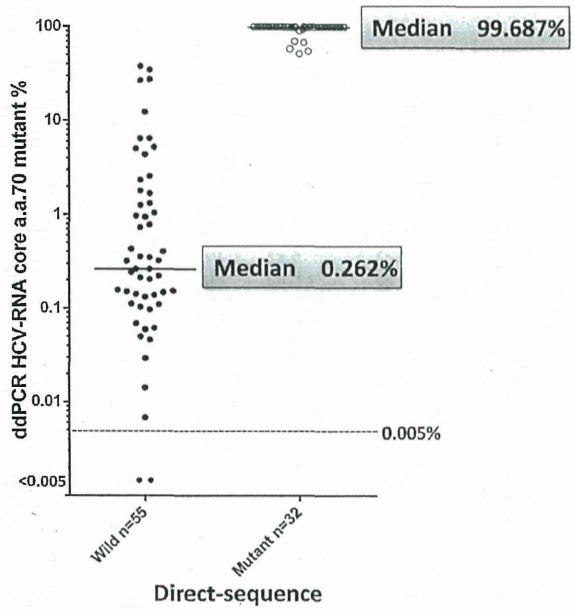


**Fig. 5.** Comparison between the results of 87 clinical specimens analyzed by direct sequencing and the ddPCR assay. The ddPCR assay detected all of the mutations that were detected by direct sequencing (wild type: $n = 55$; median, 0.262%; range, 0–37.951%; mutant: $n = 32$; median, 99.687%; range, 52.191–100%).

single mutations within a highly polymorphic genome, such as HCV and HIV because many variants in near target sequences and setting probes affected quantitation. However, Strain et al. (2013) reported that ddPCR could be used for the quantitation of HIV. In addition, there were fewer mutations in near Core a.a. 70. These data suggest that ddPCR technology is useful for the quantitation of mutations in other viruses with highly polymorphic genomes, if there are few variant sequences in near targets.

Therefore, it was hoped that this new assay could be used for the quantitation of mutations in other viral genomes, such as of mutations in HCV resistant to direct-acting antivirals (Aloia et al., 2012; Wong et al., 2013; Conteduca et al., 2014), mutations in drug-resistant HBV (Mukaide et al., 2010), and mutations associated with HBV reactivation (Dai et al., 2001; Alexopoulou et al., 2006) in patients receiving chemotherapy.

## 5. Conclusions

A novel, high-throughput and ultrasensitive assay was developed to quantitate mutations in HCV codon a.a.70. Therefore, ddPCR technology should be useful for the relative and absolute quantitation of HCV mutations as well as mutations in the polymorphic genomes of other viruses.

## Acknowledgments

This work was supported by a grant (22-108, 25-202) from the National Center for Global Health and Medicine in Japan and in part by the Japanese Ministry of Health, Labor and Welfare grant (H23-007) and the Japanese Ministry of Education, Culture, Sports, and Science (No. 22590750). The authors thank Bio-Rad Laboratories KK, Japan (Mr. Hiroto Tsuruta, Ms. Issei Iijima, Mr. Tsutomu Daito, Mr. Yukinori Yatsuda, and Mr. Keisuke Uchida) for their professional opinions and technical support. The authors thank Mr. Makoto Yamashiro (Roche Diagnostics KK Japan) for his professional opinions and technical support. The authors thank all of the members of our laboratory (Ms. Yoko Tamada, Ms. Sachiko Sato, Ms. Miki Yoshida, Ms. Mami Ohashi, Ms. Ayano Ito, Mr. Hideyuki Aoshima, Mr. Takashi Tsunoda, Ms. Hiroko Higashimoto, Mr. Youji Hirabayashi, Mr. Akio Ueda, and Mr. Isao Tada).

## Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jviromet.2014.07.006.

## References

Akuta, N., Suzuki, F., Seko, Y., Kawamura, Y., Sezaki, H., Suzuki, Y., Hosaka, T., Kobayashi, M., Hara, T., Kobayashi, M., Saitoh, S., Arase, Y., Ikeda, K., Kumada, H., 2012. Complicated relationships of amino acid substitution in hepatitis C virus core region and IL28B genotype influencing hepatocarcinogenesis. Hepatology 56, 2134–2141.

Akuta, N., Suzuki, F., Sezaki, H., Suzuki, Y., Hosaka, T., Someya, T., Kobayashi, M., Saitoh, S., Watahiki, S., Sato, J., Matsuda, M., Kobayashi, M., Arase, Y., Ikeda, K., Kumada, H., 2005. Association of amino acid substitution pattern in core protein of hepatitis C virus genotype 1b high viral load and non-virological response to interferon-ribavirin combination therapy. Intervirology 48, 372–380.

Alexopoulou, A., Theodorou, M., Dourakis, S.P., Karayiannis, P., Sagkana, E., Papanikolopoulos, K., Archimandritis, A.J., 2006. Hepatitis B virus reactivation in patients receiving chemotherapy for malignancies: role of precore stop-codon and basic core promoter mutations. J. Viral. Hepat. 13, 591–596.

Aloia, A.L., Locarnini, S., Beard, M.R., 2012. Antiviral resistance and direct-acting antiviral agents for HCV. Antivir. Ther. 17, 1147–1162.

Becker-andr, M., Hahlbrock, K., 1989. Absolute mRNA quantification using the polymerase chain reaction (PCR). A novel approach by a PCR aided transcript titration assay (PATITY). Nucleic Acids Res., 9437–9446.

Bossler, A., Gunsolly, C., Pyne, M.T., Rendo, A., Rachel, J., Mills, R., Miller, M., Sipley, J., Hillyard, D., Jenkins, S., Essmyer, C., Young, S., Lewinski, M., Rennert, H., 2011. Performance of the COBAS® AmpliPrep/COBAS TaqMan® automated system for hepatitis C virus (HCV) quantification in a multi-center comparison. J. Clin. Virol. 50, 100–103 (the official publication of the Pan American Society for Clinical Virology).

Conteduca, V., Sansonno, D., Russi, S., Pavone, F., Dammacco, F., 2014. Therapy of chronic hepatitis C virus infection in the era of direct-acting and host-targeting antiviral agents. J. Infect. 68, 1–20.

Dai, M.S., Lu, J.J., Chen, Y.C., Perng, C.L., Chao, T.Y., 2001. Reactivation of precore mutant hepatitis B virus in chemotherapy-treated patients. Cancer 92, 2927–2932.

Edelmann, a., Eichenlaub, U., Lepek, S., Krüger, D.H., Hofmann, J., 2013. Performance of the MagNA Pure 96 system for cytomegalovirus nucleic acid amplification testing in clinical samples. J. Clin. Microbiol. 51, 1600–1601.

Gilliland, G., Perrin, S., Blanchard, K., Bunn, H.F., 1990. Analysis of cytokine mRNA and DNA: detection and quantitation by competitive polymerase chain reaction. Proc. Nat. Acad. Sci. U.S.A. 87, 2725–2729.

Glenn, T.C., 2011. Field guide to next-generation DNA sequencers. Mol. Ecol. Res. 11, 759–769.

Grakoui, A., Wychowski, C., Lin, C., Feinstone, S.M., Rice, C.M., 1993. Expression and identification of hepatitis C virus polyprotein cleavage products. J. Virol. 67, 1385–1395.

Higuchi, R., Fockler, C., Dollinger, G., Watson, R., 1993. Kinetic PCR analysis: real-time monitoring of DNA amplification reactions. Biotechnology 11, 1026–1030.

Hindson, B.J., Ness, K.D., Masquelier, D.a, Belgrader, P., Heredia, N.J., Makarewicz, A.J., Bright, I.J., Lucero, M.Y., Hiddessen, A.L., Legler, T.C., Kitano, T.K., Hodel, M.R., Petersen, J.F., Wyatt, P.W., Steenblock, E.R., Shah, P.H., Bousse, L.J., Troup, C.B., Mellen, J.C., Wittmann, D.K., Erndt, N.G., Cauley, T.H., Koehler, R.T., So, A.P., Dube, S., Rose, K.a, Montesclaros, L., Wang, S., Stumbo, D.P., Hodges, S.P., Romine, S., Milanovich, F.P., White, H.E., Regan, J.F., Karlin-Neumann, G.a, Hindson, C.M., Saxonov, S., Colston, B.W., 2011. High-throughput droplet digital PCR system for absolute quantitation of DNA copy number. Anal. Chem. 83, 8604–8610.

Hoff, K.J., 2009. The effect of sequencing errors on metagenomic gene prediction. BMC Genom. 10, 520.

Holland, J., Spindler, K., Horodyski, F., Grabau, E., Nichol, S., VandePol, S., 1982. Rapid evolution of RNA genomes. Science 215, 1577–1585.

Hubers, a.J., Heideman, D.aM., Yatabe, Y., Wood, M.D., Tull, J., Tarón, M., Molina, M.a, Mayo, C., Bertran-Alamillo, J., Herder, G.J.M., Koning, R., Sie, D., Ylstra, B., Meijer, G.a, Snijders, P.J.F., Witte, B.I., Postmus, P.E., Smit, E.F., Thunnissen, E., 2013. EGFR mutation analysis in sputum of lung cancer patients: a multitechnique study. Lung Cancer 82, 38–43.

Kiyosawa, K., Sodeyama, T., Tanaka, E., Gibo, Y., Yoshizawa, K., Nakano, Y., Furuta, S., Akahane, Y., Nishioka, K., Purcell, R.H., 1990. Interrelationship of blood transfusion, non-A, non-B hepatitis and hepatocellular carcinoma: analysis by detection of antibody to hepatitis C virus. Hepatology 12, 671–675.

Korenaga, M., Wang, T., Li, Y., Showalter, L.a, Chan, T., Sun, J., Weinman, S.a, 2005. Hepatitis C virus core protein inhibits mitochondrial electron transport and increases reactive oxygen species (ROS) production. J. Biol. Chem. 280, 37481–37488.

Liang, T.J., Rehermann, B., Seeff, L.B., Hoofnagle, J.H., 2000. Pathogenesis, natural history, treatment, and prevention of hepatitis C. Ann. Intern. Med. 132, 296–305.
Miura, M., Maekawa, S., Kadokura, M., Sueki, R., Komase, K., Shindo, H., Ohmori, T., Kanayama, A., Shindo, K., Amemiya, F., Nakayama, Y., Kitamura, T., Uetake, T., Inoue, T., Sakamoto, M., Okada, S., Enomoto, N., 2011. Analysis of viral amino acids sequences and the IL28B SNP influencing the development of hepatocellular carcinoma in chronic hepatitis C. Hepatol. Int., 386–396.
Miura, M., Maekawa, S., Takano, S., Komatsu, N., Tatsumi, A., Asakawa, Y., Shindo, K., Amemiya, F., Nakayama, Y., Inoue, T., Sakamoto, M., Yamashita, A., Moriishi, K., Enomoto, N., 2013. Deep-sequencing analysis of the association between the quasispecies nature of the hepatitis C virus core region and disease progression. J. Virol. 87, 12541–12551.
Morisset, D., Štebih, D., Milavec, M., Gruden, K., Žel, J., 2013. Quantitative analysis of food and feed samples with droplet digital PCR. PLoS One 8, e62583.
Moriya, K., Fujie, H., Shintani, Y., Yotsuyanagi, H., Tsutsumi, T., Ishibashi, K., Matsuura, Y., Kimura, S., Miyamura, T., Koike, K., 1998. The core protein of hepatitis C virus induces hepatocellular carcinoma in transgenic mice. Nat. Med. 4, 1065–1067.
Mukaide, M., Tanaka, Y., Shin-I, T., Yuen, M.-F., Kurbanov, F., Yokosuka, O., Sata, M., Karino, Y., Yamada, G., Sakaguchi, K., Orito, E., Inoue, M., Baqai, S., Lai, C.-L., Mizokami, M., 2010. Mechanism of entecavir resistance of hepatitis B virus with viral breakthrough as determined by long-term clinical assessment and molecular docking simulation. Antimicrob. Agents Chemother. 54, 882–889.
Nakamoto, S., Kanda, T., Yonemitsu, Y., Arai, M., Fujiwara, K., Fukai, K., Kanai, F., Imazeki, F., Yokosuka, O., 2009. Quantification of hepatitis C amino acid substitutions 70 and 91 in the core coding region by real-time amplification refractory mutation system reverse transcription-polymerase chain reaction. Scand. J. Gastroenterol. 44, 872–877.
Niederau, C., Lange, S., Heintges, T., Erhardt, A., Buschkamp, M., Hürter, D., Nawrocki, M., Kruska, L., Hensel, F., Petry, W., Häussinger, D., 1998. Prognosis of chronic hepatitis C: results of a large, prospective cohort study. Hepatology 28, 1687–1695.
Nielsen, R., Paul, J.S., Albrechtsen, A., Song, Y.S., 2011. Genotype and SNP calling from next-generation sequencing data. Nat. Rev. Genet. 12, 443–451.
Noguchi, H., Park, J., Takagi, T., 2006. MetaGene: prokaryotic gene finding from environmental genome shotgun sequences. Nucleic Acids Res. 34, 5623–5630.
Okamoto, K., Akuta, N., Kumada, H., Kobayashi, M., Matsuo, Y., Tazawa, H., 2007. A nucleotide sequence variation detection system for the core region of hepatitis C virus-1b. J. Virol. Methods 141, 1–6.
Okanoue, T., Itoh, Y., Hashimoto, H., Yasui, K., Minami, M., Takehara, T., Tanaka, E., Onji, M., Toyota, J., Chayama, K., Yoshioka, K., Izumi, N., Akuta, N., Kumada, H., 2009. Predictive values of amino acid sequences of the core and NS5A regions in antiviral therapy for hepatitis C: a Japanese multi-center study. J. Gastroenterol. 44, 952–963.
Pekin, D., Skhiri, Y., Baret, J.-C., Le Corre, D., Mazutis, L., Salem, C., Ben Millot, F., El Harrak, A., Hutchison, J.B., Larson, J.W., Link, D.R., Laurent-Puig, P., Griffiths, A.D., Taly, V., 2011. Quantitative and sensitive detection of rare mutations using droplet-based microfluidics. Lab Chip 11, 2156–2166.
Pinheiro, L.B., Coleman, V.A., Hindson, C.M., Herrmann, J., Hindson, B.J., Bhat, S., Emslie, K.R., 2012. Evaluation of a droplet digital polymerase chain reaction format for DNA copy number quantification. Anal. Chem. 84, 1003–1011.
Porcher, C., Malinge, M.C., Picat, C., Grandchamp, B., 1992. A simplified method for determination of specific DNA or RNA copy number using quantitative PCR and an automatic DNA sequencer. BioTechniques 13, 106–114.
Roche-PCR-Chapters, 2009. PCR Applications Manual, Chapters 2, General Guidelines. Roche-PCR-Chapters.
Shendure, J., Ji, H., 2008. Next-generation DNA sequencing. Nat. Biotechnol. 26, 1135–1145.
Strain, M.C., Lada, S.M., Luong, T., Rought, S.E., Gianella, S., Terry, V.H., Spina, C.a, Woelk, C.H., Richman, D.D., 2013. Highly precise measurement of HIV DNA by droplet digital PCR. PLoS One 8, e55943.
Syvänen, A.C., Bengtström, M., Tenhunen, J., Söderlund, H., 1988. Quantification of polymerase chain reaction products by affinity-based hybrid collection. Nucleic Acids Res. 16, 11327–11338.
Tadokoro, K., Kobayashi, M., Suzuki, F., Tanaka, C., Yamaguchi, T., Nagano, M., Egashira, T., Kumada, H., 2013. Comparative quantitative analysis of hepatitis C mutations at amino acids 70 and 91 in the core region by the Q-Invader assay. J. Virol. Methods 189, 221–227.
Thermo Scientific, 2013. DNA Copy Number Calculater. Thermo Scientific, http://www.thermoscientificbio.com/EktronTemplates/FullContent.aspx?id=17179928879&rdr=true&LangType=1041 (accessed 08.11.13).
Thomas, D.L., 2013. Global control of hepatitis C: where challenge meets opportunity. Nat. Med. 19, 850–858.
Vogelstein, B., Kinzler, K.W., 1999. Digital PCR. Proc. Nat. Acad. Sci. U.S.A. 96, 9236–9241.
Wenzel, J.J., Panning, M., Kaul, K.L., Mangold, K.a, Revell, P.a, Luna, R.A., Zepeda, H., Perea, L., Vazquez-Perez, J.a, Young, S., Rodic-Polic, B., Eickmann, M., Drosten, C., Jilg, W., Reischl, U., 2010. Analytical performance determination and clinical validation of the novel Roche RealTime Ready Influenza A/H1N1 Detection Set. J. Clin. Microbiol. 48, 3088–3094.
Whale, A.S., Cowen, S., Foy, C.A., Huggett, J.F., 2013. Methods for applying accurate digital PCR analysis on low copy DNA samples. PLoS One 8, e58177.
Wong, K.A., Worth, A., Martin, R., Svarovskaia, E., Brainard, D.M., Lawitz, E., Miller, M.D., Mo, H., 2013. Characterization of hepatitis C virus resistance from a multiple-dose clinical trial of the novel NS5A inhibitor GS-5885. Antimicrob. Agents Chemother. 57, 6333–6340.
Yang, J.D., Roberts, L.R., 2010. Hepatocellular carcinoma: a global view. Nat. Rev. Gastroenterol. Hepatol. 7, 448–458.

