical variables. Receiver-operator curve (ROC) analyses were performed to establish cut-off values for serum cytokine concentration. The optimum cut-off was defined as the value that maximized the area under the ROC. Spearman's correlation coefficient (*R*) was calculated to clarify the strength of relationship between the pretreatment serum cytokine concentrations and clinical parameters. Variables that achieved statistical significance ( $P < 0.05$ ) in univariate analysis were entered into multiple logistic regression analysis to identify significant independent factors. The odds ratios and 95% confidence intervals also were calculated. Data were analyzed using Ekuseru-Toukei 2008 (SSRI, Tokyo, Japan). The haplotype block among rs2107538, rs2280788, rs2280789, rs4796120 and rs3817655 variants was analyzed using SNPalyze software ver. 8.0 (Dynacom, Chiba, Japan).  $P < 0.05$  was considered significant.

## RESULTS

### Semiquantitative measurement of pretreatment serum cytokines in 31 randomly-selected patients

**A**T FIRST, TO identify cytokines/chemokines related to the treatment responses to PEG IFN/RBV therapy, semiquantitative measurement of the serum concentrations of 36 comprehensive cytokines/chemokines was performed by sandwich ELISA method by randomly selected patients. Next, to further confirm the result, cytokines showing the associations with the response were measured more precisely by bead array method in all patients.

In the first analysis, 31 patients were randomly selected from the 96 patients. The clinical characteristics of these 31 patients at the start of the therapy are shown in Table 1. Significant differences in the clinical backgrounds between those who did and those who did not



**Table 1** Baseline characteristics of the 31 patients analyzed using the sandwich ELISA method

Factor	SVR (n = 20)	Non-SVR (n = 11)	P-value
Age (years)	52 ± 11†	57 ± 10	0.25‡
Sex (male : female)	11:9	6:5	0.64§
Bodyweight (kg)	60.9 ± 9.6†	61.9 ± 13.9	0.81‡
Body mass index (kg/m <sup>2</sup> )	22.6 (18.9–31.3)¶	22.7 (17.5–26.8)	0.87††
History of IFN therapy (%)	30	36	0.78§
ALT (IU/L)	130 ± 100†	75 ± 35	0.09‡
AST (IU/L)	76 (22–331)¶	64 (24–178)	0.73††
γ-GTP (IU/L)	40 (12–289)	52 (24–137)	0.17††
Albumin (g/dL)	4.1 (3.7–4.5)	4.0 (3.0–4.7)	0.46††
Total cholesterol (mg/dL)	170 ± 24†	149 ± 33	0.06‡
HbA1c (%)	5.3 ± 0.5	5.3 ± 0.6	0.95‡
Creatinine (mg/dL)	0.71 ± 0.15	0.68 ± 0.15	0.54‡
WBC count (/μL)	4561 ± 1631	4056 ± 1277	0.38‡
Neutrophil count (/μL)	2130 (820–4200)¶	1500 (800–2700)	0.02††
Hemoglobin (g/dL)	14.5 ± 1.0†	13.8 ± 1.6	0.15‡
Platelet count (×10 <sup>4</sup> /μL)	16.4 ± 5.4	12.2 ± 3.9	0.03‡
α-Fetoprotein (ng/mL)	4.6 (1.4–28.9)¶	22.3 (11.4–79.7)	0.00005††
HCV RNA (KIU/mL)	1520 ± 1079†	2146 ± 899	0.11‡
Fibrosis (F1/F2/F3/F4)‡‡	14/1/1/2	3/2/2/3	0.02††
Activity (A1/A2/A3)‡‡	12/5/1	3/5/2	0.06††

†Mean ± standard deviation.

‡Student's *t*-test.

§Fisher's exact probability test.

¶Median (range).

††Mann-Whitney *U*-test.‡‡SVR, *n* = 18; non-SVR, *n* = 10.

Activity, the score of activity in liver biopsies; ALT, alanine aminotransferase; AST, aspartate aminotransferase; ELISA, enzyme-linked immunoassay; fibrosis, the score of fibrosis in liver biopsies; HbA1c, hemoglobin A1c; HCV, hepatitis C virus; SVR, sustained virological response; WBC, white blood cell; γ-GTP, γ-glutamyl transpeptidase.

achieve SVR were neutrophil counts, platelet counts, serum α-fetoprotein levels and the score of fibrosis in liver biopsies. Table 2 shows the difference in the cytokine/chemokine expression between the SVR and the non-SVR group. Because some cytokines/chemokines were below the measurement limit of the ELISA kit, as shown in Table 1, those cytokine/chemokines were not studied further. As shown here, the RANTES level was significantly higher in the SVR group than the non-SVR group ( $P = 0.048$ ).

