Table 2 SNP associated with interferon-induced neutropenia

dbSNP rsID	Nearest gene	Risk allele	Allele (1/2)	Stage	Case			Control			OR[a] (95 % CI)	*P* value[b]
					11	12	22	11	12	22		
rs2305482	*PSMD3*	C	C/A	GWAS-1st	23 (20.4)	52 (46.0)	38 (33.6)	26 (8.6)	143 (47.4)	133 (44.0)	1.61 (1.17–2.20)	2.95×10^{-3}
				GWAS-2nd	12 (24.5)	28 (57.1)	9 (18.4)	26 (8.6)	143 (47.4)	133 (44.0)	2.37 (1.54–3.65)	6.47×10^{-5}
				Replication	12 (24.4)	20 (40.8)	17 (34.7)	33 (9.5)	136 (39.1)	179 (51.4)	1.99 (1.30–3.06)	1.46×10^{-3}
				Combined[c]	24 (24.5)	48 (49.0)	26 (26.5)	59 (9.1)	279 (42.9)	312 (48.0)	2.18 (1.61–2.96)	3.05×10^{-7}

Data of allele distribution represent number (%). Data of subjects whose genotypes were not determined were excluded

SNP single nucleotide polymorphism

[a] Odds ratio for the allele frequency model

[b] *P* value by the Chi square test for the allele frequency model

[c] Allele distributions in GWAS-2nd and replication were combined

Predictive factors for IFN-induced neutropenia

The following analyses were carried out for rs2305482 and rs4794822 using the subjects in Case-G2 + R and Control-G + R. Neutrophil counts at baseline correlated with rs2305482 and rs4794822 genotypes (Supplementary Fig. 2), and strongly affected IFN-induced neutropenia as shown by ROC analysis (area under the curve = 0.860) (Supplementary Fig. 3). Furthermore, gender, hemoglobin level, and platelet count at baseline were also significantly associated with IFN-induced neutropenia by univariate analysis (Table 4). Therefore, we analyzed pretreatment predictive factors for IFN-induced neutropenia in logistic regression models that included the following variables: gender, neutrophil count, platelet count, and rs2305482 or rs4794822 genotypes. In addition to neutrophil count, rs2305482 CC was an independent predictive factor for IFN-induced neutropenia (OR = 2.497; 95 % CI = 1.281–4.864, $P = 0.0072$) (Table 5) as was rs4794822 CC (OR = 2.272; 95 % CI = 1.337–3.861, $P = 0.0024$) (Supplementary Table 2).

Impact of *PSMD3-CSF3* SNPs on tolerated drug doses and treatment efficacy

To evaluate the impact of *PSMD3-CSF3* SNPs on doses of drugs given, and on treatment efficacy, we selected 380 HCV genotype 1-infected patients treated with PEG-IFN/RBV for 48 weeks. They were selected as having information available on the doses of PEG-IFN/RBV that they had received (Supplementary Table 3). It was reported that rates of viral clearance were significantly reduced in patients who could not be maintained on at least 80 % of their drug doses for the duration of PEG-IFN/RBV therapy (McHutchison et al. 2002). In reference to this result, we stratified the patients into three groups according to the doses of PEG-IFN or RBV administered, as follows: <60 %, ≥60 to <80 %, ≥80 % of the planned doses for 48 weeks. The proportion of patients in the <60 % group for PEG-IFN was significantly higher in patients possessing rs2305482 CC than in those with AA/AC ($P = 0.005$), whereas there was no association for RBV (Fig. 3). The same results were found in the analysis of rs4794822 (Supplementary Fig. 4). However, the univariate analysis of pretreatment factors associated with SVR showed that there was no association between SVR and rs2305482 or rs4794822 genotypes (Supplementary Table 3).

Candidate SNP-gene association analysis in IFN-induced neutropenia

To investigate whether the SNPs associated with neutropenia affect the expression of nearby genes, we conducted