Journal of Gastroenterology and Hepatology

JGHF

doi:10.1111/jgh.12646

HEPATOLOGY

# Pretreatment prediction of the outcome of response-guided peginterferon-α and ribavirin therapy for chronic hepatitis C

Naohiko Masaki,* Masaya Sugiyama,* Noritomo Shimada,† Yasuhito Tanaka,‡ Makoto Nakamuta,§ Namiki Izumi,¶ Sumio Watanabe,** Akihito Tsubota,†† Masafumi Komatsu,‡‡ Tsutomu Masaki,§§ Nobuyuki Enomoto,¶¶ Masashi Yoneda,*** Kazumoto Murata,* Kiyoaki Ito,*,*** Kazuhiko Koike††† and Masashi Mizokami*

*The Research Center for Hepatitis and Immunology, National Center for Global Health and Medicine, †Division of Gastroenterology and Hepatology, Shinmatsudo Central General Hospital, ††Institute of Clinical Medicine and Research, The Jikei University School of Medicine, Chiba, ‡Department of Virology, Nagoya City University Graduate School of Medical Sciences, Nagoya, §Department of Gastroenterology, NHO Kyushu Medical Center, Fukuoka, ¶Department of Gastroenterology and Hepatology, Musashino Red Cross Hospital, **Department of Gastroenterology, Juntendo University, †††Department of Gastroenterology, Graduate School of Medicine, The University of Tokyo, Tokyo, ‡‡Department of Gastroenterology, Akita City Hospital, Akita, §§Department of Gastroenterology, Kagawa Medical University School of Medicine, Kagawa, ¶¶First Department of Medicine, Yamanashi University School of Medicine, Yamanashi, and ***Division of Gastroenterology, Department of Internal Medicine, Aichi Medical University School of Medicine, Aichi, Japan

**Key words**
(TA) dinucleotide repeat, chronic hepatitis C, IL28B, response-guided therapy

Accepted for publication 3 May 2014.

**Correspondence**
Naohiko Masaki, The Research Center for Hepatitis and Immunology, National Center for Global Health and Medicine, 1-7-1 Kohnodai, Ichikawa, Chiba 272-8516, Japan.
Email: nmasaki@hospk.ncgm.go.jp

Potential conflict of interests: Nothing to declare.

Financial support: This study was supported by the Grant-in-Aid from the National Center for Global Health and Medicine (21A113) (N.M.). The funding source had no involvement, such as the roles of the study design, the collection, analysis, interpretation of the data, the writing of this manuscript or the decision to submit this manuscript for publication.

## Abstract

**Background and Aim:** The accuracy for predicting virological outcomes of peginterferon-α and ribavirin therapy in patients with chronic hepatitis C is limited to approximately 80%, even with *IL28B* genotyping. Our *in vitro* study revealed that the numbers of (TA) dinucleotide repeats [(TA)n] of rs72258881, which is located in the promoter region of *IL28B* gene, might regulate *IL28B* transcription. We aimed to evaluate the usefulness of these host factors for predicting virological outcomes of this therapy in response-guided clinical settings.

**Methods:** A nationwide, multi-center prospective study in Japan determined *IL28B* (rs8099917) genotype, (TA)n of rs72258881, and amino acid substitutions of hepatitis C virus and used these for multivariate analysis together with other parameters at pretreatment.

**Results:** After enrolling 215 patients with genotype 1 and high viral load from 23 hospitals between October 2009 and February 2011, intent-to-treat analysis identified 202 patients in whom the final virological outcomes could be determined. Non-virological response by non-TT genotype was predicted with 79.7% accuracy. When combined with the (TA)n, the incidences of virological response tended to be higher in the longer (TA)n group, regardless of rs8099917 genotype. Multivariate logistic regression analysis revealed that rs8099917 non-TT genotype ($P < 0.001$), shorter (TA)n ($P = 0.011$), mutation of amino acid 70 in the virus core region ($P = 0.029$), and lower levels of serum albumin ($P = 0.036$) were independently associated with non-virological response.

**Conclusions:** *IL28B* genotype and (TA)n of rs72258881 may independently affect virological outcomes of peginterferon-α and ribavirin as host factors, even in response-guided therapy.

## Introduction

Globally as many as 150 millions of people are infected by hepatitis C virus (HCV) and every year approximately 350 000 patients die of HCV-related liver diseases, such as liver cirrhosis or hepatocellular carcinoma.[1] The standard care for the treatment of chronic hepatitis C has been PEGylated interferon-α (PEG-IFN-α) and ribavirin (P/R) with sustained virological response (SVR) rates as high as 50% achieved, even in difficult-to-treat combination of HCV genotype 1 and high viral load.[2] Recently introduced protease inhibitors, such as boceprevir[3,4] or telaprevir,[5,6] can improve the SVR rate as much as 70–80% in IFN-naïve patients. Furthermore, the era of interferon-free treatment with only-oral directly-acting antivirals has just arrived.[7–9] However, elderly HCV-infected patients are often unable to either tolerate or afford these new treatment regimens. In such circumstances, it is very

important to identify easy-to-treat patients prior to treatment. Factors that contribute to the success of drug-mediated eradication of HCV include host factors (such as age, gender, the extent of liver fibrosis, and insulin resistance[10,11]), viral factors (such as HCV genotype, viral load, and amino acid substitutions in core[12] and NS5A[13] regions), as well as drug-related factors (such as treatment regimens, adherence to these regimens, total drug doses, and the duration of treatment).[14] The most epoch-making discoveries in this field were that single nucleotide polymorphisms (SNPs) in the *IL28B* gene (rs8099917, rs12979860) can predict virological response (VR) to P/R.[15–19] However, all these findings were based primarily on retrospective studies with per protocol analysis. Furthermore, even if those factors are all concentrated, the accuracy with which therapeutic outcomes can be predicted still remains approximately 80%. We recently reported that the number of (TA) dinucleotide repeats [(TA)n] of rs72258881, which is located in the promoter region of *IL28B* gene, might regulate its transcription.[20] Here we report our efforts to verify the role of (TA)n of rs72258881, by conducting a prospective multicenter cohort study with intent-to-treat analysis, in Japanese patients infected with HCV genotype 1 who were treated by response-guided therapy (RGT) with P/R.

## Methods

***Study Design.*** From October 2009 to February 2011, 233 patients with chronic hepatitis C were prospectively enrolled from nationwide 23 hospitals in Japan (Trial Registration: UMIN-CTR000002580); however, 18 patients were considered to be ineligible and excluded from this study because of violation of the following entry criteria: (1) infection with HCV serotype 1[21] or genotype 1 (1a or 1b)[22] without co-infection with hepatitis B virus or human immunodeficiency virus; (2) pretreatment HCV RNA levels $\geq 5.0$ $\log_{10}$ IU/mL, as determined using a quantitative real-time PCR method (COBAS AmpliPrep/COBAS TaqMan HCV test; Roche Molecular Systems, Pleasanton, CA, USA); (3) standard P/R therapy according to the American Association of the Study of the Liver Diseases (AASLD) guidelines.[23] Consequently, 215 patients met the entry criteria and were treated with weekly administration of PEG-IFN-α2a (Chugai Pharmaceutical, Tokyo, Japan) and daily administration of ribavirin (Chugai Pharmaceutical), or with weekly administration of PEG-IFN-α2b (MSD Co., Tokyo, Japan) and daily administration of ribavirin (MSD Co.). Whereas the dose of PEG-IFN-α2a was 180 μg, regardless of the patient's body weight, doses of PEG-IFN-α2b were adjusted based on the patient's body weight: respective weekly doses of PEG-IFN-α2b for patients < 45 kg, ≥ 45 kg, and < 60 kg; ≥ 60 kg and < 75 kg; ≥ 75 kg and < 90 kg; and ≥ 90 kg were given 60 μg, 80 μg, 100 μg, 120 μg, and 150 μg of PEG-IFN-α2b. Respective daily doses of ribavirin for patients < 60 kg, ≥ 60 kg and < 80 kg, and ≥ 80 kg were given 600 mg, 800 mg, and 1000 mg. Dose modifications of PEG-IFN-α or ribavirin, relating to adverse events, were based on the manufacturers' recommendations.

The treatment duration was determined based on RGT according to guidelines of AASLD[23] and the Japan Society of Hepatology (JSH).[24] Patients in whom serum HCV RNA had disappeared within 12 weeks after starting therapy received a 48-week treatment regimen. Patients in whom serum HCV RNA was still detectable at 12 weeks, but not at 24 weeks after starting therapy received a 72-week extended treatment regimen.

VR was defined as achieving SVR or transient virological response (TVR); whereas SVR was defined as undetectable HCV RNA in serum 24 weeks after the cessation of treatment, TVR was defined as undetectable HCV RNA at the cessation of treatment with reappearance of HCV RNA in serum thereafter. Non-virological response (NVR), which was defined as detectable viremia throughout the 24 weeks of P/R therapy, was classified as one of two categories. The first of these, "null responder", was defined as < 2 log-unit decline in the serum levels of HCV RNA from the pretreatment baseline value within the first 12 weeks and detectable viremia at 24 weeks after the start of P/R. The second, "partial responder", was defined as ≥ 2 log-unit decline in the serum levels of HCV RNA from the pretreatment baseline value within the first 12 weeks and detectable viremia at 24 weeks after the start of P/R. Patients whose treatment was withdrawn due either to the presence of serum HCV RNA after 24 weeks of therapy or to viral breakthrough (VBT) were also included in this study for intent-to-treat analysis. VBT was included in NVR, and was subclassified into "null responder" or "partial responder", according to the above criteria. Adherence to PEG-IFN-α and ribavirin up to 12 weeks after the start of P/R were calculated.

The study protocol (Fig. 1) complied with the Helsinki Declaration and was approved by the ethics committee of each participating institution. At the time of enrollment, written informed consent was obtained for the collection and storage of serum and peripheral blood.

***DNA Extraction.*** Genomic DNA was extracted from the buffy coat fraction of patients' whole blood using a GENOMIX kit (Talent SRL; Trieste, Italy).

***IL28B genotyping.*** We previously reported that the rs8099917 polymorphism is a better predictor of the response of P/R to chronic hepatitis C in Japanese patients than any other SNPs reported near the *IL28B* gene.[25] Therefore, the rs8099917 polymorphism was genotyped using the InvaderPlus assay (Third Wave Japan, Tokyo, Japan), which combines the polymerase chain reaction (PCR) and the invasive signal amplification reaction.[26,27] In this prospective cohort study, in order to meet the requirements of RGT, both the doctors and the patients were blinded to the results of *IL28B* genotyping until final determination of virological outcomes.