### Precise measurement of serum RANTES in all 96 patients

Because the semiquantitative measurement of pretreatment serum RANTES levels in 31 randomly selected patients demonstrated their significant correlation with the SVR, we determined the precise serum RANTES levels in all 96 patients using the bead array method and

investigated the correlation between those concentrations and the treatment outcome. The clinical characteristics of the 96 patients are shown in Table 3. Significant differences were seen between those with and without SVR in platelet count, viral loads and the liver fibrosis score, but there was no apparent difference in the total doses of PEG IFN and RBV. As shown in Figure 1, the distribution of serum RANTES levels in each treatment response differed significantly; the median serum RANTES level in the SVR group was significantly higher than that in the non-SVR group. Successive ROC analysis confirmed a significant association of the serum RANTES level with SVR, and the cut-off value of 3400 pg/mL to be most appropriate (Table 4). Using the cut-off value of 3400 pg/mL, 50.9% sensitivity, 79.5% specificity, 78.4% positive predictive value and 52.5% negative predictive value (area under the ROC, 0.643) were obtained for the prediction of SVR by serum RANTES level.



Table 2 Difference in cytokine and chemokine expression between the SVR group and the non-SVR group in the 31 patients

Cytokine/chemokine	SVR (n = 20)	Non-SVR (n = 11)	P-value
RANTES	4.99 (0.25–8.32)†	1.24 (0.17–8.01)	0.048‡
MIF	1.31 (0.06–3.31)†	0.45 (0.08–2.67)	0.0630
IL-1ra	0.09 (0.00–3.30)†	0.07 (0.00–2.05)	0.2300
PAI-1	3.10 (0.35–7.34)†	2.73 (0.46–8.42)	0.3900
sICAM-1	3.18 (0.37–8.33)†	2.78 (0.74–10.3)	0.4800
IL-23	0.08 (0.01–0.78)†	0.07 (0.00–0.38)	0.5900
IL-27	0.05 (0.02–0.18)†	0.05 (0.00–0.23)	0.6500
IL-6	0.08 (0.01–3.22)†	0.10 (0.00–1.36)	0.7100
C5a	0.21 (0.01–2.72)†	0.12 (0.00–1.67)	0.7700
IFN- $\gamma$	0.07 (0.02–0.31)†	0.08 (0.00–0.40)	0.8000
CCL4	0.04 (0.01–3.08)†	0.05 (0.00–0.69)	0.8400
IL-32 $\alpha$	0.04 (0.00–0.71)†	0.07 (0.00–0.20)	0.9000
IL-8	0.16 (0.05–2.61)†	0.17 (0.03–2.21)	0.9300
IL-1 $\alpha$			N.A.
IL-1 $\beta$			N.A.
IL-2			N.A.
IL-4			N.A.
IL-5			N.A.
IL-10			N.A.
IL-12 p70			N.A.
IL-13			N.A.
IL-16			N.A.
IL-17			N.A.
IL-17E			N.A.
CCL1			N.A.
CCL2			N.A.
CCL3			N.A.
CXCL1			N.A.
CXCL11			N.A.
CXCL12			N.A.
CD154			N.A.
G-CSF			N.A.
GM-CSF			N.A.
IP-10			N.A.
TNF- $\alpha$			N.A.
sTREM-1			N.A.

†Median (range).

‡Mann-Whitney U-test.

N.A., not available; SVR, sustained virological response.

### Correlation between serum RANTES level and clinical parameters

Spearman's correlation coefficients between the pre-treatment serum RANTES level and clinical parameters in all 96 patients are shown in Table 5. As a result, a significant negative correlation with aspartate aminotransferase level and a significant positive correlation with platelet count were found, while no significant correlation was observed in other clinical parameters.

