is among the protein components of RC in siC-transfected cells (Fig. 6A). Knockdown of FASN by small interfering RNA (siF) did not decrease the amount of NS5B in CRC but resulted in an 80% decrease of the amount of NS5B in RC. The efficiency of proteinase K digestion was monitored by the cleavage of the ER membrane protein calnexin; the C terminus of calnexin resides at the cytoplasmic side of the ER membrane and is susceptible to digest by proteinase K. Western blot analysis using an antibody that recognizes the N terminus of calnexin revealed the absence of full-length calnexin, concomitant with the presence of a calnexin cleavage product, indicating complete and effective proteinase K digestion (Fig. 6A). A large portion of the NS5B and FASN proteins in CRC was digested, while a small part of these two proteins remained resistant in RC. These data indicate that FASN colocalizes with NS5B and plays a role in retaining NS5B in active RC.

To further unveil the effect of FASN on HCV RNA expression, total RNA in CRC of control and FASN knockdown cells was isolated with or without treatment by S7 nuclease. The relative amount of HCV RNA was determined by real-time RT-PCR, whereas 28S RNA was analyzed by gel electrophoresis. In accordance with a previous study (26), HCV, but not 28S RNA, can be detected in the S7 nuclease-treated active RC. HCV RNA expression in the S7 nuclease resistant RC was decreased by 56% in FASN knockdown cells (Fig. 6B). Analysis of the replicase activity in S7 nuclease-resistant RC further confirmed the decrease in the level of newly synthesized HCV RNA by FASN knockdown (Fig. 6B).

To elucidate whether FASN is directly involved in the regulation of RdRp activity during HCV replication, *in vitro* NS5B RdRp activity was compared in the presence or absence of recombinant FASN protein. The NS5B and FASN recombinant proteins were purified by a His tag affinity column (Fig. 7A and B), and the expression of these two proteins was confirmed by Western blotting (data not shown). The NS5B-specific inhibitor 2′CMA at the dosage of the 50% inhibitory concentration (IC_{50}) (32) inhibited 37% of the RdRp activity, implying that the activity assay is NS5B specific (Fig. 7C, left). The presence of FASN in the reaction mixture containing NS5B protein increased the RdRp activity 4-fold, while FASN alone elicited minimal residual RdRp activity (Fig. 7C, right). These data indicate that by interacting with NS5B, FASN increases the *in vitro* RdRp activity of NS5B.

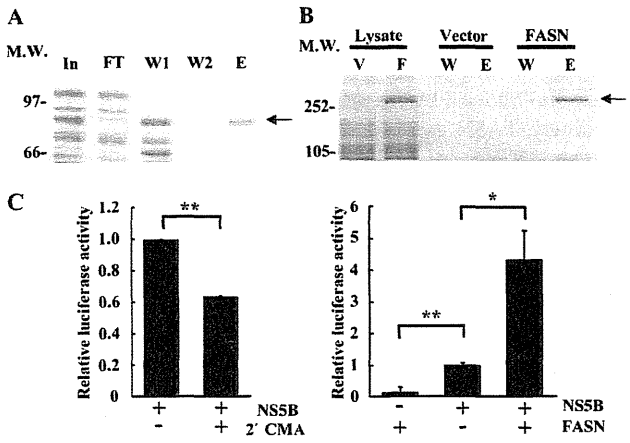

FIG 7 FASN enhances HCV NS5B RdRp activity. (A) The NS5B protein was purified as described in Materials and Methods and was then stained with Coomassie blue. The input (In) is the protein before purification, and FT, W, and E represent the flowthrough, wash, and elution fractions during NS5B purification, respectively. The arrow indicates the size of HCV NS5B. MW, molecular weight (in thousands). (B) The FASN protein (F) was purified from pcDNA-HisC-FASN-transfected cells, and the proteins were visualized by silver staining. The pcDNA3.1-HisC vector (V)-transfected cell lysates were purified in parallel as a control. W and E represent the wash and elution fractions during purification, respectively. (C) (Left) Purified NS5B was incubated alone or with the NS5B inhibitor 2′-*C*-methyladenosine (2′CMA) at 2 μM to evaluate the specificity of NS5B RdRp activity. (Right) Two picomoles of purified His-FASN was incubated with 250 nM purified NS5B to evaluate the effect of FASN on RdRp activity. An equal volume of pcDNA3.1-HisC vector-transfected cell lysates purified in parallel was used as a background control. Luciferase activity was detected at 5 min after the initiation of the reaction. The relative RdRp activity after subtraction of background luciferase activity is shown. Data are means ± standard deviations for three independent experiments (*, $P < 0.05$; **, $P < 0.01$).

