Association of serum IFN-λ3 with inflammatory and fibrosis markers in patients with chronic hepatitis C virus infection

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ABSTRACT

Background: Hepatitis C virus (HCV) is one of the major causes of liver cancer. The single nucleotide polymorphisms within the IL-28B gene, which encodes IFN-λ3, are strongly associated with the response to pegylated IFNα (PEG-IFNα) and ribavirin (RBV) therapy in chronic hepatitis C (C-CH) patients. However, the roles of IFN-λ3 in chronic HCV infection has been still elusive. In this study, we aimed to identify clinical and immunological factors influencing on IFN-λ3 and evaluated whether serum IFN-λ3 levels are involved or not in the response to PEG-IFNα/RBV therapy.

Methods: We enrolled 119 C-CH patients with HCV genotype 1 infection who underwent 48 week of PEG-IFNα/RBV therapy. As controls, 23 healthy subjects and 51 patients with non-HCV viral hepatitis were examined. Serum IFN-λ3 was quantified by chemiluminescence enzyme immunoassay and 27 cytokines or chemokines were assayed by the multiplexed BioPlex system.

Results: Serum IFN-λ3 levels were higher in C-CH patients or acute hepatitis E patients than those in healthy donors. Such levels did not differ between the IL-28B genotypes. In C-CH patients, serum IFN-λ3 was positively correlated with AST, ALT, AFP, histological activity, fibrosis index, IP-10 and platelet-derived growth factor. Multivariate analysis showed that IL-28B SNPs, fibrosis score and MIP-1α were involved in the sustained viral clearance in PEG-IFNα/RBV therapy, however, serum IFN-λ3 levels were not.

Conclusion: Serum IFN-λ3 is increased in C-CH patients regardless of IL28B genotypes. IFN-λ3 is a biomarker reflecting the activity and fibrosis of liver disease but is not correlated with the responsiveness to the PEG-IFNα/RBV therapy.

Keywords: HCV, IL-28B, Interferon-λ3, Chemokine, PEG-IFNα/ribavirin

Abbreviations

APRI, aspartate aminotransferase platelet ratio index; CI, confidence interval; CLEIA, chemiluminescence enzyme immunoassay; C.C., correlation coefficient: CXCL, C-X-C motif chemokine; DC, dendritic cells; FGF, fibroblast growth factor; FIB-4, Fibrosis-4; G-CSF, granulocyte colony stimulating factor; GM-CSF, granulocyte macrophage colony stimulating factor; GWAS, genome-wide association study; HV, healthy volunteers; IP-10, interferon gamma inducible protein 10; MCP, monocyte chemotactic protein; MIP, macrophage inflammatory protein; MVA, multivariate analysis; OR, odds ratio; PDGF-BB, platelet-derived growth factor-BB; RANTES, regulated on activation, normal T cell expressed and secreted; RBV, ribavirin; SNP, single nucleotide polymorphisms; TLR, toll-like receptor; UVA, univariate analysis; VEGF, vascular endothelial growth factor

INTRODUTION

Hepatitis C virus (HCV) is one of the leading causes of liver cirrhosis and hepatocellular carcinoma (HCC), with nearly 170 million people are infected worldwide [1]. A combination therapy with pegylated IFN-α and ribavirin (PEG-IFNα/RBV) has been used for chronic hepatitis C (C-CH) patients as standard of care, achieving sustained virological response (SVR) in 42–52 % of genotype 1 patients [2]. Even in the coming era of all oral and IFN-free regimens for the treatment of C-CH patients [3-5], PEG-IFNα/RBV therapy could hold promise for elderly patients with advanced fibrosis and high risk of HCC.

Genome-wide association studies (GWAS) including ours have demonstrated that single nucleotide polymorphisms (SNPs) upstream of the promoter region within the IL-28B gene (*IL28B*), which encodes a type III interferon (IFN-λ3), are strongly associated with the response to PEG-IFNα/RBV in C-CH patients [6-9]. Although such significant impact of IL-28B genotype is well acknowledged on the outcome of the combination therapy, the biological and clinical roles of IFN-λ3 in chronic HCV infection has been still elusive. Furthermore, it is controversial if the patients with IL-28B major genotype are capable of producing larger amount of IFN-λ3 than those with the minor type.

IFN-λs consist of a family of subtypes, such as IFN-λ1 (IL-29), IFN-λ2 (IL-28A) and IFN-λ3 (IL-28B), which are biologically active for the suppression of HCV replication [10, 11]. At the initial exposure of HCV, primary human hepatocytes *in vitro* produced IFN-λ and subsequently induced anti-viral interferon-stimulated genes [12]. It is thus rational to consider that the more IFN-λs are produced in the exposed

hosts, the more likely to protect them from HCV virulence at the primary infection. However, in chronically HCV-infected patients, it has not been proven that such scenario could be applicable for the outcome of the disease.

In order to gain insight into the role of IFN-λ3 in chronic HCV infection, we aimed to clarify the factors influencing on serum IFN-λ3 levels, including IL-28B genotype, clinical parameters and various cytokine/chemokine. For the application in the clinical practice, we evaluated whether serum IFN-λ3 levels are associated or not with the response to PEG-IFNα/RBV therapy for chronic hepatitis C patients.

