Ntcp. CCAAT/enhancer-binding protein also bound and regulated the h*NTCP* promoter (44, 48). A previous study, which was mainly based on reporter assays using a construct of the region from −188 to +83 of the h*NTCP* promoter, concluded that RAR did not affect h*NTCP* transcription (48). By using a reporter carrying a longer promoter region, our study is the first to implicate RARs in the regulation of h*NTCP* gene expression (Fig. 9). The turnover of NTCP protein was reported to be rapid, with a half-life of much less than 24 h (49). Consequently, reduction in the *NTCP* transcription by RAR inhibition could rapidly decrease the NTCP protein level and affect HBV susceptibility.

NTCP plays a major role in the hepatic influx of conjugated bile salts from portal circulation. Because NTCP knock-out mice are so far unavailable, it is not known whether loss of NTCP function can cause any physiological defect *in vivo*. However, no serious diseases are reported in individuals carrying single nucleotide polymorphisms that significantly decrease the transporter activity of NTCP (50, 51), suggesting that NTCP function may be redundant with other proteins. Organic anion transporting polypeptides are also known to be involved in bile acid transport. Moreover, an inhibition assay using Myrcludex-B showed that the IC_{50} value for HBV infection was ~0.1 nM (52), although that for NTCP transporter function was 4 nM (28), suggesting that HBV infection could be inhibited without fully inactivating the NTCP transporter (53). HBV entry inhibitors are expected to be useful for preventing *de novo* infection upon post-exposure prophylaxis or vertical transmission where serious toxicity might be avoided with a short term treatment (54). For drug development studies against HIV, down-regulation of the HIV coreceptor CCR5 by ribozymes could inhibit HIV infection both *in vitro* and *in vivo* (55). Disruption of CCR5 by zinc finger nucleases could reduce permissiveness to HIV infection and was effective in decreasing viral load *in vivo* (56). Thus, interventions to regulate viral permissiveness could become a method for eliminating viral infection (55). Our findings suggest that the regulatory mechanisms of NTCP expression could serve as targets for the development of anti-HBV agents. High throughput screening with a reporter assay using an *NTCP* promoter-driven reporter, as exemplified by this study, will be useful for identifying more anti-HBV drugs.

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

Yoshihiko Aoki, Masaya Sugiyama, Kazumoto Murata, Sachiyo Yoshio, Masayuki Kurosaki, Satoru Hashimoto, Hiroshi Yatsuhashi, et al.

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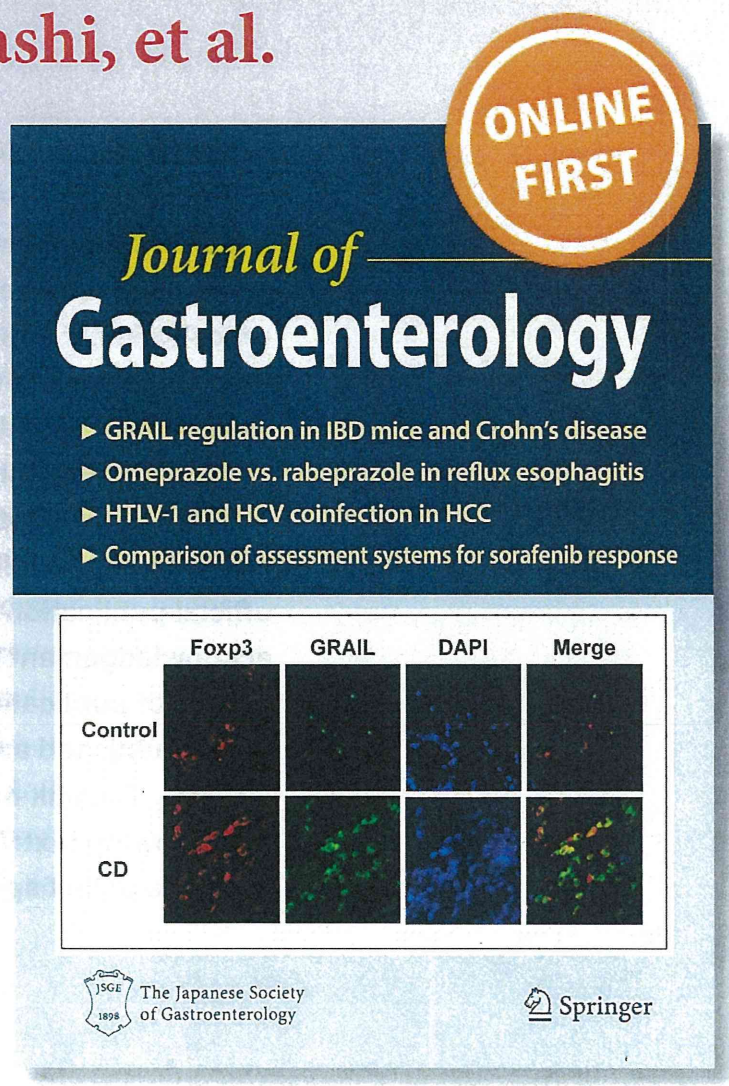