DISCUSSION

In addition to viral factors, host proteins play a pivotal role in many steps of RNA virus replication (33, 34). Nevertheless, the components of HCV RC are still not completely understood. Some of the host factors involved in HCV replication were found to be tightly linked to lipid metabolism (17, 35). In this study, FASN is revealed to interact with NS5B, with two major functional implications. The interaction with NS5B causes a portion of FASN proteins to shift from the cytoplasm to lipid rafts. On the other hand, FASN enhances NS5B-dependent RdRp activity and causes an increase in HCV replication and viral production. This study thereby demonstrates for the first time that FASN regulates HCV replication and viral production through a direct interplay with NS5B.

The main function of FASN is to catalyze the synthesis of palmitate from acetyl coenzyme A (acetyl-CoA) and malonyl-CoA, subsequently forming long-chain saturated fatty acids in the presence of NADPH (36). FASN expression is significantly increased in HCV-infected livers (37), and serum FASN concentrations are significantly increased in patients with chronic HCV infection (38, 39). FASN is also upregulated in HCV-infected Huh7 cells (18). Accordingly, a significantly higher level of free fatty acid was detected in HCV-infected than in uninfected Huh 7.5 cells (40). Consequently, more lipid droplets were formed for HCV production (41). Hence, FASN plays roles in HCV infection. However, the mechanism of FASN involvement in HCV replication is unclear. The data we present in this study indicate that both the enzymatic activity of FASN and FASN-NS5B interactions are important for the role of FASN in regulating HCV replication. Accordingly, FASN knockdown and the inhibition of FASN enzymatic activity by C75 and orlistat result in decreases in the level of HCV replication activity. Notably, we demonstrate for the first time that FASN is a component of HCV RC and, by interacting with NS5B, enhances NS5B-dependent RdRp activity. In this mode of action, an allosteric effect of FASN, but not its enzymatic activity, is likely to play a major role in modulating NS5B activity. This notion is based on the observations that the inhibition of FASN activity by C75 and orlistat or FASN knockdown alone does not result in complete inhibition of HCV replication, suggesting that both FASN activity and protein expression are involved in the regulation of HCV expression. Moreover, FASN has been reported to elicit palmitoylation activity, and the palmitoylation of NS4B is important for HCV RC formation (42, 43). Whether FASN is recruited, by interacting with NS5B, into the NS4B-associated membrane web and palmitoylates NS4B protein remains to be investigated further.

The expression of FASN has been reported to be regulated by HCV core protein (44), NS2 (45), and NS4B (46). These viral proteins enhance sterol regulatory element-binding protein 1 expression, followed by activation of the FASN promoter (47). Accordingly, FASN expression was increased in HCV-permissive cells and HCVcc-infected Huh7.5.1 cells (Fig. 4A), indicating that FASN plays a role in HCV replication. As in HCV, FASN expression can be induced by some other types of viral infection. The hepatitis B virus large surface protein accumulated on the endoplasmic reticulum leads to the unfolding of proteins and promotes FASN expression through the binding of the activated NF-Y transcription factor to the FASN promoter (48). The Epstein-Barr virus early-stage protein BRLF1 also activates FASN expression, leading to the induction of Epstein-Barr virus Z transcription for the lytic stage (49). On the other hand, the replication of two enveloped viruses, human cytomegalovirus and influenza virus, is inhibited by a fatty acid biosynthesis inhibitor that subsequently modulates the membrane composition for virus budding (50). Moreover, dengue virus NS3 interacts with FASN and recruits it to the site of RC formation, leading to membrane expansion and an increase in membrane fluidity (51). In agreement with our findings, Yang et al. have reported that HCV replication and viral entry are decreased by a FASN inhibitor and FASN RNAi (18). HCV was thought to replicate in the NS4B-induced membrane web and the viral particles were assembled on the outer surface of lipid droplets (41, 52). Hence, it was thought that HCV infection-induced FASN is involved mainly in the production of phospholipids which are participated in the formation of detergent-resistant membranes (18). Our data offer a new insight: by interacting with NS5B, FASN is recruited into HCV replication complexes and directly enhances HCV RdRp activity. Thus, promising new antiviral approaches to combat HCV infection based on the inhibition of FASN activity are worthy of consideration.