***Detection of amino acid substitutions in core and NS5A regions of HCV.*** Amino acids 70 and 91 of the HCV core region and the amino acid sequence of interferon-sensitivity determining region (ISDR: residues 2209–2248 of the NS5A region) were determined by direct sequencing, as previously reported.[12,13]

***TA repeat genotyping.*** To determine the genotype of the TA repeat polymorphism, we used GeneScan analysis to detect the fragment size of the fluorescently labeled PCR amplicon. This method requires the use of nested PCR to prevent amplification of *IL-29* region with high sequence similarity to regions within the *IL-28A* and *IL-28B* genes. The details of the nested PCR and

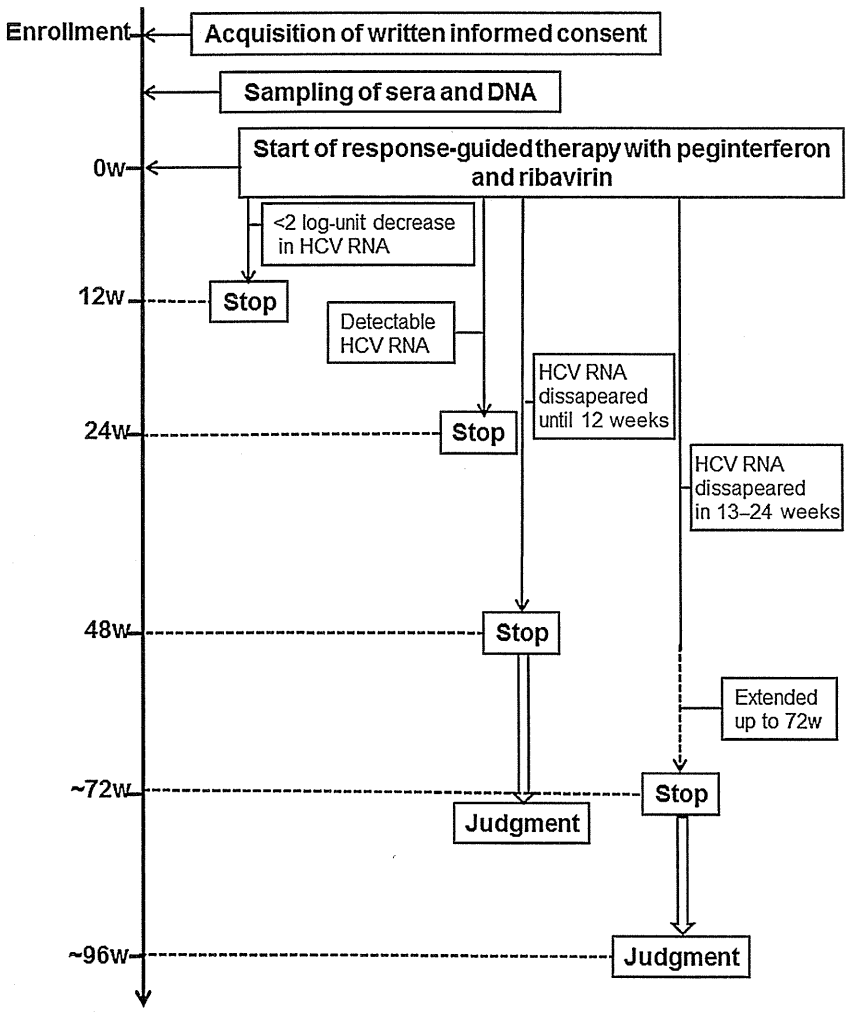


**Figure 1** Study protocol for response-guided therapy. Durations of treatments were determined depending on the virological responses to peginterferon-α and ribavirin. If the serum hepatitis C virus (HCV) RNA became undetectable within 12 weeks (w), the treatment was stopped at 48 weeks. If the serum HCV RNA disappeared between 13 to 24 weeks after treatment, the duration was extended up to 72 weeks. Both cases were considered to meet the standard of care.

GeneScan analysis may be found in the online version at the publisher's web-site as Supporting Information Table S1. The repeat number was validated by capillary sequencing as described previously.[20]

***Evaluation of liver fibrosis by a simple noninvasive index (FIB-4).*** The Fibrosis 4 (FIB-4) index that was used to evaluate liver fibrosis in each patient correlates well with hepatic fibrosis (as determined by liver biopsy) and requires only readily available clinical parameters for its determination.[28]

***Statistical Analysis.*** Quantitative variables were expressed as the mean ± standard deviation, unless otherwise specified. Categorical variables were compared using Pearson's $\chi^2$-test or Fisher's exact test. Continuous variables were compared using the Mann–Whitney *U*-test. Multivariate and simultaneous logistic regression analysis was performed to determine predictive factors for NVR, by using the variables which were found to be $P < 0.150$ by univariate analysis. In addition, a decision tree modeled these pretreatment factors to predict NVR. All *P* values were two-tailed, and $P < 0.05$ was considered statistically significant. Data analyses were performed using IBM SPSS Statistics 20 (IBM, Armonk, NY, USA).

## Results

***Treatment profiles and virological outcomes.*** In this prospective study, 215 patients infected by HCV of serotype 1 or genotype 1 were eligible. The sub-genotypes of HCV were as follows: 1a ($n = 2$), 1b ($n = 208$), 1b + 2b ($n = 1$), and indeterminate ($n = 4$). By the end of November 2012, virological outcomes had been determined in 202 patients, except for 13 patients (6%) who were lost to follow-up. Whereas all of these patients were treated with P/R, 160 patients (74%) completed standard of care (SOC) treatment for at least 48 weeks, the remaining 55 patients had to withdraw from treatment owing to either serious adverse events (SAE) in 25 patients (12%), poor response in 24 patients (11%), or other unrelated causes in 6 patients (3%). The SAE or unrelated causes responsible for the termination of the treatment were described in the small inset of Figure 2.

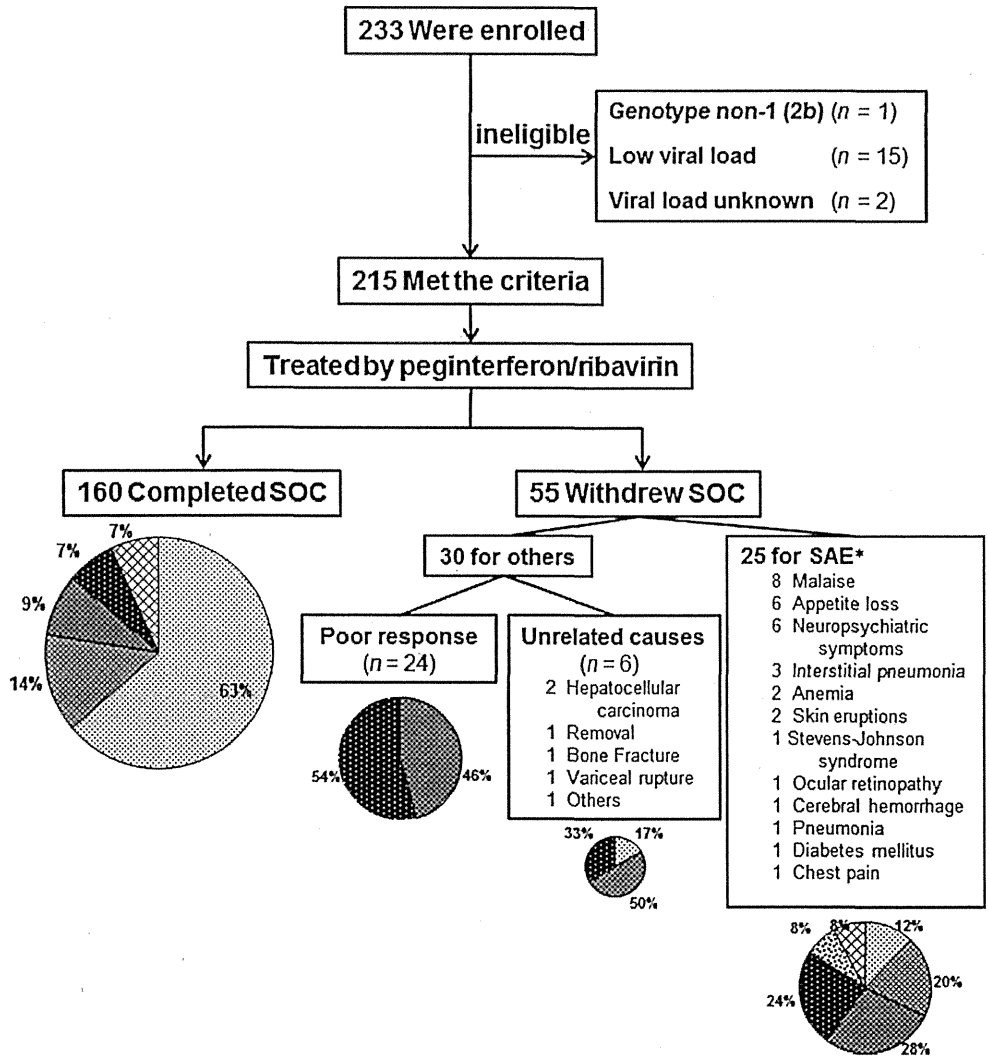


**Figure 2** Enrollment and outcomes. Among 233 patients enrolled in this study, 18 patients were ineligible for the following reasons: genotype non-1 (2b) ($n$ = 1); low viral load ($n$ = 15); and unknown viral load ($n$ = 2). Consequently, 215 patients met the entry criteria, and were treated with peginterferon and ribavirin. Among them, 160 patients completed standard of care (SOC). The remaining 55 patients were withdrawn from SOC, as detailed earlier. The virological outcomes with intent-to-treat analysis, as detailed in the Methods section, were shown as a pie chart for each group. *Serious adverse events were duplicated in some patients. RGT, response-guided therapy; SAE, serious adverse events; SVR, sustained virological response; TVR, transient virological response; NVR, non-virological response; R, responder; f/u, follow-up. ⁘, SVR; ▒, TVR; ▒, Partial R (NVR); ■, Null R (NVR); ⁘, undetemined (NVR); ◇, Lost-to-f/u.

As shown in Figure 2, if the patients completed at least 48 weeks of P/R (SOC) under conditions that observed the requirements of RGT, the SVR rate was as high as 63%, and the incidences of VR (the sum of SVR and TVR) was 77%. In the patients where the treatment was terminated for SAE or unrelated causes, the respective incidences of VR were reduced to 32% or 17%, respectively. In particular, treatment of 24 patients had to be terminated owing to the poor response to P/R, as defined by the requirements of RGT. This resulted in 11 "partial responders" (46%) and 13 "null responders" (54%). In addition, 9 cases developed viral breakthrough, and were subclassified into "partial responder": 6 of them completed SOC, while 3 stopped treatment because of the poor response to P/R (data not shown).

***Patients' characteristics and IL28B genotypes.*** Whereas 154 individuals (71.6%) had the rs8099917 TT genotype (major-homo), 60 had the TG (hetero) genotype and 1 had the GG (minor-homo) genotype. The patients were classified into two groups, TT and non-TT (TG/GG), according to their rs8099917 genotypes, and their characteristics were compared. As shown in Table 1, lower levels of γ-GTP ($P < 0.001$), higher levels

**Table 1** Comparisons of host and viral factors between *IL28B* TT and TG/GG genotypes

| Factors | TT genotype (154) | TG/GG genotype (61) | *P* value |
|---|---|---|---|
| Age (years) | 58 ± 11 (154) | 58 ± 12 (61) | 0.679 |
| Gender (male/female) | 88/66 | 31/30 | 0.448 |
| Body weight (kg) | 60.2 ± 11.2 (149) | 58.7 ± 12.5 (60) | 0.318 |
| IFN naïve/experienced | 123/31 | 47/14 | 0.711 |
| PEG-IFN-α2a/-α2b | 23/131 | 11/50 | 0.679 |
| Albumin (g/dL) | 4.1 ± 0.5 (153) | 4.1 ± 0.4 (60) | 0.721 |
| AST (U/L) | 56 ± 37 (154) | 66 ± 49 (61) | 0.332 |
| ALT (U/L) | 70 ± 52 (154) | 77 ± 59 (61) | 0.422 |
| T.Bil (mg/dL) | 0.86 ± 0.33 (150) | 0.84 ± 0.37 (61) | 0.658 |
| ALP (U/L) | 268 ± 93 (154) | 254 ± 87 (61) | 0.509 |
| γ-GTP (U/L) | 46 ± 43 (154) | 78 ± 99 (61) | **< 0.001** |
| T.Chol (mg/dL) | 172 ± 39 (151) | 160 ± 29 (58) | **0.013** |
| LDL-C (mg/dL) | 103 ± 28 (132) | 84 ± 27 (57) | **< 0.001** |
| FBS (mg/dL) | 101 ± 21 (130) | 108 ± 29 (53) | 0.076 |
| IRI (μU/mL) | 10.9 ± 9.5 (66) | 15.5 ± 20.9 (26) | 0.541 |
| AFP (ng/mL) | 14.4 ± 67.1 (141) | 17.8 ± 26.2 (57) | **0.009** |
| HCV RNA (Log IU/mL) | 6.5 ± 0.5 (154) | 6.4 ± 0.6 (61) | 0.243 |
| WBC (/μL) | 4917 ± 1367 (154) | 4940 ± 1105 (61) | 0.525 |
| Hemoglobin (g/dL) | 13.8 ± 1.3 (154) | 13.7 ± 1.7 (61) | 0.633 |
| Platelets (x10⁴/μL) | 16.5 ± 5.9 (154) | 16.9 ± 4.7 (61) | 0.347 |
| FIB-4 index | 2.82 ± 1.77 (154) | 2.80 ± 1.76 (61) | 0.994 |
| Core 70 amino acid (wild/mutant) | 109/43 | 18/41 | **< 0.001** |
| Core 91 amino acid (wild/mutant) | 102/50 | 38/21 | 0.747 |
| ISDR mutation (*n* = 0–1/2-) | 126/25 | 50/7 | 0.523 |

Data are shown as mean ± standard deviation. Figures in parentheses are the numbers of data available in each factor. Significant *P* values are shown in bold.

AFP, α-fetoprotein; ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; FBS, fasting blood sugar; HCV, hepatitis C virus; IRI, immune-reactive insulin; ISDR, interferon-sensitivity determining region; LDL-C, low-density lipoprotein cholesterol; T.Bil, total bilirubin; T.Chol, total cholesterol; WBC, white blood cell; γ-GTP, γ-glutamyl transpeptidase.

of total cholesterol (T.Chol; $P = 0.013$), higher levels of low-density lipoprotein cholesterol (LDL-C; $P < 0.001$), and lower levels of α-fetoprotein (AFP; $P = 0.009$) were significantly associated with TT genotype. The percentages of wild type of core 70 amino acid of patients with the TT genotype were significantly higher than those of patients with either TG or GG genotypes (71.7% *vs* 30.5%, $P < 0.001$).