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ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

Association of serum IFN-λ_3 with inflammatory and fibrosis markers in patients with chronic hepatitis C virus infection

Yoshihiko Aoki · Masaya Sugiyama · Kazumoto Murata · Sachiyo Yoshio · Masayuki Kurosaki · Satoru Hashimoto · Hiroshi Yatsuhashi · Hideyuki Nomura · Jong-Hon Kang · Tsutomu Takeda · Shigeko Naito · Tatsuji Kimura · Yoko Yamagiwa · Masaaki Korenaga · Masatoshi Imamura · Naohiko Masaki · Namiki Izumi · Masayoshi Kage · Masashi Mizokami · Tatsuya Kanto

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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 *IFNL3* gene, which encodes interferon (IFN)-λ_3, are strongly associated with the response to pegylated IFN-α (PEG-IFN-α) plus ribavirin (RBV) therapy in chronic hepatitis C (C-CH) patients. However, the roles of IFN-λ_3 in chronic HCV infection are still elusive. In this study, we aimed to identify clinical and immunological factors influencing IFN-λ_3 and evaluated whether serum IFN-λ_3 levels are involved or not involved in the response to PEG-IFN-α plus RBV therapy.

Methods We enrolled 119 C-CH patients with HCV genotype 1 infection who underwent 48 weeks of PEG-IFN-α plus RBV therapy. As controls, 23 healthy subjects and 56 patients with non-HCV viral hepatitis were examined. Serum IFN-λ_3 was quantified by chemiluminescence enzyme immunoassay, and 27 cytokines or chemokines were assayed by the multiplexed BioPlex system.

Results Serum IFN-λ_3 levels were higher in C-CH patients or acute hepatitis E patients than in healthy volunteers. Such levels did not differ between the *IFNL3* genotypes. In C-CH patients, serum IFN-λ_3 was positively correlated with aspartate aminotransferase, alanine aminotransferase, α-fetoprotein, histological activity, fibrosis index, IFN-γ-inducible protein 10, and platelet-derived growth factor. Multivariate analysis showed that *IFNL3* single nucleotide polymorphisms, fibrosis score, and macrophage inflammatory protein 1α were involved in the

Y. Aoki and M. Sugiyama contributed equally to this work.

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Y. Aoki · M. Sugiyama · K. Murata · S. Yoshio · Y. Yamagiwa · M. Korenaga · M. Imamura · N. Masaki · M. Mizokami · T. Kanto (✉)
Department of Hepatic Diseases, The Research Center for Hepatitis and Immunology, National Center for Global Health and Medicine, 1-7-1 Kohnodai, Ichikawa, Chiba 272-8516, Japan
e-mail: kantot@hospk.ncgm.go.jp

M. Kurosaki · N. Izumi
Department of Gastroenterology and Hepatology, Musashino Red Cross Hospital, Tokyo, Japan

S. Hashimoto · H. Yatsuhashi
National Hospital Organization Nagasaki Medical Center, Clinical Research Center, Omura, Japan