Increased FASN expression and activity are found in several cancers, including HCV-associated HCC (53, 54). Our data demonstrate that NS5B, FASN, and caveolin-1 (Cav-1) can interact with each other in HCV replicon cells (data not shown). Cav-1 is a palmitoylated lipid raft protein and has been found to interact with FASN in prostate cancer cells to promote tumor growth and survival (55). Recently, Cav-1 was also reported to promote the motility and invasion of hepatocellular carcinoma (HCC) and metastasis in a mouse model though an unknown mechanism (56, 57). We therefore postulate that following HCV infection, FASN interacts with NS5B and, together with Cav-1, is recruited to lipid rafts, followed by the induction of signaling leading to hepatocellular carcinoma.

In conclusion, we demonstrate for the first time that FASN interacts with NS5B, leading to an increase in HCV RdRp activity. Furthermore, inhibition of FASN reduces HCV replication and viral production. This study thereby contributes to our understanding of the role of FASN in the control of HCV replication and offers new insight for developing novel anti-HCV therapeutic approaches.

ACKNOWLEDGMENTS

We thank Jing-Hsiung Ou for providing HCV subgenome replicon cells, Robert T. Schooley for providing Huh7.5.1 cells and plasmid JC1-Luc2A, Charles Rice for providing plasmid J6/JFH(p7-Rlu2A), and Takaji Wakita for providing a polyclonal antibody against NS5B. We also thank Chien-Ling Huang, Yung-Ju Yeh, and Chia-Fan Lin for technical support.

This work was supported by grants NSC97-2320-B-039-026-MY3, NSC94-2320-B-039-008-, and NSC 93-2314-B-039-029- from the National Science Council (J.-C.C.).