***Virological response, IL28B genotypes, and (TA)n of rs72258881.*** Intent-to-treat analysis of the entire cohort indicated SVR, TVR, and NVR rates of 49.3%, 12.6%, and 32.1%, respectively. As shown in Figure 3a, serum HCV RNA disappeared significantly earlier in patients with TT genotype than in those with either of the TG or GG genotype. Assessment of the usefulness of the rs8099917 non-TT genotype to predict NVR among 202 patients in whom the final virological outcome could be determined indicate a sensitivity of 63.8% (44/[44 + 25]); specificity of 88.0% (117/[117 + 16]); positive predictive value of 73.3% (44/[44 + 16]); negative predictive value of 82.4% (117/[25 + 117]), and an accuracy of 79.7% ([44 + 117]/202).

As shown in Figure 3b, the (TA)n of rs72258881 varied from 11 to 18, with the most frequent numbers of repeats being 12 ($n = 147$; 68.4%). Given that more than 12 repeats were found in 67 patients (31.2%), the cohort was divided into 2 × 2 groups, according to rs8099917 genotype (TT or non-TT) and (TA)n ($n = 11$–12 or $n = 13$–18), and the incidences of VR were calculated as [SVR + TVR]/[SVR + TVR + NVR]. As shown in Figure 3c, the incidences of VR tended to be higher in the group with longer (TA)n than in the group with shorter (TA)n, regardless of the rs8099917 genotype. In particular, in patients with the non-TT genotype, the longer (TA)n might increase VR more than the twofold, relative to that for patients with a shorter (TA)n [compare 18.4% in $(TA)_{11-12}$ *vs* 40.9% in $(TA)_{13-18}$, $P = 0.074$].

***Factors associated with NVR.*** After excluding 13 patients who were lost to follow-up, we attempted to identify the variables that were associated with final virological outcome. As shown in Table 2, univariate analysis indicated that nine variables were significantly different or tended to be different between VR and NVR. NVR was associated with higher levels of serum AST, γ-GTP, and FIB-4, but with lower levels of T.Chol. Whereas the core 70-mino acid mutation was a viral factor related to NVR, the rs8099917 non-TT genotype and shorter (TA)n were host factors related to NVR. For nine variables for which univariate analysis indicated that the *P* value less than 0.15, multivariate logistic

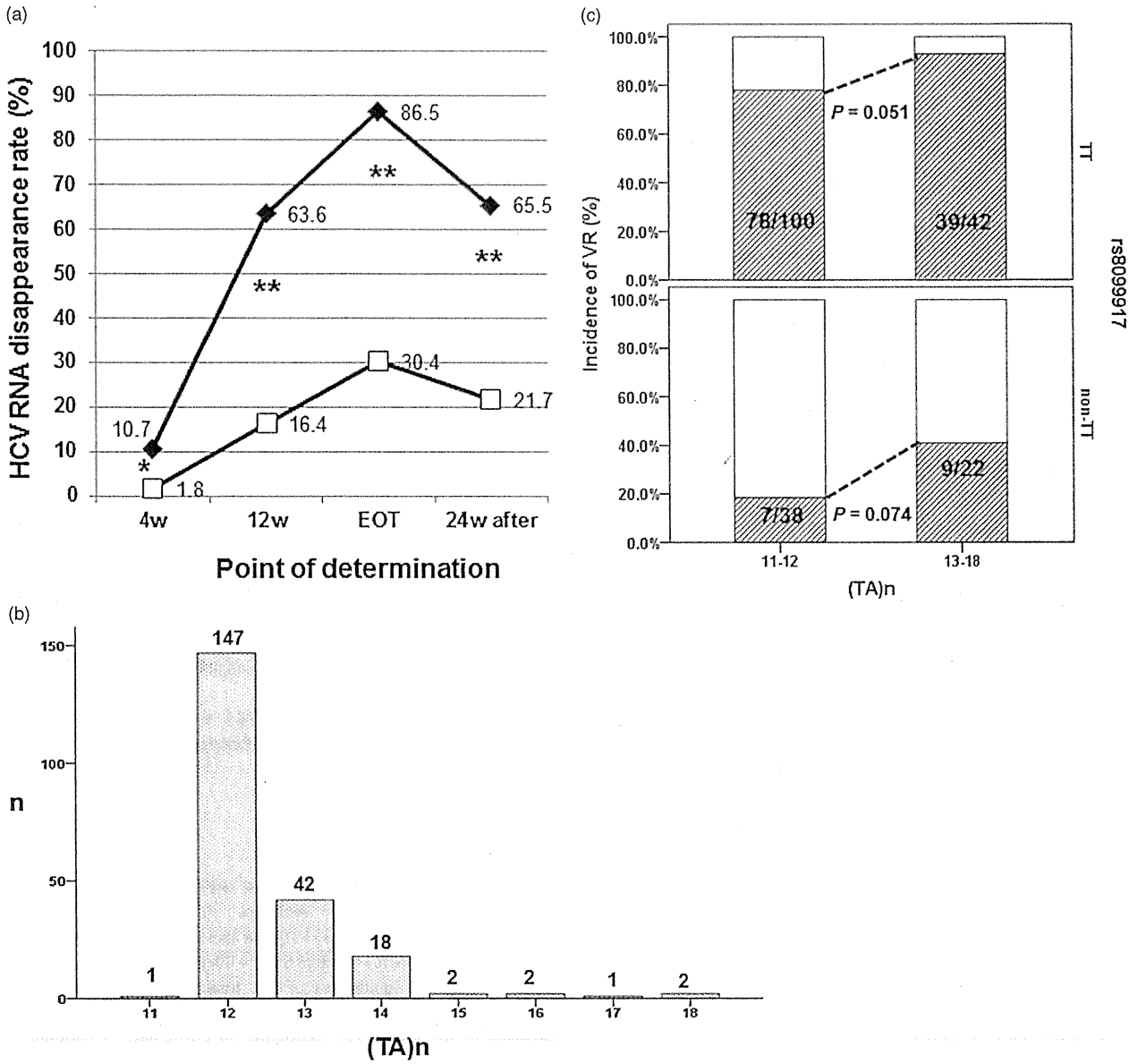


**Figure 3** Genotype of rs8099917, (TA) dinucleotide repeat [(TA)n] of rs72258881 and virological response. (a) Hepatitis C virus (HCV) RNA disappearance rate. Serum HCV RNA disappeared significantly earlier in patients with TT genotype than in those with TG/GG genotypes. $^{*}P < 0.05$, $^{**}P < 0.001$. The abbreviation used was: EOT, end of treatment; HCV, hepatitis C virus; w, weeks. —◆—, TT genotype; —□—, TG/GG genotype. (b) Distributions of (TA)n in this cohort. The most frequent (TA)n was 12 ($n$ = 147: 68.4%). In 67 patients (31.2%), the numbers were more than 12. (c) The incidences of virological response (VR) in four groups stratified by rs8099917 genotype and (TA)n. The longer (TA)n might favor virological responses to PEGylated interferon-α and ribavirin, regardless of the IL*28*B genotype.

regression analysis identified four variables for the prediction of NVR: HCV core 70 amino acid mutation, rs8099917 non-TT genotype, shorter (TA)n, and the lower levels of serum albumin. Finally, a decision tree temporarily modeled 22 pretreatment factors (see the legend to Fig. 4) to predict NVR. For this purpose, 13 patients who were lost to follow-up were excluded from the analysis, in order to avoid their influence on final decision. As shown in Figure 4, if the rs8099917 genotype was primarily selected as the predictive factor ($P < 0.001$, $\chi^2 = 57.647$), then the shorter (TA)n attributed to the NVR in patients with the TT genotype ($P = 0.023$, $\chi^2 = 5.166$). In cases with the shorter (TA)n ($n$ = 11 or 12), the presence of the core 70 amino acid mutation in HCV might significantly increase the incidences of NVR ($P = 0.008$, $\chi^2 = 9.115$). On the other hand, in the patients with non-TT genotype, higher viral load was the second most powerful determinant of NVR ($P = 0.001$, $\chi^2 = 15.645$).

**Table 2** Univariate and multivariate analyses of patients with chronic hepatitis C treated with pegylated interferon-α and ribavirin with respect to VR and NVR

| Variable | Univariate analysis | | | Multivariate analysis | | | |
|---|---|---|---|---|---|---|---|
| | VR (133) | NVR (69) | *P* value | B | *P* value | Odds ratio | 95% CI |
| Gender (Male/Female) | 74/59 | 38/31 | 1.000 | | | | |
| Age (years) | 58 ± 11 (133) | 59 ± 11 (69) | 0.973 | | | | |
| Body weight (kg) | 59.9 ± 11.5 (130) | 59.7 ± 11.6 (68) | 0.992 | | | | |
| Hx. of IFN treatment (naïve/experienced) | 108/25 | 52/17 | 0.363 | | | | |
| PEG-IFN-α2a/-α2b | 18/115 | 12/57 | 0.533 | | | | |
| Albumin (g/dL) | 4.2 ± 0.5 (133) | 4.0 ± 0.4 (67) | 0.148 | 0.118 | **0.036** | 1.125 | 1.008–1.256 |
| AST (U/L) | 52 ± 34 (133) | 70 ± 50 (69) | **0.022** | −0.001 | 0.919 | 0.999 | 0.983–1.016 |
| ALT (U/L) | 66 ± 47 (133) | 81 ± 64 (69) | 0.244 | | | | |
| T.Bil (mg/dL) | 0.88 ± 0.32 (129) | 0.81 ± 0.35 (69) | 0.085 | 0.102 | 0.083 | 1.108 | 0.987–1.243 |
| ALP (U/L) | 258 ± 80 (133) | 273 ± 109 (69) | 0.378 | | | | |
| γ-GTP (U/L) | 45 ± 43 (133) | 75 ± 95 (69) | **< 0.001** | −0.002 | 0.508 | 0.998 | 0.991–1.005 |
| T.Chol (mg/dL) | 172 ± 39 (130) | 161 ± 32 (66) | **0.016** | 0.004 | 0.556 | 1.004 | 0.991–1.017 |
| HCV RNA (Log IU/mL) | 6.5 ± 0.6 (133) | 6.6 ± 0.5 (69) | 0.384 | | | | |
| WBC (/μL) | 4982 ± 1248 (133) | 4784 ± 1271(69) | 0.275 | | | | |
| Hemoglobin (g/dL) | 13.9 ± 1.2 (133) | 13.7 ± 1.8 (69) | 0.308 | | | | |
| Platelets (× $10^4$/μL) | 17.0 ± 5.9 (133) | 16.0 ± 5.0 (69) | 0.377 | | | | |
| FIB-4 index | 2.7 ± 1.7 (133) | 3.1 ± 1.8 (69) | **0.035** | 0.055 | 0.755 | 1.056 | 0.749–1.490 |
| Core 70 amino acid (wild/mutant) | 93/37 | 23/45 | **< 0.001** | −0.914 | **0.029** | 0.401 | 0.177–0.910 |
| Core 91 amino acid (wild/mutant) | 92/38 | 42/26 | 0.205 | | | | |
| ISDR mutation (*n* = 0–1/2-) | 108/20 | 59/8 | 0.528 | | | | |
| rs8099917 (TT/non-TT) | 117/16 | 25/44 | **< 0.001** | −2.735 | **< 0.001** | 0.065 | 0.025–0.171 |
| (TA)n (*n* = 11–12/13–18) | 85/48 | 53/16 | 0.079 | −1.226 | **0.011** | 0.294 | 0.114–0.757 |
| PEG-IFN adherence (%) | 95.5 ± 10.3 (132) | 89.7 ± 17.3 (64) | 0.093 | | | | |
| Ribavirin adherence (%) | 94.8 ± 10.9 (133) | 90.3 ± 17.7 (64) | 0.232 | | | | |

Data are shown as mean ± standard deviation. Figures in parentheses are the numbers of data available in each variable. Multivariate and simultaneous logistic regression analysis was performed to determine predictive factors for NVR, by using nine variables which were found to be $P < 0.150$ by univariate analysis (albumin, AST, T.Bil, γ-GTP, T.Chol, fib-4 index, Core 70 amino acid, rs8099917, (TA)n). In addition, PEG-IFN adherence and ribavirin adherence were excluded from this analysis, since these two variables could not be available at pretreatment. The corresponding references in categorical variables were as follows: wild (Core 70 amino acid); TT (rs8099917); *n* = 13–18 [(TA)n]. Significant *P* values are shown in bold. The calculated values for serum albumin and T.Bil by multivariate logistic regression analysis correspond to those per 0.1 of increase.
ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; CI, confidence interval; HCV, hepatitis C virus; Hx., history; ISDR, interferon-sensitivity determining region; NVR, non-virological response; PEG-IFN, pegylated interferon; T.Bil, total bilirubin; T.Chol, total cholesterol; VR, virological response; WBC, white blood cell; γGTP, γ-glutamyl transpeptidase.