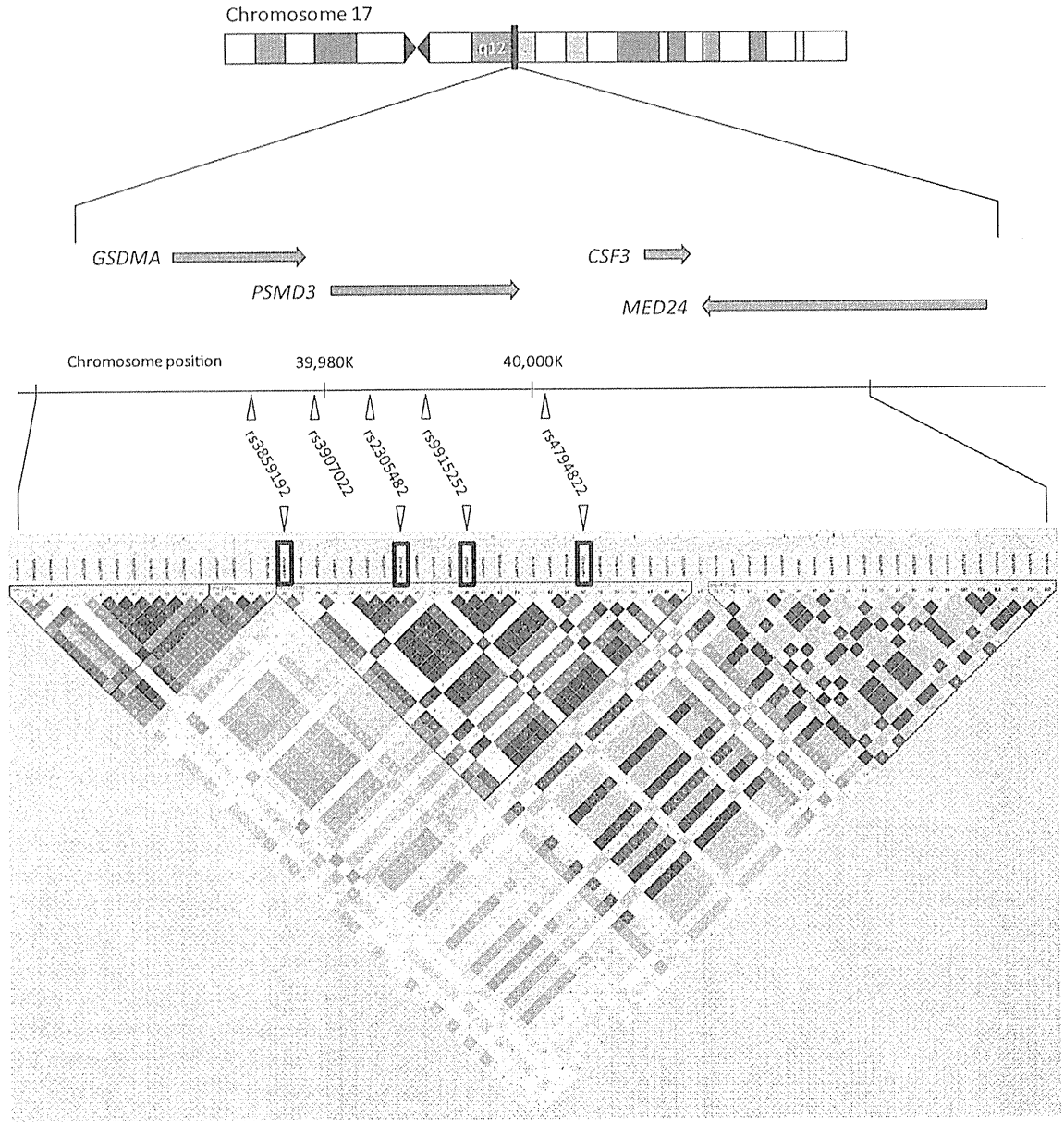

Fig. 2 Position on chromosome and pairwise linkage disequilibrium (r^2) diagrams in the HapMap JPT around the *PSMD3-CSF3* locus

an eQTL analysis. The C allele of rs2305482, a risk for neutropenia, was associated with higher expression levels of *PSMD3* in the populations of LWK: Luhya in Webuye, Kenya (rho = 0.30, $P = 0.006$), and MEX: Mexican ancestry in Los Angeles, California (rho = 0.36, $P = 0.015$) (Supplementary Fig. 5a), whereas it was associated with lower expression levels of *CSF3* in CHB: Han Chinese in Beijing, China, in the probe of ILMN_1655639 (rho = −0.48, $P = 5.5 \times 10^{-6}$) (Supplementary Fig. 5b), and in MEX in that of ILMN_1706852 (rho = −0.33, $P = 0.028$) (Supplementary Fig. 5c).

CSF3 encodes a cytokine, known as G-CSF which is produced by different type of cells such as macrophages, monocytes, stromal cells in the bone marrow, fibroblast, and endothelial cells. The eQTL analysis is based on the whole-genome gene expression variations in lymphoblastoid cell lines derived from HapMap individuals. Therefore, it was still necessary to analyze gene expression in G-CSF producing cells, as well as expression at the protein level. Hence, we measured serum G-CSF levels at baseline and week 2 or 4 (at the time of minimum neutrophil counts) in 127 CHC patients receiving IFN-based therapy. There were no differences in serum G-CSF levels at baseline and the time of minimum neutrophil counts as well as in their changes according to rs2305482 or rs4794822 genotypes (Supplementary Fig. 6a, b). In addition, neutrophil counts

Table 3 Association of SNPs located in *PSMD3-CSF3* with interferon-induced neutropenia

dbSNP rsID	Nearest gene	Risk allele	Allele (1/2)	Case-G2 + R[a] ($n = 100$)			Control-G + R[b] ($n = 656$)			OR[c] (95 % CI)	*P* value[d]
				11	12	22	11	12	22		
rs9915252	*PSMD3*	G	G/C	23 (24.0)	47 (49.0)	26 (27.1)	57 (8.9)	276 (43.3)	304 (47.7)	2.13 (1.57–2.89)	9.64×10^{-7}
rs4794822	*PSMD-CSF3*	C	C/T	42 (42.9)	45 (45.9)	11 (11.2)	130 (21.2)	308 (50.2)	176 (28.7)	2.24 (1.63–3.07)	3.63×10^{-7}
rs3907022	*GSDMA-PSMD*	A	A/G	41 (41.8)	45 (45.9)	12 (12.2)	129 (21.3)	306 (50.6)	170 (28.1)	2.11 (1.54–2.89)	2.31×10^{-6}
rs3859192	*GSDMA*	C	C/T	37 (37.8)	44 (44.9)	17 (17.3)	123 (19.9)	313 (50.7)	181 (29.3)	1.82 (1.34–2.48)	1.04×10^{-4}

Data of allele distribution represent number (%). Data of subjects whose genotypes were not determined were excluded

SNP single nucleotide polymorphism

[a] Case-G2 + R: Case-G2 plus Case-R

[b] Control-G + R: Control-G plus Control-R

[c] Odds ratio for the allele frequency model

[d] *P* value by the Chi square test for the allele frequency model

did not correlate with serum G-CSF levels at baseline and the time of minimum neutrophil counts (Supplementary Fig. 7a), and there was no difference in the changes of serum G-CSF levels from baseline to the time of minimum neutrophil counts between patients with minimum neutrophil counts of $\geq 1{,}000/mm^3$ and $<600/mm^3$ (Supplementary Fig. 7b).