REFERENCES

1. Lindenbach BD, Rice CM. 2005. Unravelling hepatitis C virus replication from genome to function. Nature 436:933–938.
2. Egger D, Wolk B, Gosert R, Bianchi L, Blum HE, Moradpour D, Bienz K. 2002. Expression of hepatitis C virus proteins induces distinct membrane alterations including a candidate viral replication complex. J. Virol. 76:5974–5984.
3. Moradpour D, Penin F, Rice CM. 2007. Replication of hepatitis C virus. Nat. Rev. Microbiol. 5:453–463.
4. Samuel D, Feray C. 2000. Recurrent hepatitis C after liver transplantation: clinical and therapeutical issues. J. Viral Hepat. 7:87–92.
5. Thomson BJ, Finch RG. 2005. Hepatitis C virus infection. Clin. Microbiol. Infect. 11:86–94.
6. Poenisch M, Bartenschlager R. 2010. New insights into structure and replication of the hepatitis C virus and clinical implications. Semin. Liver Dis. 30:333–347.
7. Hwang SJ, Lee SD. 2011. Hepatic steatosis and hepatitis C: still unhappy bedfellows? J. Gastroenterol. Hepatol. 26(Suppl. 1):96–101.
8. Negro F. 2010. Abnormalities of lipid metabolism in hepatitis C virus infection. Gut 59:1279–1287.
9. Jiang J, Luo G. 2009. Apolipoprotein E but not B is required for the formation of infectious hepatitis C virus particles. J. Virol. 83:12680–12691.
10. Liu S, McCormick KD, Zhao W, Zhao T, Fan D, Wang T. 2012. Human apolipoprotein E peptides inhibit hepatitis C virus entry by blocking virus binding. Hepatology 56:484–491.
11. Mancone C, Steindler C, Santangelo L, Simonte G, Vlassi C, Longo MA, D'Offizi G, Di Giacomo C, Pucillo LP, Amicone L, Tripodi M, Alonzi T. 2011. Hepatitis C virus production requires apolipoprotein A-I and affects its association with nascent low-density lipoproteins. Gut 60:378–386.
12. Gastaminza P, Cheng G, Wieland S, Zhong J, Liao W, Chisari FV. 2008. Cellular determinants of hepatitis C virus assembly, maturation, degradation, and secretion. J. Virol. 82:2120–2129.
13. Huang H, Sun F, Owen DM, Li W, Chen Y, Gale M, Jr., Ye J. 2007. Hepatitis C virus production by human hepatocytes dependent on assembly and secretion of very low-density lipoproteins. Proc. Natl. Acad. Sci. U. S. A. 104:5848–5853.
14. Kapadia SB, Chisari FV. 2005. Hepatitis C virus RNA replication is regulated by host geranylgeranylation and fatty acids. Proc. Natl. Acad. Sci. U. S. A. 102:2561–2566.
15. Wang C, Gale M, Jr, Keller BC, Huang H, Brown MS, Goldstein JL, Ye J. 2005. Identification of FBL2 as a geranylgeranylated cellular protein required for hepatitis C virus RNA replication. Mol. Cell 18:425–434.
16. Ye J, Wang C, Sumpter R, Jr, Brown MS, Goldstein JL, Gale M, Jr. 2003. Disruption of hepatitis C virus RNA replication through inhibition of host protein geranylgeranylation. Proc. Natl. Acad. Sci. U. S. A. 100:15865–15870.
17. Su AI, Pezacki JP, Wodicka L, Brideau AD, Supekova L, Thimme R, Wieland S, Bukh J, Purcell RH, Schultz PG, Chisari FV. 2002. Genomic analysis of the host response to hepatitis C virus infection. Proc. Natl. Acad. Sci. U. S. A. 99:15669–15674.
18. Yang W, Hood BL, Chadwick SL, Liu S, Watkins SC, Luo G, Conrads TP, Wang T. 2008. Fatty acid synthase is up-regulated during hepatitis C virus infection and regulates hepatitis C virus entry and production. Hepatology 48:1396–1403.
19. Choi J, Lee KJ, Zheng Y, Yamaga AK, Lai MM, Ou JH. 2004. Reactive oxygen species suppress hepatitis C virus RNA replication in human hepatoma cells. Hepatology 39:81–89.
20. Cheng JC, Chang MF, Chang SC. 1999. Specific interaction between the hepatitis C virus NS5B RNA polymerase and the 3′ end of the viral RNA. J. Virol. 73:7044–7049.
21. Weng LP, Wu CC, Hsu BL, Chi LM, Liang Y, Tseng CP, Hsieh LL, Yu JS. 2008. Secretome-based identification of Mac-2 binding protein as a potential oral cancer marker involved in cell growth and motility. J. Proteome Res. 7:3765–3775.
22. Craggs JK, Ball JK, Thomson BJ, Irving WL, Grabowska AM. 2001.