H. Nomura
The Center for Liver Diseases, Shin-Kokura Hospital, Kitakyushu, Japan

J.-H. Kang
Center for Gastroenterology, Teine Keijinkai Hospital, Sapporo, Japan

T. Takeda
Department of Gastroenterology, Juntendo University School of Medicine, Tokyo, Japan

S. Naito · T. Kimura
Institute of Immunology Co. Ltd, Tokyo, Japan

M. Kage
Department of Diagnostic Pathology, Kurume University Medical School, Fukuoka, Japan

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sustained viral clearance in PEG-IFN-α plus RBV therapy; however, serum IFN-λ_3 levels were not involved.
Conclusion Serum IFN-λ_3 levels are increased in C-CH patients regardless of the *IFNL3* genotype. IFN-λ_3 is a biomarker reflecting the activity and fibrosis of liver disease, but is not correlated with the responsiveness to PEG-IFN-α plus RBV therapy.

Keywords Hepatitis C virus · IL-28B · Interferon-λ_3 · Chemokine · Pegylated interferon-α plus ribavirin

Abbreviations

APRI	Aspartate aminotransferase platelet ratio index
ALT	Alanine aminotransferase
AST	Aspartate aminotransferase
B-CH	Chronic hepatitis B
C-CH	Chronic hepatitis C
FIB-4	Fibrosis-4
HBV	Hepatitis B virus
HCC	Hepatocellular carcinoma
HCV	Hepatitis C virus
HIV	Human immunodeficiency virus
HV	Healthy volunteer
IFN	Interferon
IP-10	Interferon-γ-inducible protein 10
MIP	Macrophage inflammatory protein
PDGF-BB	Platelet-derived growth factor BB
PEG-IFN-α	Pegylated interferon-α
RANTES	Regulated on activation, normally T cell expressed, and secreted
RBV	Ribavirin
SNP	Single nucleotide polymorphism
SVR	Sustained virological response

Introduction

Hepatitis C virus (HCV) is one of the leading causes of liver cirrhosis and hepatocellular carcinoma (HCC), with nearly 170 million people infected worldwide [1]. A combination therapy with pegylated interferon (IFN)-α (PEG-IFN-α) and ribavirin (RBV) has been used for chronic hepatitis C (C-CH) patients as the 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-IFN-α plus RBV therapy could hold promise for elderly patients with advanced fibrosis and high risk of HCC.

Genome-wide association studies, including ours, have demonstrated that single nucleotide polymorphisms (SNPs) upstream of the promoter region within the *IFNL3* gene (also known as *IL28B*), which encodes a type III IFN (IFN-λ_3), are strongly associated with the response to PEG-IFN-α plus RBV therapy in C-CH patients [6–9]. Although such significant impact of the *IFNL3* genotype on the outcome of the combination therapy is well acknowledged, the biological and clinical roles of IFN-λ_3 in chronic HCV infection are still elusive. Furthermore, it is controversial if patients with the *IFNL3* major genotype are capable of producing larger amounts of IFN-λ_3 than those with the minor genotype.

The IFN-λ family consists of several subtypes, such as IFN-λ_1 (IL-29), IFN-λ_2 (IL-28A), and IFN-λ_3 (IL-28B), which are biologically active for the suppression of HCV replication [10, 11]. On initial exposure to HCV, primary human hepatocytes in vitro produced IFN-λ and subsequently induced antiviral IFN-stimulated genes [12]. It is thus rational to consider that the more IFN-λ family members are produced in the exposed hosts, the more likely they are to protect the hosts from HCV virulence in the primary infection. However, in chronically HCV-infected patients, it has not been proven that such a scenario could be applicable for the outcome of the disease.

To gain insight into the role of IFN-λ_3 in chronic HCV infection, we aimed to clarify the factors influencing serum IFN-λ_3 levels, including *IFNL3* genotype, clinical parameters, and various cytokines and chemokines. For application in clinical practice, we evaluated whether serum IFN-λ_3 levels are associated or not associated with the response to PEG-IFN-α plus RBV therapy for C-CH patients.