## Discussion

This is the first prospective evaluation of the usefulness of the rs8099917 SNP of the *IL28B* gene to predict virological outcome of RGT with P/R in the patients with chronic hepatitis C. Given that RGT has been accepted by AASLD,[2,23] European Association for the Study of the Liver (EASL),[29] and JSH[24] as the standard interferon-based treatment, it is often challenging to perform conventional per-protocol analysis in real clinical settings. With intent-to-treat analysis, the rates of SVR, TVR, and NVR were 49.3%, 12.6%, and 32.1%, respectively. The SVR rate in our cohort was as much as 10% higher than that in a report based on non-RGT with intent-to-treat analysis.[30] This might substantiate the value of RGT for the treatment of chronic hepatitis C. Differences between the TT genotype and non-TT genotype of rs8099917 in several background features are of interest. For instance, serum levels of γ-GTP and AFP were higher, while those of T.Chol and LDL-C were lower in patients with the non-TT genotype, compared with those with the TT genotype. Especially for the association of γ-GTP and LDL-C levels with *IL28B* genotypes, quite similar results were recently reported for HALT-C study[31] and our retrospective study,[32] respectively. However, the precise mechanisms by which *IL28B* genotypes affect levels of γ-GTP and LDL-C have yet to be elucidated. The current study confirmed that the core 70 amino acid mutation is more frequently associated with the non-TT genotype than with TT genotype.[33] The rs8099917 genotype could clearly differentiate between the effects of P/R on the disappearance of serum HCV RNA, which ultimately leads to SVR rate at 65.5% and 21.7%, in the TT and non-TT genotypes, respectively. However, given the less than 80% accuracy in predicting NVR in non-TT genotypes, there is likely a fair level of discrepancy between predicted and actual virological outcome. We have recently reported that the (TA) dinucleotide repeat of rs72258881, which lies in the promoter region of *IL28B* gene, can affect the transcriptional activity in a (TA)n length-dependent manner *in vitro*.[20] The current study has indicated that in clinical settings, increases in (TA)n tended to increase the incidences of VR in patients regardless of rs8099917 genotypes.

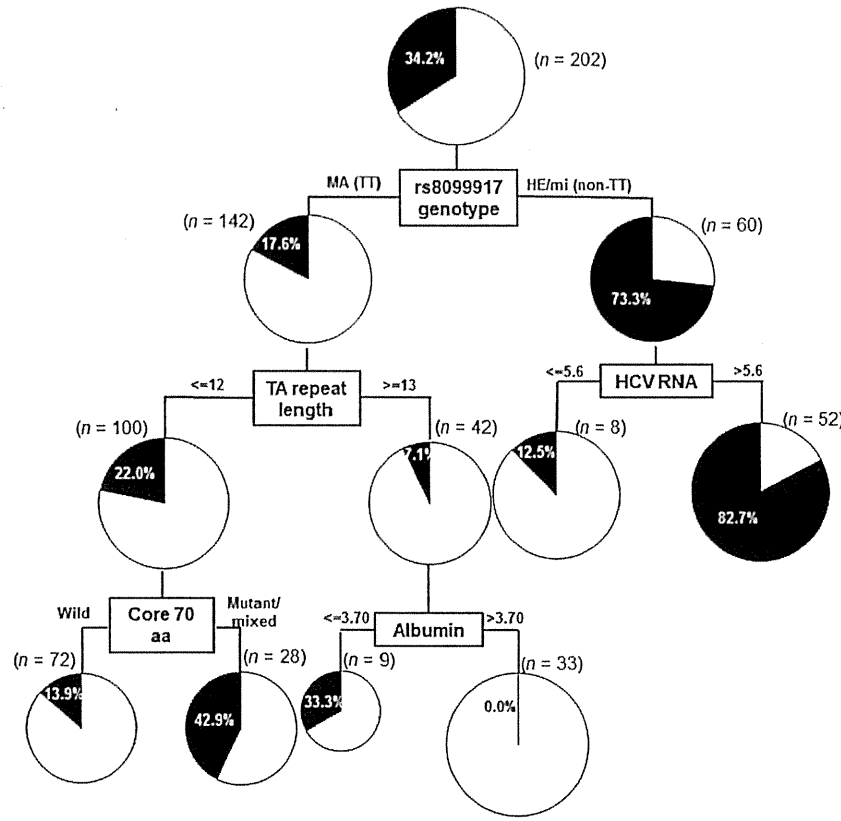


**Figure 4** Decision tree analysis for the virological outcomes. Boxes indicate the factors used for splitting and the cut-off value for the split. Pie charts indicate the rate of non-virological response for each group of patients after splitting. A total of 202 patients were included in this analysis, after excluding 13 patients who were lost to follow-up, in order to avoid the influence on final decision. Among 22 pretreatment factors (gender, prior history of interferon, pegylated interferon regimen, age, body weight; serum albumin, aspartate aminotransferase, alanine aminotransferase, total bilirubin, alkaline phosphatase, γ-glutamyl transpeptidase, total cholesterol; white blood cell, hemoglobin, platelets; FIB-4; serum levels of hepatitis C virus (HCV) RNA (reverse transcription–polymerase chain reaction), core 70/91 amino acid mutation, interferon-sensitivity determining region mutation; rs8099917 genotype, TA repeat length) tested for their abilities to predict non-virological responses, determinations of (TA)n of rs72258881 and/or the HCV core 70 amino acid substitution were useful, especially for patients with the TT genotype. In the patients with a non-TT genotype, HCV viral load was the second most important determinant of virological response. MA, major-homo; HE, hetero; mi, minor-homo; aa, amino acid. The units used to measure levels of albumin and HCV RNA were g/dL and Log IU/mL, respectively.

Although the current study did not directly assess the expression of *IL28B* gene at the mRNA or protein levels, further investigation of mechanistic basis of the length-dependent effects of (TA)n on VR might help to elucidate how the responsiveness of patients to P/R is regulated by their levels of *IL28B* expression.

Univariate analysis indicated that NVR was associated with significantly higher levels of serum AST, γ-GTP, and FIB-4, together with lower levels of T.Chol, than for VR. These findings are consistent with those of our previous retrospective study that involved per-protocol analysis.[32] Regarding the viral factor, the core 70 amino acid mutation was significantly correlated with NVR, as reported previously.[12] Multivariate logistic regression analysis revealed that rs8099917 non-TT genotype, shorter (TA)n, core 70 amino acid mutation, and the lower levels of serum albumin were independently associated with NVR, in this prospective cohort with RGT. A decision tree analysis might demonstrate more clearly the clinical implications to measure simultaneously these host and viral factors at pretreatment.

There are several limitations to this study. First, the prospective study design prompted us to evaluate the virological response by performing intent-to-treat analysis, rather than strict per-protocol analysis. Especially, in patients for whom treatment needed to be terminated prematurely owing to SAE or other unrelated causes, virological outcomes were worse than expected. Nonetheless, the results of this study appear to reflect the real clinical settings used for the treatment of chronic hepatitis C. Another limitation was that the cohort contained fewer patients with non-TT genotype of rs8099917 than those with TT genotype. This might explain the unexpected observation that decision tree analysis did not select (TA)n as a predictive factor in the group with non-TT genotype. External validation is needed to establish whether the results of our prospective study could apply more generally. However, a

requirement for such studies is that the indications of current and future direct-acting antiviral agents for chronic hepatitis C should be further clarified in clinical settings.

In conclusion, the *IL28B* genotype and (TA)n of rs72258881 may independently affect the virological outcomes of RGT with P/R for chronic hepatitis C. At a minimum, when considering P/R-based regimens for chronic hepatitis C, pretreatment determinations of both genotypes as well as the core 70 amino acid mutation of HCV are promising cost-effective tools to predict VR.

## Acknowledgments

The authors thank Dr. Yasuyuki Kohjima (NHO Kyushu Medical Center), Dr. Kyoko Fukuhara and Dr. Ken-ichi Ikejima (Juntendo University), Dr. Kunio Nakane (Akita City Hospital), Dr. Hirohito Yoneyama (Kagawa Medical University School of Medicine), Dr. Shinya Maekawa (Yamanashi University School of Medicine), Dr. Haruhisa Nakao (Aichi Medical University School of Medicine), Dr. Hiroshi Mitsui and Dr. Naoaki Hashimoto (Tokyo Teishin Hospital), Dr. Takeya Tsutsumi and Dr. Hiroshi Yotsuyanagi (The University of Tokyo), Dr. Hideaki Miura and Dr. Haruki Yamada (Social Insurance Chuo General Hospital), Dr. Keiichi Hirata and Dr. Shigeki Hayashi (NHO Disaster Medical Center), Dr. Koichi Takaguchi (Kagawa Prefectural Central Hospital), Dr. Shogo Ohkoshi and Dr. Yutaka Aoyagi (Niigata University School of Medicine), Dr. Akihisa Miyazaki (Juntendo University, Nerima Hospital), Dr. Hiroshi Adachi (Tonami General Hospital), Dr. Tsutomu Ogawa (Takamatsu Red Cross Hospital), Dr. Masaaki Korenaga, Dr. Keisuke Hino (Kawasaki Medical School Hospital), Dr. Hideyuki Nomura (SHIN-KOKURA Hospital), Dr. Mikio Yanase (National Center for Global Health and Medicine Hospital) for assistance with the collection of clinical samples.

## References

1 World Health Organization. *WHO Fact sheet N°164*, Updated April 2014. Hepatitis C. Cited 19 June 2014. Available from URL: http://www.who.int/mediacentre/factsheets/fs164/en/index.html
2 Ghany MG, Nelson DR, Strader DB, Thomas DL, Seeff LB; American Association for Study of Liver Diseases. 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**: 1433–43.
3 Poordad F, McCone J Jr, Bacon BR *et al.* Boceprevir for untreated chronic HCV genotype 1 infection. *N. Engl. J. Med.* 2011; **364**: 1195–206.
4 Bacon BR, Gordon SC, Lawitz E *et al.* Boceprevir for previously treated chronic HCV genotype 1 infection. *N. Engl. J. Med.* 2011; **364**: 1207–17.
5 Jacobson IM, McHutchison JG, Dusheiko G *et al.* Telaprevir for previously untreated chronic hepatitis C virus infection. *N. Engl. J. Med.* 2011; **364**: 2405–16.
6 McHutchison JG, Manns MP, Muir AJ *et al.* Telaprevir for previously treated chronic HCV infection. *N. Engl. J. Med.* 2010; **362**: 1292–303.
7 Chayama K, Takahashi S, Toyota J *et al.* Dual therapy with the nonstructural protein 5A inhibitor, daclatasvir, and the nonstructural protein 3 protease inhibitor, asunaprevir, in hepatitis C virus genotype 1b-infected null responders. *Hepatology* 2012; **55**: 742–8.
8 Gane EJ, Stedman CA, Hyland RH *et al.* Nucleotide polymerase inhibitor sofosbuvir plus ribavirin for hepatitis C. *N. Engl. J. Med.* 2013; **368**: 34–44.
9 Poordad F, Lawitz E, Kowdley KV *et al.* Exploratory study of oral combination antiviral therapy for hepatitis C. *N. Engl. J. Med.* 2013; **368**: 45–53.
10 Guedj H, Guedj J, Negro F *et al.* The impact of fibrosis and steatosis on early viral kinetics in HCV genotype 1-infected patients treated with Peg-IFN-alfa-2a and ribavirin. *J. Viral Hepat.* 2012; **19**: 488–96.
11 Harrison SA, Hamzeh FM, Han J, Pandya PK, Sheikh MY, Vierling JM. Chronic hepatitis C genotype 1 patients with insulin resistance treated with pioglitazone and peginterferon alpha-2a plus ribavirin. *Hepatology* 2012; **56**: 464–73.
12 Akuta N, Suzuki F, Sezaki H *et al.* Association of amino acid substitution pattern in core protein of hepatitis C virus genotype 1b high viral load and non-virological response to interferon-ribavirin combination therapy. *Intervirology* 2005; **48**: 372–80.
13 Enomoto N, Sakuma I, Asahina Y *et al.* Mutations in the nonstructural protein 5A gene and response to interferon in patients with chronic hepatitis C virus 1b infection. *N. Engl. J. Med.* 1996; **334**: 77–81.
14 Manns MP. Adherence to combination therapy: influence on sustained virologic response and economic impact. *Gastroenterol. Clin. North Am.* 2004; **33**: 11–24.
15 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**: 1105–9.
16 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**: 1100–4.
17 Thomas DL, Thio CL, Martin MP *et al.* Genetic variation in IL28B and spontaneous clearance of hepatitis C virus. *Nature* 2009; **461**: 798–801.
18 Ge D, Fellay J, Thompson AJ *et al.* Genetic variation in IL28B predicts hepatitis C treatment-induced viral clearance. *Nature* 2009; **461**: 399–401.
19 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**: 1338–45.
20 Sugiyama M, Tanaka Y, Wakita T, Nakanishi M, Mizokami M. Genetic variation of the IL-28B promoter affecting gene expression. *PLoS ONE* 2011; **6**: e26620.
21 Tsukiyama-Kohara K, Yamaguchi K, Maki N *et al.* Antigenicities of group I and II hepatitis C virus polypeptides—molecular basis of diagnosis. *Virology* 1993; **192**: 430–7.
22 Ohno T, Mizokami M, Wu RR *et al.* New hepatitis C virus (HCV) genotyping system that allows for identification of HCV genotypes 1a, 1b, 2a, 2b, 3a, 3b, 4, 5a, and 6a. *J. Clin. Microbiol.* 1997; **35**: 201–7.
23 Ghany MG, Strader DB, Thomas DL, Seeff LB, American Association for the Study of Liver Diseases. Diagnosis, management, and treatment of hepatitis C: an update. *Hepatology* 2009; **49**: 1335–74.
24 Izumi N, Nishiguchi S, Hino K *et al.* Management of hepatitis C; Report of the Consensus Meeting at the 45th Annual Meeting of the Japan Society of Hepatology (2009). *Hepatol. Res.* 2010; **40**: 347–68.
25 Ito K, Higami K, Masaki N *et al.* The rs8099917 polymorphism, determined by a suitable genotyping method, is a better predictor for response to pegylated interferon-alpha/ribavirin therapy in Japanese patients than other SNPs associated with IL28B. *J. Clin. Microbiol.* 2011; **49**: 1853–60.