Development of a strand-specific RT-PCR based assay to detect the replicative form of hepatitis C virus RNA. J. Virol. Methods 94:111–120.
23. Cheng JC, Yeh YJ, Tseng CP, Hsu SD, Chang YL, Sakamoto N, Huang HD. 2012. Let-7b is a novel regulator of hepatitis C virus replication. Cell. Mol. Life Sci. 69:2621–2633.
24. Wakita T, Pietschmann T, Kato T, Date T, Miyamoto M, Zhao Z, Murthy K, Habermann A, Krausslich HG, Mizokami M, Bartenschlager R, Liang TJ. 2005. Production of infectious hepatitis C virus in tissue culture from a cloned viral genome. Nat. Med. 11:791–796.
25. Gao L, Aizaki H, He JW, Lai MM. 2004. Interactions between viral nonstructural proteins and host protein hVAP-33 mediate the formation of hepatitis C virus RNA replication complex on lipid raft. J. Virol. 78: 3480–3488.
26. Quinkert D, Bartenschlager R, Lohmann V. 2005. Quantitative analysis of the hepatitis C virus replication complex. J. Virol. 79:13594–13605.
27. Lahser FC, Malcolm BA. 2004. A continuous nonradioactive assay for RNA-dependent RNA polymerase activity. Anal. Biochem. 325:247–254.
28. Mosley RT, Edwards TE, Murakami E, Lam AM, Grice RL, Du J, Sofia MJ, Furman PA, Otto MJ. 2012. Structure of hepatitis C virus polymerase in complex with primer-template RNA. J. Virol. 86:6503–6511.
29. Shi ST, Lee KJ, Aizaki H, Hwang SB, Lai MM. 2003. Hepatitis C virus RNA replication occurs on a detergent-resistant membrane that cofractionates with caveolin-2. J. Virol. 77:4160–4168.
30. Kuhajda FP, Pizer ES, Li JN, Mani NS, Frehywot GL, Townsend CA. 2000. Synthesis and antitumor activity of an inhibitor of fatty acid synthase. Proc. Natl. Acad. Sci. U. S. A. 97:3450–3454.
31. Kridel SJ, Axelrod F, Rozenkrantz N, Smith JW. 2004. Orlistat is a novel inhibitor of fatty acid synthase with antitumor activity. Cancer Res. 64: 2070–2075.
32. Migliaccio G, Tomassini JE, Carroll SS, Tomei L, Altamura S, Bhat B, Bartholomew L, Bosserman MR, Ceccacci A, Colwell LF, Cortese R, De Francesco R, Eldrup AB, Getty KL, Hou XS, LaFemina RL, Ludmerer SW, MacCoss M, McMasters DR, Stahlhut MW, Olsen DB, Hazuda DJ, Flores OA. 2003. Characterization of resistance to non-obligate chain-terminating ribonucleoside analogs that inhibit hepatitis C virus replication in vitro. J. Biol. Chem. 278:49164–49170.
33. Ahlquist P, Noueiry AO, Lee WM, Kushner DB, Dye BT. 2003. Host factors in positive-strand RNA virus genome replication. J. Virol. 77: 8181–8186.
34. Moriishi K, Matsuura Y. 2007. Host factors involved in the replication of hepatitis C virus. Rev. Med. Virol. 17:343–354.
35. Alvisi G, Madan V, Bartenschlager R. 2011. Hepatitis C virus and host cell lipids: an intimate connection. RNA Biol. 8:258–269.
36. Jayakumar A, Tai MH, Huang WY, al-Feel W, Hsu M, Abu-Elheiga L, Chirala SS, Wakil SJ. 1995. Human fatty acid synthase: properties and molecular cloning. Proc. Natl. Acad. Sci. U. S. A. 92:8695–8699.
37. Fujino T, Nakamuta M, Yada R, Aoyagi Y, Yasutake K, Kohjima M, Fukuizumi K, Yoshimoto T, Harada N, Yada M, Kato M, Kotoh K, Taketomi A, Maehara Y, Nakashima M, Enjoji M. 2010. Expression profile of lipid metabolism-associated genes in hepatitis C virus-infected human liver. Hepatol. Res. 40:923–929.
38. Aragones G, Alonso-Villaverde C, Oliveras-Ferraros C, Beltran-Debon R, Rull A, Rodriguez-Sanabria F, Camps J, Martin AV, Menendez JA, Joven J. 2010. Infection with HIV and HCV enhances the release of fatty acid synthase into circulation: evidence for a novel indicator of viral infection. BMC Gastroenterol. 10:92. doi:10.1186/1471-230X-10-92.
39. Joven J, Espinel E, Rull A, Beltran-Debon R, Aragones G, Rodriguez-Gallego E, Camps J, Pedro-Botet J, Sans T, Menendez JA, Alonso-Villaverde C. 2011. Serum fatty acid synthase concentration is increased in patients with hepatitis viral infection and may assist in the prediction of liver steatosis. J. Clin. Virol. 51:199–201.
