with the TT genotype at SNP rs8099917 were reported to have lower expression levels of ISGs in the liver during the pretreatment period as compared with subjects with the TG or GG genotypes [8]. Several *in vitro* studies support a direct role of IFN-λs in the control of HCV replication through the innate immune pathway. In a cell culture system, Marcello *et al.* [9] showed that IFN-λ1 inhibited HCV replication with similar kinetics to that of IFN-α, although IFN-λ1 induced stronger upregulation of ISGs and this effect lasted longer. Moreover, combinations of IFN-λ1 and IFN-α had a greater inhibitory effect on HCV replication compared with the individual agents [10].

As suggested by the studies performed to date, a change in IFN-λ3 expression might be a key mechanism by which IL-28B SNPs determine the response to PEG-IFN and RBV. Considering that IFN-λ1 plays a direct role in the control of HCV replication and that IFN-λ1 enhances the antiviral activity of IFN-α, it seems reasonable to speculate that IFN-λ3 plays a similar antiviral role. Therefore, in this study, we investigated the direct antiviral role of IFN-λ3 alone and in combination with IFN-α using an HCV replicon system. In addition, we used microarray analysis to investigate the influence of IFN-λ3, alone or in combination with IFN-α, on the regulation of ISG-mediated antiviral pathways.

MATERIALS AND METHODS

Cell culture and HCV replicon

The human hepatoma cell lines OR6 and Huh7.5.1 were maintained in Dulbecco's modified Eagle's medium (DMEM; Sigma, St. Louis, MO, USA) supplemented with 10% foetal bovine serum (FBS) at 37°C in 5% CO_2. JFH-1-infected Huh7.5.1 cells were grown in DMEM supplemented with 10% FBS. The OR6 cell line, harbouring full-length genotype 1b HCV RNA and co-expressing *Renilla* luciferase (ORN/C-5B/KE) [11], was established in the presence of 500 μg/mL G418 (Promega, Madison, WI, USA).

Reagents

IL-28A (IFN-λ2), IL-28B (IFN-λ3) and IL-29 (IFN-λ1) were obtained from R&D Systems (Minneapolis, MN, USA). IL-28A and IL-29 are recombinant proteins generated from an NSO-derived murine myeloma cell line, and IL-28B is a recombinant protein generated from the CHO cell line. Interferon alpha-2b (INTRON®A 300 IU) was obtained from Schering-Plough Corporation (Kenilworth, NJ, USA).

Reporter plasmids and luciferase assay

HCV replication in OR6 replicon cells was determined by monitoring *Renilla* luciferase activity (Promega). To monitor IFN signalling directed by the interferon-stimulated response element (ISRE), the plasmids pISRE-luc expressing firefly luciferase were cotransfected using FuGENE®6 Transfection Reagent (Roche, Indianapolis, IN, USA) following the manufacturer's protocol. Luciferase activity was quantified using the dual-luciferase assay system (Promega) and a GloMax 96 Microplate Luminometer (Promega). Assays were performed in triplicate, and the results were expressed as mean ± SD percentage of the control values.

Quantification of HCV core protein and RNA

We quantified HCV core protein in culture supernatant using Lumipulse Ortho HCV Ag (Ortho Clinical Diagnostics, Tokyo, Japan) as specified by the manufacturer. The principle of the measurement method is based on the chemiluminescent enzyme immunoassay (CLEIA) [12].

Real-time reverse transcription polymerase chain reaction (RT-PCR)

Intracellular genomic JFH-1 HCV RNA as well as cellular mRNA of IFI-6, IFIT1 (ISG56), DDX60, OAS2, Mx1 and USP18 was quantified by TaqMan RT-PCR. The cells were lysed and subjected to reverse transcription without purification of RNA using a Cells-to-Ct kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's protocol. Quantitative PCR was performed in triplicate using a 7500 Real-Time PCR System (Applied Biosystems) and TaqMan Gene Expression Assays (Applied Biosystems) according to the manufacturer's instructions. The sequences of the sense and antisense primers and the TaqMan probe for 5′UTR region of HCV were 5′-TGCGG AACCGGTGAGTACA-3′, 5′-CTTAAGGTTTAGGATTCGTGCT CAT-3′ and 5′-(FAM)CACCCTATCAGGCAGTACCACAAGG CC(TAMRA)-3′, respectively. TaqMan probes for IFI-6, IFIT1 (ISG56), DDX60, OAS2, Mx1 and USP18 were purchased from Applied Biosystems. Primers for 18s rRNA (Applied Biosystems) were used as internal control.

Microarray analysis

OR6 replicon cells were harvested by centrifugation after exposure to 0.01 ng/mL IFN-α, 10 ng/mL IFN-λ3 or a combination of both for 6, 12, 24 and 48 h. Total RNA was isolated from cells using the RNeasy Mini Kit (Qiagen, Valencia, CA, USA). On-column DNase digestion was performed using the RNase-Free DNase Set (Qiagen). Quality control of extracted RNA was performed with the 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA).

