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.

Acknowledgements: The authors are indebted to Dr. L. Prokunina-Olsson for providing the IFNλ4 plasmid, Dr. Rongtuan Lin for p50 and p65 plasmid and to Dr. M. Hijikata for HuS/E-2 cells.

Conflict of interest: Dr. Asahina and Dr. Kakinuma belong to a donation-funded department funded by Chugai Pharmaceutical Co. Ltd., Toray Industries Inc., Bristol-Myers Squibb, Dainippon Sumitomo Pharma Co. Ltd., and Merck Sharp & Dohme.

Financial Support: This study was supported by grants from the Japanese Ministry of Education, Culture, Sports, Science, and Technology; the Japanese Ministry of Welfare, Health, and Labor; the Japan Society for the Promotion of Science; and the Japan Health Sciences Foundation.

20141225

Reference

1 Alter MJ. Epidemiology of hepatitis C. Hepatology 1997;26:62S-65S.

2 Ghany MG, Strader DB, Thomas DL, et al. Diagnosis, management, and treatment of hepatitis C: an update. Hepatology 2009;49:1335-1374.

3 Ghany MG, Nelson DR, Strader DB, et al. An update on treatment of genotype 1 chronic hepatitis C virus infection: 2011 practice guideline by the American Association for the Study of Liver Diseases. Hepatology 2011;54:1433-1444.

4 Forestier N, Zeuzem S. Triple therapy with telaprevir: results in hepatitis C virus-genotype 1 infected relapsers and non-responders. Liver Int 2012;32 Suppl 1:44-50.

5 Tanaka Y, Nishida N, Sugiyama M, et al. Genome-wide association of IL28B with response to pegylated interferon-alpha and ribavirin therapy for chronic hepatitis C. Nat Genet 2009;41:1105-1109.

6 Ge D, Fellay J, Thompson A, et al. Genetic variation in IL28B predicts hepatitis C treatment-induced viral clearance. Nature 2009;461:399-401.

7 Suppiah V, Moldovan M, Ahlenstiel G, et al. IL28B is associated with response to chronic hepatitis C interferon-alpha and ribavirin therapy. Nat Genet 2009;41:1100-1104.

8 Rauch A, Kutalik Z, Descombes P, et al. Genetic Variation in IL28B Is Associated With Chronic Hepatitis C and Treatment Failure: A Genome-Wide Association Study. Gastroenterology 2010;138:1338-U1173.

9 Kotenko S. The family of IL-10-related cytokines and their receptors: related, but to what extent? Cytokine Growth Factor Rev 2002;13:223-240.

10 Sheppard P, Kindsvogel W, Xu W, et al. IL-28, IL-29 and their class II cytokine receptor IL-28R. Nat Immunol 2003;4:63-68.

20141225

11 Osterlund P, Pietilae T, Veckman V, et al. IFN regulatory factor family members differentially regulate the expression of type III IFN (IFN-lambda) genes. J Immunol 2007;179:3434-3442.

12 Ank N, Iversen M, Bartholdy C, et al. An important role for type III interferon (IFN-lambda/IL-28) in TLR-induced antiviral activity. J Immunol 2008;180:2474-2485.

13 Ank N, West H, Bartholdy C, et al. Lambda interferon (IFN-lambda), a type III IFN, is induced by viruses and IFNs and displays potent antiviral activity against select virus infections in vivo. J Virol 2006;80:4501-4509.

14 Marcello T, Grakoui A, Barba-Spaeth G, et al. Interferons alpha and lambda inhibit hepatitis C virus replication with distinct signal transduction and gene regulation kinetics. Gastroenterology 2006;131:1887-1898.

15 Dolganiuc A, Kodys K, Marshall C, et al. Type III Interferons, IL-28 and IL-29, Are Increased in Chronic HCV Infection and Induce Myeloid Dendritic Cell-Mediated FoxP3+Regulatory T Cells. PLoS One 2012;7.

16 Li M, Liu X, Zhou Y, et al. Interferon-lambda s: the modulators of antivirus, antitumor, and immune responses. J Leukoc Biol 2009;86:23-32.

17 Asahina Y, Nakagawa M, Kakinuma S, et al. Polymorphism Near the Interleukin-28B Gene and Anti-Hepatitis C Viral Response. J Clin Transl Hepatol 2013;1:39-44.

18 Asahina Y, Izumi N, Hirayama I, et al. Potential relevance of cytoplasmic viral sensors and related regulators involving innate immunity in antiviral response. Gastroenterology 2008;134:1396-1405.

19 Asahina Y, Tsuchiya K, Muraoka M, et al. Association of gene expression involving innate immunity and genetic variation in interleukin 28B with antiviral response. Hepatology 2012;55:20-29.

20141225

20 Sarasin-Filipowicz M, Oakeley EJ, Duong FH, et al. Interferon signaling and treatment outcome in chronic hepatitis C. Proc Natl Acad Sci U S A. 2008;105:7034-7039.

21 Prokunina-Olsson L, Muchmore B, Tang W, et al. A variant upstream of IFNL3 (IL28B) creating a new interferon gene IFNL4 is associated with impaired clearance of hepatitis C virus. Nat Genet 2013;45:164-171.

22 Amanzada A, Kopp W, Spengler U, et al. Interferon-λ4 (IFNL4) Transcript Expression in Human Liver Tissue Samples. PLoS One 2013;8:e84026.

23 Sakamoto N, Nakagawa M, Tanaka Y, et al. Association of IL28B Variants With Response to Pegylated-Interferon Alpha Plus Ribavirin Combination Therapy Reveals Intersubgenotypic Differences Between Genotypes 2a and 2b. J Med Virol 2011;83:871-878.

24 Aly H, Watashi K, Hijikata M, et al. Serum-derived hepatitis C virus infectivity in interferon regulatory factor-7-suppressed human primary hepatocytes. J Hepatol 2007;46:26-36.

25 Tasaka M, Sakamoto N, Nakagawa M, et al. Hepatitis C virus non-structural proteins responsible for suppression of the RIG-I/Cardif-induced interferon response. J Gen Virol 2007;88:3323-3333.

26 Nakagawa M, Sakamoto N, Tanabe Y, et al. Suppression of hepatitis C virus replication by cyclosporin A is mediated by blockade of cyclophilins. Gastroenterology 2005;129:1031-1041.

27 Langhans B, Kupfer B, Braunschweiger I, et al. Interferon-lambda serum levels in hepatitis C. J Hepatol 2011;54:859-865.

28 Murata K, Sugiyama M, Kimura T, et al. Ex vivo induction of IFN-λ3 by a TLR7 agonist determines response to Peg-IFN/Ribavirin therapy in chronic hepatitis C patients. J Gastroenterol 2013.

20141225

29 Stone AE, Giugliano S, Schnell G, et al. Hepatitis C virus pathogen associated molecular pattern (PAMP) triggers production of lambda-interferons by human plasmacytoid dendritic cells. PLoS Pathog 2013;9:e1003316.