40. Woodhouse SD, Narayan R, Latham S, Lee S, Antrobus R, Gangadharan B, Luo S, Schroth GP, Klenerman P, Zitzmann N. 2010. Transcriptome sequencing, microarray, and proteomic analyses reveal cellular and metabolic impact of hepatitis C virus infection in vitro. Hepatology 52: 443–453.
41. Miyanari Y, Atsuzawa K, Usuda N, Watashi K, Hishiki T, Zayas M, Bartenschlager R, Wakita T, Hijikata M, Shimotohno K. 2007. The lipid droplet is an important organelle for hepatitis C virus production. Nat. Cell Biol. 9:1089–1097.
42. Ueno K. 2000. Involvement of fatty acid synthase in axonal development in mouse embryos. Genes Cells 5:859–869.
43. Yu GY, Lee KJ, Gao L, Lai MM. 2006. Palmitoylation and polymerization of hepatitis C virus NS4B protein. J. Virol. 80:6013–6023.
44. Jackel-Cram C, Babiuk LA, Liu Q. 2007. Up-regulation of fatty acid synthase promoter by hepatitis C virus core protein: genotype-3a core has a stronger effect than genotype-1b core. J. Hepatol. 46:999–1008.
45. Oem JK, Jackel-Cram C, Li YP, Zhou Y, Zhong J, Shimano H, Babiuk LA, Liu Q. 2008. Activation of sterol regulatory element-binding protein 1c and fatty acid synthase transcription by hepatitis C virus non-structural protein 2. J. Gen. Virol. 89:1225–1230.
46. Park CY, Jun HJ, Wakita T, Cheong JH, Hwang SB. 2009. Hepatitis C virus nonstructural 4B protein modulates sterol regulatory element-binding protein signaling via the AKT pathway. J. Biol. Chem. 284:9237–9246.
47. Jackel-Cram C, Qiao L, Xiang Z, Brownlie R, Zhou Y, Babiuk L, Liu Q. 2010. Hepatitis C virus genotype-3a core protein enhances sterol regulatory element-binding protein-1 activity through the phosphoinositide 3-kinase-Akt-2 pathway. J. Gen. Virol. 91:1388–1395.
48. Foo NC, Yen TS. 2000. Activation of promoters for cellular lipogenic genes by hepatitis B virus large surface protein. Virology 269:420–425.
49. Li Y, Webster-Cyriaque J, Tomlinson CC, Yohe M, Kenney S. 2004. Fatty acid synthase expression is induced by the Epstein-Barr virus immediate-early protein BRLF1 and is required for lytic viral gene expression. J. Virol. 78:4197–4206.
50. Munger J, Bennett BD, Parikh A, Feng XJ, McArdle J, Rabitz HA, Shenk T, Rabinowitz JD. 2008. Systems-level metabolic flux profiling identifies fatty acid synthesis as a target for antiviral therapy. Nat. Biotechnol. 26: 1179–1186.
51. Heaton NS, Perera R, Berger KL, Khadka S, Lacount DJ, Kuhn RJ, Randall G. 2010. Dengue virus nonstructural protein 3 redistributes fatty acid synthase to sites of viral replication and increases cellular fatty acid synthesis. Proc. Natl. Acad. Sci. U. S. A. 107:17345–17350.
52. Miyanari Y, Hijikata M, Yamaji M, Hosaka M, Takahashi H, Shimotohno K. 2003. Hepatitis C virus non-structural proteins in the probable membranous compartment function in viral genome replication. J. Biol. Chem. 278:50301–50308.
53. Menendez JA, Lupu R. 2007. Fatty acid synthase and the lipogenic phenotype in cancer pathogenesis. Nat. Rev. Cancer 7:763–777.
54. Wu JM, Skill NJ, Maluccio MA. 2010. Evidence of aberrant lipid metabolism in hepatitis C and hepatocellular carcinoma. HPB (Oxford) 12:625–636.
55. Di Vizio D, Adam RM, Kim J, Kim R, Sotgia F, Williams T, Demichelis F, Solomon KR, Loda M, Rubin MA, Lisanti MP, Freeman MR. 2008. Caveolin-1 interacts with a lipid raft-associated population of fatty acid synthase. Cell Cycle 7:2257–2267.
56. Cokakli M, Erdal E, Nart D, Yilmaz F, Sagol O, Kilic M, Karademir S, Atabey N. 2009. Differential expression of Caveolin-1 in hepatocellular carcinoma: correlation with differentiation state, motility and invasion. BMC Cancer 9:65. doi:10.1186/1471-2407-9-65.
57. Tse EY, Ko FC, Tung EK, Chan LK, Lee TK, Ngan ES, Man K, Wong AS, Ng IO, Yam JW. 2012. Caveolin-1 overexpression is associated with hepatocellular carcinoma tumourigenesis and metastasis. J. Pathol. 226: 645–653.