Discussion

The present GWAS first showed a strong association between genetic variant and IFN-induced neutropenia, namely, with rs2305482 in *PSMD3* on chromosome 17. Although neutrophil counts at baseline were associated with the rs2305482 genotype and the incidence of neutropenia during IFN-based therapy, the logistic regression analysis revealed that the rs2305482 genotype was independently associated with IFN-induced neutropenia.

Intriguingly, the *PSMD3-CSF3* locus was reported to be associated with total white blood cell (WBC) counts based on GWAS of populations with European ancestry (Crosslin et al. 2012; Soranzo et al. 2009) and in Japanese (Kamatani et al. 2010). These findings were replicated in African Americans (Reiner et al. 2011). Moreover, another GWAS by Okada et al. (2010) showed that rs4794822 in *PSMD3-CSF3* was associated with neutrophil counts in 14 different groups of diseases in Japanese patients who were not undergoing chemotherapy. In the present study, rs4794822 as well as rs2305482 was also associated with pretreatment neutrophil counts in CHC patients (Supplementary Fig. 2). However, there have been no reports showing an association between *PSMD3-CSF3* variants and reduction of WBC or neutrophil counts following treatments such as IFN and chemotherapy. The pairwise LD diagram for *PSMD3-CSF3* by HapMap JPT shows that rs4794822 is in strong LD with rs2305482, which we identified here (Fig. 2). In the present study, both rs2305482 and rs4794822 were associated with IFN-induced neutropenia. Collectively, previous reports together with our results imply that the *PSMD3-CSF3* locus is associated with neutropenia in CHC patients under IFN-based therapy as well as with neutrophil counts in healthy individuals and patients without bone marrow suppressive therapy.

In further clinical investigation, the rs2305482 and rs4794822 genotypes were associated with the doses of PEG-IFN that could be given to HCV genotype 1-infected patients treated with PEG-IFN/RBV (Fig. 3; Supplementary Fig. 4). Unfortunately, we could not collect the detailed information about the reason for the reduction of PEG-IFN in this group. However, we highly suppose that these SNPs affected the doses of PEG-IFN through neutropenia in some cases, since neutropenia is one of the major

Table 4 Univariate analysis of pretreatment factors associated with interferon-induced neutropenia

	Case-G2 + R[a] ($n = 100$)	Control-G + R[b] ($n = 656$)	P value[c]
Gender, male/female	45/55	378/278	0.018
Age, years	58.1 (9.3)	56.9 (10.4)	0.262
Neutrophil count, /mm^3	1,614 (735)	2,742 (979)	<0.001
Hemoglobin, g/dL	13.5 (1.5)	14.2 (1.5)	<0.001
Platelet count, $\times 10^9$/L	136 (46)	163 (57)	<0.001
ALT, IU/L	79.1 (69.7)	83.5 (74.3)	0.574
HCV RNA, log IU/ml	6.0 (0.9)	6.1 (0.8)	0.164
Liver fibrosis, F0-2/F3-4/ND	46/16/38	397/157/102	0.674
rs2305482, AA + AC/CC/ND	74/24/2	591/59/6	<0.001
rs4794822, TT + TC/CC/ND	56/42/2	484/130/42	<0.001

Data are expressed as number for categorical data or the mean (standard deviation) for non-categorical data

ALT alanine transaminase, *ND* not determined

[a] Case-G2 + R: Case-G2 plus Case-R

[b] Control-G + R: Control-G plus Control-R

[c] Categorical variables were compared between groups by the Chi square test and non-categorical variables by the Student's t test

Table 5 Logistic regression analysis of pretreatment factors associated with interferon-induced neutropenia

	OR (95 % CI)	P value
Gender, female	1.229 (0.734–2.059)	0.4331
Neutrophil count, /mm^3	0.998 (0.997–0.998)	<0.0001
Platelet count, $\times 10^9$/L	1.005 (0.953–1.059)	0.8604
rs2305482, CC	2.497 (1.281–4.864)	0.0072

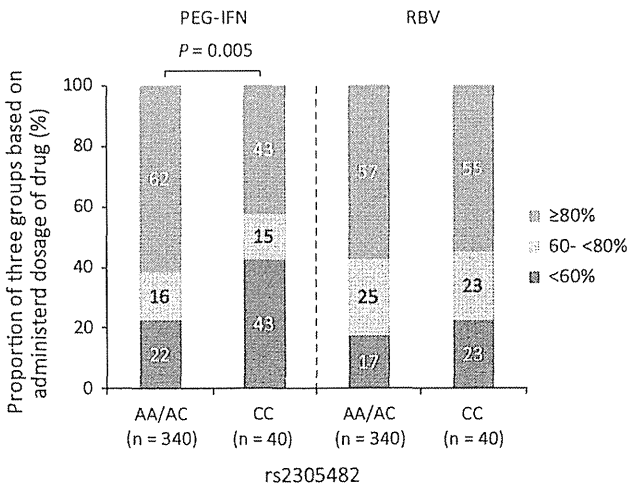

Fig. 3 Administered doses of PEG-IFN and RBV according to rs2305482 genotypes. The patients were stratified into three groups according to the doses of PEG-IFN or RBV administered, as follows: <60 %, ≥60 to <80 %, ≥80 % of the planned doses for 48 weeks. The proportion of patients receiving <60 % of the PEG-IFN doses was significantly higher in patients with rs2305482 CC than in those with AA/AC ($P = 0.005$, by the Chi square test). *PEG-IFN* pegylated interferon, *RBV* ribavirin

reasons for the dose reduction of PEG-IFN in PEG-IFN/RBV therapy. While, there were no associations between SVR and rs2305482 or rs4794822 genotypes (Supplementary Table 3).