The RNA was then amplified and labelled using the Ambion® WT Expression Kit and GeneChip® WT Terminal Labelling and Control Kit (Affymetrix, Santa Clara, CA, USA). cDNA was synthesized, labelled and hybridized to the GeneChip® array according to the manufacturer's protocol, starting with 200-ng total RNA. The GeneChips were finally washed

and stained using the GeneChip® Fluidics Station 450 (Affymetrix) and then scanned with the GeneChip® Scanner 3000 7G (Affymetrix).

Affymetrix CEL files were imported into GeneSpring GX v.11.5 (Agilent Technologies, Santa Clara, CA, USA) analysis software. Data were normalized using robust multichip average analysis (RMA).

Dimethylthiazol carboxymethoxyphenyl sulphophenyl tetrazolium (MTS) assays

To evaluate the cell viability, dimethylthiazol carboxymethoxyphenyl sulphophenyl tetrazolium (MTS) assays were performed using a CellTiter 96 AQueous One Solution Cell Proliferation Assay kit (Promega) according to the manufacturer's instructions.

Statistical analyses

Statistical analyses were performed using an unpaired, two-tailed Student's *t*-test. *P* values of < 0.05 were considered to be statistically significant.

RESULTS

IFN-λ1, IFN-λ2 and IFN-λ3 demonstrate antiviral activity against HCV

To determine the antiviral effect of IL-29 (IFN-λ1), IL-28A (IFN-λ2) and IL-28B (IFN-λ3) against HCV, OR6/ORN/C-5B/KE cells were seeded in 96-well plates for 24 h and then treated with IFN-λ1, IFN-λ2, IFN-λ3 or IFN-α at various concentrations for another 24, 48 and 72 h. In this system, the *Renilla* luciferase activity reflects the amount of HCV RNA synthesized. As shown in Fig. 1, at concentrations of 1 ng/mL or more, all IFN-λs led to a concentration- and time-dependent decrease in luciferase activity of the OR6/C-5B replicon. IFN-λ3 at 10 ng/mL inhibited HCV replication (32% reduction, $P < 0.05$) to a similar extent as 0.01 ng/mL IFN-α (49% reduction, $P < 0.05$) by 48 h.

We also assessed the effects of IFN-λ1, IFN-λ2 and IFN-λ3 on Huh7.5.1/JFH-1 cells. JFH-1 cells were seeded in 96-well plates for 24 h and then treated with IFN-λ1, IFN-λ2, IFN-λ3 or IFN-α at various doses for another 48 h. To determine their antiviral effect, HCV core protein in the medium and intracellular HCV RNA were measured by CLEIA and quantitative real-time RT-PCR, respectively. HCV RNA quantitative PCR assays were multiplexed for 18s ribosomal RNA to control for the amount of input RNA. As shown in Fig. 2, all IFN-λs inhibited HCV replication in JFH-1 cells in a concentration-dependent manner. Similarly, all of the IFN-λs caused suppression of HCV core protein secretion into the cell culture medium (Figure S1).

In C-5B system, there was no evident cytotoxicity below 100 ng/mL in any interferons except for IFN-λ1 (Figure S2). On the other hand, cytotoxicity was observed in lesser concentrations by those IFNs in JFH-1 system. However, as demonstrated in Fig. 2 and Figure S3, antiviral effect exceeded the cytotoxicity in the JFH-1 system.

Synergistic inhibition of HCV replication by IFN-λ3 and IFN-α in combination

We examined whether the combination of IFN-λ3 and IFN-α induces greater antiviral activity as compared with the individual cytokines alone. OR6/ORN/C-5B/KE cells were treated with the combinations of IFN-λ3 and IFN-α at various concentrations for 48 h. As shown in Fig. 3a, the relative concentration–inhibition curves of IFN-α were plotted for each fixed concentration of IFN-λ3, and the curves shifted to the left with increasing concentrations of IFN-λ3. The results indicate a synergistic effect of IFN-λ3 and IFN-α against HCV replication. We confirmed the synergistic effect of IFN-λ3 and IFN-α by isobologram (Fig. 3b). The inhibitory effects of the combination were quantified according to the method of Chou *et al.* using the CalcuSyn software program (Biosoft, Cambridge, UK). At the ED_{50} of each drug, the combination index was 0.40-0.61, indicating significant synergism. We also assessed the effect of the combination on Huh7.5/JFH-1 cells by HCV RNA quantitative PCR assays and HCV core protein secretion. At the ED_{50} of each drug, the combination index was 0.55 and 0.48, respectively (Table S1). The cytotoxicity was not observed at the range of concentration tested (Fig. S2E, S3E).

IFN-λ3 induces ISRE promoter activity

We used the ISRE luciferase reporter assay to assess activity downstream of the JAK-STAT signalling pathway. The ISRE-firefly luciferase plasmid was transfected into OR6/ORN/C-5B/KE cells for 24 h, and these cells were cultured with various concentrations of IFN-λ3 and IFN-α for another 12, 24 or 48 h. Firefly and *Renilla* luciferase activity was then measured.

IFN-λ3 induced ISRE luciferase activity in a time-dependent manner; activity was elevated threefold after treatment with 100 ng/mL IFN-λ3 for 48 h (Fig. 4). In contrast, IFN-α induced ISRE luciferase more rapidly, producing maximal activation of the response to IFN-α at 12 h. The combination of IFN-λ3 and IFN-α induced ISRE luciferase activity similarly to IFN-λ3 alone.