MATERIALS AND METHODS

Study subjects

One hundred nineteen Japanese patients with chronic hepatitis C (C-CH) (genotype 1b and high viral load) were enrolled in the study. All patients were negative for hepatitis B virus (HBV) and human immunodeficiency virus (HIV) and did not have any other chronic liver diseases, such as alcoholic, autoimmune and fatty liver disease. The presence of hepatocellular carcinoma (HCC) was ruled out by ultrasonography or computed tomography examinations. They had been followed at National Center for Global Health and Medicine Kohnodai Hospital, National Hospital Organization Nagasaki Medical Center, Shin-Kokura Hospital and Musashino Red Cross Hospital. They were treated with PEG-IFN-α2b (subcutaneously once a week; 1.5 μg/kg body weight) or PEG-IFN- α2a (180 μg once a week) plus ribavirin (RBV) (600–1,000 mg daily depending on body weight) for 48 weeks according to the guidelines of Japan Society of Hepatology [13]. Virological response to the combination therapy was defined according to the practical guidelines of the American Association for the Study of Liver Diseases [14]. All patients attained adherence to PEG-IFN-α and RBV more than 80% of the estimated total dose. Liver biopsy was carried out before the start of the therapy. Histological activity and fibrosis were determined according to METAVIR scoring system [15]. Serum samples were collected from them before starting PEG-IFN-α/RBV treatment and were stored at -80C. In some patients, the samples were obtained 24 weeks after the cessation of the therapy (at the end of follow up).

As controls, sera was obtained from 10 healthy subjects without HCV, HBV and HIV

infection (male: female, 5:5, mean age ± SD: 45 ± 12 years). In comparison of serum IFN-λ levels between C-CH patients with those with other type of liver diseases, 6 patients with chronic HBV infection (B-CH, HBeAg-negative) were examined as well. They were not treated with IFN or nucleot(s)ide analogues for HBV infection. In addition, we compared serum IFN-λ3 levels among patients with acute viral hepatitis with various etiology, such as hepatitis A (A-AH), hepatitis B (B-AH) or hepatitis E (E-AH), the diagnosis of which was determined by serological examinations at Teine Keijinkai Hospital and Kurume University Hospital. The serum samples were obtained from them at the time of active liver inflammation (ALT levels > 2 x upper limit of normal range). As the representatives for non-invasive fibrosis markers, Fibrosis-4 (FIB-4) and aspartate aminotransferase platelet ratio index (APRI) were calculated as reported previously [16, 17].

The study protocol was conformed to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the ethical committee of each institute. Written informed consent was obtained from all patients.

IL28B genotyping

The subjects were evaluated for SNPs near *IL28B* gene (rs8099917) using the Invader Plus assay (Invader Chemistry, Madison, WI, USA) as previously reported [18]. The TT, TG and GG genotypes were determined accordingly.

Measurement of serum IFN-λ3

Serum levels of IFN-λ3 were evaluated by the newly developed chemiluminescence enzyme immunoassay (CLEIA) system as reported previously [19]. The system enables

to quantify serum IFN-λ3 specifically without any overlap from IFN-λ1 and IFN-λ2. The threshold of the assay is 10 pg/mL and its range is 10-1000 pg/mL.

Simultaneous measurement of multiple chemokines and cytokines

In order to quantify multiple chemokines and cytokines simultaneously in limited volume of samples, we used BioPlex 3D system (BioPlex Pro Human GI 27Plex; Bio-Rad, Hercules, CA, USA) for the study. In this system, 27 chemokines and cytokines were measurable, such as basic FGF, Eotaxin, G-CSF, GM-CSF, IFN-γ, IL-10, IL-12, IL-13, IL-15, IL-17, IL-1β, IL-1rα, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IP-10, MCP-1, MIP-1α, MIP-1β, PDGF-BB, RANTES, TNF-α, and VEGF. The detection range and thresholds are shown in Supplementary Table (**Table S1**). For the measurement of IP-10, ELISA (R&D Systems, MN, USA) was performed as well

Statistical analyses

Continuous variables were compared between groups using the Wilcoxon's signed rank test, Mann-Whitney U-test, and categorical data were compared using the chi-squared test or Fisher's exact test. The correlation between cytokines, chemokines and clinical markers were evaluated by Spearman's correlation coefficient. The p value < 0.05 was considered to be significant. The logistic regression was used for multivariate analyses. All statistical analyses were performed with PRISM and SPSS software.

RESULTS

Serum IFN-λ3 levels are increased in patients with chronic HCV infection.