PSMD3 encodes the proteasome 26S subunit, non-ATPase 3, a member of the 26S proteasome family, and is involved in the control of cell cycle transition via the ubiquitin–proteasome pathway (Bailly and Reed 1999). *CSF3* encodes G-CSF, which controls the production, differentiation, and function of granulocytes (Nagata et al. 1986). Recombinant G-CSF is widely used to treat patients with severe neutropenia during chemotherapy. Therefore, we hypothesize that *PSMD3-CSF3* variants may influence neutrophil counts through affecting the process of endogenous G-CSF synthesis during IFN-based therapy or other bone marrow suppressive therapies. However, eQTL analysis by Okada et al. (2010) showed that rs4794822 was significantly associated with the expression level of *PSMD3*, rather than that of *CSF3* in the JPT and CHB populations. Our eQTL analysis showed that the risk allele for neutropenia at rs2305482 correlated with higher expression levels of *PSMD3* in LWK and MEX populations (Supplementary Fig. 5a), whereas with lower expression levels of *CSF3* in MEX and especially in CHB populations (Supplementary Fig. 5b, c). However, these results were not replicated in the other probe of *CSF3*. Additionally, we analyzed serum G-CSF levels in CHC patients receiving IFN-based therapy. Although serum G-CSF levels were thought to be increased in response to neutropenia regardless of rs2305482 and rs4794822 genotypes, there was no evidence that they were lower in patients with a risk allele of these SNPs at baseline and during the neutropenic period (Supplementary Fig. 6). Moreover, neutrophil counts did not correlate with serum

G-CSF levels at baseline and the time of minimum neutrophil counts (Supplementary Fig. 7a). Further functional analyses of these genes and polymorphisms are required to elucidate the reason for the association between *PSMD3-CSF3* and IFN-induced neutropenia as well as neutrophil counts in healthy individuals.

In previous reports, *PLBC4*, *DARC*, *CXCL2*, and *CDK5* loci have also been associated with neutrophil or WBC counts in healthy individuals or patients who were not under chemotherapy (Crosslin et al. 2012; Kamatani et al. 2010; Okada et al. 2010; Reiner et al. 2011). However, there were no associations with these loci discernible in our GWAS.

The important limitation of this study is that the association between rs2305482 and IFN-induced neutropenia was not statistically significant in a genome-wide level. Thompson et al. (2012) also identified no genetic determinants of IFN-induced neutropenia during PEG-IFN/RBV therapy at the level of genome-wide significance by their GWAS. Unlike our study design, they analyzed the association between the reduction of neutrophil counts at week 4 and any SNPs. Indeed, we analyzed the association between the reduction of neutrophil counts at week 2 or 4 and rs2305482 or rs4794822, but there was no significant association. Therefore, further independent replication analyses which are designed in the similar way as our study are desirable.

IFN-free therapies are expected to be useful especially in IFN-resistant patients and may become the standard of care in the near future. However, combination therapies of DAA and IFN will continue to be used for some time. Our findings contribute to our understanding of the genetic factors influencing IFN-induced neutropenia. Furthermore, these genetic variants may be associated with neutropenia during chemotherapies for various malignant diseases as well as IFN-based therapy for CHC. Therefore, genetic testing of these variants might be useful for establishing personalized doses of such therapies to minimize drug-induced adverse events. Additionally, our results might contribute to the elucidation of the mechanism of drug-induced neutropenia.

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Conflict of interest The following authors are currently conducting research sponsored by the companies: Yasuhito Tanaka, Keisuke Hino, and Yoshito Itoh by Merck Sharp & Dohme, Corp., Chugai Pharmaceutical Co., Ltd., and Bristol-Myers Squibb; Nobuyuki Enomoto, Shuhei Nishiguchi, and Eiji Tanaka by Merck Sharp & Dohme, Corp. and Chugai Pharmaceutical Co., Ltd.; Naoya Sakamoto by Chugai Pharmaceutical Co., Ltd, Bristol-Myers Squibb, Merck Sharp & Dohme, Corp., and Otsuka Pharmaceutical Co., Ltd.; Hiroshi Yatsuhashi by Chugai Pharmaceutical Co., Ltd.; Akihiro Tamori by Merck Sharp & Dohme, Corp.; Satoshi Mochida by Merck Sharp & Dohme, Corp., Chugai Pharmaceutical Co., Ltd., Bristol-Myers Squibb, and Toray Medical Co., Ltd. The other authors have no conflict of interest.

Compliance with ethical standards All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. This article does not contain any studies with animals performed by any of the authors.

Informed consent Informed consent was obtained from all individual participants included in the study.

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Impaired induction of *IL28B* and expression of *IFNλ4* associated with non-response to interferon-based therapy in chronic hepatitis C

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ABSTRACT

Background: Interferon (IFN) λ plays an important role in innate immunity to protect against hepatitis C viral (HCV) infection. Single nucleotide polymorphisms (SNPs) near *IL28B* (*IFNλ3*) are strongly associated with treatment response to IFNα therapy in chronic hepatitis C (CHC) patients. Recently, IFNλ4 related to *IL28B*-unfavorable allele was discovered. However, the impact of IFNλs on CHC is unknown. We aimed to investigate the mechanism underlying responsiveness to IFN-based therapy in CHC associated with SNPs near *IL28B*.

Methods: We evaluated the basal mRNA levels and ex-vivo induction of *IFNλ* expression including *IFNλ4* in peripheral blood mononuclear cells (PBMCs) from 50 CHC patients treated with PEG-IFNα/RBV. Furthermore, we investigated the effect of *IFNλ4* on induction of *IL28B* in vitro.