Materials and methods

Study subjects

One hundred nineteen Japanese patients with 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 HCC was ruled out by ultrasonography or computed tomography examinations. The patients had been followed at the National Center for Global Health and Medicine Kohnodai Hospital, the National Hospital Organization Nagasaki Medical Center, Shin-Kokura Hospital, and Musashino Red Cross Hospital. They were treated with PEG-IFN-α_{2b} (subcutaneously once a week; 1.5 μg/kg body weight) or PEG-IFN-α_{2a} (180 μg once a week) plus RBV (600–1,000 mg daily depending on body weight) for 48 weeks according to the guidelines of the 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 plus RBV therapy exceeding 80 % of the estimated total dose. Liver biopsy was performed before the start of the therapy. Histological activity and fibrosis were determined according to the METAVIR scoring system [15]. Serum samples were collected from the patients before PEG-IFN-α plus RBV treatment started and were stored at −80 °C. In some patients, the samples were obtained 24 weeks after the cessation of the therapy (at the end of follow-up).

As controls, serum was obtained from 23 healthy subjects without HCV, HBV, and HIV infection (male-to-female ratio, 5:5, mean age ± standard deviation, 45 ± 12 years). In the comparison of serum IFN-λ levels between C-CH patients and patients with other types of liver diseases, 11 patients with chronic HBV infection (three HBeAg-positive patients and eight HBeAg-negative patients) were examined as well. They were not treated with IFN or nucleot(s)ide analogues for HBV infection. In addition, we compared serum IFN-λ_3 levels among patients with acute viral hepatitis of various causes, such as acute hepatitis A, acute hepatitis B, or acute hepatitis E, the diagnosis of which was determined by serological examinations at Teine Keijinkai Hospital and Kurume University Hospital. The serum samples were obtained from the patients at the time of active liver inflammation [alanine aminotransferase (ALT) levels more than two times the upper limit of the normal range]. As representatives for noninvasive fibrosis markers, the fibrosis-4 (FIB-4) score and the aspartate aminotransferase (AST) platelet ratio index (APRI) were calculated as reported previously [16, 17].

The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the Institutional Review Board at the National Center for Global Health and Medicine (approval ID and date, NCGM-G-001379-00, March 14, 2013) and the ethical committee of each institute. Written informed consent was obtained from all patients.

IFNL3 genotyping

The subjects were evaluated for SNPs near the *IFNL3* 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-λ_3

Serum levels of IFN-λ_3 were evaluated by the newly developed chemiluminescence enzyme immunoassay system as reported previously [19]. The system enables one to quantify serum IFN-λ_3 specifically without any overlap from IFN-λ_1 and IFN-λ_2. The threshold of the assay is 10 pg/mL and its range is 10–1,000 pg/mL.

Simultaneous measurement of multiple chemokines and cytokines

To quantify multiple chemokines and cytokines simultaneously in the limited volume of the samples, we used the 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 fibroblast growth factor, eotaxin, granulocyte colony stimulating factor, granulocyte–macrophage colony stimulating factor, IL-1β, IL-1 receptor antagonist, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12, IL-13, IL-15, IL-17, IFN-γ, IFN-γ-inducible protein 10 (IP-10), monocyte chemotactic protein 1, macrophage inflammatory protein (MIP)-1α, MIP-1β, platelet-derived growth factor BB (PDGF-BB), regulated on activation, normally T cell expressed, and secreted (RANTES), TNF-α, and vascular endothelial growth factor. The detection range and thresholds are given in 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 signed-rank test and the Mann–Whitney *U* test, and categorical data were compared using the χ^2 test or Fisher's exact test. The correlations between cytokines, chemokines, and clinical markers were evaluated by Spearman's correlation coefficient. A p value below 0.05 was considered to be significant. Logistic regression was used for multivariate analyses. All statistical analyses were performed with PRISM and SPSS.