30 Thomas E, Gonzalez VD, Li Q, et al. HCV infection induces a unique hepatic innate immune response associated with robust production of type III interferons. Gastroenterology 2012;142:978-988.

31 Onoguchi K, Yoneyama M, Takemura A, et al. Viral infections activate types I and III interferon genes through a common mechanism. J Biol Chem 2007;282:7576-7581.

32 Makowska Z, Heim M. Interferon signaling in the liver during hepatitis C virus infection. Cytokine 2012;59:460-466.

33 Booth D, George J. Loss of function of the new interferon IFN-lambda 4 may confer protection from hepatitis C. Nat Genet 2013;45:119-120.

34 Lupberger J, Felmlee DJ, Baumert TF. Interferon-lambda polymorphisms and hepatitis C virus clearance revisited. Hepatology 2013;58:439-441.

35 Yoshio S, Kanto T, Kuroda S, et al. Human blood dendritic cell antigen 3 (BDCA3)(+) dendritic cells are a potent producer of interferon-λ in response to hepatitis C virus. Hepatology 2013;57:1705-1715.

20141225

Figure legends

Fig. 1. Comparison of *IFNλs* expression levels between chronic hepatitis C patients with rs12979860 CC or CT/TT. (a) Baseline mRNA levels of *IL29*, *IL28A,* and *IL28B* in PBMCs expressed relative to the internal control (/int.cont.). (b) Fold changes in *IL29*, *IL28A,* and *IL28B* expression in PBMCs stimulated for 8 h with poly(I:C) (10 μg/ml) after a 12-h pretreatment with IFNα-2b (100 IU/ml). Columns represent means ± SEM.

Fig. 2. Impact of *IFNλs* expression levels on therapy response in chronic hepatitis C patients. Fold changes in *IL29*, *IL28A*, and *IL28B* expression in PBMCs stimulated with IFNα-2b and poly(I:C). IFNλ induction levels were compared between (a) SVR (sustained virological responders), relapsers, and NR (non-virological responders) for peg-IFNα/ RBV (P/R) therapy. (b) VR (virological responders) and NR in patients with distinct IL28B genotypes (rs12979860 CC or CT/TT). (c) SVR for P/R, SVR for protease inhibitor (PI) plus P/ R triple therapy, and non-SVR for the triple therapy. Columns represent means ± SEM.

Fig. 3. Impact of *IFNλ4* on *IFNλs* expression and therapy response. Relationship of *IFNλ4* expression with (a) baseline expression of *IFNλs*, (b) *IFNλs* induction and (c) therapy response were compared in chronic hepatitis C patients with distinct *IL28B* genotypes (rs12979860 CC or CT/TT). The *IL28B*-unfavorable (CT/TT) group were subdivided into undetectable (−) or detectable (+) *IFNλ4* mRNA patients. (a) Baseline expressions of *IL29*, *IL28A*, and *IL28B* in PBMC. (b) Fold changes in *IL29*, *IL28A*, and *IL28B* expression in PBMCs stimulated f with IFNα-2b and poly(I:C). (c) Virological non-response rates for PEG-IFNα/ RBV therapy. Columns represent means ± SEM.

20141225

Fig. 4. Manipulating *IFNλ4* expression regulates *IL28B* induction and promoter activity. (a) Fold inductions of *IL28B* mRNA in BLCs transfected with *IFNλ4* and treated with IFNα (100U/ml). (b) Fold inductions of *IL28B* mRNA in HEK293T cells co-transfected with *IFNλ4* and IRF7 (control, 100ng, 500ng, 1000ng). Induction rates were expressed as fold change relative to control-transfected cells. (c) Fold inductions of *IL28B* promoter activity in HEK293/IL28B-Luc cells transfected with IFN*λ4* and treated with IFNα (0, 10, 100, 1000 IU/ml). (d, e) Fold inductions of *IL28B* promoter activity in HEK293/IL28B-Luc cells co-transfected with IFNλ4 and (d) IRF7 (control, 200ng, 500ng) or (e) p50:p65 (control, 200ng). Luciferase activities and cell viabilities were expressed as fold change relative to untreated or control-transfected cells. The error bars indicate standard deviation. *P<0.05.

20141225

Table 1. Characteristics of patients analyzed for IFNλ expression levels.

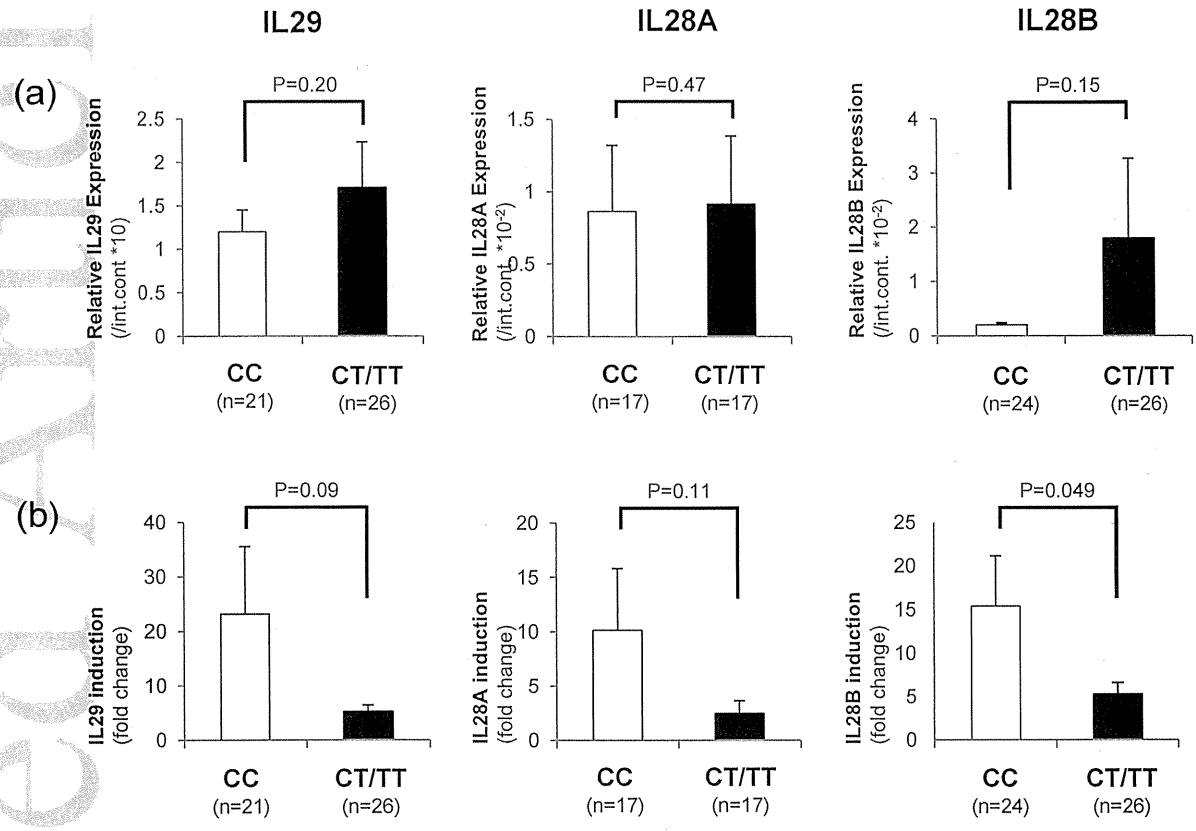
Characteristic	(n = 50)
Age median (range), year	64 (29-79)
Sex, n (%) male/female	19 (38) / 31 (62)
ALT median (range), IU/L	22 (5-157)
γGTP median (range), IU/L	23 (10-343)
LDL-C median (range), mg/dL	100 (38-169)
Hemoglobin median (range), g/dL	13.4 (9.3-16.8)
Platelet count median (range), $\times 10^4/\mu L$	15.5 (5.2-23.6)
Fibrosis stage, n (%)	
F1,2 / F3,4	28 (70) / 12 (30)
Viral load median (range), log IU/mL*	6.8 (4.8-7.6)
HCV core 70 a.a. n(%)†	
wild / mutant / ND	15 (30) / 21 (42) / 14 (28)
HCV core 91 a.a. n (%)	
wild / mutant / ND	18 (36) / 18 (36) / 14 (28)
ISDR substitutions, n (%)‡	
0,1 / 2≦ / ND	26 (52) / 6 (12) / 18 (36)
IL28B SNP (rs8099917), n (%)	
TT / TG, GG	27 (54) / 23 (46)
IL28B SNP (rs12979860), n (%)	
CC / CT, TT	24 (48) / 26 (52)
IL28B SNP (ss469415590), (%)	
TT / ΔG	24 (48) / 26 (52)
Effect of previous therapy, n (%)	
SVR / Relapse / NR	18 (36) / 14 (28) / 18 (36)