Results: When PBMCs were stimulated with IFNα and poly(I:C), *IL28B* induction was significantly lower in patients with *IL28B*-unfavorable genotype (rs12979860 CT/TT) than those with *IL28B*-favorable genotype (rs12979860 CC; p = 0.049). *IL28B* induction was lower in non-responders than in relapsers (p = 0.04), and it was also lower in non-SVR patients for triple therapy including NS3 protease inhibitors. *IFNλ4* mRNA was detected in 12 of 26 patients with *IL28B*-unfavorable SNP and *IFNλ4* expression was associated with lower *IL28B* induction in patients with *IL28B*-unfavorable genotype (p = 0.04) and non-response to

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IFNα therapy (p = 0.003). Overexpression of *IFNλ4* suppressed *IL28B* induction and promoter activation.

Conclusions: Impaired induction of *IL28B,* related to *IFNλ4* expression in PBMCs of *IL28B*-unfavorable patients, is associated with non-response to IFNα-based therapy for HCV infection.

Keywords: hepatitis C virus, peripheral blood mononuclear cells, pegylated interferon, NS3 protease inhibitor, type III interferon

Abbreviations: HCV, Hepatitis C virus; IFN, interferon; CHC, chronic hepatitis C; PEG-, pegylated; RBV, ribavirin; DAA, direct-acting antiviral agents; SNP, single nucleotide polymorphism; IL, interleukin; TLR, Toll like receptor; RLR, RIG-I like receptor; ISG, IFN-stimulated gene; PBMC, peripheral blood mononuclear cells; SVR, sustained virological responder; VR, virological responder; NR, non-responder; poly (I:C), polyinosinic-polycytidylic acid; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; BLC, immortalized B lymphocytes; IRF7, interferon regulatory transcription factor 7; ISRE, IFN-stimulated response element; STAT, signal transducers and activator of transcription; BDCA3, blood dendritic cell antigen 3; DC, dendric cell; ALT, alanine aminotransferase; γ-GTP, γ-glutamyl transpeptidase; LDL-C, low-density lipoprotein cholesterol; ISDR, IFN sensitivity determining region.

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Introduction

Hepatitis C virus (HCV) infection is a common cause of chronic hepatitis, which progresses to liver cirrhosis and hepatocellular carcinoma [1]. Interferon (IFN)-based therapy has been used to treat chronic hepatitis C (CHC) over the last two decades and the combination therapy with direct-acting antiviral agents (DAAs) improved the treating effect. However, non-responders [2] to previous pegylated interferon α (PEG-IFNα) plus ribavirin (RBV) therapy respond poorly to the triple therapy containing HCV NS3/4A serine protease inhibitors [3, 4]. Moreover, although IFN-free regimen using NS5A inhibitors or NS5B polymerase inhibitors is developed, triple or quadruple therapy including PEG-IFN may still be required to suppress DAA-resistant viruses or difficult-to-treat genotype. Therefore, IFNα responsiveness of host innate immunity remains essential for achieving a good prognosis, and determining the mechanisms responsible for non-response to IFNα is crucial.

In a recent genome-wide association study, single nucleotide polymorphisms (SNPs) located near *interleukin 28B* (*IL28B*) encoding type III IFN (IFNλ3) were found to be strongly associated with the virological response to PEG-IFNα/RBV therapy in CHC patients [5-8]. IFNλ3 is induced by viral infection through stimulation of Toll-like receptors (TLR) and RIG-I like receptors (RLR) [9-12], and it is also induced by type-I IFN signaling [13]. This interferon stimulates the expression of IFN-stimulated genes (ISGs), including numerous antiviral [13, 14] and immunoregulatory genes [15, 16]. Therefore, IFNλ3 induction may play essential roles in the innate antiviral response [17].

Recently, it was reported that high baseline expression levels of intrahepatic RLR, and lower responsiveness of ISGs to exogenous IFN, were significantly associated with unfavorable *IL28B* SNP and poor treatment outcome in CHC patients [18, 19, 20]. Furthermore, RNA sequencing using primary human hepatocytes revealed that unfavorable allele of dinucleotide polymorphisms near *IL28B* generate *IFNλ4* [21]. The ability of IFNλ4 to

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induce ISGs was reported in human liver tissue samples [22]. Based on these findings, we hypothesized that preactivation of IFN signaling by *IFNλ4* prevents further induction of antiviral genes by exogenous type I IFN, particularly the type I IFN-mediated induction of IFNλs. However, the expression of *IFNλ4* has never been documented in clinical blood samples from CHC patients.

The study aimed to determine the contribution of IFNλ family (IL29 [IFNλ1], IL28A [IFNλ2], IL28B [IFNλ3] and IFNλ4) to the poor response of CHC patients to anti-HCV therapy, and to clarify the mechanisms associated with SNPs near *IL28B*. Since peripheral blood mononuclear cells (PBMCs) are major sources of IFNλ [9, 10], we measured the expression level and investigated the ex vivo induction of *IFNλs* in PBMCs derived from CHC patients receiving PEG-IFNα/RBV therapy. Furthermore, we studied the impact of *IFNλ4* on *IL28B* expression in vitro.

Methods

Patients and Clinical samples. This study included 50 CHC patients with genotype 1b HCV treated with PEG-IFNα-2b/RBV at the Tokyo Medical and Dental University Hospital. Eleven of these patients were re-treated with telaprevir (TVR) or simeprevir (SMV). Exclusion parameters were alcoholic liver injury, autoimmune hepatitis, and decompensated liver cirrhosis. No patient tested positive for hepatitis B surface antigen or anti-human immunodeficiency virus antibody, or had received immunomodulatory therapy before enrollment. The clinical characteristics of the patients immediately before blood collection are shown in Table 1.Written informed consent was obtained from all patients, and this study was approved by the ethical committee of Tokyo Medical and Dental University in accordance with the Declaration of Helsinki.