### Univariate and multivariate analysis of factors related to SVR

Univariate and multivariate analyses were performed successively in order to clarify the factors related to SVR. The viral factors included in the analysis were the ISDR and core a.a. 70 and 91, along with the host factor, IL-28B SNP. Those factors, conventional clinical background factors and serum RANTES levels were subjected to univariate and multivariate analysis. In the univariate



Table 3 Baseline characteristics of all patients analyzed using the bead array method ( $n = 96$ )

Factor	SVR ( $n = 57$ )	Non SVR ( $n = 39$ )	P-value
Age (years)	53 $\pm$ 10†	57 $\pm$ 8	0.08‡
Sex (male : female)	34:23	23:16	0.56§
Bodyweight (kg)	60.6 $\pm$ 10.5†	57.8 $\pm$ 7.8	0.17‡
Body-mass index (kg/m <sup>2</sup> )	22.9 $\pm$ 2.8	22.1 $\pm$ 2.2	0.15‡
History of IFN therapy (%)	25	28	0.74§
ALT (IU/L)	68 (19–413)¶	64 (20–215)	0.25††
AST (IU/L)	58 (21–331)	62 (21–178)	0.80††
$\gamma$ -GTP (IU/L)	37 (11–289)	50 (13–167)	0.12††
Albumin (g/dL)	4.1 $\pm$ 0.3†	4.1 $\pm$ 0.4	0.93‡
Total cholesterol (mg/dL)	166 $\pm$ 30	158 $\pm$ 31	0.25‡
HbA1c (%)	5.2 (4.7–6.6)¶	5.3 (4.5–7.4)	0.47††
Creatinine (mg/dL)	0.72 $\pm$ 0.15†	0.69 $\pm$ 0.16	0.39††
WBC count (/ $\mu$ L)	4497 $\pm$ 1247	4501 $\pm$ 1281	0.99‡
Neutrophil count (/ $\mu$ L)	2243 $\pm$ 857	2144 $\pm$ 825	0.57‡
Hemoglobin (g/dL)	14.1 $\pm$ 1.2	14.2 $\pm$ 1.2	0.87‡
Platelet count ( $\times 10^9$ / $\mu$ L)	15.1 (7–29)¶	13.2 (6.9–19.7)	0.03††
$\alpha$ -Fetoprotein (ng/mL)	4.8 (1.3–137.1)	9.0 (1.4–79.7)	0.05††
HCV RNA (KIU/mL)	1300 (100–5000)	2400 (620–5000)	0.0002‡
Fibrosis (F1/F2/F3/F4)‡‡	35/6/5/6	11/13/5/6	0.006††
Activity (A1/A2/A3)‡‡	27/18/7	12/20/3	0.26††
PEG IFN dose (%)	92 (40–113)¶	73 (27–147)	0.23††
RBV dose (%)	97 (44–147)	100 (33–135)	0.38††

†Mean  $\pm$  standard deviation.‡Student's *t*-test.

§Fisher's exact probability test.

¶Median (range).

††Mann-Whitney's *U*-test.‡‡SVR,  $n = 52$ ; non-SVR,  $n = 35$ .

Activity, the score of activity in liver biopsies; ALT, alanine aminotransferase; AST, aspartate aminotransferase; fibrosis, the score of fibrosis in liver biopsies; HbA1c, hemoglobin A1c; HCV, hepatitis C virus; PEG IFN, pegylated interferon; RBV, ribavirin; SVR, sustained virological response; WBC, white blood cell;  $\gamma$ -GTP,  $\gamma$ -glutamyl transpeptidase.

analysis, significant differences were observed for the ISDR mutation, core a.a. 70, viral loads, platelet counts, IL-28B SNP and serum *RANTES* levels. When multivariate analysis was carried out with these factors, the serum *RANTES* level was extracted as an independent factor related to SVR (Table 6).

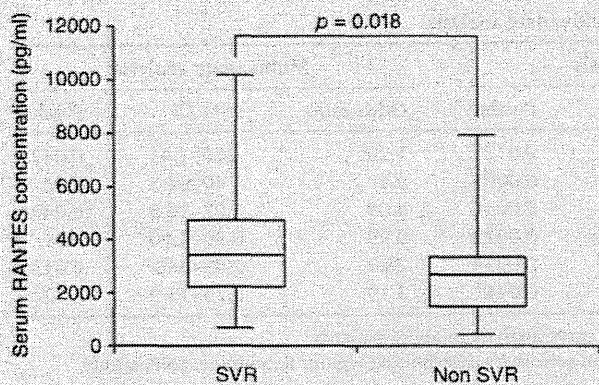
### ***RANTES* haplotyping and serum *RANTES* level**