ALT, alanine aminotransferase; γ-GTP, γ-glutamyl transpeptidase; LDL-C, low-density lipoprotein cholesterol; HCV, Hepatitis C virus; ISDR, IFN sensitivity determining region; SVR, sustained virological responder; VR, virological responder; NR, non-responder; ND, not determined.

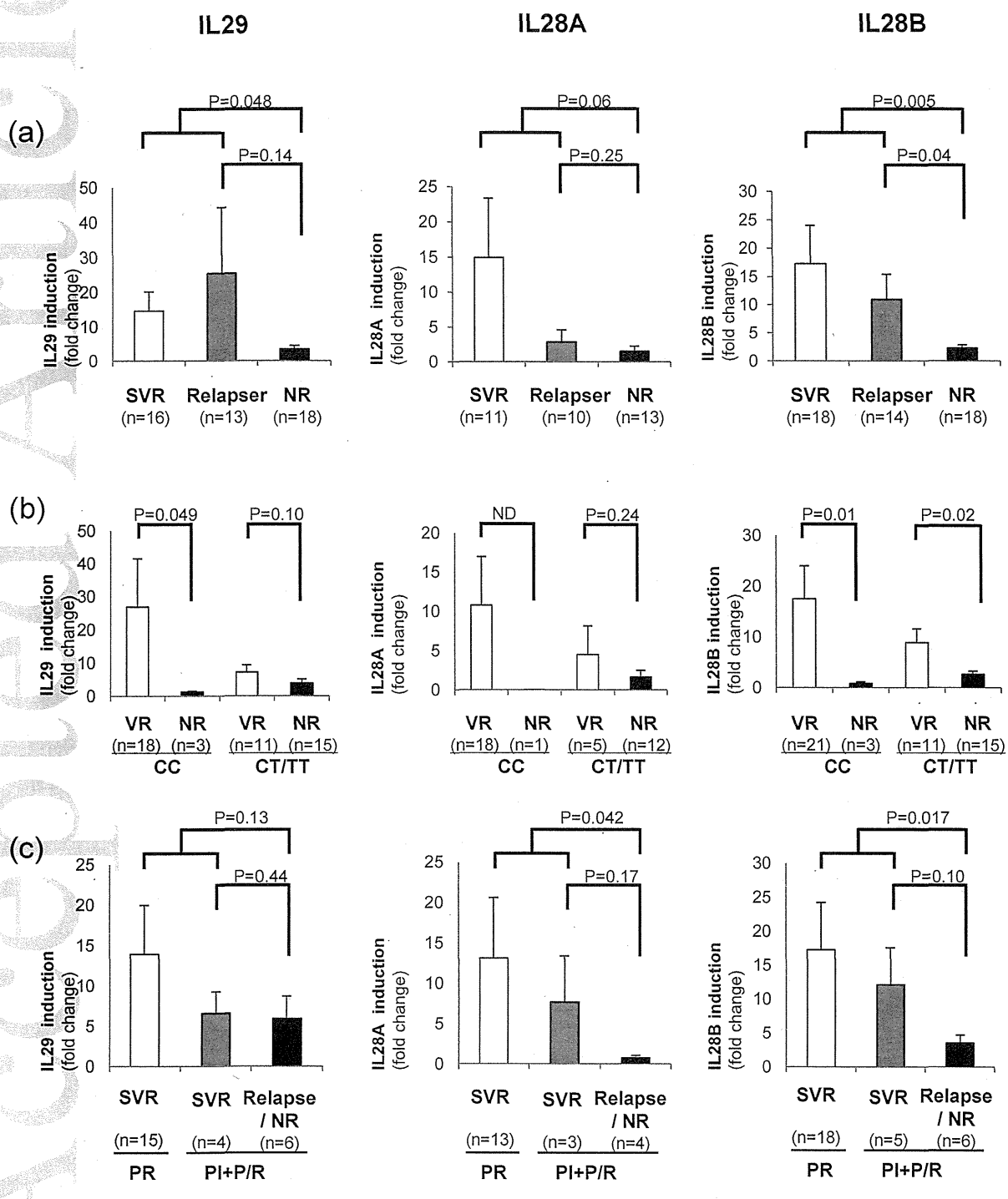
*HCV viral load was analyzed among Relapsers and Non-responders.

†HCV core amino acid (aa) 70R and 91L are considered wild type, while substituted amino acids are considered mutants.