The clinical backgrounds of chronic hepatitis C (C-CH) patients are shown in **Table 1**. First, we compared serum IFN-λ3 levels among patients with C-CH or HBV infection (B-CH) and uninfected healthy volunteers (HV). Such levels in the C-CH group were significantly higher than those in the B-CH or in the HV groups (**Fig 1A**). The levels in the B-CH were increased, however, the significance of which was much less than those in the C-CH group (**Fig 1A**). Next, we compared serum IFN-λ3 levels between patients with IL-28B TT genotype and those with TG/GG (non-TT) genotype in the C-CH group. Although some patients in the TT group showed relatively higher levels of IFN-λ3 than those in the non-TT, such difference between the TT and non-TT did not reach significance (**Fig 1B**). Third, we compared serum IFN-λ3 levels before and after the combination therapy in the relevant cases. In patients who successfully eradicated HCV (sustained virological response, SVR), serum IFN-λ3 levels were significantly decreased at 24 weeks after the therapy. In contrast, such levels did not change in those who failed to do so (transient virological response and no virological response, TVR and NVR groups, respectively) (**Fig 1C**). Fourth, we compared serum IFN-λ3 levels among patients with various causes of acute viral hepatitis. The IFN-λ3 levels in the acute hepatitis E group (E-AH) were higher than those in the HV patients **(Fig 1D**). The IFN-λ3 levels in the B-AH group tended to be higher than those in the HV, however, statistical analysis was not performed because of the limited samples (N=2). No significant difference was observed between the A-AH and the HV groups. These results indicate that serum IFN-λ3 levels are increased in patients with chronic

hepatitis C or acute hepatitis E.

Serum IFN-λ3 levels may be related to liver inflammation or fibrosis in patients with chronic hepatitis C.

In order to explore the clinical significance of IFN-λ3 in chronic HCV infection, we simultaneously examined 27 chemokines and cytokines in serum by means of BioPlex system, which allows to measure multiple factors at high sensitivity in small volume of samples (10 μl/sample). In comparison with the results of healthy donors, we found that the levels of some chemokine in the C-CH were higher than those in the HV group, such as IP-10, MIP-1α, MIP-1β, RANTES and PDGF-BB (**Fig 2, Supplementary Fig 1**).

Next, we examined whether serum IFN-λ3 levels are correlated or not with clinical parameters or immunological markers in the C-CH group. The IFN-λ3 levels were weakly and positively correlated with AST, ALT, AFP and histological activity (A-score) **(Table 2**). These results indicate that the increase of serum IFN-λ3 in patients with chronic hepatitis C is related to liver inflammation. The indices of FIB-4 or APRI are the representatives of non-invasive marker of liver fibrosis. The levels of serum IFN-λ3 were positively correlated with APRI, but not with FIB-4 (**Table 2**). With regard to the chemokines of displaying higher results in the C-CH, the levels of IP-10 and PDGF-BB were positively correlated with the IFN-λ3 levels (**Table 2**). Such chemokines are reported to be involved in early stage of liver fibrosis [20-22]. Thus, serum levels of IFN-λ3 may be related to the fibrotic markers as well.

Pretreatment serum IFN-λ3 is not related to sustained virological response to

PEG-IFNα/RBV therapy in patients with chronic hepatitis C.

Because the genotype of IL-28B is a strong predictor of PEG-IFNα/RBV therapy for chronic hepatitis C, we sought to examine the clinical value of serum IFN-λ3 in patients who underwent the combination therapy. In comparison of the clinical and immunological factors between the SVR and non-SVR groups, univariate analysis revealed that AST, IL28B genotype, F-score and MIP-1α were associated with the SVR (**Table 3**). However, serum IFN-λ3 or IP-10 levels were not different between the SVR and non-SVR groups (**Table 3**). Subsequently, multivariate analysis including such factors of significance ($p<0.05$ by UVA) showed that IL-28B SNPs, F-score and MIP-1α were involved in the SVR (**Table 3**). These results suggest that serum IFN-λ3 fails to be a predictive marker for SVR in PEG-IFNα/RBV therapy.

DISCUSSION

In this study, we demonstrated that serum IFN-λ3 levels were higher in patients with chronic hepatitis C than those in uninfected or HBV-positive patients, the levels of which did not differ regardless of IL-28B genotypes. Serum IFN-λ3 levels were correlated with clinical and immunological markers of liver inflammation and fibrosis, suggesting that the production of IFN-λ3 may be regulated by not only the presence or absence of HCV but also by the status of liver disease. It is well acknowledged that IL-28B genotype is a strong predictor of SVR in a PEG-IFNα/RBV therapy for chronic hepatitis C [7-9]. However, serum IFN-λ3 fail to be a surrogate marker for IL-28B genotype in the combination therapy.

At the primary HCV infection, IFN-λ is produced from hepatocytes that subsequently induce anti-viral interferon-stimulated genes [23]. In addition to hepatocytes, dendritic cells (DCs) or macrophages are capable of producing IFN-λ in response to HCV [24]. For sensing HCV, hepatocytes and $BDCA3^{+}$DCs mainly utilize TLR3 and RIG-I and plasmacytoid DCs do TLR7, respectively [24, 25]. It is yet to be clarified which cells, hepatocytes or DCs are equipped with stronger potential to secrete IFN-λ at a single cell level. However, it is rational to consider that serum IFN-λ3 levels in patients are determined by the sum of IFN-λ3 sporadically released from both types of cells. Therefore, it is plausible that the amount of IFN-λ released from hepatocytes or DCs are influenced by the environment of producers, such as inflammation and fibrosis. Of interest is the finding that serum IFN-λ3 levels were higher in patients with acute hepatitis E, but not in acute hepatitis A patients. It is reported that DCs localized in intestine are capable of producing IFN-λ in response to Rotavirus to protect host from

infection [26]. Although both HEV and HAV are RNA virus transmissible by entero-fecal route, such difference in serum IFN-λ3 levels suggest that distinct mechanisms of recognition of HEV and HAV by hosts.