IFN-α and IFN-λ3 induce expression of similar genes in HCV 1b replicon cells

OR6/ORN/C-5B/KE cells were stimulated for 6, 12, 24 and 48 h with 0.01 ng/mL IFN-α, 10 ng/mL IFN-λ3 or a combination of both, while controls were left unstimulated for the same time interval. Induction of gene expression by IFNs was analysed in microarray experiments.

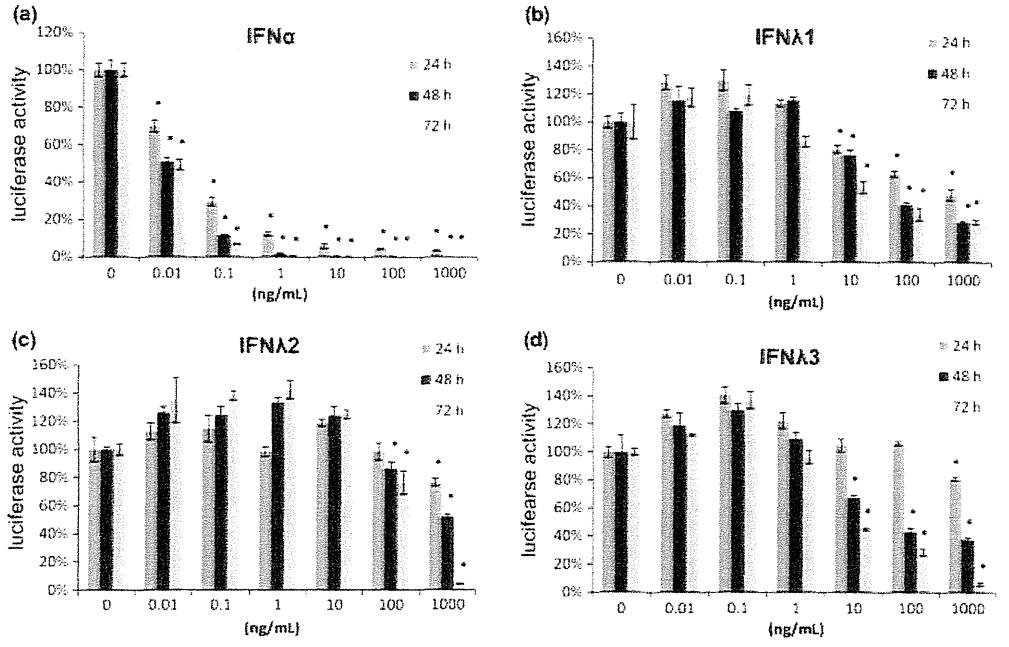

Fig. 1 IFN-α and IFN-λs inhibit HCV replicon in OR6 cells. Specific inhibition of the replication of a full-length HCV genotype 1b replicon by (a) IFN-α and (b) IFN-λ1, (c) IFN-λ2, (d) IFN-λ3 were quantified on the basis of luciferase activity. Symbols show the mean value of triplicate wells; error bars show the SD. *: $P < 0.05$ *vs* control (IFN 0 ng/mL) of each time point.

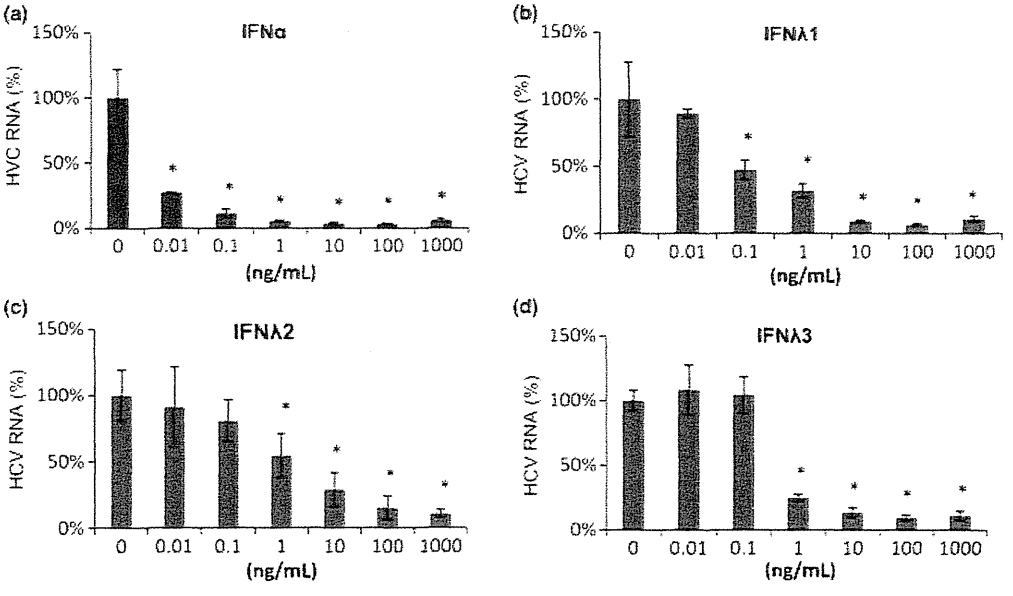

Fig. 2 IFN-α and IFN-λs inhibit HCV replicon in Huh7.5.1 cells. JFH1-infected Huh7.5.1 cells were incubated with various concentrations of (a) IFN-α and (b) IFN-λ1, (c) IFN-λ2, (d) IFN-λ3. After 48 h of treatment, total RNA was isolated and reverse transcribed, after which quantitative PCR was performed. Symbols show the mean value of triplicate wells; error bars show the SD. *: $P < 0.05$ *vs* control (IFN 0 ng/mL).