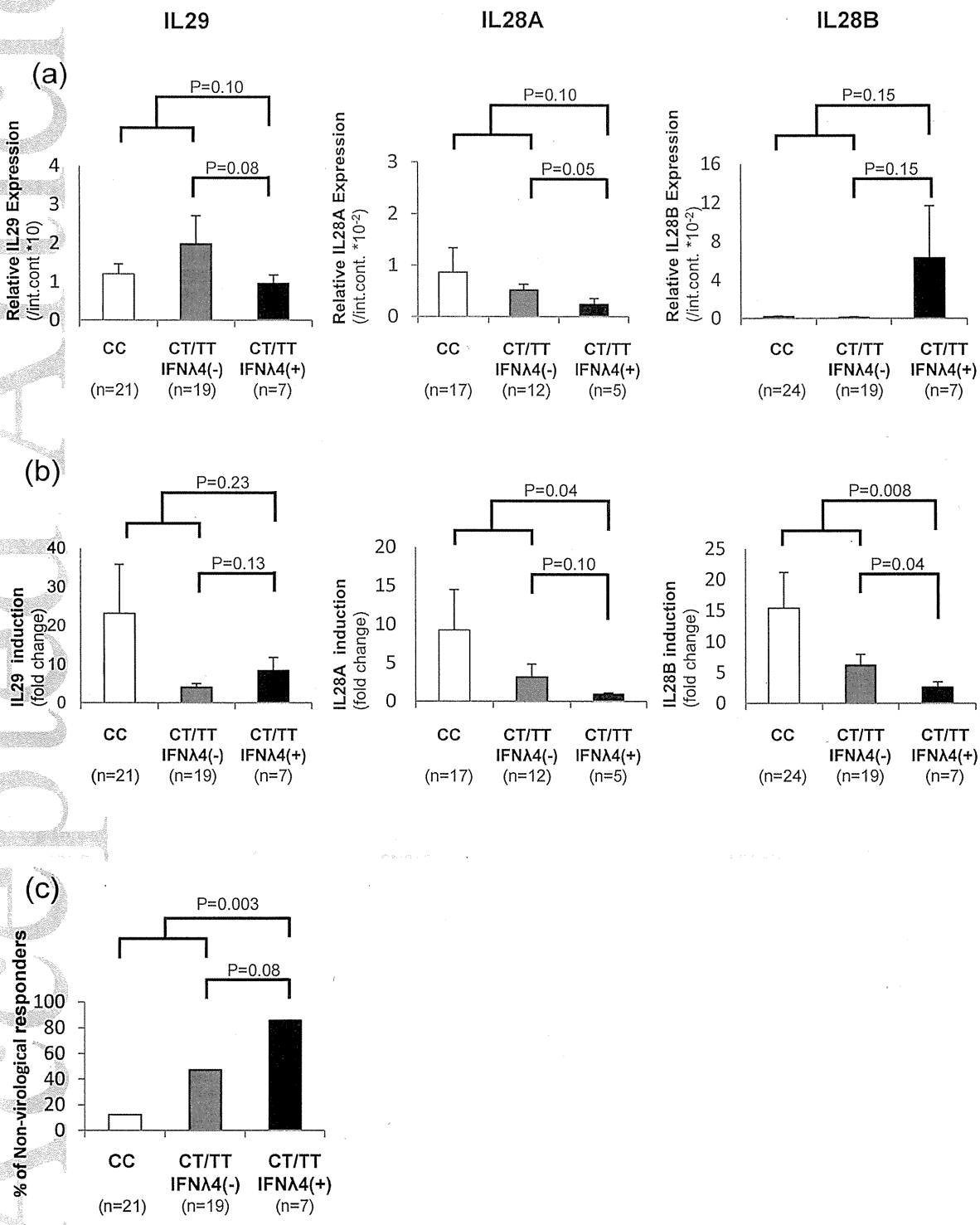
20141225



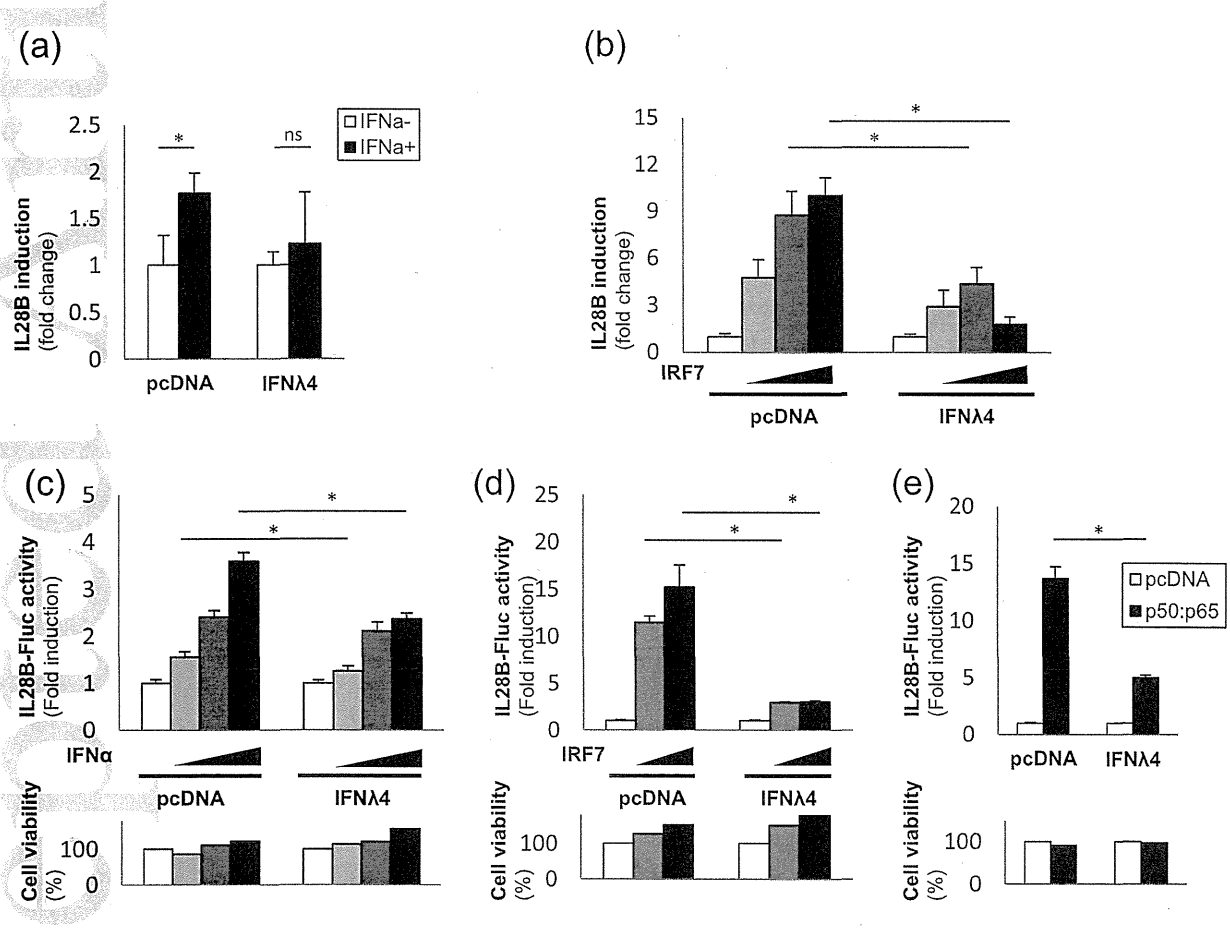
jgh_12902_f1.pdf



jgh_12902_f2.pdf



jgh_12902_f3.pdf



jgh_12902_f4.pdf

Hum Genet (2015) 134:279–289
DOI 10.1007/s00439-014-1520-7

ORIGINAL INVESTIGATION

Genome-wide association study identifies a *PSMD3* variant associated with neutropenia in interferon-based therapy for chronic hepatitis C