The regulatory mechanisms of transcription and translation of IFN-λ3 have not been well documented. The IL-28B SNPs (rs8099917) are located in 8.9 kb upstream of the promoter region of *IL-28* gene [8, 9, 11]. Taking such localization into consideration, it is less likely that the genetic variation gives some impact on transcriptional level of IL-28B. With regard to the relationship between IL-28B genotype and its transcripts, controversial results have been reported thus far. Some groups declared that IFN-λ3 mRNA levels in PBMC were higher in patients with IL-28B major genotype than those in the minor type [9]. In contrast, others showed that such levels of IL-28B in hepatocytes were comparable regardless of IL-28B SNPs. In search for some genetic factors influencing on IL-28B transcription, Sugiyama et al. reported the existence of variable length of TA repeat in the promoter of IL-28B gene [27]. Other investigators showed that certain structure of 3'-UTR in IL-28B gene is involved in the durability/stability of the gene [28]. Nevertheless, the contribution of such factors is not enough to fill in the gap, suggesting that certain other regulatory factors for IL-28B are still undisclosed.

The report concerning with serum IFN-λ in chronic hepatitis C patients is limited. Langhans et al. showed that serum levels of IFN-λ, which includes IFN-λ2 and IFN-λ3, were higher in patients with IL-28B major type than those in the minor type [29]. One of the limitations of their study seems to be the lack of specificity for the measurement of IFN-λ3. Since the homology of IL-28A and IL-28B is quite high, it is difficult to quantify specifically IFN-λ3 by excluding the contamination of IFN-λ2. In

order to exclude such possibility, we used the newly-developed CLEIA for IFN-λ3, which enables to quantify IL-28B without any influence of IL-28A at the range of 0-1000 pg/mL. By means of this system, we found that serum levels of IFN-λ3 are not statistically different between the patients with the IL-28B major and those with the minor type.

At the primary HCV exposure, the significance of IFN-λs as an anti-viral protein is evident. However, such impact of IFN-λ3 in chronically HCV-infected patients are still elusive. In the above-mentioned paper, Langhans et al. reported that serum IFN-λ level in patients who had spontaneously cleared HCV was higher than those in chronic HCV infection [29], implying that higher level of IFN-λ has somewhat contributed to HCV eradication. In this study, we aimed to clarify the significance of IFN-λ3 in patients with chronic HCV infection with different approaches. Firstly, we searched the factors influencing on serum IFN-λ3 quantity by the correlation analysis with clinical markers and multiple cytokines/chemokines. We found that AST, ALT, AFP and histological activity (A-scores) were positively correlated with serum IFN-λ3 levels. In addition, one of the non-invasive fibrosis markers, APRI, was weakly correlated with serum IFN-λ3. Among the chemokines examined in this study, serum IFN-λ3 was positively correlated with IP-10 and PDGF-BB. IP-10/CXCL10 is induced in HCV-infected hepatocytes as one of the interferon-stimulated genes, which attracts CXCR3-positive T cells and NK cells and subsequently activates inflammation. IP-10 is also reported to be involved in early stage of liver fibrosis [30, 31]. Similar fibrotic function was reported as well for PDGF-BB, which is reported to be increased in patients with advanced/fibrosis stages of HBV infection [32, 33]. These reports support the notion that IFN-λ3 is related to liver inflammation and fibrosis. Secondly, we

examined whether serum IFN-λ3 and chemokine are involved or not in the SVR to PEG-IFNα / RBV therapy for chronic hepatitis C patients. We confirmed that IL-28B genotypes, F-score and MIP-1α are associated with SVR in this cohort, but failed to do so with IP-10 and serum IFN-λ3. Several studies showed that pretreatment IP-10 levels could be a predictor of SVR in PEG-IFNα/RBV therapy for chronic hepatitis C [34], the significance of which became stronger in combined with IL-28B genotypes [35, 36]. One of the reasons why the IP-10 levels failed to be significant in this study may be a bias for the enrollment of patients from multiple hospitals and medical centers.

In summary, serum IFN-λ3 is increased in patients with chronic HCV infection regardless of IL-28B genotypes, the level of which is associated with liver inflammation and fibrosis. The biological role and clinical impact of IFN-λ3 in patients with chronic HCV infection needs to be further investigated.

FIGURE LEGENDS

Fig 1: Serum IFN-λ3 levels are increased in patients with chronic HCV infection or acute HEV infection.