Results

Serum IFN-λ_3 levels are increased in patients with chronic HCV infection

The clinical backgrounds of C-CH patients are shown in Table 1. First, we compared serum IFN-λ_3 levels among patients with C-CH or chronic hepatitis B (B-CH) and uninfected healthy volunteers (HVs). Such levels in the C-CH group were significantly higher than those in the B-CH group or the HV group (Fig. 1a). The levels in the B-CH group were increased, but the significance of this was much less than in the C-CH group (Fig. 1a). When we compared serum IFN-λ_3 levels in B-CH patients between

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Table 1 Clinical backgrounds of the patients with chronic hepatitis C virus (*HCV*) infection

Factors	Values
Number	119 (69 male, 50 female)
Age (years)	56.5 ± 10.1
WBC (/mm^3)	5,120 ± 1,575
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
IFNL3 rs8099917 (TT/non-TT)	100:19

Alb albumin, *AFP* α-fetoprotein, *ALT* alanine aminotransferase, *AST* aspartate aminotransferase, *Hb* hemoglobin, *Plt* platelets, *T-bil* total bilirubin, *T-chol* total cholesterol, *TP* total protein, *WBC* white blood cells

HBeAg-positive and HBeAg-negative patients, we found no difference between them (2.5 ± 0.9 pg/mL vs 1.8 ± 1.7 pg/mL, respectively). Next, we compared serum IFN-λ_3 levels between patients with the *IFNL3* TT genotype and those with the TG/GG (non-TT) genotype in the C-CH group. Although some patients in the TT group showed relatively higher levels of IFN-λ_3 than those in the non-TT group, this difference between the TT and non-TT groups did not reach significance (Fig. 1b). Third, we compared serum IFN-λ_3 levels before and after the combination therapy in the relevant cases. In patients who successfully eradicated HCV (SVR), serum IFN-λ_3 levels were significantly decreased at 24 weeks after the therapy. In contrast, such levels did not change in those patients who failed to eradicate HCV (transient virological response and no virological response groups, respectively) (Fig. 1c). Fourth, we compared serum IFN-λ_3 levels among patients with various causes of acute viral hepatitis. Unfortunately, serum samples from acute hepatitis C patients were not available in this study. The IFN-λ_3 levels in the acute hepatitis E group were higher than those in the HVs (Fig. 1d). The IFN-λ_3 levels in the acute hepatitis B group tended to be higher than those in the HVs; however, statistical analysis was not performed because of the limited number of samples ($N = 2$). No significant difference was observed between the acute hepatitis A and HV groups. These results indicate that serum IFN-λ_3 levels are increased in patients with C-CH or acute hepatitis E.

Serum IFN-λ_3 levels may be related to liver inflammation or fibrosis in patients with C-CH

To explore the clinical significance of IFN-λ_3 in chronic HCV infection, we simultaneously examined 27 chemokines and cytokines in serum by means of the BioPlex system, which allows one to measure multiple factors at high sensitivity in a small volume of samples (10 μL per sample). In comparison with the results for HVs, we found that the levels of some chemokines in the C-CH group were higher than those in the HV group, such as IP-10, MIP-1α, MIP-1β, RANTES, and PDGF-BB (Figs. 2, S1).

Next, we examined whether serum IFN-λ_3 levels are correlated or not correlated with clinical parameters or immunological markers in the C-CH group. The IFN-λ_3 levels were weakly and positively correlated with AST, ALT, and α-fetoprotein levels and histological activity (Table 2). These results indicate that the increase of serum IFN-λ_3 levels in patients with C-CH is related to liver inflammation. The FIB-4 score and the APRI are representatives of noninvasive markers of liver fibrosis. The levels of serum IFN-λ_3 were positively correlated with the APRI, but not with the FIB-4 score (Table 2). With regard to the chemokines displaying higher values in the C–CH group, the levels of IP-10 and PDGF-BB were positively correlated with the IFN-λ_3 levels (Table 2). Such chemokines are reported to be involved in the early stage of liver fibrosis [20–22]. Thus, serum levels of IFN-λ_3 may be related to the fibrotic markers as well. To clarify the mechanisms causing the increase of serum IFN-λ_3 levels in B-CH patients, we examined the correlations between serum IFN-λ_3 levels and clinical markers and fibrosis indices. Serum IFN-λ_3 levels were correlated with the levels of AST ($r = 0.64$, $p = 0.03$) and total cholesterol ($r = -0.76$, $p = 0.03$), FIB-4 score ($r = 0.65$, $p = 0.03$), and APRI ($r = 0.76$, $p = 0.007$) (Table S2). In addition, serum IFN-λ_3 levels tended to be higher in HBV-positive patients with liver cirrhosis or HCC (3.0 ± 3.1 pg/mL in liver cirrhosis patients and 4.1 ± 4.7 pg/mL in HCC patients, respectively) (Fig. S2). These results show that serum IFN-λ_3 levels are related to liver inflammation and fibrosis not only in C-CH patients but also in B-CH patients.