Etsuko Iio · Kentaro Matsuura · Nao Nishida · Shinya Maekawa · Nobuyuki Enomoto · Mina Nakagawa · Naoya Sakamoto · Hiroshi Yatsuhashi · Masayuki Kurosaki · Namiki Izumi · Yoichi Hiasa · Naohiko Masaki · Tatsuya Ide · Keisuke Hino · Akihiro Tamori · Masao Honda · Shuichi Kaneko · Satoshi Mochida · Hideyuki Nomura · Shuhei Nishiguchi · Chiaki Okuse · Yoshito Itoh · Hitoshi Yoshiji · Isao Sakaida · Kazuhide Yamamoto · Hisayoshi Watanabe · Shuhei Hige · Akihiro Matsumoto · Eiji Tanaka · Katsushi Tokunaga · Yasuhito Tanaka

Received: 2 October 2014 / Accepted: 8 December 2014 / Published online: 17 December 2014

Abstract Cytopenia during interferon-based (IFN-based) therapy for chronic hepatitis C (CHC) often necessitates reduction of doses of drugs and premature withdrawal from therapy resulting in poor response to treatment. To identify genetic variants associated with IFN-induced neutropenia, we conducted a genome-wide association study (GWAS) in 416 Japanese CHC patients receiving IFN-based therapy. Based on the results, we selected 192 candidate single nucleotide polymorphisms (SNPs) to carry out a replication analysis in an independent set of 404 subjects. The SNP rs2305482, located in the intron region of the *PSMD3* gene on chromosome 17, showed a strong association when the results of GWAS and the replication stage were combined (OR = 2.18, $P = 3.05 \times 10^{-7}$ in the allele frequency model). Logistic regression analysis showed that rs2305482 CC and neutrophil count at baseline were independent predictive factors for IFN-induced neutropenia (OR = 2.497, $P = 0.0072$ and OR = 0.998, $P < 0.0001$, respectively). Furthermore, rs2305482 genotype was associated with the doses of pegylated interferon (PEG-IFN) that could be tolerated in hepatitis C virus genotype 1-infected patients treated with PEG-IFN plus ribavirin, but not with treatment efficacy. Our results suggest that genetic

E. Iio and K. Matsuura equally contributed to this work (shared first authorship).

Electronic supplementary material The online version of this article (doi:10.1007/s00439-014-1520-7) contains supplementary material, which is available to authorized users.

E. Iio · K. Matsuura · Y. Tanaka (✉)
Department of Virology and Liver Unit, Nagoya City University Graduate School of Medical Sciences, Kawasumi, Mizuho, Nagoya 467-8601, Japan
e-mail: ytanaka@med.nagoya-cu.ac.jp

E. Iio · K. Matsuura
Department of Gastroenterology and Metabolism, Nagoya City University Graduate School of Medical Sciences, Nagoya 467-8601, Japan

N. Nishida · N. Masaki
The Research Center for Hepatitis and Immunology, National Center for Global Health and Medicine, Ichikawa 272-8516, Japan

N. Nishida · K. Tokunaga
Department of Human Genetics, Graduate School of Medicine, The University of Tokyo, Tokyo 113-0033, Japan

S. Maekawa · N. Enomoto
First Department of Internal Medicine, University of Yamanashi, Chuo 409-3898, Japan

M. Nakagawa · N. Sakamoto
Department of Gastroenterology and Hepatology, Tokyo Medical and Dental University, Tokyo 113-0034, Japan

N. Sakamoto · S. Hige
Department of Internal Medicine, Hokkaido University Graduate School of Medicine, Sapporo 060-0814, Japan

H. Yatsuhashi
Clinical Research Center, National Nagasaki Medical Center, Omura 856-8562, Japan

M. Kurosaki · N. Izumi
Division of Gastroenterology and Hepatology, Musashino Red Cross Hospital, Musashino 180-0023, Japan

testing for this variant might be useful for establishing personalized drug dosing in order to minimize drug-induced adverse events.

Introduction

Chronic hepatitis C virus (HCV) infection is a significant risk factor for progressive liver fibrosis and hepatocellular carcinoma. Antiviral treatment improves the natural course in chronic hepatitis C (CHC) (George et al. 2009; Yoshida et al. 2004). Newly-developed treatments involving direct-acting antivirals (DAAs), including nonstructural (NS) 3/4A protease inhibitors have shown promising outcomes in combination with pegylated interferon (PEG-IFN) plus ribavirin (RBV) in several clinical trials. Thus, >70 % of patients infected with HCV genotype 1 are reported to achieve sustained virological responses (SVR) (Jacobson et al. 2011; Poordad et al. 2012; Zeuzem et al. 2011). Furthermore, interferon-free (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, IFN-based regimens have been standard-of-care therapies over the last couple of decades.

IFN-based therapies are associated with various adverse effects. Cytopenia is common due to bone marrow suppression cased by IFN or DAA and hemolysis by RBV. This is particularly the case in patients with advanced hepatic fibrosis, but can sometimes also occur in those with mild fibrosis. This then often necessitates dose reduction or premature withdrawal from therapy, resulting in poor response to treatment. For instance, 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). Therefore, pre-treatment prediction of possible adverse effects in order to avoid them and undergo therapy safely is desirable.

Recent genome-wide association studies (GWASs) have identified two important host genetic variants influencing CHC treatment: (1) single nucleotide polymorphisms (SNPs) near the interleukin-28B (*IL28B*) gene, which are strongly associated with response to therapy for chronic HCV genotype 1 infection (Ge et al. 2009; Suppiah et al. 2009; Tanaka et al. 2009), and (2) SNPs in the inosine triphosphatase (*ITPA*) gene, which accurately predict RBV-induced anemia in European–American (Fellay et al. 2010) and Japanese population (Ochi et al. 2010). We validated the association between this *ITPA* genetic variant and RBV-induced anemia (Sakamoto et al. 2010), and reported that the *ITPA* genotype affects the tolerated doses of RBV and treatment response in a stratified group (Kurosaki et al. 2011; Matsuura et al. 2014). Additionally, our GWAS showed that *DDRGK1/ITPA* variants are strongly associated with IFN-induced thrombocytopenia as well as anemia during PEG-IFN/RBV therapy (Tanaka et al. 2011). Thompson et al. (2012) also reported that the *ITPA* genetic variant was associated with anemia and thrombocytopenia during PEG-IFN/RBV therapy. However they identified no genetic determinants of IFN-induced neutropenia at the level of genome-wide significance by their GWAS in populations of European Americans, African Americans and Hispanics.