A: Serum IFN-λ3 levels in patients with chronic hepatitis C (C-CH, N=119), chronic hepatitis B (B-CH, N=6) and healthy volunteers (HV, N=23) were quantified by CLEIA method as described in Materials and Methods. *: $p<0.05$, **: $p<0.01$, ***: $p<0.0001$ by Mann-Whitney U-test

B: Serum IFN-λ3 levels in the C-CH group were compared between the patients with IL-28B TT (rs8099917) (N=100) and those with non-TT (TG/GG) (N=19) genotype.

C: Serum IFN-λ3 levels in C-CH patients were compared before and 24 weeks after the PEG-IFN-α and ribavirin therapy.

SVR: sustained virological response (N=21), TVR: transient virological response (N=10), NVR: non virological response (N=5), *: $p<0.05$ by Wilcoxon's signed rank test

D: Serum IFN-λ3 levels in acute hepatitis patients with various etiology were quantified by CLEIA as described in Materials and Methods.

HV; healthy volunteers (N=23), A-AH; acute hepatitis A (N=34), B-AH; acute hepatitis B (N=2), E-AH; acute hepatitis E (N=9). All samples were collected from patients whose ALT levels were 2 times higher than upper limit of normal range (>2xULN).

**: $p<0.0001$ by Mann-Whitney U-test

Fig 2: Several chemokine are increased in patients with chronic HCV infection.

Twenty-seven chemokines and cytokines in serum from chronic hepatitis C patients (C-CH) and healthy donors (HV) were assayed by means of BioPlex method. IP-10 was measured by ELISA. Representative results of chemokine that showed statistical significance between the groups are shown, such as IP-10, MIP-1α, MIP-1β, RANTES and PDGF-BB.

*p<0.005, **p<0.001, ***p<0.0001 by Mann-Whitney U test

Supplementary Figure Legends

Fig S1: Multiple cytokine/chemokine analysis in patients with chronic HCV infection and uninfected healthy volunteers

Serum cytokine and chemokine were quantified by means of multiplexed Bio-Plex system as described in Materials and Methods. IP-10 was assayed by ELISA and are shown in Fig2. IL-2 was not measurable in this study.

C-CH: chronic hepatitis C patients, HV: healthy volunteers

*p<0.005, **p<0.001, ***p<0.0001 by Mann-Whitney U test

ACKNOELEDGEMENT

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Conflict of interest

Shigeko Naito and Tatsuji Kimura are employee of the Institute of Immunology Co., Ltd. All other authors have nothing to declare.

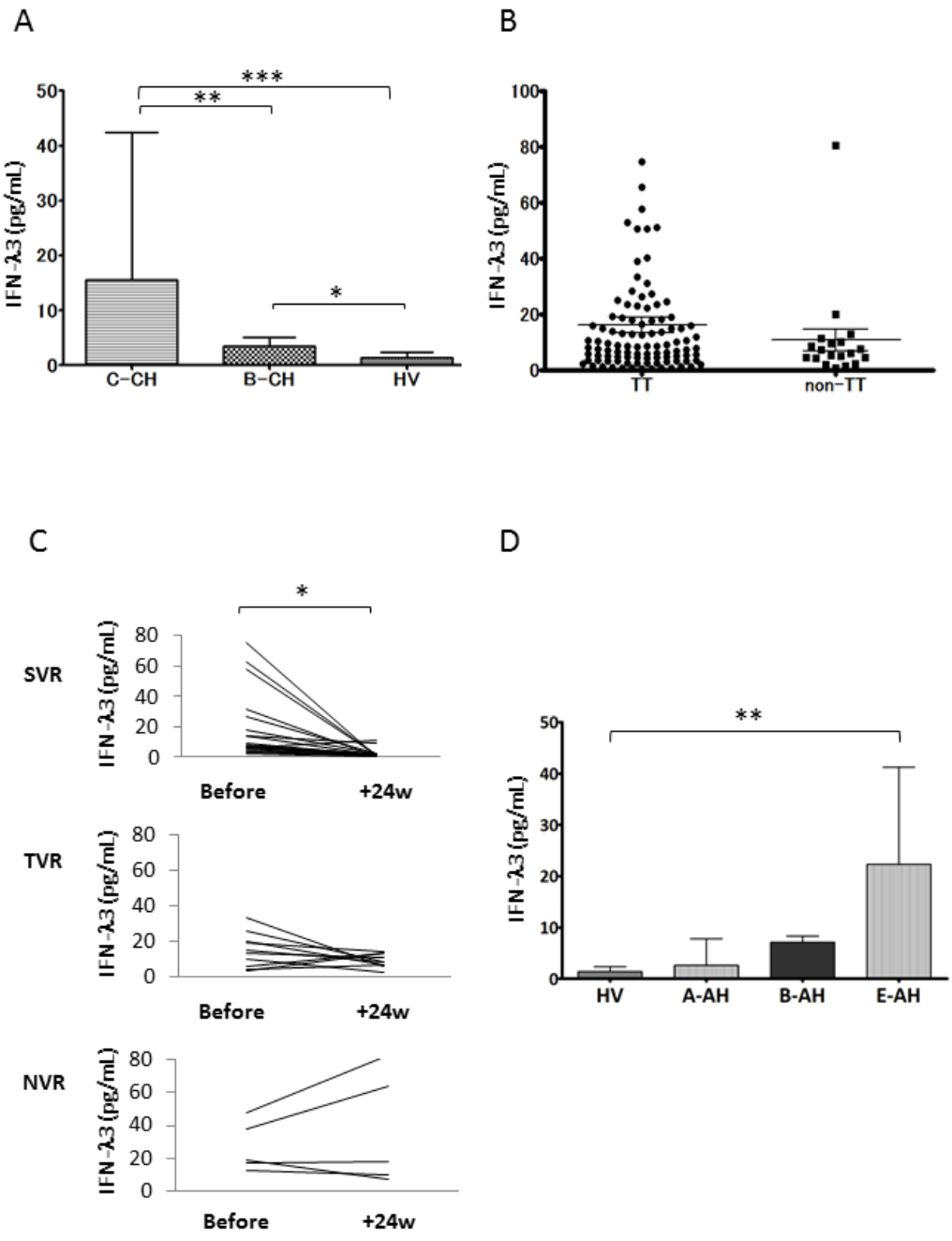
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Fig 1