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Blood samples were collected from each patient during off-therapy periods for gene expression analysis. Human genomic DNA was extracted from whole blood, and SNPs located near the *IL28B* gene (rs8099917, rs12979860, and ss469415590) were analyzed using the TaqMan SNP genotyping assay (Applied Biosystems, Carlsbad, CA) [21, 23]. HCV core mutations and IFN-stimulated response element (ISDR) substitutions were determined before the therapy.

Definitions of responsiveness to therapy. The present study used the definition of response to therapy outlined by the AASLD Practice Guideline for Diagnosis, Management, and Treatment of Hepatitis C [2, 3].

Generation of *IL28B* mRNA-specific RT-qPCR systems. For the quantification of *IL28B* mRNA expression, we developed an original real-time quantitative PCR assay that distinguishes *IL28B* from *IL28A*. Gene-specific PCR primers were designed to anneal directly to the cDNA sequences of each gene (Supplementary Table 1).

Real-time detection RT-PCR analysis for *IL28A*, *IL28B*, and *IL29*. Immediately after blood collection, PBMCs were separated by gradient centrifugation with Ficoll-Conray, and incubated in the RPMI 1640 medium (Sigma, St. Louis, MO) with 10% fetal calf serum at 37°C under 5% CO_2. The cells were treated with recombinant IFNα-2b (100 IU/ml) (Schering-Plough, Kenilworth, NJ) for 12 h prior to polyinosinic-polycytidylic acid (poly(I:C)) (Sigma) treatment (10 μg/ml) for 8 h. PBMC RNA was extracted using the RNeasy Mini Kit (Quiagen, Valencia, CA). Total cell RNA (200 ng) was used to generate 10 μl of cDNA from each sample using SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA). The mRNA expression levels were measured using a ABI 7500 real-time PCR system (Applied Biosystems), and a QuantiTect SYBR Green PCR kit (Quiagen) or TaqMan Universal PCR Master Mix (Applied Biosystems). Expression levels were normalized to the

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expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) or β-actin. The sequences of the primer sets are provided in Supplementary Table 1.

Analysis of *IFNλ4* mRNA expression. Total cell RNA was pre-treated with DNase-I (Nippon Gene, Tokyo, Japan), followed by RT with SuperScript II, and PCR analysis was performed for 45 cycles using 4 sets of primers (Supplementary Table 1). Primer set #1 could detect 1 copy of IFNλ4 per assay, whereas primer sets #2, 3, and 4 could detect 10 copies of IFNλ4 per assay (Supplementary Fig. 1). The PCR products corresponding to the size of spliced *IFNλ4* mRNA were extracted and the sequences were confirmed. Only the amplicon with ss469415590-ΔG (*) is defined as IFNλ4 (Supplementary Figs. 1, 2).

Generation of the *IL28B* promoter-reporter and stably expressing cell lines. The promoter sequences of human *IL28B* (-1129/+111) were subcloned and the DNA fragment was inserted into the pGL3-basic vector (Invitrogen). The reporter plasmid was transfected into HEK293 cells with pcDNA3.1 (Invitrogen). After cell culture in the presence of the selective antibiotic G418 (Nacalai Tesque, Kyoto, Japan), transfected colonies were isolated to establish a cell line stably expressing the IL28B-Fluc-reporter (HEK293/ IL28B-luc).

Cell culture. HEK293T, Huh7, HepG2, and HeLa cells were maintained in Dulbecco's modified Eagle's Medium (Sigma) supplemented with 10% fetal calf serum (37°C; 5% CO_2). The maintenance medium for the IL28B-promoter-reporter-harboring cell line (HEK293/IL28B-luc) was supplemented with 500 μg/ml of G418 (Nacalai Tesque). Immortalized B lymphocytes (BLC) were generated in-house from human PBMCs by EBV transformation, and maintained in RPMI1640 medium (Sigma) with 10% feral calf serum and 200 ng/ml Cyclosporin-A (Sigma). The HuS/E-2 cells were kindly provided by Dr. Hijikata (Kyoto University, Kyoto, Japan) and cultured as previously described [24].

Expression plasmids and transfections. The expression construct for IFNλ4 (p179) was kindly provided by Dr. Prokunina-Olsson (National Cancer Institute, Bethesda, MD). The

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DNA fragments of IRF7 were inserted into the vector pcDNA4/TO/myc-His (Invitrogen). The expression plasmids for p50 and p65 were kindly provided by Dr. Rongtuan Lin (Lady Davis Institute for Medical Research, Baltimore, MD). pcDNA4/TO/myc-His vector (Invitrogen) was used as control for mock transfection.

BLC were transfected with *IFNλ4* plasmids or control plasmids by electroporation using Gene Pulser Xcell Electroporation System (BIO RAD, Hercules, CA). After 24 h, cells were treated with mock, recombinant IFNα-2b (100 IU/ml) (Schering-Plough) for 24h. *IFNλ4* plasmids and *IRF7* plasmids or control plasmids were co-transfected into HEK293T cells with Lipofectamine LTX reagent (Invitrogen) and Opti-MEM medium, according to the manufacturer's instructions. Total RNA was extracted and quantified by real-time qRT-PCR.

Luciferase assays. *IFNλ4* or control plasmids were transfected into HEK293/IL28B-luc cells and the cells were treated with IFNα for 24h next day. HEK293/IL28B-luc cells were co-transfected with IFNλ4 plasmids and IRF7, p50: p65 or control plasmids and incubated for 24h. MTS viability and single luciferase assays were conducted by 1420 Multilabel Counter (ARVO MX, PerkinElmer, Boston, MA) using a CellTiter 96 AQueous One Solution System (Promega, Madison, WI) and a Bright-Glo Luciferase Assay System (Promega), as previously described [25, 26].