26 Lyamichev VI, Kaiser MW, Lyamicheva NE *et al.* Experimental and theoretical analysis of the invasive signal amplification reaction. *Biochemistry* 2000; **39**: 9523–32.
27 Hall JG, Eis PS, Law SM *et al.* Sensitive detection of DNA polymorphisms by the serial invasive signal amplification reaction. *Proc. Natl. Acad. Sci. U.S.A.* 2000; **97**: 8272–7.
28 Sterling RK, Lissen E, Clumeck N *et al.* Development of a simple noninvasive index to predict significant fibrosis in patients with HIV/HCV coinfection. *Hepatology* 2006; **43**: 1317–25.
29 European Association for the Study of the Liver. EASL Clinical Practice Guidelines: management of hepatitis C virus infection. *J. Hepatol.* 2011; **55**: 245–64.
30 McHutchison JG, Lawitz EJ, Shiffman ML *et al.* Peginterferon alfa-2b or alfa-2a with ribavirin for treatment of hepatitis C infection. *N. Engl. J. Med.* 2009; **361**: 580–93.
31 Everhart JE, Wright EC. Association of γ-glutamyltransferase (GGT) activity with treatment and clinical outcomes in chronic hepatitis C (HCV). *Hepatology* 2013; **57**: 1725–33.
32 Saito H, Ito K, Sugiyama M *et al.* Factors responsible for the discrepancy between IL28B polymorphism prediction and the viral response to peginterferon plus ribavirin therapy in Japanese chronic hepatitis C patients. *Hepatol. Res.* 2012; **42**: 958–65.
33 Akuta N, Suzuki F, Hirakawa M *et al.* Amino acid substitution in hepatitis C virus core region and genetic variation near the interleukin 28B gene predict viral response to telaprevir with peginterferon and ribavirin. *Hepatology* 2010; **52**: 421–9.

## Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

**Table S1** Methods of TA repeat genotyping.

Journal of Medical Virology 86:1130–1133 (2014)

# The Interaction of a Single-Nucleotide Polymorphism With Age on Response to Interferon-α and Ribavirin Therapy in Female Patients With Hepatitis C Infection

**Jo Nishino,[1] Masaya Sugiyama,[2] Nao Nishida,[2] Katsushi Tokunaga,[3] Masashi Mizokami,[2] and Shuhei Mano[4]***

[1]*Center for Information Biology, National Institute of Genetics, Mishima, Japan*
[2]*The Research Center for Hepatitis and Immunology, National Center for Global Health and Medicine, Ichikawa, Japan*
[3]*Department of Human Genetics, School of Medicine, The University of Tokyo, Tokyo, Japan*
[4]*The Institute of Statistical Mathematics, Tachikawa, Japan*

Older female patients exhibit a poor response to the current standard treatment for hepatitis C, interferon-α, and ribavirin (PEG-IFN-α/RBV). In this study, we reported that the combination of age and the genotype of a novel SNP can predict response to standard treatment ($P = 7.31 \times 10^{-8}$). The model incorporating genotype of the novel SNP, rs1287948, predicts response more accurately (AUC = 0.934; 95% CI = 0.881–0.988) in women as compared with the model using age and the previously identified SNP, rs8099917. ***J. Med. Virol. 86:1130–1133, 2014.*** © 2014 Wiley Periodicals, Inc.

**KEY WORDS:** hepatitis C; SNP; prediction; response to treatment; interaction with age

## INTRODUCTION

In late 2009, genome-wide association studies (GWAS) were used to identify two single-nucleotide polymorphisms (SNPs) located near the *IL28B* (*IFNλ3*) gene, rs12979860, and rs8099917, strongly associated with the response to pegylated-interferon-α plus ribavirin (PEG-IFN-α/RBV) therapy for hepatitis C; the identity of the SNPs and their association with treatment response has been independently verified by three separate groups [Ge et al., 2009; Suppiah et al., 2009; Tanaka et al., 2009]. The SNP genotypes are recognized as important pretreatment predictors of the response to PEG-IFN-α/RBV therapy, as described in the present guidelines for the management of hepatitis C [Ghany et al., 2011; Editors of the Drafting Committee for Hepatitis Management Guidelines: The Japan Society of Hepatology, 2013]. Since the identification of these SNPs, the molecular mechanism by which the *IL28B* (*IFNλ3*) gene influences the difference in response to PEG-IFN-α/RBV treatment has been investigated but has not yet been clarified. Recently a new dinucleotide variant near the *IL28B* gene ss469415590 (TT or ΔG), which is strongly linked to rs12979860 and rs8099917, has been discovered [Prokunina-Olsson et al., 2013]. Interestingly, the ΔG allele creates a novel interferon gene, *IFNL4*, and is thought to reduce responses to type I (including IFN- α) and type III interferons [Prokunina-Olsson et al., 2013]. Meanwhile, it is known that the response to PEG-IFN-α/RBV in older women is poor [Sezaki et al., 2009]. A genetic signature associated with the poor responder phenotype might exist and may be correlated with age. In this paper, we report that the result of a genome-wide interaction analysis between SNP status and age in female patients treated with PEG-IFN-α/RBV therapy. These results will lead to further improvement in prediction accuracy when assessing the probable response to Hepatitis C treatment and may also lead to further understanding of

Grant sponsor: Health Labor Sciences research grant from the Ministry of Health Labor and Welfare (H23 jituyouka(nanbyou)-006 and H23-kannen-005); Grant sponsor: National Center for Global Health and Medicine (H22-302)

Conflict of interest: none.

*Correspondence to: Shuhei Mano, Ph.D., The Institute of Statistical Mathematics, 10-3 Midori-cho, Tachikawa, Tokyo 190-8562, Japan.
E-mail: smano@ism.ac.jp

Accepted 7 March 2014

DOI 10.1002/jmv.23939
Published online 1 April 2014 in Wiley Online Library (wileyonlinelibrary.com).

the molecular mechanism by which treatment response in altered older women.

## MATERIALS AND METHODS

### Study Patients

In a previous study conducted by our group, genotypes of 142 Japanese patients (67 male and 75 female) treated with PEG-IFN-α/RBV therapy were used in a GWAS [Tanaka et al., 2009]. The genotypes were derived from an Affymetrix SNP 6.0 array for 900,000 SNPs. The present study uses the same SNP data and the corresponding demographic information from the 75 women who were members of the original group of 142 patients. Of the 75 female patients, 29 patients exhibited a virologic response (VR) while 46 patients exhibited a null virologic response (NVR). A total of 672,883 SNPs on autosomal or X chromosomes met our criteria: SNP call rate ≥95%, minor allele frequency ≥1%, and a Hardy–Weinberg equilibrium $P$-value ≥0.001 for all 75 female patients; the identified SNPs were used for the subsequent analyses. As stated previously [Tanaka et al., 2009], informed consent was obtained from all patients and the study protocol conforms to the relevant ethical guidelines as reflected in a priori approval by the ethics committees of all the participating universities and hospitals.

### Genome-Wide Interaction Analysis

In order to determine whether there was an effect modifier of relation between age and the response to PEG-IFN-α/RBV, a genome-wide interaction analysis (i.e., 672,883 SNP—age interaction tests) was performed using a logistic regression model. The genotype of each SNP was regarded as the number of alleles in each individual (i.e., 0, 1, or 2). The likelihood ratio test, which uses the deviance reduction due to including the SNP—age interaction as the $\chi^2$ test statistics, was employed.

### Detailed Analyses for Detected Interaction

After the genome-wide interaction analysis, detailed analyses including a newly detected SNP, rs12879468, and/or age (see Model in Table I) were conducted using the logistic regression with the $\chi^2$—based likelihood test as described above.

To assess the improvement of the predictive performance by adding the rs1287948 SNP—age interaction to the model having age and rs1287948 as main effects (see Model 3 and 4 in Table I), receiver operator characteristic (ROC) curves based on the predicted probabilities and corresponding area under the curves (AUC) were used. The confidence intervals for each AUC and the $P$-value for the difference between the two ACUs were obtained using DeLong's method [DeLong et al., 1988].

It was also investigated whether prediction accuracy could be improved by adding the novel rs1287948 SNP to the model, which includes only age and the preexisting SNP, rs8099917 that was identified in previous studies [Suppiah et al., 2009; Tanaka et al., 2009]. That is, the two models: Logit = ext.Geno + Age (the existing model) versus Logit = ext.Geno + Age + Geno + Age × Geno, where Geno is the number of G alleles for rs1287948 and ext.Geno is the number of T alleles for rs8099917 in individuals, were compared with each other. For the analysis, ROC, and AUC were used and the confidence interval of each AUC and the $P$-value for the difference between two AUCs were obtained using DeLong's method [DeLong et al., 1988].

## RESULTS

Using genome-wide interaction analysis, we was found that the rs1287948 SNP located on chromosome 1 exhibited a strong interaction with age ($P = 7.31 \times 10^{-8}$) regarding the response to PEG-IFN-α/RBV and reached genome-wide significance with a Bonferroni-corrected significance level of $P = 7.43 \times 10^{-8}$ (=0.05/672,883). No other SNP showed significant evidence of interaction. Furthermore, there was no interaction of rs1287948 SNP with age in 67 male patients ($P = 0.48$).

Table I presents the results of fitting a series of logistic regression models involving genotypes of rs1287948 and/or age. Note that rs1287948 could be found only when the interaction term (Age × Geno) was considered (see the $P$-values in the rows of Model 1, 3, and 4). Figure 1 shows the probability of the predicted response and the observed response to PEG-IFN-α/RBV therapy according to rs1287948 genotype. A clear SNP—age interaction for rs1287948 (i.e., the reverse effect of age in the two different genotypes, CG and GG, of rs1287948) can be seen.

TABLE I. Summary of Models Assessing the Effect of rs1287948 on PEG-IFN-α/RBV Therapy

| | Model Logit (response) | Deviance | Model compared | Likelihood ratio | $P$-value |
|---|---|---|---|---|---|
| | Const. | 100.085 | | | |
| 1: | Geno | 99.719 | vs. Const. | 0.366 | 0.545 |
| 2: | Age | 92.854 | vs. Const. | 7.231 | $7.17 \times 10^{-3}$ |
| 3: | Geno + age | 92.070 | vs. Model 2 | 0.784 | 0.376 |
| 4: | Geno + age + age × geno | 63.088 | vs. Model 3 | 28.982 | $7.31 \times 10^{-8}$ |

Geno, number of G allele for rs1287948 in individuals (0:GG,1:GC). Note that genotype CC does not exist in the sample.

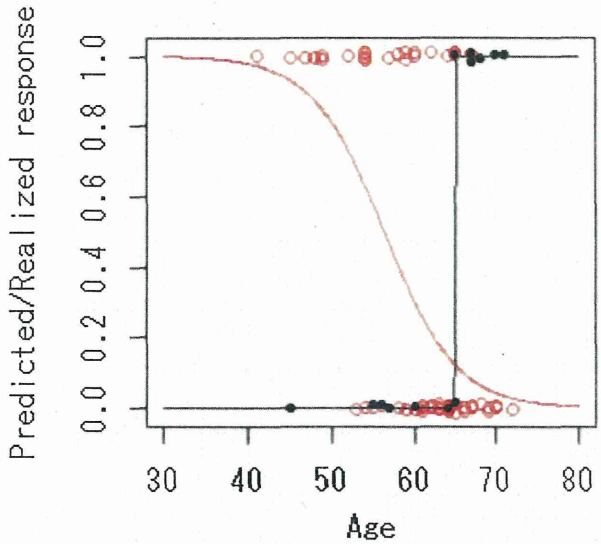


Fig. 1. Predicted or observed response according to genotype of rs1287948. Lines show the probability of the predicted response as calculated using logistic models stratified by the genotypes. Circles and dots show observed response (vertically jittered for clarity). Red: rs1287948 genotype GG. Black: rs1287948 genotype CG. Note that genotype CC does not exist in the sample.

Figure 2 shows the AUCs for Model 3 and Model 4 in Table I. For the no-interaction model (Model 3), the AUC is 0.704 (95% confidence interval, CI = 0.586–0.822). Adding the interaction term (Model 4), the AUC was improved to 0.867 (95% CI, 0.784–0.949). The *P*-value for the difference between the two AUCs was $P = 0.000826$. Thus, the rs1287948 SNP—age interaction is considered to be an effective predictor of response to PEG-IFN-α/RBV therapy.

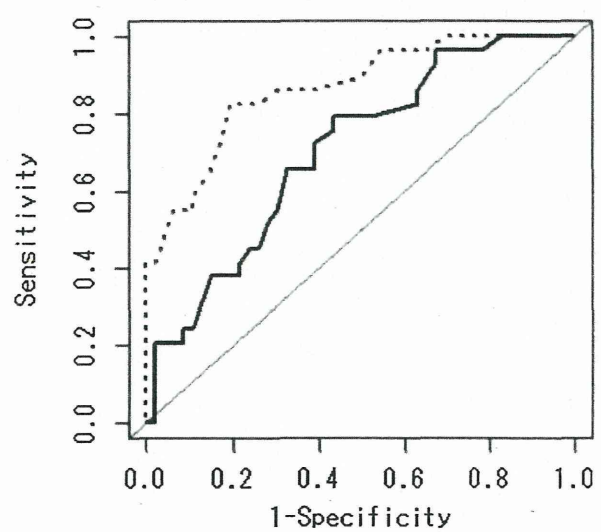


Fig. 2. Prediction improvement using the rs1287948 SNP—age interaction. The ROC curves for the prediction of response to PEG-IFN-α/RBV therapy for the models with (dashed line) and without (solid line) the genotype of rs1287948-age interaction.

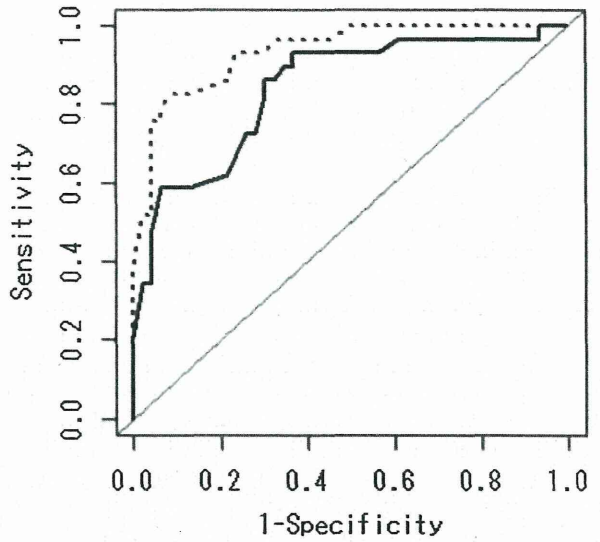


Fig. 3. Comparison of models with or without including rs1287948. The ROC curves for the prediction of response to PEG-IFN-α/RBV therapy for the models with (dash line) and without (solid line) the rs1287948.