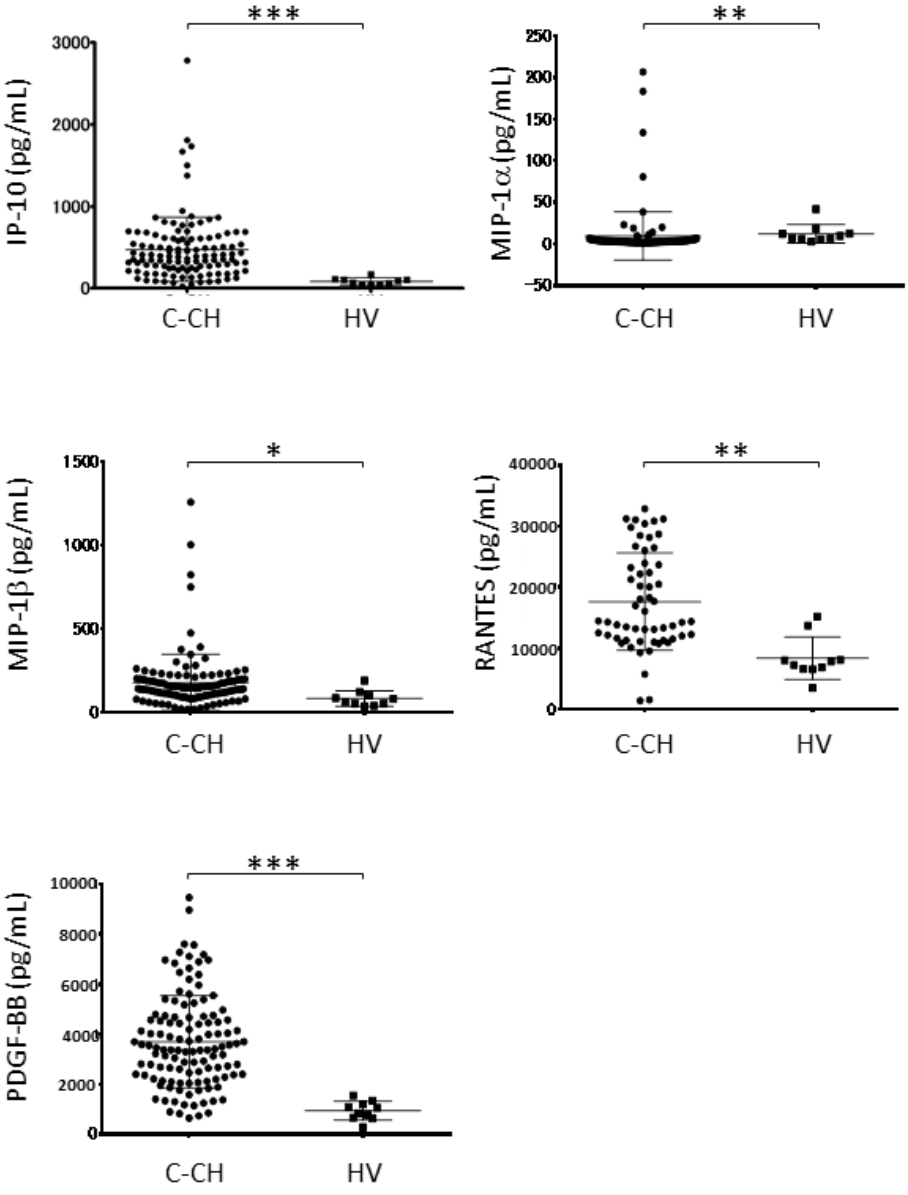
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