Pretreatment serum IFN-λ_3 is not related to SVR to PEG-IFN-α plus RBV therapy in patients with C-CH

Because the *IFNL3* genotype is a strong predictor of the efficacy of PEG-IFN-α plus RBV therapy for C-CH, we sought to examine the clinical value of serum IFN-λ_3 in patients who underwent the combination therapy. In a comparison of the clinical and immunological factors between the SVR and non-SVR groups, univariate analysis

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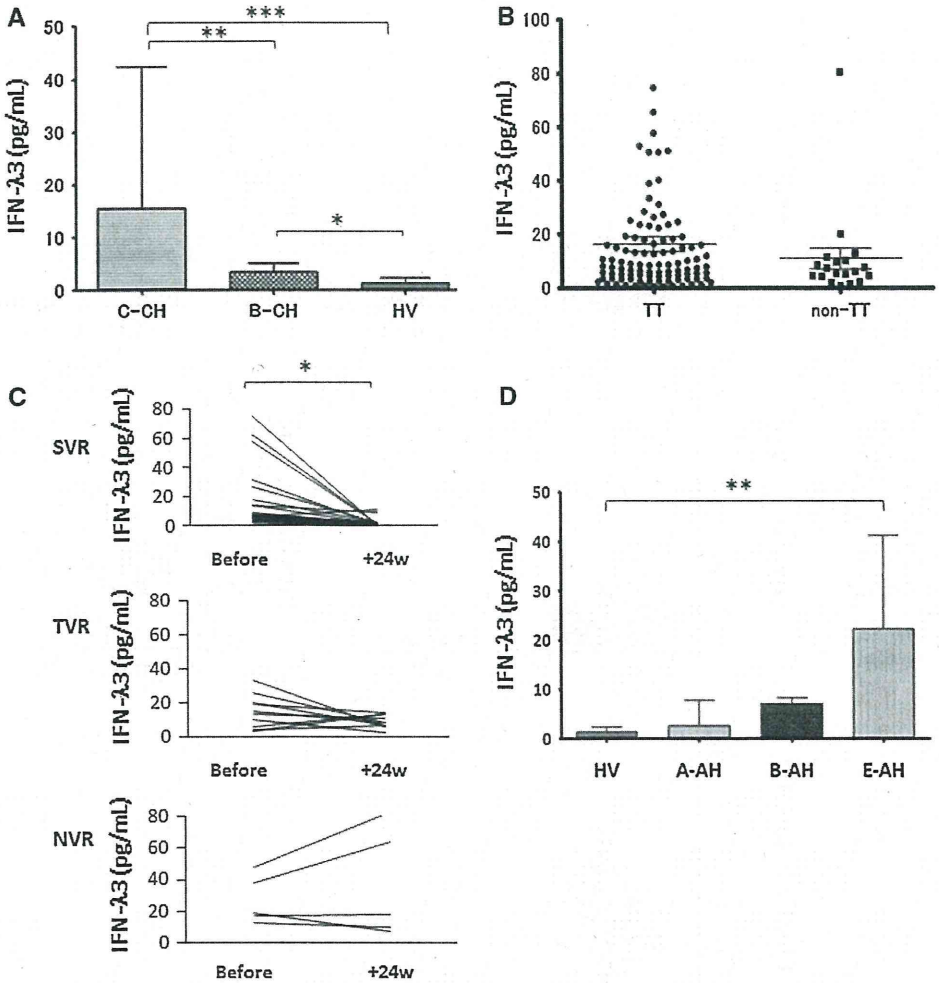