Figure 3 shows the improvement of the predictive performance by adding the rs1287948 SNP into the existing model including only age and the preexisting SNP, rs8099917 (for more details, see Material and Methods section). For the existing model, the AUC was 0.839 (95% CI, 0.744–0.933). After including the novel rs1287948 SNP, the AUC improved to 0.934 (95% CI, 0.881–0.988). The *P*-value for the difference between the two AUCs is $P = 0.0167$. Therefore, it was concluded that adding the rs8099917 and its interaction term with age improve the prediction accuracy compared with the existing model.

The rs1287948 SNP is located in a non-coding region of chromosome 1 and is 108 kbp downstream from the *PARP1* gene. PARP1 is important for repairing single-strand breaks and, as such, PARP1 inhibitors are currently being investigated as potential anti-cancer agents in patients carrying BRCA mutations [Rouleau et al., 2010]. Therefore, the rs1287948 SNP—age interaction involvement in the response to PEG-IFN-α/RBV therapy could be related to the function of PARP1.

## DISCUSSION

In this paper, the impact of the rs1287948 SNP—age interaction on the response to PEG-IFN-α/RBV therapy was investigated. Although the importance of the analysis of gene–gene (SNP-SNP) interaction is recognized, there are few practical or successful GWASs [Cordell, 2010; Wu et al., 2009; Bansal et al.,

2010]. Statistically detecting the interaction between SNPs is difficult because there are a large number of combinations of SNPs. Therefore it is more practical to first identify those SNPs that show a strong marginal effect before testing the interactions between them. Using such methods, SNPs with interactive effects but without marginal effects are missed. Conversely, studies of the interactions between SNPs and demographic or clinical characteristics are easy to conduct; the exhaustive test of all characteristic values and SNPs interactions can be done without first identifying an SNP with a strong marginal effect; this is a merit of the analysis. In fact, the rs1287948 SNP was identified using tests for the SNP—age interaction and could not be found by its marginal effect (see Table I). In addition, GWAS is becoming an useful tool in pharmacogenomics and personalized medicine [Madian et al., 2012]. In GWAS for pharmacogenomics, testing interactions between SNPs and demographic or clinical characteristics can and should be done, as the information gained leads to more effective personalized medicine.

## ACKNOWLEDGMENTS

The authors thank Yasuhito Tanaka and Takayuki Yamada for comments on the early stages of this work.

## REFERENCES

Bansal V, Libiger O, Torkamani A, Schork NJ. 2010. Statistical analysis strategies for association studies involving rare variants. Nat Rev Genet 11:773-L 785.

Cordell HJ. 2009. Detecting gene–gene interactions that underlie human diseases. Nat Rev Genet 10:392–404.

DeLong ER. DeLong DM, Clarke-Pearson DL. 1988. Comparing the areas under two or more correlated receiver operating characteristic curves: A nonparametric approach. Biometrics 44:837–845.

Editors of the Drafting Committee for Hepatitis Management Guidelines: The Japan Society of Hepatology. 2013. Guidelines for the Management of Hepatitis C Virus Infection: May 2012, The Japan Society of Hepatology. Hepatol Res 43:1–34.

Ge D, Fellay J, Thompson AJ, Simon JS, Shianna KV, Urban TJ, Heinzen EL, Qiu P, Bertelsen AH, Muir AJ, Sulkowski M, McHutchison JG, Goldstein DB. 2009. Genetic variation in IL28B predicts hepatitis C treatment-induced viral clearance. Nature 461:399–401.

Ghany MG, Nelson DR, Strader DB, Thomas DL, Seeff LB. 2011. 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 54:1433–1444.

Madian AG, Wheeler HE, Jones RB, Dolan ME. 2012. Relating human genetic variation to variation in drug responses. Trends Genet 28:487–495.

Prokunina-Olsson L, Muchmore B, Tang W, Pfeiffer RM, Park H, Dickensheets H, Hergott D, Porter-Gill P, Mumy A, Kohaar I, Chen S, Brand N, Tarway M, Liu L, Sheikh F, Astemborski J, Bonkovsky HL, Edlin BR, Howell CD, Morgan TR, Thomas DL, Rehermann B, Donnelly RP, O'Brien TR. 2013. A variant upstream of IFNL3 (IL28B) creating a new interferon gene IFNL4 is associated with impaired clearance of hepatitis C virus. Nat Genet 45:164–171.

Rouleau M, Patel A, Hendzel MJ, Kaufmann SH, Poirier GG. 2010. PARP inhibition: PARP1 and beyond. Nat Rev Cancer 10:293–301.

Sezaki H, Suzuki F, Kawamura Y, Yatsuji H, Hosaka T, Akuta N, Kobayashi M, Suzuki Y, Saitoh S, Arase Y, Ikeda K, Miyakawa Y, Kumada H. 2009. Poor response to pegylated interferon and ribavirin in older women infected with hepatitis C virus of genotype 1b in high viral loads. Dig Dis Sci 54:1317–1324.

Suppiah V, Moldovan M, Ahlenstiel G, Berg T, Weltman M, Abate ML, Bassendine M, Spengler U, Dore GJ, Powell E, Riordan S, Sheridan D, Smedile A, Fragomeli V, Müller T, Bahlo M, Stewart GJ, Booth DR. George J for the Hepatitis C Study. 2009. IL28B is associated with response to chronic hepatitis C interferon-α and ribavirin therapy. Nat Genet 41:1100–1104.

Tanaka Y, Nishida N, Sugiyama M, Kurosaki M, Matsuura K, Sakamoto N, Nakagawa M, Korenaga M, Hino K, Hige S, Ito Y, Mita E, Tanaka E, Mochida S, Murawaki Y, Honda M, Sakai A, Hiasa Y, Nishiguchi S, Koike A, Sakaida I, Imamura M, Ito K, Yano K, Masaki N, Sugauchi F, Izumi N, Tokunaga K, Mizokami M. 2009. Genome-wide association of IL28B with response to pegylated interferon-α and ribavirin therapy for chronic hepatitis C. Nat Genet 41:1105–1109.

Wu TT, Chen YF, Hastie T, Sobel E, Lange K. 2009. Genome-wide association analysis by lasso penalized logistic regression. Bioinformatics 25:714–721.

OPEN ACCESS Freely available online

PLOS ONE

# IL28B Polymorphisms and Clinical Implications for Hepatitis C Virus Infection in Uzbekistan

**Dinara Khudayberganova[1,2], Masaya Sugiyama[1]*, Naohiko Masaki[1], Nao Nishida[1], Motokazu Mukaide[1], Dildora Sekler[2], Renat Latipov[2], Kan Nataliya[2], Suyarkulova Dildora[2], Said Sharapov[2], Guzal Usmanova[2], Mahmarajab Raxmanov[2], Erkin Musabaev[2], Masashi Mizokami[1]**

**1** Department of Hepatic Diseases, The Research Center for Hepatitis and Immunology, National Center for Global Health and Medicine, Ichikawa, Japan, **2** Institute of Virology, Ministry of Public Health of Uzbekistan, Tashkent, Uzbekistan

## Abstract

***Aims:*** Genome-wide association studies highlighted single nucleotide polymorphisms (SNPs) within the IFNL3/IL28B locus predict the treatment outcome for patients with HCV. Furthermore, SNPs in newly discovered IFNL4 are shown to have population-specific correlation with spontaneous clearance of HCV. The aim of this study was to examine the prevalence and clinical significance of the outlined SNPs in a population from Central Asia, a multi-ethnic region with a developing economy and a high prevalence of HCV infection.

***Methods:*** One hundred and thirty-five chronic HCV patients from Uzbekistan were enrolled. DNA specimens were extracted from peripheral blood mononuclear cells and the IFNL3 SNPs (rs8099917, rs12979860) were genotyped by the Invader Plus assay, the TaqMan assay, and by direct sequence analysis. The IFL4 region (ss469415590) was sequenced.

***Results:*** Of the 135 patients that completed 24 or 48 weeks of treatment with Peg-IFN-α plus RBV, 87.4% were of Central Asian (CA) ancestry and 12.6% were of Eastern European (EE) ancestry. A non-virological response was observed in 21.2% of CA and in 35.3% of EE, respectively ($p<0.32$). The rs12979860 was strongly associated with treatment response (OR, 5.2; 95% CI, 1.9–14.6; $p<0.004$) in the overall sample; however, SNP rs8099917 was the most predictive of outcome for CA group (OR, 6.9; 95% CI, 2.6–18.0; $p<0.002$). The allele frequency of IFNL4 SNP, ss469415590, was identical with that of rs12979860 in all samples.

***Conclusions:*** SNPs in IFNL3 and IFNL4 can be used to predict HCV treatment outcome in a population of Central Asian ancestry.

**Citation:** Khudayberganova D, Sugiyama M, Masaki N, Nishida N, Mukaide M, et al. (2014) IL28B Polymorphisms and Clinical Implications for Hepatitis C Virus Infection in Uzbekistan. PLoS ONE 9(3): e93011. doi:10.1371/journal.pone.0093011

**Editor:** Wenyu Lin, Harvard Medical School, United States of America

**Received** January 3, 2014; **Accepted** February 27, 2014; **Published** March 24, 2014



**Funding:** Grant-in-Aid for Scientific Research (B) from Japan Society for the Promotion of Science (JSPS) (23390117). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

**Competing Interests:** The authors have declared that no competing interests exist.

* E-mail: m.sugiyama@hospk.ncgm.go.jp

## Introduction

Chronic Hepatitis C virus (HCV) infection is a global healthcare problem, with thestimated number of people positive for anti-hepatitis C virus antibodies increasing from >122 million to >185 million between 1990 and 2005 [1]. Central and Eastern Asia, North Africa, and the Middle East are thought to have the highest prevalence of anti-HCV antibodies (>3.5%) [1]. Although successful implementation of direct-acting antiviral therapy was recently reported in Western countries, combined treatment with pegylated interferon-alpha (PEG-IFN-α) plus ribavirin (RBV) is still the most effective treatment for patients with chronic hepatitis C in central Asia [2,3]. However, this treatment is both costly and associated with significant adverse side effects, resulting in poor compliance. Furthermore, approximately half of treated patients fail to achieve a sustained virological response (SVR). Of the various host (age, sex, race, fibrosis stage) and viral (genotype, viral load) factors (reviewed in ref 2, 4) associated with the effectiveness of IFN-based therapy, the recently discovered genetic polymorphisms (SNPs) of *interleukin 28B (IL28B)* has been reported. The SNPs had the most significant predictive value for treatment outcomes in several countries [4–6]. The polymorphism in *IL28B* forms a cluster of single nucleotide polymorphisms (SNPs) that appear to delineate a genetic haplotype within a very low recombination fragment containing the *IL28B* gene. Among all the SNPs within this cluster, rs12979860 and rs8099917 are the strongest markers of the haplotype, and consistently predict treatment outcomes for patients receiving IFN-based regimens [7–10]. Recently, a study examining a cohort of African Americans identified a novel *interferon lambda 4 (IFNL4)* gene located in an immediate proximity to the *IL28B*, and suggested that it was associated with HCV clearance [11]. The IFNL4 SNP improved the prediction rate of IFN-based regimens in African Americans, and more recently in Caucasians and Japanese [12–20].

**Table 1.** Summary of results of genotyping by three different methods.

| Total n = 135 | | No.(%) of cases with genotype by: | | | |
|---|---|---|---|---|---|
| SNP | Genotype | Direct sequencing | Invader | TaqMan | Concordance |
| rs12979860 | CC | 57 | 57 | 57 | |
| | CT | 64 | 64 | 64 | 1 |
| | TT | 14 | 14 | 14 | |
| rs8099917 | TT | 90 | 89 | 89 | |
| | TG | 40 | 40 | 40 | 0.992 |
| | GG | 5 | 6 | 6 | |

doi:10.1371/journal.pone.0093011.t001

Uzbekistan is one of the most populous countries in Central Asia. The HCV infection prevalence in the general population is very high, at >6.4%, and is >20% in "high-risk" groups [21]. The most prevalent HCV genotypes are HCV-1b followed by 3a [22,23]. Because the population of Uzbekistan comprises individuals from many different genetic backgrounds, the aim of this study was to examine the prevalence and clinical relevance of IL28B and IFNL4 polymorphisms in the context of the ethnic ancestry background of populations in this country.

## Methods

### Study population

Outpatients with chronic HCV infection treated with PEG-IFN-α plus RBV at the Institute of Virology Ministry of Public Health of Uzbekistan between May, 2009 and December, 2011 were enrolled in the study. The study protocol was approved by the Institutional Review Board and Institute of Virology Ministry of Public Health of Uzbekistan. Written informed consent was obtained from all patients. This study conforms to the provisions of the Declaration of Helsinki (as revised in Seoul, Korea, October 2008). The patients and their physicians completed a written questionnaire, which was used to collect socioeconomic, demographic, clinical, and laboratory data. The data were then subjected to a per-protocol analysis. The diagnosis of HCV infection was based on the detection of anti-HCV antibodies. The viral load was determined using the AmpliSens HCV-Monitor-FL (InterLabService Ltd., Moscow) Real-Time PCR kit, which has a detection range limits of 300–$10^8$ IU/mL (equivalent to $1\times10^3$–$3\times10^8$, HCV RNA copies/mL).

Patients consisting of 135 subjects received the full treatment course (see below). Data derived from the patients that received at least 80% of the prescribed drug dose were used for the outcome association study. Patients with end-stage kidney disease, hepatocellular carcinoma, or decompensated liver cirrhosis (as defined by a Child-Pugh score greater than 6) were excluded. The ethnic background of each individual was assessed according to the patient questionnaire; patients of Uzbek, Kyrgyz, Kazakh and Tajik ethnicities were included into the Central Asian ancestry (CA) group, patients of Russian and Tatar ethnicities were included into the Eastern Europe (EE) group Other ethnic minorities were excluded from the study.

