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Serum Chemokine Levels Are Associated with the Outcome of Pegylated Interferon and Ribavirin Therapy in Patients with Chronic Hepatitis C

Running title: Chemokines and antiviral therapy in CH-C

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Abstract

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

Methods: A total of 6 serum chemokines were quantified before, during, and after pegylated interferon and ribavirin treatment in 79 genotype 1 chronic HCV patients using a multiple bead array system. Viral ISDR and core region variants were determined by direct sequencing.

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

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

Keywords: chemokines, pegylated interferon, ribavirin, ISDR, core, MIP-1β

Introduction

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

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

In the present study, we sought to determine the levels of six chemokines in patients with chronic HCV-1b infection who underwent treatment with PEG-IFN and ribavirin using a broad-spectrum bead-based multiplex immunoassay.

Methods

Subjects

A total of 79 treatment-naïve patients with chronic hepatitis C [40 men and 39 women; median age 60 years (range: 17 - 74)] were seen at Shinshu University Hospital or its affiliated hospitals in the Nagano Interferon Treatment Research Group. Alanine aminotransferase (ALT), aspartate aminotransferase (AST), and γ-glutamyl transpeptidase (γGTP) were tested using standard methods [8]. All patients, who were infected with genotype 1b HCV, received PEG-IFNα-2b (PegIntron, Schering-Plough K.K., Tokyo, Japan; 1.5μg/kg of body weight) and ribavirin (Rebetol, Schering-Plough K.K.; 600 - 1000 mg/day) adjusted to body weight for 48 weeks as described previously [9]. The pretreatment median value for ALT was 54 (range: 22-389) IU/L, AST was (range: 20-288), and HCV RNA was 1700 (11-5100) KIU/mL, as measured by COBAS AMPLICOR assays (Roche Diagnostic Systems, Tokyo, Japan). A group of 26 healthy

individuals [13 men and 13 women; mean age 54 years (range: 28 - 60)] with hepatitis B and C–negative serologies and normal transaminases was used as the control. All patients and controls were negative for the antibody to the human immunodeficiency virus. The protocol of this study was approved by the ethics committee of Shinshu University School of Medicine and all patients provided written informed consent. All serum samples were immediately stored at -70°C and remained in storage until testing.

Definition of Treatment Outcome

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

Detection of amino acid substitutions in core and ISDR regions

The sequences of 1-191 amino acids (aa) in the core protein and 2209-2248 aa in the NS5A region of genotype 1b HCV were determined by direct sequencing using stored serum samples obtained before therapy, as reported previously. Nucleotide and aa sequences were compared with the nucleotide sequences of genotype 1b HCV-J [10]. Substitutions of aa70 arginine (Arg70) and glutamine (Gln70) or aa91 leucine (Leu91) and methionine (Met91) [11] and the number of aa substitutions in the ISDR were defined as wild-type (0), intermediate-type (1), and mutant-type (≥2) [12]. Of the 79 patients, 75 were determined to have substitutions at aa70 and aa91, and 76 could be sequenced for their ISDR.

Detection of Chemokines

Six chemokines (macrophage inflammatory protein [MIP]-1α, MIP-1β, eotaxin, IP-10, RANTES, and IL-8) were quantified using Luminex® Multiplex Cytokine Kits (Procarta Cytokine assay kit) from serum samples obtained before the start of treatment, 4 weeks after the start of treatment, and 24 weeks after the completion of treatment,

according to manufacturer's instructions.[13]

Statistical Analysis

The Mann-Whitney *U* test and Kruskal-Wallis test were used to analyze continuous variables as appropriate. The Wilcoxon signed-rank test and the Friedman test were used to evaluate changes in serum chemokine levels over time. Spearman's rank order correlations were used to evaluate the relationship between each pair of markers. The chi-square test with Yate's correction was used for the analysis of categorical data. In cases where the number of subjects was less than 5, Fisher's exact test was used. A *P* value of ≤ 0.05 was considered significant. To predict treatment outcome, each cutoff point for continuous variables was determined by receiver-operating characteristic (ROC) curve analysis. Statistical analyses were performed using SPSS version 18.0J (SPSS, Chicago, IL).

Results

Of the 79 patients receiving PEG-IFN and ribavirin therapy, 31 (39%) achieved an SVR, 23 (29%) relapsed, and 25 (32%) did not respond to treatment and were termed null viral responders (NVR). When stratified into 3 groups based on treatment outcome, patients with an NVR had a higher female ratio (*P* = 0.030) (**Table 1**). Before treatment, the median AST and γGTP levels in the SVR group were significantly lower than those in the relapsed and NVR groups. Substitutions of aa 70 in the core region (*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

nonresponders.

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 signed-rank 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 Gln 70 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 since 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 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 (Figure 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 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 a sustained virological response. Serum cytokines have also been associated with pathogenesis in HCV infection. Since 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 transendothelial 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 curve 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 human immunodeficiency virus 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 nonresponders 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. Since 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. Since we could not perform multivariate statistical analysis, it was difficult to draw a definitive conclusion on the most relevant chemokine. Hence, ROC curve 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 literature 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 noninvasive markers of a sustained viral response 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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Figure Legend

Figure 1. Receiver-operating characteristic (ROC) curves for serum chemokine levels on treatment outcome

The areas under the curve for MIP-1α, MIP-1β, Eotaxin, IP-10, RANTES, and IL-8 were 0.612, 0.756, 0.629, 0.623, 0.739, and 0.530, respectively.