Fig. 1 Serum interferon-λ_3 (*IFN-λ3*) levels are increased in patients with chronic hepatitis C virus infection or acute hepatitis E virus infection. **a** Serum IFN-λ_3 levels in patients with chronic hepatitis C (*C–CH*; $N = 119$), patients with chronic hepatitis B (*B-CH*; $N = 11$), and healthy volunteers (*HV*; $N = 23$) were quantified by the chemiluminescence enzyme immunoassay (CLEIA) method as described in "Materials and methods." *One asterisk* $p < 0.05$, *two asterisks* $p < 0.01$, *three asterisks* $p < 0.0001$ by the Mann–Whitney *U* test. **b** Serum IFN-λ_3 levels in the C-CH group were compared between the patients with the *IFNL3* TT (rs8099917) genotype ($N = 100$) and those with non-TT (TG/GG) genotype ($N = 19$). **c** Serum IFN-λ_3 levels in C-CH patients were compared before and 24 weeks after the pegylated interferon-α plus ribavirin therapy. *SVR* sustained virological response ($N = 21$), *TVR* transient virological response ($N = 10$), *NVR* nonvirological response ($N = 5$), *one asterisk* $p < 0.05$ by Wilcoxon's signed-rank test. **d** Serum IFN-λ_3 levels in acute hepatitis patients of various causes were quantified by CLEIA as described in "Materials and methods." All samples were collected from patients whose alanine aminotransferase levels were two times higher than the upper limit of the normal range. *HV* healthy volunteers ($N = 23$), *A-AH* acute hepatitis A patients ($N = 34$), *B-AH* acute hepatitis B patients ($N = 2$), *E-AH* acute hepatitis E patients ($N = 9$), *two asterisks* $p < 0.0001$ by the Mann–Whitney *U* test

revealed that AST, *IFNL3* genotype, fibrosis score, and MIP-1α were associated with the SVR (Table 3). However, serum IFN-λ_3 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 univariate analysis) showed that *IFNL3* SNPs, fibrosis score, and MIP-1α were involved in the SVR (Table 3). These results suggest that serum IFN-λ_3 fails to be a predictive marker for SVR in PEG-IFN-α plus RBV therapy.

Discussion

In this study, we demonstrated that serum IFN-λ_3 levels were higher in patients with C-CH than in uninfected or HBV-positive patients, the levels in whom did not differ regardless of the *IFNL3* genotype. Serum IFN-λ_3 levels were correlated with clinical and immunological markers of liver inflammation and fibrosis, suggesting that the production of IFN-λ_3 may be regulated by not only the presence or absence of HCV but also by the status of liver

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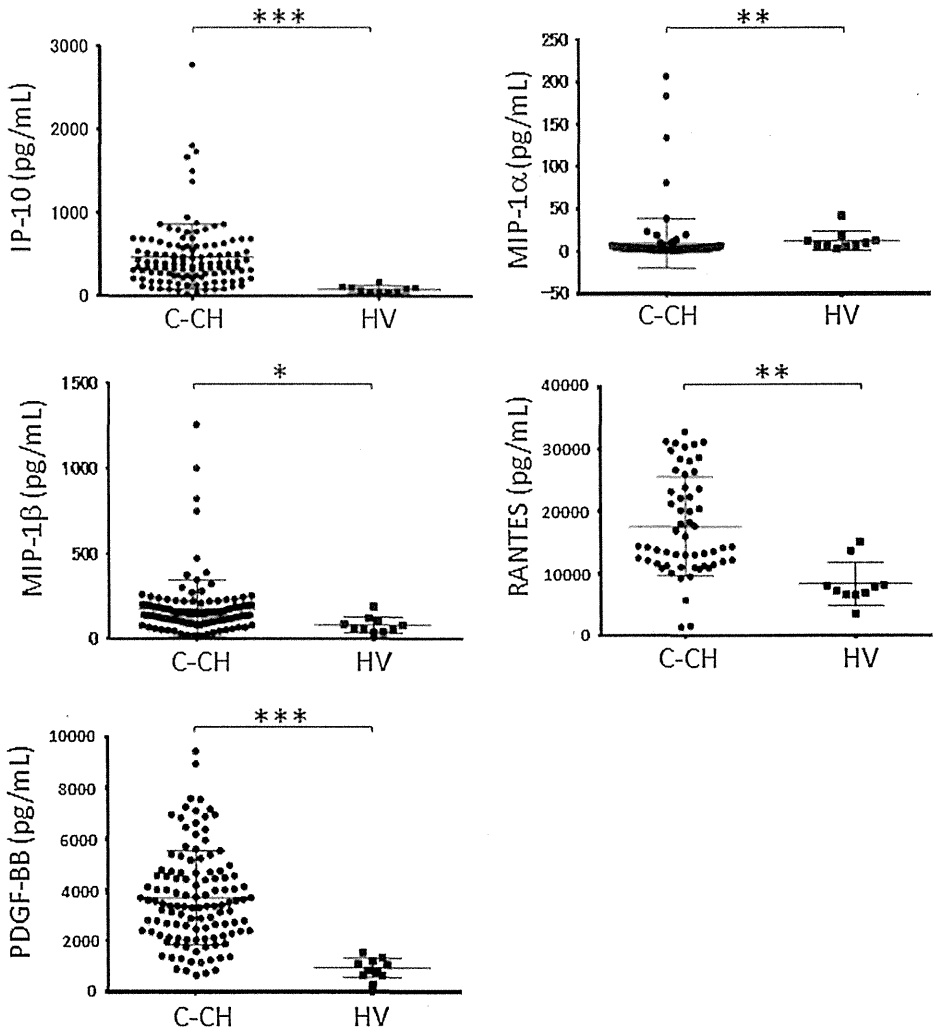