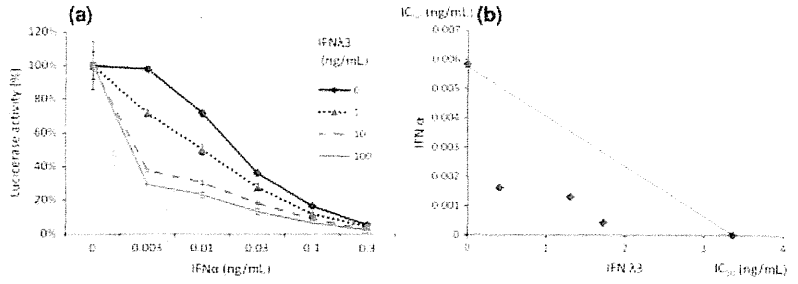

Fig. 3 Synergistic inhibitory effect of IFN-λ3 with IFN-α on hepatitis C virus replication. OR6/ORN/C-5B/KE cells were treated with combinations of IFN-λ3 with IFN-α at various concentrations. (a) The relative concentration–inhibition curves of IFN-α plotted for each fixed concentration of IFN-λ3 (0, 1, 10 and 100 ng/mL). (b) Classic isobologram for IC_{50} of IFN-λ3 with IFN-α in combination.

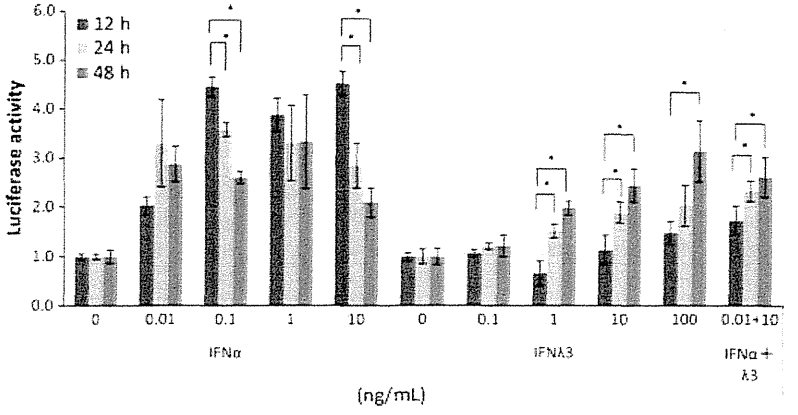

Fig. 4 IFN-stimulated response element (ISRE) promoter activity induced by IFN-α, IFN-λ3 or combination of IFN-α and IFN-λ3. OR6/ORN/C-5B/KE cells transfected with ISRE-firefly luciferase were cultured with various concentrations of IFN-α alone, IFN-λ3 alone or 0.01 ng/mL IFN-α plus 10 ng/mL IFN-λ3. ISRE-firefly luciferase activity at 24 h after transfection. Symbols show the mean value of triplicate wells; error bars show the SD. *: $P < 0.05$.

At all time points, the IFN-λ3-treated samples showed a tendency for the induction of a larger number of genes than samples treated with IFN-α. However, as shown in Table 1 listing the top 25 genes that were upregulated by both IFN-α and IFN-λ3 at 12 h, most of the upregulated genes are previously identified ISGs and the genes with high ranks were similar irrespective of the type of IFN or time point.

The time course of ISGs regulation differs between IFN-α and IFN-λ3

By microarray analysis, ISGs were more rapidly induced after the addition of IFN-α *vs* IFN-λ3 (data not shown). To confirm the rapid induction of ISGs by IFN-α, six ISGs, that is, IFI-6, IFIT1 (ISG56), DDX60, OAS2, Mx1 and USP18, were quantified for time-dependent expressional change by real-time RT-PCR. Expression of most of the genes upregulated by IFN-α peaked at 12 h and fell thereafter. In contrast, expression of IFN-λ3-induced genes peaked at 24 h and lasted up to 48 h. Combination of IFN-α and IFN-λ3 induced ISG with peak effects occurring at 12–24 h and lasting up to 48 h (Fig. 5).

DISCUSSION

In this study, we demonstrated that IFN-λ family members have distinctive time-dependent antiviral activities in an HCV replicon system and that IFN-λ3 and IFN-α have a synergistic effect in combination. Moreover, we attempted to identify the antiviral mechanism of IFN-λ3 by conducting a cDNA microarray analysis.

In previous studies, anti-HCV activity of IFN-λ1, IFN-λ2 and IFN-λ3 was reported in JFH-1 and OR6/C-5B systems [13]. Time-dependent anti-HCV activity has also been observed with IFN-λ1 [9]. In this study, we confirmed the previous results and added the further finding that time-dependent antiviral activity is not limited to IFN-λ1, but rather is common among all IFN-λs.