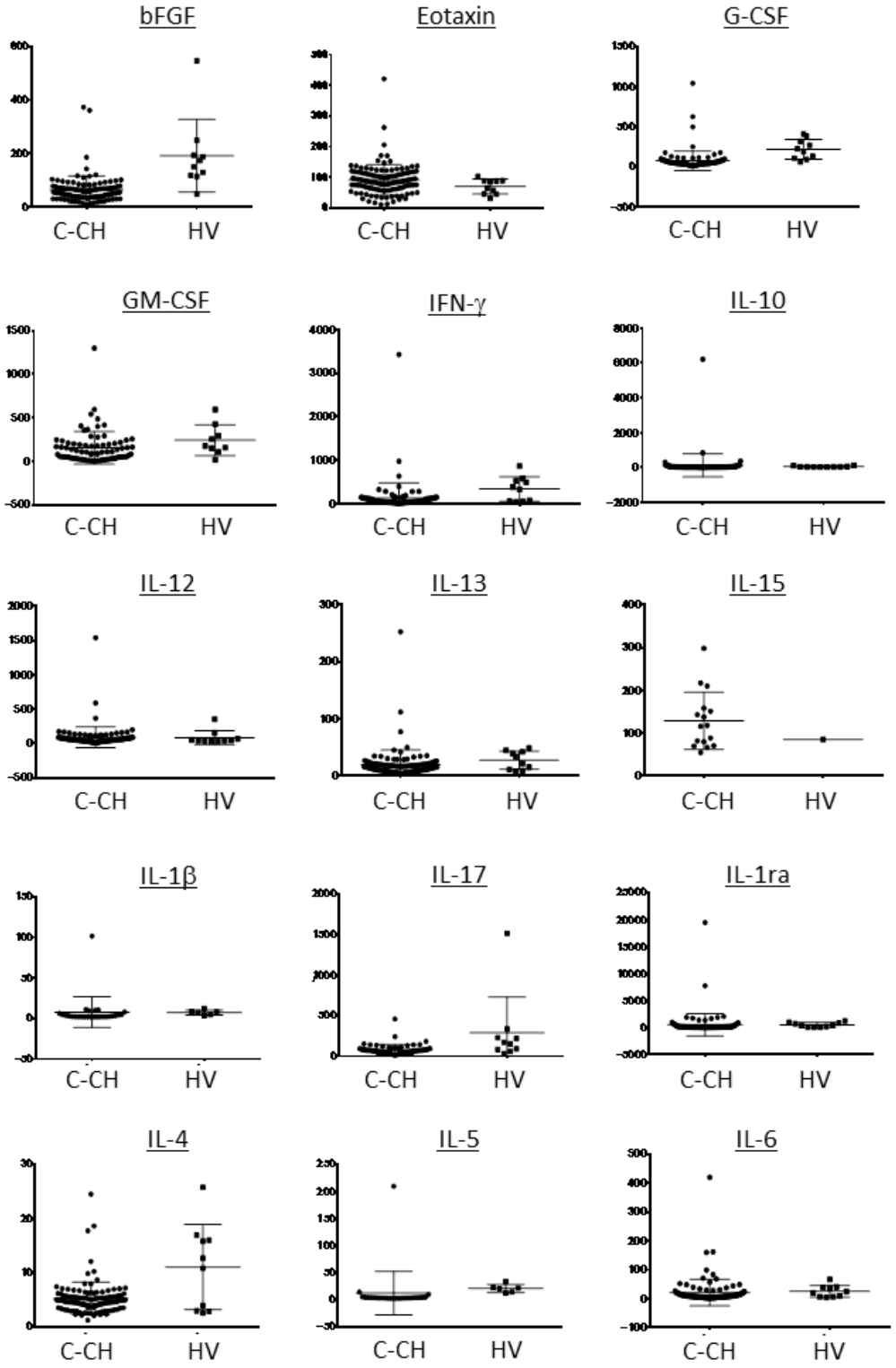
Fig 2