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
γGTP (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 aa 70 (Arg70/Gln70)	22/6	14/8	11/14	0.034
Core aa 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

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

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 – 11487.0)	31.1 (21.3 – 80.6)	< 0.001
RANTES	31755.3 (17.9 – 83248.0)	3460.0 (191.5 – 40001.0)	< 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).

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	83248.0 (31755.3 – 83248.0)	8633.4 (3469.1 – 22498.6)	30970.8 (3638.7 – 83248.0)
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). Abbreviations: SVR, sustained virological response; TR, transient response; NVR, null virological 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	83248.0 (57501.7 – 83248.0)	83248.0 (31037.0 – 83248.0)	83248.0 (17542.9 – 83248.0)	0.091
	Non-SVR	14670.7 (3730.4 – 55199.4)	25377.2 (11272.6 – 83248.0)	21707.6 (8746.5 – 83248.0)	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

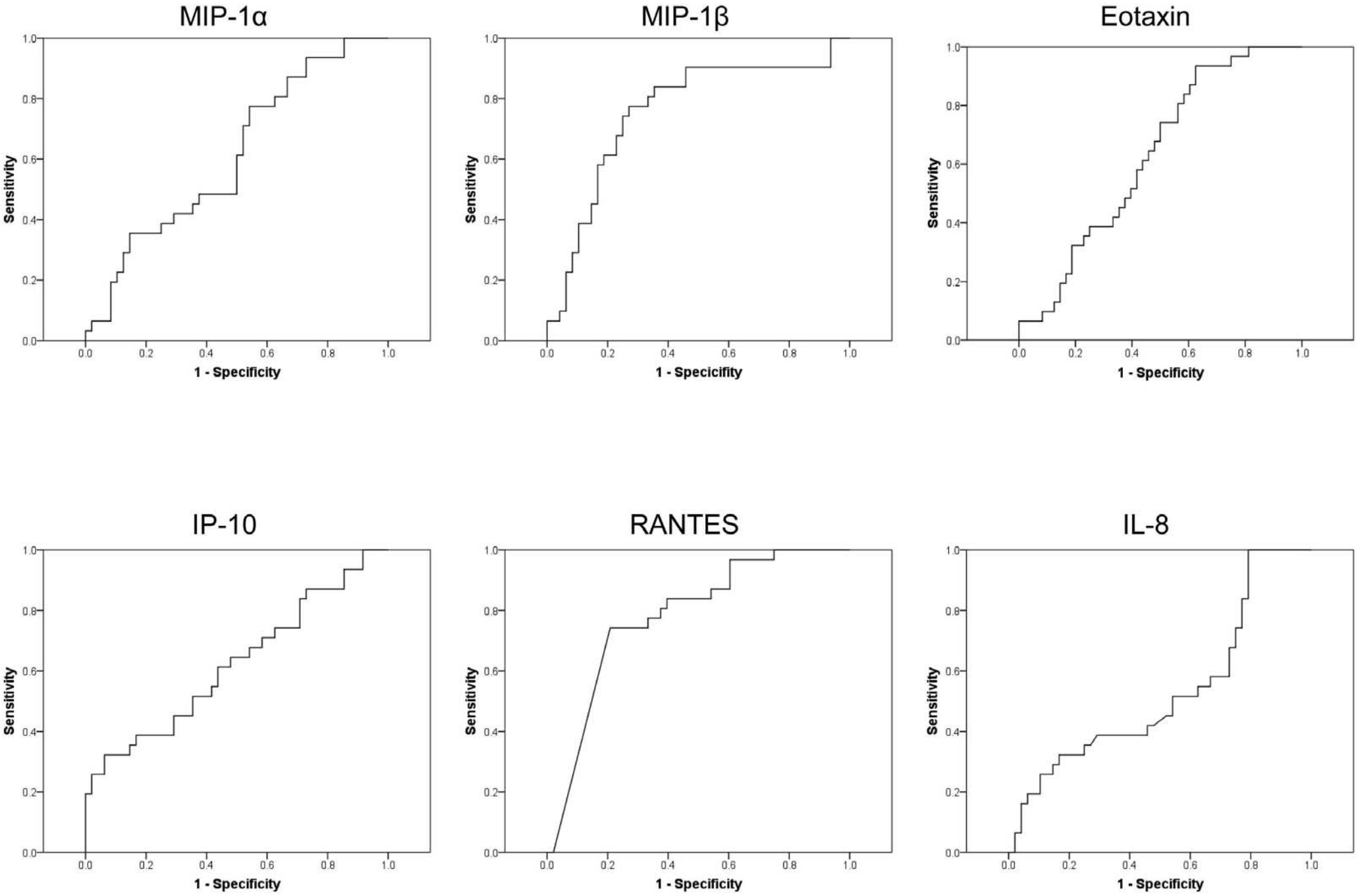
Data are expressed as median (interquartile range) values (pg/mL). Abbreviations: SVR, sustained virological response.

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	83248.0 (6309.0 – 83248.0)	29705.6 (6713.2 – 83248.0)	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).

Figure 1


MIP-1α
MIP-1β
Eotaxin
IP-10
RANTES
IL-8
Sensitivity
1 - Specificity
1 - Specicifity
0.0
0.2
0.4
0.6
0.8
1.0