Because a high serum *RANTES* level was an independent factor predicting SVR, we sought to examine further the role of the *RANTES* gene and tried to clarify the association of the SNP of the gene with the serum levels. First, we determined how many and which SNP in the *RANTES* gene should be investigated to represent all *RANTES* haplotypes found in the Japanese population. Reference to the HapMap project database ([\[snp.cshl.org\]\(http://snp.cshl.org\)\) made it clear that the information from five unique SNP was required to determine the majority of haplotypes found in the Japanese population. Therefore, to determine the \*RANTES\* haplotype of each patient, we investigated these five SNP in the 65 of the 96 patients available for the haplotype analysis. The \*RANTES\* haplotypes were finally divided into three types \(named R1, R2 and R3 for convenience\), as shown in Figure 2\(a\). However, the \*RANTES\* gene haplotype and serum \*RANTES\* level did not show any clear correlation \(Fig. 2b\).](http://</a></p>
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### **DISCUSSION**

FROM THE ANALYSIS of 36 cytokine and chemokine species, we discovered that a high pretreatment serum *RANTES* level was significantly related to SVR





**Figure 1** Difference in serum RANTES levels between the sustained virological response (SVR) group and the non-SVR group. Ninety-six patients who underwent the standard therapy for 48 weeks were analyzed for serum RANTES level using the bead array method. They were divided into the SVR ( $n = 57$ ) and non-SVR groups ( $n = 39$ ) and their serum RANTES levels compared. Box and whisker plots show the distributions of serum RANTES levels for the SVR and non-SVR groups. The boxes represent the 25th to 75th percentile and horizontal lines within the box show the median values. The ends of the whiskers show the minimum and maximum values of all the data. *P*-values were obtained using Mann-Whitney's *U*-test.

following PEG IFN/RBV combination therapy of patients infected with genotype 1b HCV. In particular, a high serum RANTES level was an independent factor contributing to SVR in the multivariate analysis, even among other treatment-restricting factors as the HCV ISDR, core a.a. 70, viral loads, platelets or IL-28B SNP. On the other hand, a systematic haplotyping study did not reveal any correlation between the RANTES haplotype and serum RANTES level.

RANTES, also known as CC-chemokine ligand (CCL)5, is classified as a chemotactic T-helper (Th)1-

**Table 4** Association between the serum RANTES level and SVR rate in all 96 patients analyzed using the bead array method

Cytokine/chemokine	Serum concentration	SVR rate	<i>P</i> -value
RANTES	≥3400 pg/mL†	78% (29/37)	0.002‡
	<3400 pg/mL†	47% (28/59)	

†A cut-off value of 3400 pg/mL was determined by receiver-operator curve analysis in all 96 patients.

‡Fisher's exact probability test.

SVR, sustained virological response.

**Table 5** Spearman's correlation coefficient (*R*) between the pretreatment serum RANTES level and clinical parameters ( $n = 96$ )

Clinical parameters	Serum RANTES level	
	<i>R</i>	<i>P</i> -value
Platelet count	-0.30	0.0025
Aspartate aminotransferase	-0.24	0.0200
White blood cell	-0.15	0.1600
Total cholesterol	-0.11	0.2700
Alanine aminotransferase	-0.088	0.3900
α-Fetoprotein	-0.088	0.4100
Neutrophil count	-0.064	0.5400
Hemoglobin A1c	-0.056	0.6300
γ-Glutamyl transpeptidase	-0.047	0.6500
Albumin	-0.021	0.7900
Hemoglobin	-0.025	0.8000
Creatinine	-0.00098	0.9900

type chemokine.<sup>23</sup> In chronic hepatitis C, RANTES is significantly upregulated in the infected liver, and considered to play a role in recruiting T cells to portal and periportal regions, regulating liver inflammation and innate and adaptive immunity through interactions with CC-chemokine receptor (CCR)5, CCR1 and CCR3 expressed on activated T cells.<sup>24</sup> The serum RANTES level is significantly upregulated in the early stages of fibrosis in chronic hepatitis and its upregulation becomes weaker in advanced chronic disease.<sup>14</sup> HCV-encoded proteins are considered to affect RANTES production, for example, exposure of peripheral blood mononuclear cells to the HCV envelope 2 (E2) protein induces the release of RANTES,<sup>25</sup> the HCV NS3/4A proteins suppress RANTES promoter activity<sup>26</sup> and the HCV core protein may either induce or inhibit the expression of RANTES in various cell types.<sup>27</sup> A recent *in vitro* study has shown that human hepatoma cells secrete RANTES via the Toll-like receptor (TLR)3-mediated recognition of HCV dsRNA and activation of the nuclear factor (NF)-κB pathway, suggesting that the hepatocytes themselves may serve as the source of RANTES.<sup>15</sup>