Hepatology Research

Hepatology Research 2013; 43: 35–43 doi: 10.1111/j.1872-034X.2012.01056.x

Special Report

A multicenter survey of re-treatment with pegylated interferon plus ribavirin combination therapy for patients with chronic hepatitis C in Japan

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Aim: This study aimed to clarify the factors associated the efficacy of re-treatment with pegylated interferon (PEG IFN) plus ribavirin combination therapy for patients with chronic hepatitis C who had failed to respond to previous treatment.

Methods: One hundred and forty-three patients who had previously shown relapse ($n = 79$), non-response ($n = 34$) or intolerance ($n = 30$) to PEG IFN plus ribavirin were re-treated with PEG IFN plus ribavirin.

Results: Twenty-five patients with intolerance to previous treatment completed re-treatment and the sustained virological response (SVR) rates were 55% and 80% for hepatitis C virus (HCV) genotype 1 and 2, respectively. On re-treatment of the 113 patients who completed the previous treatment, the SVR rates were 48% and 63% for genotype 1 and 2, respectively. Relapse after previous treatment and a low baseline HCV RNA level on re-treatment were associated with SVR in genotype 1 ($P < 0.001$). Patients with the interleukin-28B major genotype responded significantly better and earlier to re-treatment, but the difference in the SVR rate did not reach a significant level between the major and minor genotypes ($P = 0.09$). Extended treatment of 72 weeks raised the SVR rate among the patients who attained complete early virological response but not rapid virological response with re-treatment (72 weeks, 73%, 16/22, vs 48 weeks, 38%, 5/13, $P < 0.05$).

Conclusion: Relapse after previous treatment and a low baseline HCV RNA level have predictive values for a favorable response of PEG IFN plus ribavirin re-treatment for HCV genotype 1 patients. Re-treatment for 72 weeks may lead to clinical improvement for genotype 1 patients with complete early virological response and without rapid virological response on re-treatment.

Key words: chronic hepatitis C, pegylated interferon and ribavirin combination therapy, re-treatment

INTRODUCTION

PEGYLATED INTERFERON (PEG IFN) plus ribavirin combination therapy can show antiviral efficacy for patients with chronic hepatitis C (CH-C). However, a sustained virological response (SVR), which is defined as undetectable serum hepatitis C virus (HCV) RNA at 24 weeks after the treatment, remains at 50% for patients with HCV genotype 1 and 80% for those with HCV genotype 2 treated with PEG IFN plus ribavirin.[1–6] The number of patients who fail to achieve a SVR increases over time, requiring urgent action to eradicate HCV in them.