Hence, to identify genetic variants associated with IFN-induced neutropenia, we conducted a GWAS in Japanese CHC patients.

Y. Hiasa
Department of Gastroenterology and Metabology, Ehime University Graduate School of Medicine, Toon 791-0295, Japan

T. Ide
Division of Gastroenterology, Department of Medicine, Kurume University, Kurume 830-0011, Japan

K. Hino
Department of Hepatology and Pancreatology, Kawasaki Medical School, Kurashiki 701-0114, Japan

A. Tamori
Department of Hepatology, Osaka City Graduate School of Medicine, Osaka 545-8585, Japan

M. Honda · S. Kaneko
Department of Gastroenterology, Kanazawa University Graduate School of Medicine, Kanazawa 920-0934, Japan

S. Mochida
Division of Gastroenterology and Hepatology, Internal Medicine, Saitama Medical University, Iruma 350-0495, Japan

H. Nomura
The Center for Liver Disease, Shin-Kokura Hospital, Kitakyushu 803-8505, Japan

S. Nishiguchi
Department of Internal Medicine, Hyogo College of Medicine, Nishinomiya 663-8131, Japan

C. Okuse
Department of Gastroenterology and Hepatology, St. Marianna University School of Medicine, Kawasaki 216-8511, Japan

Y. Itoh
Molecular Gastroenterology and Hepatology, Kyoto Prefectural University of Medicine, Kyoto 602-0841, Japan

H. Yoshiji
Third Department of Internal Medicine, Nara Medical University, Kashihara 634-8522, Japan

I. Sakaida
Department of Gastroenterology and Hepatology, Yamaguchi University Graduate School of Medicine, Ube 755-8505, Japan

Materials and methods

Patients

From 2007 to 2012, samples for the GWAS were obtained from 416 CHC patients who were treated at 22 hospitals (liver units with hepatologists) throughout Japan. In the following stage of replication analysis, samples were collected in an independent set of 404 Japanese CHC patients. Most patients were treated with PEG-IFN-α2b (1.5 μg/kg body weight subcutaneously once a week) or PEG-IFN-α2a (180 μg once a week) plus RBV (600–1,000 mg daily according to body weight) for 48 weeks for HCV genotype 1 and 24 weeks for genotype 2. Treatment duration was extended in some patients up to 72 weeks for genotype 1 and 48 weeks for genotype 2 according to physicians' preferences. Other patients were treated with PEG-IFN-α2a or IFN monotherapy, or IFN-α2b plus RBV in standard doses of the regimens. The doses of drugs were reduced according to the recommendations on the package inserts or the clinical conditions of the individual patients. Erythropoietin or other growth factors were not given. Patients chronically infected with hepatitis B virus or human immunodeficiency virus, or with other causes of liver disease such as autoimmune hepatitis and primary biliary cirrhosis, were excluded from this study. Written informed consent was obtained from all individual participants in this study and the study protocol conformed to the ethics guidelines of the Declaration of Helsinki and was approved by the institutional ethics review committees.

Inclusion criteria of neutropenia

In the initial stage of GWAS, we defined the inclusion criteria of the case group as minimum neutrophil counts of $<750/mm^3$ at week 2 or 4 during IFN-based therapy, since the dose reduction of IFN is recommended at those levels on the package inserts. Thereafter we did it as minimum neutrophil counts of $<600/mm^3$ at week 2 or 4 in the following GWAS and the replication stages.

SNP genotyping and data cleaning

We conducted two stages of GWAS using the Affymetrix Genome-Wide Human SNP Array 6.0 (Affymetrix, Inc. Santa Clara, CA) according to the manufacturer's instructions. The cut-off value was calculated to maximize the difference, which was also close to median change. At GWAS, the average overall call rate of patients in the case and the control group reached 98.66 and 98.79 %, respectively. We then applied the following thresholds for SNP quality control (QC) in data cleaning: SNP call rate ≥95 % for all samples, minor allele frequency (MAF) ≥1 % for all samples. A total of 601,578 SNPs on autosomal chromosomes passed the QC filters and were used for association analysis. All cluster plots of SNPs showing $P < 0.0001$ in association analyses by comparing allele frequencies in both groups were checked by visual inspection and SNPs with ambiguous genotype calls were excluded. In the replication study, the genotyping of 192 candidate SNPs in an independent set of 404 Japanese HCV-infected patients was carried out using the DigiTag2 assay (Nishida et al. 2007). Successfully genotyped SNPs in the replication analysis had a >95 % call rate, and cleared Hardy–Weinberg equilibrium (HWE) $P \geq 0.001$. One SNP could not be genotyped, and hence we obtained data on 191 SNPs including rs9915252. Three SNPs, rs4794822, rs3907022, and rs3859192 located around the proteasome 26S subunits non-ATPase 3 (*PSMD3*) gene and rs8099917 near the *IL28B* gene were genotyped by TaqMan SNP Genotyping Assays (Applied Biosystems, Carlsbad, CA) following the manufacturer's protocol.

Laboratory and histological tests

Blood samples were obtained at baseline and at appropriate periods after the start of therapy and for hematologic tests, blood chemistry, and HCV RNA. Fibrosis was evaluated on a scale of 0–4 according to the METAVIR scoring system. The SVR was defined as an undetectable HCV RNA level by Roche COBAS Amplicor HCV Monitor test, v.2.0 (Roche Molecular Diagnostics, Pleasanton, CA) with a lower detection limit of 50 IU/ml or Roche COBAS AmpliPrep/COBAS TaqMan HCV assay (Roche Molecular Diagnostics, Pleasanton, CA) with a lower detection limit of 15 IU/ml 24 weeks after the completion of therapy. Serum granulocyte colony-stimulating factor (G-CSF) levels were analyzed using Human G-CSF Quantikine ELISA Kit (R&D Systems, Inc., Minneapolis, MN).

K. Yamamoto
Department of Gastroenterology and Hepatology, Okayama University Graduate School of Medicine, Dentistry, and Pharmaceutical Sciences, Okayama 700-0914, Japan

H. Watanabe
Department of Gastroenterology, Yamagata University Faculty of Medicine, Yamagata 990-9585, Japan

S. Hige
Department of Gastroenterology, Sapporo Kosei General Hospital, Sapporo 060-0033, Japan

A. Matsumoto · E. Tanaka
Department of Medicine, Shinshu University School of Medicine, Matsumoto 390-8621, Japan

Expression quantitative trait locus analysis

Expression quantitative trait locus analysis (eQTL) was conducted using the web-based tool, Genevar (http://www.sanger.ac.uk/resources/software/genevar) (Yang et al. 2010). We evaluated the correlations between rs2305482 genotypes and the expression of transcripts of *PSMD3* or colony-stimulating factor 3 (*CSF3*) by the Spearman's rank correlation coefficient.