Fig S1-1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IL-5
IL-6
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HV

Fig S1-2

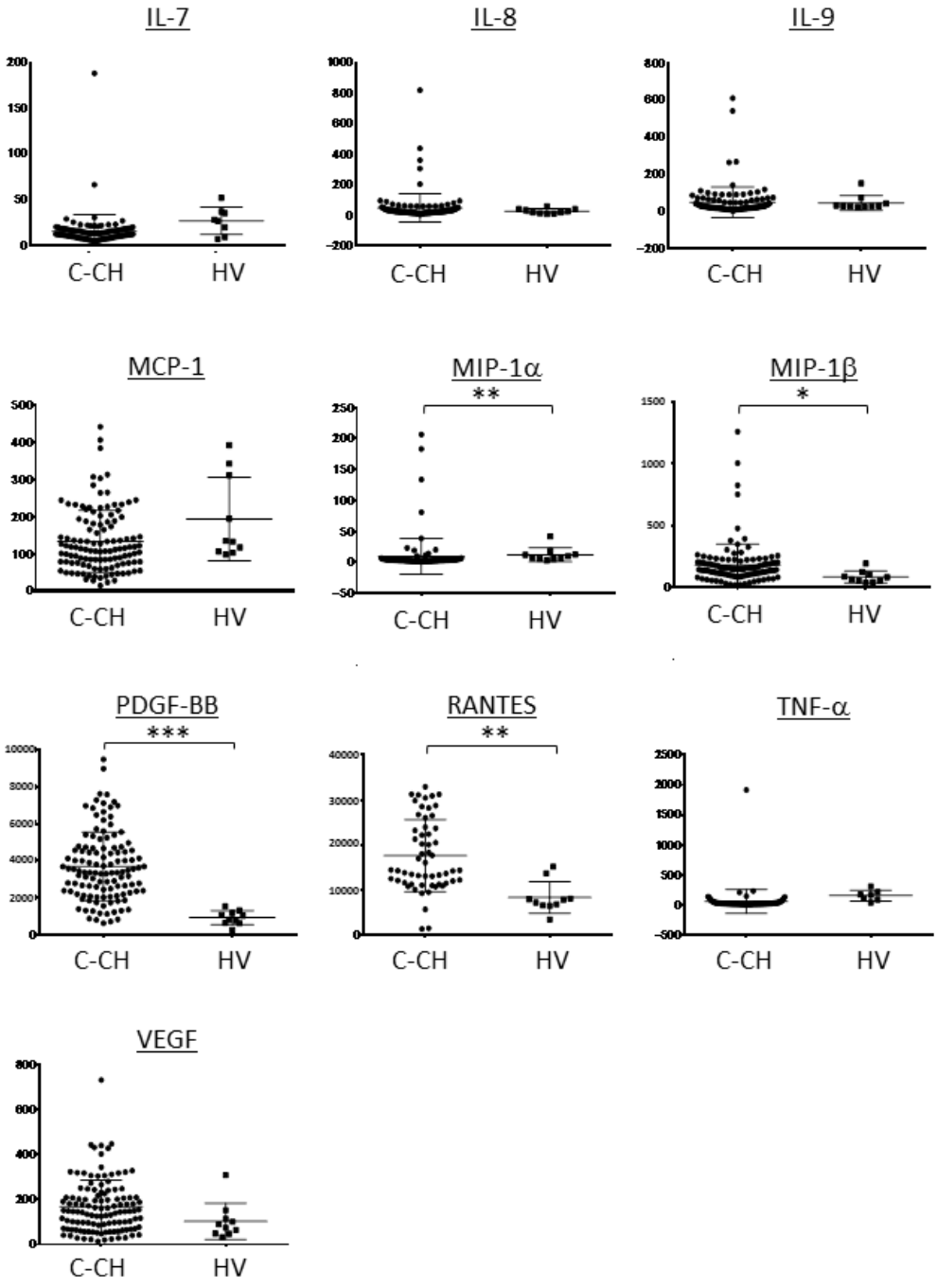

Table 1: Clinical backgrounds of the patients with chronic HCV infection

Factors	C-CH
N (M : F)	119 (69 : 50)
Age	56.5 ± 10.1
WBC (/mm^3)	5120 ± 1575
Hb (g/dL)	14.4 ± 1.5
Plt (×10^4/mm^3)	17.7 ± 5.2
TP (g/dl)	7.5 ± 0.5
Alb (g/dL)	4.2 ± 0.4
AST (U/L)	54.7 ± 38.3
ALT (U/L)	71.5 ± 54.2
T-Bil (mg/dL)	0.8 ± 0.3
T-chol (mg/dL)	176.6 ± 37.0
AFP (ng/mL)	9.7 ± 13.4
HCV RNA (Log IU/mL)	6.3 ± 0.6
Activity (A0/A1/A2/A3)	1/68/33/2
Fibrosis (F1/F2/F3/F4)	48/36/16/4
IL28B rs8099917 (TT : non-TT)	100 : 19

C-CH : chronic hepatitis C patients

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Table S1: The range and thresholds of cytokines and chemokines measured by means of BioPlex™ system

Factors	Range (pg/mL)	Threshold (pg/mL)
IL-1b	3.2 – 3,261	0.6
IL-1ra	81.1 – 70,487	5.5
IL-2	2.1 – 17,772	1.6
IL-4	2.2 – 3,467	0.7
IL-5	3.1 – 7,380	0.6
IL-6	2.3 – 18,880	2.6
IL-7	3.1 – 6,0014	1.1
IL-8	1.9 – 26,403	1.0
IL-9	2.1 – 7,989	2.5
IL-10	2.2 – 8,840	0.3
IL-12	3.3 – 13,099	3.5
IL-13	3.7 – 3,137	0.7
IL-15	2.1 – 2,799	2.4
IL-17	4.9 – 12,235	3.3
Basic FGF	27.2 – 7,581	1.9
Eotaxin	40.9 – 5,824	2.5
G-CSF	2.4 – 11,565	1.7
GM-CSF	63.3 – 6,039	2.2
IFN-γ	92.6 – 52,719	6.4
MCP-1	2.1 – 1,820	1.1
MIP-1α	1.4 – 836	1.6
MIP-1β	2.0 – 1,726	2.4
PDGF-BB	7.0 – 51,933	2.9
RANTES	2.2 – 8,617	1.8
TNF-α	5.8 – 95,484	6.0
VEGF	5.5 – 56,237	3.1
IP-10	7.8 – 500	1.67

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