Statistical analyses. The data were analyzed using the Welch's *t* test for continuous variables and the chi-square test for categorical data. p values < 0.05 were considered statistically significant.

Results

Genotype of *IL28B* SNP and expression of *IL29*, *IL28A*, and *IL28B* mRNA in PBMC. Three SNPs near the *IL28B* gene (rs8099917, rs12979860, and ss469415590) were genotyped. The number of patients with each genotype is shown in Table 1. In agreement with a recent

report from the HapMap Project in Asia [21], the genotype of ss469415590 was completely correlated with that of rs12979860 in this study, while 3 of 50 patients have different genotype between ss469415590 and rs8099917. Baseline mRNA expression levels of *IL29*, *IL28A*, and *IL28B* were not influenced by the rs12979860 genotype (Fig. 1A). However, when PBMCs were stimulated with IFNα and poly(I:C), the induction of *IL28B* expression was significantly lower in patients with the *IL28B*-unfavorable genotype (rs12979860 CT/TT) than in those without (rs12979860 CC) ($p = 0.049$) (Fig. 1B).

Relationship of therapy response with *IL29*, *IL28A,* and *IL28B* mRNA levels in PBMC. We assessed the relationship between the expression level of the *IFNλ*s and the virological response to PEG-IFNα/RBV therapy. At baseline, there was no significant difference in *IFNλ*s expression between the SVR, relapser, and NR patients (data not shown). On the other hand, the induction of *IL28B* expression by IFNα and poly(I:C) decreased with the patients' response to therapy (Fig. 2A). The mRNA levels of NR patients were significantly lower than those for relapsers ($p = 0.04$) as well as VR ($p=0.005$). The induction of IL29 expression of NR patients were lower than those for VR ($p=0.048$). In contrast, the induction of *IL28A* did not reveal any association between mRNA levels and treatment response.

When the *IL28B* induction levels of VR and NR patients were further stratified by genotype, it was significantly lower in NR than in VR patients in both rs12979860 CC and CT/TT subgroups ($p = 0.01$ and 0.02, respectively) (Fig. 2B).

Furthermore, 11 of 32 non-SVR patients were re-treated with NS3 protease inhibitor (TVR or SMV) plus PEG-IFNα/RBV triple therapy; 2 of them were IL28B-favorable and 9 were unfavorable. Even treated with NS3 protease inhibitor, it should be noted that IL28B inductions in non-SVR of the triple therapy were significantly lower than those in SVR of the PEG-IFNα/RBV therapy or triple therapy ($p=0.017$). IL28B inductions in non-SVR were also

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lower than those in SVR of triple therapy (3.5 vs 12.1 fold induction). IL28A inductions in non-SVR of the triple therapy were also significantly lower than those in SVR (p=0.042) (Fig. 2C).

Impact of *IL28B* genotype and induction on *IFNλ4* mRNA expression. We measured the expression level of *IFNλ4* in PBMCs derived from CHC patients. Because we could not detect *IFNλ4* mRNA in PBMCs with RNA sequencing nor the previously reported TaqMan real-time quantitative RT-PCR system [21], we designed a new highly sensitive RT-PCR system using 4 sets of primers. The detection threshold was as low as 1–10 copies/assay (Supplementary Table 1; Supplementary Fig. 1A). This RT-PCR assay allowed us to confirm the full length mRNA sequence of *IFNλ4* in poly(I:C)-treated HepG2, HeLa, HEK293T cells, and BLC from ss469415590-ΔG/ΔG patients by amplicon sequencing (Supplementary Figs. 1B,C).

Using this system, we tested PBMCs from 47 CHC patients for the presence of *IFNλ4* mRNA. Among the 23 patients with *IL28B*-unfavorable rs12979860 [T] and ss469415590 [ΔG]-allele, *IFNλ4* mRNA was detected in 12 patients (7 in non-stimulated PBMCs and 8 in IFN-poly(I:C)-stimulated PBMCs). In marked contrast, *IFNλ4* mRNA was not detected in any of the *IL28B*-favorable patients (Supplementary Fig. 2). There was no significant difference in baseline expression of *IFNλ*s between patients with or without detectable *IFNλ4* expression (Fig. 3A). However, the induction of *IL28B* expression by IFN-poly(I:C) was significantly lower in patients with *IFNλ4* mRNA than those without detectable *IFNλ4* ($p = 0.008$) (Fig. 3B). Even among *IL28B*-unfavorable patients (rs12979860 CT/TT), *IL28B* induction levels were significantly lower in *IFNλ4*-positive patients ($p = 0.04$) (Fig. 3B). Although induction of *IL28A* was lower in *IFNλ4*-positive patients than *IFNλ4*-negative patients ($p = 0.04$), there was no significant relation between *IFNλ4* expression and the induction of *IL28A* and *IL29* among *IL28B*-favorable patients.

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Association between *IFNλ4* expression and clinical response to antiviral therapy. The rate of virological non-response was significantly higher in patients with *IFNλ4* mRNA than in all those without detectable *IFNλ4* (p = 0.003; Fig. 3C). Among the *IL28B*-unfavorable patients (rs12979860 CT/TT), the virological non-response rate also tended to be higher in patients expressing *IFNλ4* (p = 0.08).