Table 1 Top 25 genes that were upregulated by both IFN-α and IFN-λ3 at 12 h

Gene bank ID	Gene symbol	Gene description	IFN-α 0.01 ng/mL fold increase	IFN-λ3 10 ng/mL fold increase	IFN-α+IFN-λ3 fold increase
BC007091	IFIT1	Interferon-induced protein with tetratricopeptide repeats 1	4.01	4.49	4.87
BC049215	OAS2	2′-5′-oligoadenylate synthetase 2, 69/71kDa	3.06	3.88	4.48
M33882	MX1	Myxovirus (influenza virus) resistance 1	3.24	3.29	3.69
AF095844	IFIH1	Interferon induced with helicase C domain 1	2.73	3.02	3.54
BC038115	DDX60	DEAD (Asp-Glu-Ala-Asp) box polypeptide 60	2.70	2.92	3.51
BC011601	IFI6	Interferon, alpha-inducible protein 6	3.07	3.24	3.42
BC042047	HERC6	Hect domain and RLD 6	2.56	2.75	3.34
AF442151	RSAD2	Radical S-adenosyl methionine domain containing 2	1.32	2.59	3.28
U34605	IFIT5	Interferon-induced protein with tetratricopeptide repeats 5	2.47	2.91	3.25
AY730627	OAS1	2′,5′-oligoadenylate synthetase 1, 40/46kDa	2.32	2.57	3.05
AB006746	PLSCR1	Phospholipid scramblase 1	2.37	2.51	3.03
AF307338	PARP9	Poly (ADP-ribose) polymerase family, member 9	2.39	2.46	2.94
M87503	IRF9	Interferon regulatory factor 9	2.61	2.59	2.85
AK297137	IFIT3	Interferon-induced protein with tetratricopeptide repeats 3	1.90	2.36	2.79
AK290655	EIF2AK2	Eukaryotic translation initiation factor 2-alpha kinase 2	2.47	2.45	2.77
BX648758	PARP14	Poly (ADP-ribose) polymerase family, member 14	2.07	2.25	2.66
BC132786	DDX58	DEAD (Asp-Glu-Ala-Asp) box polypeptide 58	1.83	2.17	2.59
AF445355	SAMD9	Sterile alpha motif domain containing 9	2.07	2.08	2.56
	DDX60L	DEAD (Asp-Glu-Ala-Asp) box polypeptide 60-like	1.63	1.92	2.39
BC014896	USP18[1] USP41	Ubiquitin-specific peptidase 18/ubiquitin-specific peptidase 41	1.52	1.78	2.11
AB044545	OAS3	2′-5′-oligoadenylate synthetase 3, 100kDa	1.44	1.57	2.10
BC010954	CXCL10	Chemokine (C-X-C motif) ligand 10	0.76	1.66	1.99
BC014896	USP18	Ubiquitin-specific peptidase 18	1.33	1.55	1.99
AL832618	IFI44L	Interferon-induced protein 44-like	0.58	1.31	1.95

We also assessed whether IFN-λ3 and IFN-α in combination could produce additive or synergistic effects on antiviral activity. In previous studies, additive antiviral activity against HCV was reported with the combination of IFN-λ1 and IFN-α [9, 10]. However, there have been no previous reports on the combined effects of IFN-λ3 and IFN-α. In this study, the focus was on IFN-λ3, because IFN-λ3 is suspected to be the key molecule, mediating the effect of SNPs in the IL-28B gene region on the anti-HCV response to IFN-α. As shown in Fig. 3 and Table S1, synergistic induction of anti-HCV activity occurred in both the OR6/C-5B and Huh7.5/JFH-1 HCV replicon systems. Synergy was demonstrated by the combination index values (Table S1).

Although it has been reported that the upregulated genes induced by IFN-λ are similar to those induced by IFN-α [9, 14–16], there have been no previous reports on

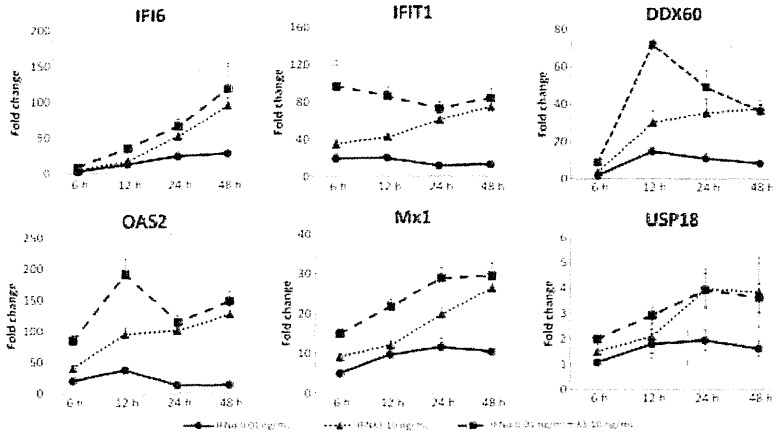

Fig. 5 Time course of ISG expression induced by 0.01 ng/mL IFN-α, 10 ng/mL IFN-λ3 or 0.01 ng/mL IFN-α plus 10 ng/mL IFN-λ3. Expression of the ISGs – IFI-6, *IFIT1* (ISG56), *DDX60*, *OAS2*, *Mx1* and *USP18* – in OR6/ORN/C-5B/KE cells treated 6, 12, 24 and 48 h were determined by qRT-PCR. Results are presented as the relative fold induction. Symbols show the mean value of triplicate wells; error bars show the SD. *Solid lines* represent 0.01 ng/mL IFN-α alone, whereas *fine dashed lines* show 10 ng/mL IFN-λ3 alone, and *coarse dashed lines* show the combination of the 2 cytokines.