Statistical analysis

In the GWAS and the replication stages, the observed association between a SNP and neutropenia induced by IFN-based therapy was assessed by the Chi square test with a two-by-two contingency table in three genetic models: the allele frequency model, the dominant-effect model and the recessive-effect model. Significance levels after Bonferroni correction for multiple testing were $P = 8.31 \times 10^{-8}$ (0.05/601,578) in the GWAS stage and $P = 2.62 \times 10^{-4}$ (0.05/191) in the replication stage. Categorical variables were compared between groups by the Chi square test, and non-categorical variables by the Student's t test or the Mann–Whitney U test. Multivariate logistic regression analysis with stepwise forward selection was performed with $P < 0.05$ in univariate analysis as the criteria for model inclusion. To evaluate the discriminatory ability of neutrophil counts at baseline to predict neutropenia during IFN-based therapy, receiver operating characteristic curve (ROC) curve analysis was conducted. Changes of serum G-CSF levels from baseline to the period with neutropenia during IFN-based therapy were compared by the repeated measure analysis of variance (ANOVA). Correlations between neutrophil counts and serum G-CSF levels were analyzed using Pearson's correlation coefficient test. $P < 0.05$ was considered significant in all tests.

Results

Genetic variants associated with IFN-induced neutropenia

We conducted two stages of GWAS by changing the terms of neutrophil counts, followed by the replication analysis (Fig. 1). The characteristics of the patients in each group for the GWAS and the replication stage are summarized in Table 1. At the first stage of GWAS (GWAS-1st), we genotyped 416 Japanese CHC patients with minimum neutrophil counts of <750/mm^3 (Case-G1, $n = 114$) and ≥1,000/mm^3 (Control-G, $n = 302$) at week 2 or 4 during IFN-based therapy. Here there may still be mixed with undesirable samples that should be removed from the case group. Therefore, we designed and carried out the second stage of GWAS (GWAS-2nd) comparing the patients with more severe neutropenia to the control group: in patients with minimum neutrophil counts of <600/mm^3 (Case-G2, $n = 50$) and ≥1,000/mm^3 (Control-G, $n = 302$) at week 2 or 4 using the same samples as used in GWAS-1st. Supplementary Fig. 1 shows a genome-wide view of the single-point association data based on allele frequencies in GWAS-1st and GWAS-2nd. No association between SNPs and IFN-induced neutropenia reached a genome-wide level of significance [Bonferroni criterion $P < 8.31 \times 10^{-8}$ (0.05/601,578)]. Therefore, we selected the candidate SNPs principally

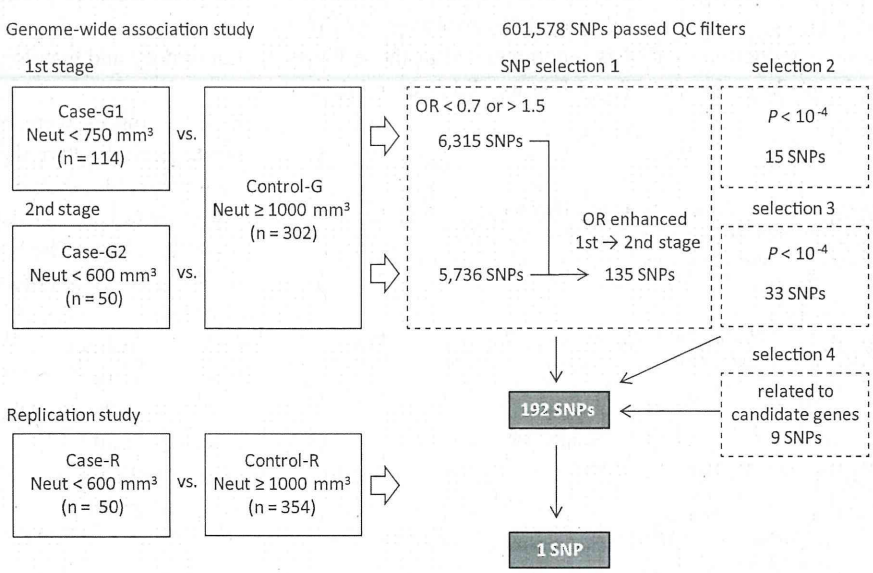


Fig. 1 Outline of the study design. *Neut* neutrophil counts, *SNP* single nucleotide polymorphism, *QC* quality control, *OR* odds ratio

Table 1 Clinical characteristics of patients in GWAS and the replication study

	GWAS			Replication study	
	Case-G1 ($n = 114$)	Case-G2 ($n = 50$)	Control-G ($n = 302$)	Case-R ($n = 50$)	Control-R ($n = 354$)
At baseline					
Gender, male/female	48/66	21/29	170/132	24/26	208/146
Age, years	57.9 (8.7)	57.1 (8.3)	57.2 (11.2)	59.1 (10.2)	56.7 (9.6)
Neutrophil count, /mm^3	1,800 (777)	1,662 (897)	2,750 (984)	1,570 (552)	2,724 (985)
Hemoglobin, g/dL	13.6 (1.3)	13.5 (1.3)	14.2 (1.5)	13.6 (1.6)	14.3 (1.5)
Platelet count, $\times 10^9$/L	141 (42)	132 (46)	164 (54)	140 (47)	162 (60)
ALT, IU/L	82.9 (88.6)	70.4 (53.1)	81.5 (77.9)	87.8 (82.7)	85.2 (71.1)
HCV genotype, 1/2/ND	95/18/1	40/10/0	250/51/1	45/5/0	277/77/0
HCV RNA, log IU/mL	5.9 (0.8)	5.9 (1.0)	6.1 (0.8)	6.1 (0.9)	6.1 (0.8)
Liver fibrosis, F0-2/F3-4/ND	62/22/30	25/10/15	168/70/64	21/6/23	229/87/38
rs8099917, TT/TG + GG/ND	74/39/1	35/15/0	189/109/4	31/17/2	278/70/6
Regimen					
PEG-IFN + RBV/IFN + RBV/PEG-IFN/IFN mono	112/0/0/2	48/0/0/2	277/9/9/7	44/4/2/0	351/0/3/0
At week 4					
Neutrophil count, /mm^3	606 (126)	496 (104)	1,551 (501)	501 (89)	1,533 (484)

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

GWAS genome-wide association study, *ALT* alanine transaminase, *ND* not determined, *PEG-IFN* pegylated interferon, *IFN* mono, interferon monotherapy, *RBV* ribavirin

by comparing between GWAS-1st and GWAS-2nd as follows. There were 6,315 and 5,736 SNPs with odds ratios (ORs) <0.7 or >1.5 at GWAS-1st and GWAS-2nd, respectively. Of these, the ORs of 135 SNPs were more notable at GWAS-2nd than at GWAS-1st. In addition to the 135 SNPs, we selected 15 and 33 SNPs with $P < 10^{-4}$ at GWAS-1st and GWAS-2nd, and added 9 SNPs which are located around the candidate genetic regions identified by the GWAS stage and are non-synonymous or related to diseases in previous reports. Consequently, we carried out the replication analysis focusing on this total of 192 SNPs.