Suppression of *IL28B* induction by *IFNλ4* in vitro. The mechanism behind the lower induction of *IL28B* mRNA in CHC patients expressing *IFNλ4* was investigated by testing whether the expression of *IL28B* is influenced by overexpression of *IFNλ4* in vitro. When IFNλ4 was overexpressed, baseline expression of IL28B was significantly increased in HEK293, BLC (Supplementary Fig. 3). However, as shown in Fig. 4A, IL28B expression was increased by IFNα (1.8 fold induction, p=0.012) but that induction was suppressed in the presence of IFNλ4 (1.2 fold induction, p=0.28) in BLC. As *IL28B* promoter is known to be activated by the transcription factors such as IRF7 and NFκB [11, 31], we next evaluated IL28B induction by IRF7. *IL28B* mRNA was induced by IRF7 in dose dependent manner and the induction levels were suppressed by *IFNλ4* overexpression significantly (Fig. 4B). *IL28B* promoter activities induced by IFNα, IRF7 and p50:p65 were also inhibited by *IFNλ4* overexpression (Fig. 4C-E).

Discussion

The present study shows that the inducibility of *IL28B* expression is associated with virological responsiveness to IFNα in CHC patients, and it is also related to the *IL28B* genotype. Furthermore, we detected *IFNλ4* mRNA in PBMCs using an original sensitive RT-PCR system. *IFNλ4* suppressed *IL28B* induction and associated with virological non-responses to IFNα-based antiviral therapy.

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Earlier studies reported the lower production of *IL28B* in blood cells of *IL28B*-unfavorable CHC patients [5, 27]. However, the relationship between *IL28B* genotype and expression level remained controversial, probably due to the very low expression level of *IL28B*. In the present study, there was no significant difference in baseline expression level between the *IL28B* genotypes. However, stimulation to PBMCs with IFNα and poly(I:C) raised *IL28B* expression, and this induction was significantly lower in *IL28B*-unfavorable CHC patients. More importantly, the degree of *IL28B* induction was positively correlated to the responsiveness to PEG-IFNα/RBV therapy.

Our findings are consistent with a previous study showing ex vivo induction of *IL28B* by TLR7 agonists [28], and we further confirmed *IL28B* inducibility using IFNα and poly(I:C), which mimic exogenous IFNα administration in HCV patients. Because IFNλ is an essential element of innate anti-HCV responses [16, 29, 30], our data suggest that inadequate induction of IL28B is primarily responsible for virological non-response to IFNα-based therapy.

To elucidate the mechanisms responsible for the genotype-specific inducibility of *IL28B*, we focused on *IFNλ4*. We report, for the first time, the presence of *IFNλ4* mRNA in PBMCs derived from CHC patients with the *IL28B*-unfavorable allele. We could not detect *IFNλ4* mRNA with the previously reported TaqMan real-time RT-PCR system [21]. *IFNλ4* expression was confirmed with a highly sensitive RT-PCR system we designed for this study, which could detect even a single copy of *IFNλ4* mRNA per assay. Although *IFNλ4* mRNA was not detected in 16 of the 23 unstimulated PBMC samples of CHC patients with the *IL28B*-unfavorable genotype, we cannot exclude the presence of *IFNλ4* mRNA under the detection limit of this RT-PCR system in these patients. However, it is important to mention that detectable level of *IFNλ4* expression was associated with NR and more severe impairment of *IL28B* induction. These data suggest that the baseline expression of *IFNλ4* in

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PBMCs is responsible for the non-response to IFNα treatment through suppression of *IL28B* induction.

Our in vitro experiments in cell lines demonstrated that *IL28B* induction by IFNα, IRF7 or NFκB was suppressed by IFNλ4 overexpression. These data are consistent with the relationship between *IFNλ4* and *ISG* induction [20-22]. Our finding of base line *IL28B* induction by *IFNλ4* is also reasonable because *IFNλ* promoters contain IFN-stimulated response element (ISRE) sites [11, 31] that could be activated by IFNλ4 through STAT1 and STAT2 phosphorylation [21]. IFNλ4 may pre-activate IL28B promoter through ISRE activation, and moreover, it may influence NFκB-induced promoter activity by unknown mechanism. Our in vitro data support our observation in the clinical samples, and suggest that the expression of *IFNλ4* in immune cells of *IL28B*-unfavorable CHC patients may weakly induce basal *IL28B* expression, which may be insufficient for HCV eradication [32]. But it may prevent additional induction of *IL28B* by exogenous IFNα treatment through impairment of *IL28B* promoter activity. The molecular mechanism by which *IFNλ4* suppresses *IL28B* mRNA induction and promoter activation should be further investigated, although *IFNλ4* may also have important functions affecting IFN regulation [20, 33, 34].

The lower induction of *IL28B* might be caused by the decrease of the frequency of IFNλs producing cells. However, in the present study, because we measured the expression of *IFNλ*s in all PBMCs, we could not specify the subset of IFNλ4 producer cells. A recent study demonstrated that blood dendritic cell antigen 3 $(BDCA3)^+$ dendritic cells (DCs) produce IFNλ3 and expression levels of *IL28B* from BDCA3+ DCs were significantly higher in subjects with *IL28B* major than those with minor type in response to HCV infection [35]. In their experiment, large volumes of blood samples (i.e., 400 ml) were required to sort very small populations of BDCA3+DC (0.054% of all PBMCs), but obtaining such a large amount

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of blood per patient was ethically impossible in our study. We also considered that *IFNλ4* mRNA levels might be higher when analyzed in those specific IFNλ producer cells.

In conclusion, the induction of *IL28B* mRNA expression by ex vivo stimulation with IFNα and poly(I:C) in PBMCs was significantly associated with virological responsiveness in CHC patients treated with IFNα-based therapy. The impaired induction of *IL28B* was associated with the expression of *IFNλ4,* generated by unfavorable dinucleotide polymorphisms near the *IL28B* gene. These data improve our understanding of IFN resistance and may lead to the development of new antiviral therapies targeting the IFNλ induction system.

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