the genes induced by IFN-α and IFN-λ in combination. In cDNA microarray analysis, as demonstrated in Table 1, the most strongly upregulated genes induced by IFN-α/IFN-λ3 alone or in combination were almost identical, and most of them were ISGs. As no genes showed upregulation specific to IFN-λ3, we speculate that IFN-α and IFN-λ3 share a similar antiviral intracellular mechanism at the molecular level.

Unexpectedly in microarray analyses, synergistic upregulation of ISGs was not observed. In the same manner, TaqMan real-time RT-PCR analysis showed that the combination of IFN-α and IFN-λ3 did not upregulate ISGs synergistically (Fig. 5). In addition to cDNA microarray analysis, ISRE reporter assays were performed to determine the activation of components of the JAK-STAT pathway common to both type I and III IFNs. As shown in Fig. 4, each IFN upregulated ISRE activity, and the combination of IFN-λ3 and IFN-α did not synergistically enhance ISRE activity either.

Meanwhile, the peak time of the induction of ISG expression differs for IFN-α and IFN-λ1 [9, 17]; peak gene expression occurs earlier with IFN-α than with IFN-λ1. In our study, we confirmed that the peak induction of gene expression occurred later (24 h) and lasted longer (24–48 h) with IFN-λ3 than with IFN-α (12 h). Importantly, gene expression appeared early (12 h) and was prolonged (48 h) by the combination of both IFNs. Similarly to the peak time difference between IFN-α and IFN-λ3 seem for ISG expression, a time-dependent increase in ISRE activation was observed with the combination of both IFNs. While the precise mechanism remains to be clarified, differential regulation of the time-dependent induction of ISG gene expression could be one of the mechanisms underlying the synergistic antiviral effect. One of the molecules contributing to time-dependent ISG upregulation is the ISG known as ubiquitin-specific peptidase 18 (USP18), which has been reported to bind to IFNAR2 and inhibit the interaction of Jak1 with its receptor, thereby preventing IFN-α signalling while leaving IFN-λ signalling unaffected [18, 19]. Actually, expression of USP18 is specifically upregulated with IFN-λ3 in this study as shown in Fig. 5. If the ISGs upregulated by IFN-α are downregulated by USP18, it is plausible that the expression of genes induced by IFN-α decreases early, while expression of genes induced by IFN-λ lasts longer.

A number of clinical studies have confirmed that SNPs around the IL-28B gene are associated with the response to PEG-IFN and RBV therapy, and as previously indicated, various investigations have been performed to clarify the underlying mechanism. Specifically, increased IL-28B mRNA expression in PBMC [2, 3], high serum concentrations of IFN-λ1 (IL-29) [20], low expression of ISGs in the liver prior to IFN treatment [8, 21] and high upregulation of ISG expression by IFN treatment [8, 22] were found in subjects with IL-28B SNP genotypes associated with SVR (rs12979860 CC and rs8099917 TT). Although the functional role of IFN-λ3 still needs to be investigated more thoroughly, if IFN-λ3 expression change is the essential difference in determining the clinical treatment response to PEG-IFN and RBV therapy and if its expression is decreased in patients with the specific IL-28B genotype, which is associated with non-SVR, it is possible that exogenous administration of IFN-λ3 might improve IFN-α-induced viral clearance and that such treatment would be beneficial for patients with the IFN-resistant IL-28B genotype.

In present study, the OR6-cultured cells harboured the rs8099917 TT genotype, and recombinant IFN-λ3 (IL-

28B) protein used in the experiment was derived from cells with the rs8099917 TT genotype (data not shown). Therefore, the viral responses and/or cellular gene expression change in cells and/or proteins with different IL-28B genotypes *in vitro* should be determined in future studies.

In conclusion, we demonstrated that IFN-α and IFN-λ3 synergistically enhance anti-HCV activity *in vitro*. Although the ISGs upregulated by IFN-α and IFN-λ3 were similar, differences in time-dependent upregulation of these genes, especially prolonged ISGs expression by IFN-λ3, might contribute to their synergistic antiviral activity.

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CONFLICT OF INTEREST

Shinya Maekawa and Taisuke Inoue belong to a donation-funded department that is funded by MSD co. ltd.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Figure S1: IFN-α and IFN-λs inhibit HCV core protein secretion. JFH1-infected Huh7.5.1 cells were incubated with various concentrations of IFN-α and IFN-λ1, -λ2, -λ3. After 48 h of treatment, HCV core protein in the medium was measured. Symbols show the mean value of triplicate wells; error bars show the SD. *: $p < 0.05$ vs. control (IFN 0ng/ml).