**Table 2.** Summary of population completed antiviral treatment for chronic HCV.

| | Central Asian | | | East European | | | Overall | | |
|---|---|---|---|---|---|---|---|---|---|
| | VR | NVR | p | VR | NVR | p | VR | NVR | p |
| n. | 93 | 25 | | 11 | 6 | | 104 | 31 | |
| Age (mean years old ± SE) | 39.7±1.5 | 39.7±3.2 | 0.437 | 45.2±3.6 | 39.3±5 | 0.358 | 40.6±1.3 | 37.9±2.9 | 0.346 |
| Baseline HCV viral load (mean ×106±SE) | 3.1±8.9 | 4.2±2.3 | 0.609 | 1.6±6 | 1.2±5 | 0.663 | 2.9±6.7 | 3.4±1.7 | 0.727 |
| HCV genotype (1/non-1) | 77/16 | 22/3 | 0.759 | 11 | 6 | | 88/16 | 28/3 | 0.673 |
| Treatment duration (mean months±SE) | 7.6±0.2 | 8±0.5 | | 8.3±0.8 | 6.0±1.2 | | 7.7±0.5 | 7.6±0.8 | |
| Drug configuration (IFN/Peg IFN) | 38/55 | 14/11 | 0.176 | 7/4 | 5/1 | 0.6 | 59/45 | 12/19 | 0.101 |
| IL28B (rs8099917) | | | | | | | | | |
| MA: n(%) | 71(76.4) | 8(32) | <0.001 | 8(72.7) | 3(50) | 0.6 | 79 (76) | 11 (35.5) | <0.001 |
| HE&MI: n(%) | 22(23.6) | 17(68) | | 3(27.3) | 3(50) | | 25 (24) | 20 (64.5) | |
| IL28B (rs12979860) | | | | | | | | | |
| MA: n(%) | 47(50.5) | 4(16) | 0.003 | 5(45.5) | 1(16.6) | 0.333 | 52 (50) | 5 (16.1) | <0.001 |
| HE&MI: n(%) | 46(49.5) | 21(84) | | 6(54.5) | 5(83.4) | | 52 (50) | 26 (83.9) | |
| IFNL4 (ss469415590) | | | | | | | | | |
| MA: n(%) | 47(50.5) | 4(16) | 0.003 | 5(45.5) | 1(16.6) | 0.333 | 52 (50) | 5 (16.1) | <0.001 |
| HE&MI: n(%) | 46(49.5) | 21(84) | | 6(54.5) | 5(83.4) | | 52 (50) | 26 (83.9) | |

doi:10.1371/journal.pone.0093011.t002

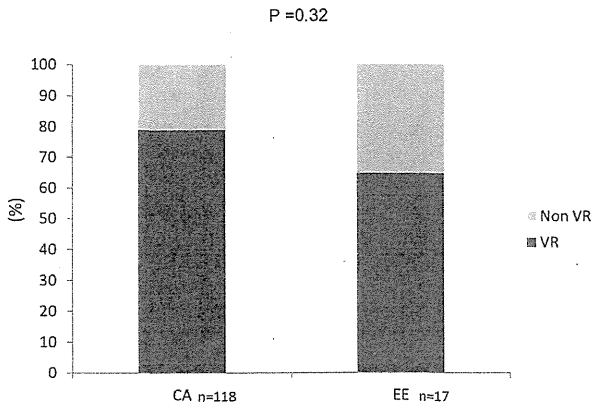


**Figure 1. Different HCV treatment outcomes in groups of individuals of central Asian (CA) or eastern European (EE) ancestry.** Treatment outcome was measured in terms of virological and non-virological response (VR and NVR, respectively) (see text for details).
doi:10.1371/journal.pone.0093011.g001

### Treatment for hepatitis C

Patients were treated with a weekly dose of PEG-IFN-α (1.5 mcg/kg) coupled with a daily dose RBV (1000 mg/day for patients up to 75 kg, and 1,250 mg/day for those over 75 kg). The viral load was determined by real-time reverse transcription-polymerase chain reaction (RT-PCR) prior to the start of treatment. On-treatment viral kinetics were evaluated at Weeks 4 and 12. To evaluate the power of the SNP genotype as a predictor of responses to antiviral treatment, all patients were classified into one of two groups: (I) non-responders (including those who still had detectable HCV RNA levels at Weeks 4 and 12 or at the post-treatment follow-up [24 weeks after treatment]); and (II) responders (including those with no detectable HCV RNA both during and/or after treatment).

Treatment was stopped if a patient failed to achieve a 2log (or greater) reduction in viral load after 12 weeks.

### IL28B genotyping

Whole blood was collected from all participants and centrifuged to separate the buffy coat. Genomic DNA was extracted from the buffy coat (containing peripheral blood mononuclear cells) using a QIAamp DNA Mini Kit (QIAGEN, Venlo, Netherlands).

All patients were genotyped for the SNPs rs8099917, rs12979860, rs8103142, and rs11881222 using a probe-based assay as previously described [24]. Two different probe-based assays, Invader Plus and the TaqMan probe assay, were used, and their sensitivity and specificity were compared with those of direct sequencing. For direct sequencing, the region of genomic DNA around rs12979860 was amplified using primers t63_L (5′-GGAAGGAGCAGTTGCG-3′), t63_R (5′-GGCTGTGGGT-CCTGT-3′), t64_L (5′-GACAGGAACGGGTGTATG-3′), and t64_R (5′-AGCTCTGATGTTGGGAAAG-3′).

**Table 3.** SNPs showed statistical significance in predicting treatment outcome in studied population.

| Ethnic origin | ss469415590 TT | rs8099917 TT | rs12979860 CC |
|---|---|---|---|
| | OR (95% CI) | OR (95% CI) | OR (95% CI) |
| Central Asian | 5.364 (1.7–16.8) * | 6.858 (2.6–18.0) * | 5.364 (1.7–16.8) * |
| East EU | 4.167 (0.4–48.4) | 2.667 (0.3–21.3) | 4.167 (0.4–48.4) |
| Overall | 5.2 (1.9–14.6) * | 5.745 (2.4–13.6) * | 5.2 (1.9–14.6) * |

*(p≤0.05).
doi:10.1371/journal.pone.0093011.t003

### Statistical analysis

Data were analyzed using SPSS 17.0 (SPSS for Windows, Chicago, IL). Categorical variables were expressed as numbers and percentages and continuous variables with a normal distribution were expressed as the mean and standard deviation. The Chi-squared and Fisher's exact tests were used where appropriate, and $p<0.05$ was considered statistically significant. Statistical odds ratios (OR) for treatment prediction were derived by logistic regression analysis.

## Results

### Comparison of the genotyping assays

Genotyping of *IL28B* was performed using the Invader Plus and TaqMan probe-based assays [24], and by direct sequencing. There was 100% concordance between the two assays, and there was 99.2% agreement between the two assays and direct sequencing (i.e., a discrepancy of 0.8%) (**Table 1**). Therefore, we used the broadly-prevalent TaqMan probe assay to examine the association between SNPs and treatment responses in the present study.

### Association between SNPs and treatment responses

The characteristics of each patient group are summarized in Table 2. One hundred thirty five patients (87.5% CA, 12.5% EE) completed either 24 or 48 weeks of treatment with Peg-IFN-α plus RBV. There was no significant difference between the groups in terms of age, gender, HCV viral load, and viral genotype (**Table 2**). There was no statistically significant difference between the percentages of CA and EE that showed a NVR (21.2% and 35.2%, respectively; $p<0.32$) (**Fig. 1**). However, there was a significant difference in the prevalence of SNPs within *IL28B* and *IFNL4* between VR and NVR in each ethnic. To evaluate the clinical applicability of individual SNPs, we calculated the predictive ORs for each SNP between VR and NVR in each ethnic (**Table 3**). All of the identified SNPs (favorable genotype) predicted positive response to treatment outcome in the overall study population and in the CA population, but not in the EE population. Interestingly, the polymorphism identified in the newly discovered *IFNL4* gene, ss469415590 [11], showed a strong linkage with the rs12979860 SNP around *IFNL3* in the overall study population; therefore, each had equal predictive value (**Table 3**). The most informative marker to predict VR of HCV treatment outcome was rs8099917 (OR, 5.75; 95% CI, 2.4–13.6, $p<0.001$), followed by rs12979860/ss469415590 (OR, 5.2;95% CI, 1.9–14.6; $p=0.002$). The predictive values of the SNPs are shown here for the entire studied population inclusive all HCV genotypes. There was no significant difference in predictive power (OR) of the SNPs when population was analyzed in the context of different HCV genotypes (1 vs non-1), however statistical power of the analysis was lower, most likely due to the smaller size of the non-1 genotype infected patients in this study.

### Genetic differences between ethnic groups

The alleles associated with all of the tested SNPs were in the Hardy-Weinberg equilibrium. The rs12979860, rs8103142, and rs1188122 SNPs showed high linkage disequilibrium (LD) in both

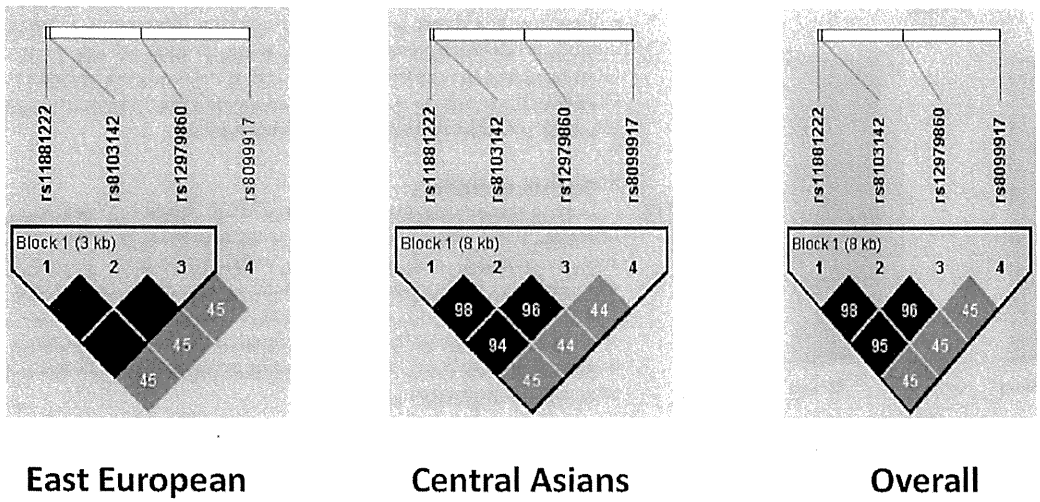


**Figure 2. Linkage disequilibrium diagram showing clustering of the studied SNPs.** The diagram was generated using HaploView software (available through the HapMap project).
doi:10.1371/journal.pone.0093011.g002

ethnic groups (**Fig. 2**). There was no difference in the frequency of rs8099917 alleles between the CA and EE populations; however, a minor allele of the rs12979860 SNP was observed more frequently in the EE group (0.32 *vs.* 0.44; $p<0.002$) (**Fig. 3**). The rs8099917 SNP had a higher predictive value than rs12979860/ss469415590 in the CA population (**Table 3**), whereas the reverse tended to be true in the EE population, although the differences were not statistically significant.

## Discussion

The aim of this study was to examine the prevalence and clinical significance of SNPs within the *IFNL3/IL28B* and *IFNL4* alleles in a population of HCV-infected patients in Central Asia. We also evaluated the ability of these SNPs to predict responses to anti-HCV treatments in this population. We found that rs12979860 and rs8099917 were informative markers of treatment response in Uzbekistan with different ethnicity. The rs8099917 genotype TT was the most common in the overall study population (67.8%), followed by the rs12979860 genotype CC (49%). This is the first report showing the distribution and linkage between the recently described *IFNL4* ss469415590 SNP [11] and the *IL28B* rs12979860 SNP in HCV-infected individuals in Uzbekistan.

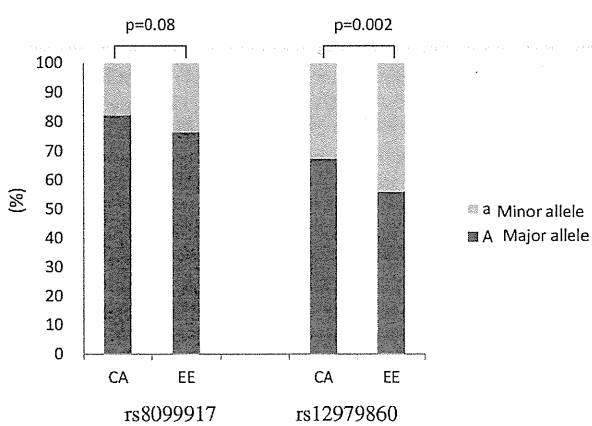


**Figure 3. Allele frequencies of the tested SNPs (n = 135).** The capital letter "A" represents ancestral ("major") alleles and lower case letter "a" represents mutant alleles ("minor"). CA: population with central Asian ancestry; EE: population with eastern European ancestry.
doi:10.1371/journal.pone.0093011.g003

According to the Human Haplotype Mapping project, only 15–19% of Caucasians carry the rs8099917 G allele. Notably, the GG genotype of rs8099917 was identified in 3.6% of patients in the present study, a lower prevalence than that observed in other countries [7–10]. These results agree with those of a previous study showing the variability of allele frequencies (2–31%) between different ethnic groups [25].

The rs8099917 SNP was a better predictor of treatment outcome in subjects of CA ancestry than the rs12979860/ss469415590 SNPs (**Table 3**). However, the reverse tended to be true for patients with EE ancestry. This is in agreement with the results of a previous study that examined populations of Western European ancestry [7,10]. A greater number of individuals of Eastern European ancestry must be examined to confirm the trend observed in the present study. Finally, although previous reports show that combined polymorphisms may show increased predictive value in terms of a SVR [26], no significant improvements were noted for the populations examined herein. Interestingly, the degree of LD between rs12979860 and the two SNPs within the IL28-encoding gene identified herein was slightly different in the two populations studied (Fig. 2), i.e., a strong LD was observed among patients of EE ancestry. One possible explanation for this is the smaller size of this patient population. Thus, we need to confirm our findings in a larger cohort. Another interesting observation is that, differently from a previously described Japanese population [9], we found a very low LD between rs8099917 and SNPs within the IL28B-encoding region; nevertheless both the favorable genotype of rs12979860 and rs8099917 were independent predictors of treatment outcome, suggesting the possibility of different mechanisms of involvement of the genetic regions around IL28B.

Predictive power of the SNPs, particularly of the IFNL4 ss469415590 variation reported here was in the range of that reported among Caucasians with HCV/1b [14,15]. The fact that