Fig. 2 The levels of several chemokines are increased in patients with chronic hepatitis C virus infection. Twenty-seven chemokines and cytokines in serum from chronic hepatitis C patients (*C-CH*) and healthy volunteers (*HV*) were assayed by means of the BioPlex method. Interferon-γ-inducible protein 10 (*IP-10*) was measured by ELISA. Representative results for chemokines that showed statistical significance between the groups are shown, such as IP-10, macrophage inflammatory protein 1α (*MIP-1α*), macrophage inflammatory protein 1β (*MIP-1β*), regulated on activation, normally T cell expressed, and secreted (*RANTES*), and platelet-derived growth factor BB (*PDGF-BB*). * $p < 0.005$, ** $p < 0.001$, *** $p < 0.0001$ by the Mann–Whitney *U* test

disease. It is well acknowledged that *IFNL3* genotype is a strong predictor of SVR in PEG-IFN-α plus RBV therapy for C-CH [7–9]. However, serum IFN-λ_3 fails to be a surrogate marker for *IFNL3* genotype in the combination therapy.

On primary HCV infection, IFN-λ is produced by hepatocytes that subsequently induce antiviral IFN-stimulated genes [23]. Parallel reduction of serum IFN-λ_3 levels in C-CH patients who attained SVR by PEG-IFN-α plus RBV treatment indicates that the presence of HCV is involved in the production of IFN-λ_3. In addition to hepatocytes, dendritic cells or macrophages are capable of producing IFN-λ in response to HCV [24]. For sensing HCV, hepatocytes and BDCA3$^+$ dendritic cells mainly utilize Toll-like receptor 3 and retinoic acid inducible gene I, and plasmacytoid dendritic cells utilize Toll-like receptor 7 [24, 25]. It is yet to be clarified which cells—hepatocytes or dendritic cells—have stronger potential to secrete IFN-λ at the single-cell level. However, it is rational to consider that serum IFN-λ_3 levels in patients are determined by the sum of IFN-λ_3 sporadically released from both types of cells. Therefore, it is plausible that the amount of IFN-λ released from hepatocytes or dendritic cells is influenced by the environment of the producers, such as inflammation and fibrosis. A positive correlation observed between serum IFN-λ_3 levels and AST levels, FIB-4 score, and APRI in B-CH patients may support such a possibility. In this study, serum IFN-λ_3 levels in the B-CH group were higher than those in HVs. However, this difference was slim compared with the difference between the C-CH group and HVs, suggesting that the difference in their genome structure, either RNA or DNA virus, may influence IFN-λ_3 production by infected cells. Of interest is the finding that serum IFN-λ_3 levels were higher in patients with acute hepatitis E than in patients with acute hepatitis A. It is reported that dendritic cells localized in the