Figure S2: The dimethylthiazol carboxymethoxyphenyl sulfophenyl tetrazolium assay was performed after OR6/ORN/C-5B/KE cells were cultured with various concentrations of (A) IFN-α, (B) IFN-λ1, (C) IFN-λ2, (D) IFN-λ3 and (E) combination of IFN-α and IFN-λ3 for 48 h. Symbols show the mean value of triplicate wells; error bars show the SD. *: $p < 0.05$ vs. control (IFN 0ng/ml).

Figure S3: The dimethylthiazol carboxymethoxyphenyl sulfophenyl tetrazolium assay was performed after Huh7.5.1/JFH-1 cells were cultured with various concentrations of (A) IFN-α, (B) IFN-λ1, (C) IFN-λ2, (D) IFN-λ3 and (E) combination of IFN-α and IFN-λ3 for 48 h. Symbols show the mean value of triplicate wells; error bars show the SD. *: $p < 0.05$ vs. control (IFN 0ng/ml).

Table S1: Combination index after 48hr stimulation by CalucSyn.

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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.[1] Therapy leading to HCV eradication is the only treatment with proven efficacy in decreasing the occurrence of hepatocellular carcinoma.[2] 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%[3,4] 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,[5] 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,[6,7] 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.[8–11] 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.[12,13] 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.[14] In chronic HCV infection, chemokines such as *RANTES* (regulated on activation normal T-cell expressed and secreted), macrophage inflammatory protein (*MIP*)-1α, *MIP-1β* and interferon-γ 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.[14,15] Upregulation of several serum chemokines, such as eotaxin, *IP-10* and *RANTES* also has been reported in HCV infection, possibly reflecting hepatic inflammation.[16] 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-1β*, *RANTES* or *IP-10* have been investigated previously for their association with the treatment response.[16–20] 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-α-2b 1.5 μg/kg bodyweight, once weekly s.c., and RBV 600–800 mg daily p.o. for 48 weeks).[21] 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°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-γ, anti-IL-1α, anti-IL-1β, 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-32α, anti-IP-10, anti-CXCL11, anti-CCL2, anti-MIF, anti-CCL3, anti-CCL4, anti-PAI-1, anti-RANTES, anti-CXCL12, anti-TNF-α, 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

AT 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^2)	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^{-4}/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-γ	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-32α	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-1α			N.A.
IL-1β			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-α			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 ± 10†	57 ± 8	0.08‡
Sex (male : female)	34:23	23:16	0.56§
Bodyweight (kg)	60.6 ± 10.5†	57.8 ± 7.8	0.17‡
Body- mass index (kg/m^2)	22.9 ± 2.8	22.1 ± 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††
γ-GTP (IU/L)	37 (11–289)	50 (13–167)	0.12††
Albumin (g/dL)	4.1 ± 0.3†	4.1 ± 0.4	0.93‡
Total cholesterol (mg/dL)	166 ± 30	158 ± 31	0.25‡
HbA1c (%)	5.2 (4.7–6.6)¶	5.3 (4.5–7.4)	0.47††
Creatinine (mg/dL)	0.72 ± 0.15†	0.69 ± 0.16	0.39††
WBC count (/μL)	4497 ± 1247	4501 ± 1281	0.99‡
Neutrophil count (/μL)	2243 ± 857	2144 ± 825	0.57‡
Hemoglobin (g/dL)	14.1 ± 1.2	14.2 ± 1.2	0.87‡
Platelet count (×10^{-4}/μL)	15.1 (7–29)¶	13.2 (6.9–19.7)	0.03††
α-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 ± 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; γ-GTP, γ-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 (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).

DISCUSSION

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

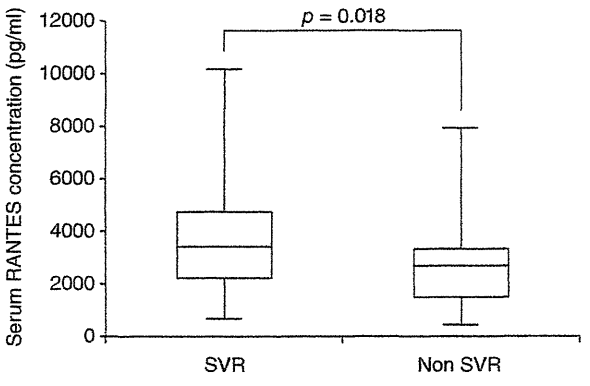


Figure 1 Difference in serum *RANTES* levels between the sustained virological response (SVR) group and the non-SVR group. Ninety-six patients who underwent the standard therapy for 48 weeks were analyzed for serum *RANTES* level using the bead array method. They were divided into the SVR ($n = 57$) 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-type chemokine.[23] 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.[24] 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.[14] 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*,[25] the HCV NS3/4A proteins suppress *RANTES* promoter activity[26] and the HCV core protein may either induce or inhibit the expression of *RANTES* in various cell types.[27] 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*)-*kB* pathway, suggesting that the hepatocytes themselves may serve as the source of *RANTES*.[15]

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 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	*P*-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	
	R	*P*-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

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

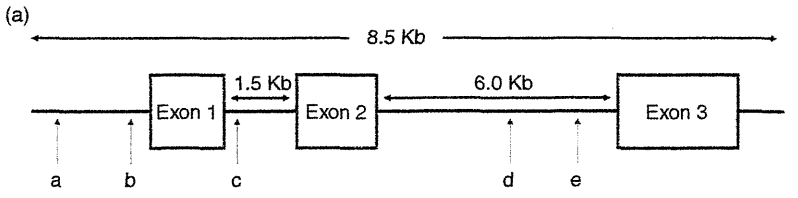
†The cut-off value of 3400 pg/mL was determined by receiver–operator curve analysis in all 96 patients.
a.a., amino acids; CI, confidence interval; IL, interleukin; ISDR, interferon sensitivity-determining region; R, arginine; SNP, single nucleotide polymorphisms.