In the subsequent replication analysis, we carried out genotyping of the 192 candidate SNPs in an independent set of 404 Japanese HCV-infected patients with minimum neutrophil counts of <600/mm^3 (Case-R, $n = 50$) and ≥1,000/mm^3 (Control-R, $n = 354$) at week 2 or 4 during IFN-based therapy (Table 1; Fig. 1). The results in the replication stage combined with GWAS-2nd are shown in Supplementary Table 1. Several SNPs such as rs11743919 and rs2457840 showed strong associations with low P value, however, the MAF of them were <5 %. In general, low frequent SNPs tend to show unsettled associations, especially in statistical analysis with small number of samples. Therefore, we excluded these SNPs from the final candidates. Consequently, we determined the SNP rs2305482, located in the intron of *PSMD3* gene on chromosome 17, as the most promising candidate, which showed a strong association with IFN-induced neutropenia in the combined results of GWAS-2nd and the replication stage (OR = 2.18; 95 % CI = 1.61–2.96, $P = 3.05 \times 10^{-7}$ in the allele frequency model) (Table 2).

Association of SNPs located in *PSMD3-CSF3* with neutropenia

A previous GWAS showed that rs4794822 located between the *PSMD3* and *CSF3* genes was associated with neutrophil counts in Japanese patients including 14 different disease groups (Okada et al. 2010). As shown in Fig. 2, rs4794822 is in strong linkage disequilibrium (LD) with rs2305482 which we identified in the present study. Thus, the pairwise LD (r^2) in the HapMap JPT: Japanese in Tokyo, Japan, is 0.66. Because the SNP rs4794822 is not included in the Affymetrix Genome-Wide Human SNP Array 6.0, we additionally genotyped it together with three other SNPs (rs9915252, rs3859192 and rs3907022) located in the same LD block around the *PSMD3* gene (Fig. 2). The allele frequency of each SNP was compared between patients with minimum neutrophil counts of <600/mm^3 (Case-G2 + R: Case-G2 plus Case-R, $n = 100$) and ≥1,000/mm^3 (Control-G + R: Control-G plus Control-R, $n = 656$) at week 2 or 4 during IFN-based therapy. This showed that, rs4794822 was also strongly associated with neutropenia during IFN-based therapy (OR = 2.24; 95 % CI = 1.63–3.07, $P = 3.63 \times 10^{-7}$ in the allele frequency model) (Table 3).

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

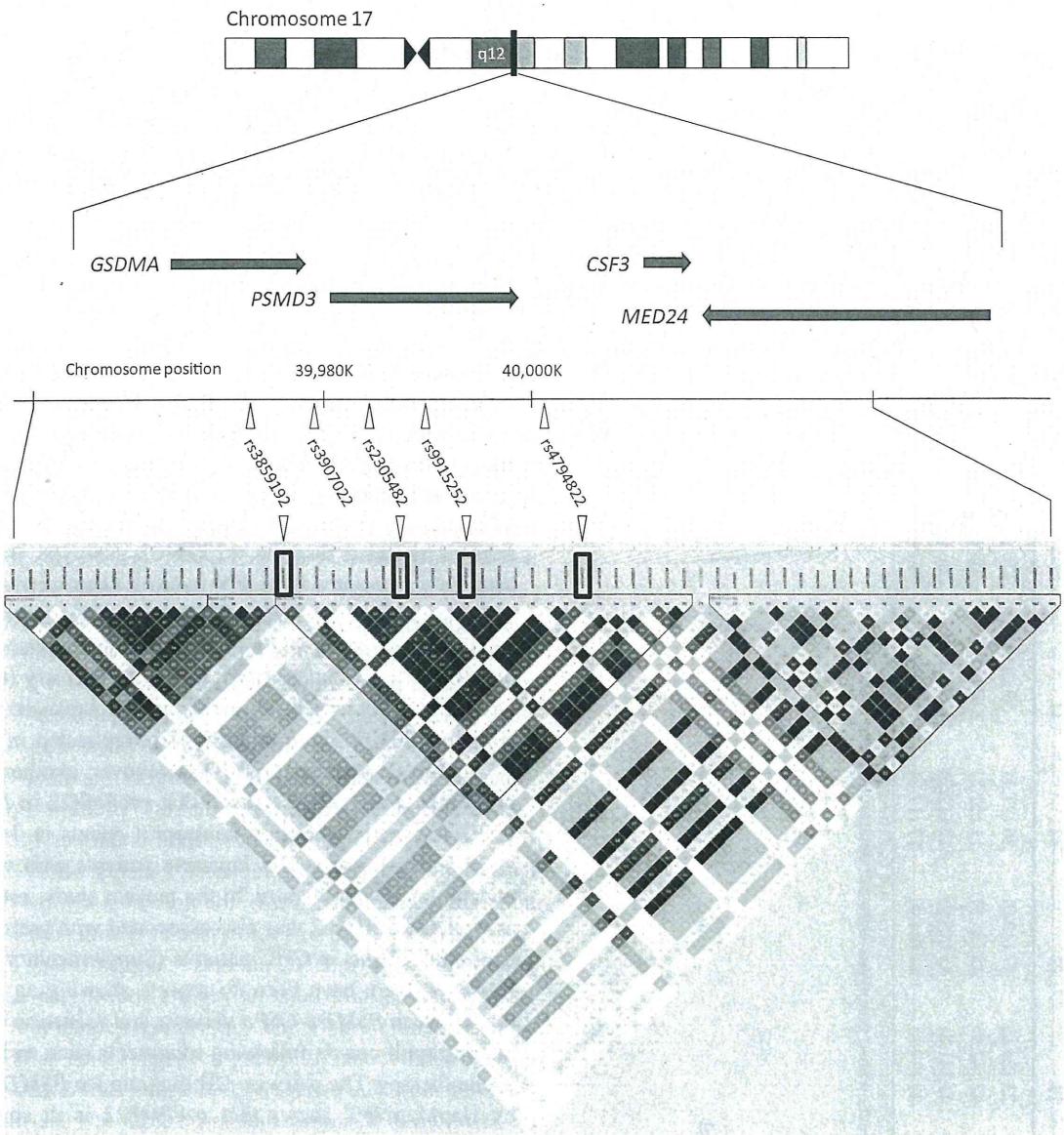


Fig. 2 Position on chromosome and pairwise linkage disequilibrium (r^2) diagrams in the HapMap JPT around the *PSMD3-CSF3* locus

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