In this study, we showed the close association between the serum RANTES level and SVR in the PEG IFN/RBV combination therapy by analyzing 31 randomly selected, primary test patients and then all 96 patients. In addition to the association with SVR, we also searched the association between RANTES and the initial viral response because SVR could be influenced by the initial viral dynamics, and revealed that complete early viral response (HCV RNA negative at 12 weeks



Table 6 Factors associated with SVR analyzed by univariate and multivariate analysis

Characteristic	Subcategory	Univariate analysis			Multivariate analysis		
		Odds ratio	95% CI	P-value	Odds ratio	95% CI	P-value
Platelet count		1.13	1.03–1.25	0.012	1.20	1.00–1.41	0.042
IL-28B SNP	T/T or not	16.0	3.37–76.2	0.0005	9.48	1.40–64.3	0.02
RANTES	≥3400†	4.01	1.58–10.2	0.0036	4.09	1.02–16.5	0.048
Viral loads		0.99	0.99–0.99	0.0012	0.99	0.99–1.00	0.51
ISDR mutation	≥2	21.7	2.76–170	0.0034	28.2	2.05–388	0.013
Core a.a. 70	R or not	2.52	1.03–6.20	0.044	3.19	0.73–13.9	0.12

†The cut-off value of 3400 pg/ml. was determined by receiver-operator curve analysis in all 96 patients.

a.a., amino acids; CI, confidence interval; IL, interleukin; ISDR, interferon sensitivity-determining region; R, arginine; SNP, single nucleotide polymorphisms.

after commencement of therapy) was also significantly correlated with high pretreatment serum RANTES level ( $P = 0.015$ , data not shown). Moreover, we could also show that high serum RANTES levels correlated with the clinical background factors low alanine aminotransferase values and high platelet counts, suggesting that the patients with high RANTES levels have less severe hepatitis. A previous study also showed a tendency for correlation between the serum RANTES level and SVR in PEG IFN/RBV therapy, but this correlation did not reach significance.<sup>16</sup> Although the reason for this discrepancy is not known, we speculate that a difference in drug dosage may have contributed. In our study, most of the patients received a sufficient dose of both PEG IFN and RBV, as shown in Table 3. However, the previous study lacks information regarding drug dosage, suggesting that the study group comprised a heterogeneous population.

Then, what is the mechanism of the association between high serum RANTES levels and high SVR? Because RANTES is a chemotactic Th1-type chemokine, it may be speculated that a high serum RANTES level reflects activation and preservation of the Th1-type immune responses needed to suppress viral replication and so enhances viral elimination by PEG IFN/RBV therapy. Although it is also possible that a high RANTES level could be simply a reflection of early stages of the disease, we suggest that it could have a more direct role in achieving SVR, because multivariate analysis extracted a high serum RANTES level as a variable contributing to SVR independently of the platelet count, which reflects the stage of disease. Importantly, our result also demonstrated that the serum RANTES level was a factor contributing to SVR independently of other treatment-restricting factors, including the IL-28B SNP and the viral factors of NS5A and core. This independent contribution of a high serum RANTES level among

other variables indicates its importance and potency in improving the prediction of the treatment efficacy.

Concerning the association between the serum RANTES level and RANTES haplotype, we could not find a significant correlation in the HCV-infected patients, although there was a tendency that patients with the R3 haplotype had higher serum RANTES levels. In patients with coronary artery disease and type 1 diabetes mellitus, and in healthy volunteers, the serum RANTES level has been reported to correlate with the RANTES gene SNP. Specifically, those patients and healthy volunteers with the A allele in the RANTES promoter polymorphism at position -403 (rs2107538) had lower serum RANTES levels than those with the G allele.<sup>28,29</sup> On the other hand, in previous reports of chronic hepatitis C, no evident correlation was reported between the RANTES SNP at position -403 (rs2107538) and serum RANTES level.<sup>30</sup> In this study, through more systematic haplotyping analysis based upon the HapMap Database, we tried to determine the correlation between the serum RANTES level and the RANTES gene SNP in chronic hepatitis C in more detail. However, we could not find any association and the result shows that the serum RANTES level is not primarily determined by the RANTES haplotype in chronic hepatitis C. The result seems strange at first, however, it is understandable considering that RANTES expression is modulated by multiple factors in chronic hepatitis C, including viral components and the stage of liver disease, as described before.

However, there are some limitations in our study. Namely, the number of investigated patients was rather small, and included patients for the analysis were limited to those with genotype 1b HCV infection. Therefore, it is considered that additional independent studies including the analysis of other genotypes would