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.[16] 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.[28,29] 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.[30] 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



RANTES haplotype	a	b	c	d	e	Haplotype frequency
	rs2107538	rs2280788	rs2280789	rs4796120	rs3817655	
R1	G	C	T	A	A	0.599
R2	A	C	C	G	T	0.197
R3	A	G	C	A	T	0.189

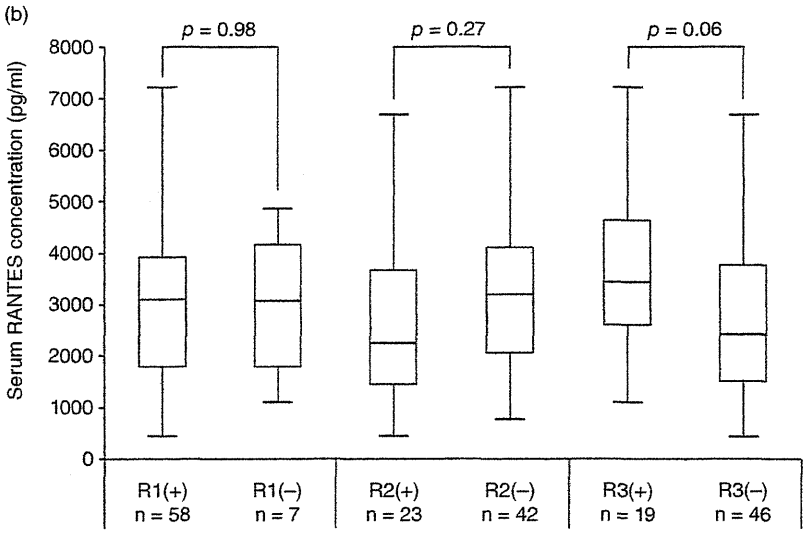


Figure 2 *RANTES* haplotypes and serum *RANTES* level. (a) *RANTES* haplotypes in the patients studied. The human *RANTES* gene spans 8.5 kb on chromosome 17q11-q12 and has the characteristic three exon and two intron organization of the CC chemokine family.[22] Exons are shown as open boxes while introns are shown as solid lines. Five single nucleotide polymorphisms (SNP) (rs2107538/rs2280788/rs2280789/rs4796120/rs3817655) were selected on the basis of data from the HapMap project (http://snp.cshl.org) to obtain complete coverage of the *RANTES* gene in the Japanese population. The locations of SNP variants are indicated by arrows. After the analysis of five *RANTES* SNP in 65 hepatitis C virus patients, haplotypes were determined using SNPAlyze software ver. 8.0 (Dynacom, Chiba, Japan) and divided into three groups on the basis of linkage disequilibrium. These were designated R1, R2 and R3 on the basis of haplotype frequency. (b) Serum *RANTES* level and *RANTES* haplotype. The correlation between serum the *RANTES* level and *RANTES* haplotype was investigated. Box and whisker plots shows distributions of serum *RANTES* levels for the haplotypes R1(+), R1(−), R2(+), R2(−), R3(+) and R3(−). The boxes represent the 25th to 75th percentile and horizontal lines within the boxes 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. R1(+), the patients with the R1 haplotype; R1(−), the patients with a non-R1 haplotype; R2(+), the patients with the R2 haplotype; R2(−), the patients with a non-R2 haplotype; R3(+), the patients with the R3 haplotype; R3(−), the patients with a non-R3 haplotype.

further clarify the correlation. On the other hand, we could not show an association of pretreatment cytokines/chemokine concentrations with the treatment response to PEG IFN/RBV therapy for the other 35 cytokine and chemokine species investigated in this study. Recently, the serum level of *IP-10* was reported to be strongly associated with the response to PEG IFN/RBV therapy and baseline *IP-10* levels were elevated in patients infected with HCV genotype 1 or 4 who did not achieve an SVR after completion of interferon therapy.[19,20] In our study, however, *IP-10* was not extracted as a molecule associated with treatment responses. Actually, due to the measurement limit of the ELISA kit used, several cytokines and chemokines, including *IP-10*, were undetectable in this study, as shown in Table 2, raising the possibility that some cytokines and chemokines associated with SVR were not extracted. Therefore, our study cannot exclude the possibility of other cytokine/chemokines making a contribution to treatment efficacy.

In conclusion, we found that a high pretreatment serum *RANTES* level was related to the efficacy of PEG IFN/RBV therapy in genotype 1b HCV, independent of other treatment-restricting factors, and prediction of treatment outcome could be improved with the measurement of the pretreatment serum *RANTES* level.

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