Recently, addition of the first-wave protease inhibitor telaprevir to PEG IFN plus ribavirin combination therapy, which has been reported to improve antiviral efficacy, has become commercially available, but this

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Received 18 April 2012; revision 19 May 2012; accepted 21 May 2012.

triple therapy increases side-effects, especially severe anemia and skin rash.[7–11] Second-wave protease inhibitors, such as TMC435, which not only improve antiviral efficacy but also decrease side-effects, have been developed and are undergoing clinical trials.[12] Also, IFN-free regimens, such as protease inhibitor and polymerase inhibitor combination therapy, have been developed.[13,14] In Japan, HCV carriers are increasing in an aging population, and large numbers of patients are ineligible for triple therapy with telaprevir due to potential anemia. That is why re-treatment with PEG IFN plus ribavirin is a possible choice for patients who failed to achieve SVR to previous antiviral therapy or patients ineligible for triple therapy with telaprevir who must wait until next-generation antiviral therapies, such as triple therapy with second-wave protease inhibitors or IFN-free regimens, become commercially available.

As for re-treatment with PEG IFN plus ribavirin, some studies have been reported but the subjects and treatment protocols were varied.[15–20] According to past reports, the previous treatment response is associated with the efficacy of the re-treatment[17,20] and the SVR rates in re-treatment ranged 4–23%.[16–18] Recently, host factors, such as single nucleotide polymorphisms (SNP) located near the interleukin (IL)-28B gene, and virus factors, such as the amino acid substitutions in the HCV core region, were revealed to have a strong impact on SVR in PEG IFN plus ribavirin combination therapy for naïve CH-C patients.[21–26] Moreover, response-guided therapy which extends treatment duration until 72 weeks for patients with a slow virological response can raise the SVR rate for naïve CH-C patients.[27–29] However, the value of IL-28B SNP has been uncertain in re-treatment and the most appropriate treatment duration in re-treatment is still unclear. Although it remains obscure which factors are associated with SVR in re-treatment with standard PEG IFN plus ribavirin therapy as pointed out above, some patients do respond to re-treatment and it is very important to be able to identify them. Such findings will be valuable for optimizing the antiviral treatment for CH-C patients by making it possible to decide which patients should be considered for re-treatment with PEG IFN plus ribavirin therapy and which should wait for next-generation antiviral treatment.

In the present study, we tried to determine which patients could benefit from re-treatment and to identify the factors associated with SVR in re-treatment, including the host genome SNP and treatment duration.

METHODS

Patients

THIS RETROSPECTIVE, MULTICENTER study was conducted by the Study Group of Antiviral Therapy for Difficult-to-Treat Chronic Hepatitis C supported by the Ministry of Health, Labor and Welfare, Japan. This study was conducted with 143 CH-C patients, 113 patients (genotype 1, $n = 86$; genotype 2, $n = 27$) who had previously completed PEG IFN-α-2b plus ribavirin combination therapy but had failed to attain SVR, and 30 patients (genotype 1, $n = 22$; genotype 2, $n = 8$) who had previously discontinued this combination therapy due to adverse events.

Treatment

For the previous treatment, patients had been treated with PEG IFN-α-2b (PEGINTRON; MSD, Whitehouse Station, NJ, USA) plus ribavirin (REBETOL; MSD). For re-treatment with PEG IFN plus ribavirin, patients were treated PEG IFN-α-2a (PEGASYS; Roche, Basel, Switzerland) plus ribavirin (COPEGUS; Roche) or PEG IFN-α-2b plus ribavirin. In principle, as a starting dose, PEG IFN was given once weekly at a dose of 180 μg of PEG IFN-α-2a and 1.5 μg/kg of PEG IFN-α-2b and ribavirin was given at a total dose of 600–1000 mg/day based on bodyweight (bodyweight, ≤60 kg, 600 mg; 60–80 kg, 800 mg; ≥80 kg, 1000 mg), according to the standard treatment protocol for Japanese patients and the decision of the investigator at the participating clinical center. Dose modification followed, as a rule, the manufacturer's drug information on the intensity of the hematological adverse effects.

Laboratory tests and virological assessment

Examination of peripheral blood, transaminase and the serum HCV RNA level were tested at the start of treatment, weeks 4, 12 and 24, end of treatment (EOT), and 24 weeks after the treatment. Sequences of the IFN-sensitivity determining region (ISDR) and the core region of HCV were determined at start of the previous treatment, and the number of mutations in the ISDR, the amino acid substitutions at core 70 and 91, glutamine (Gln) or histidine (His) at core 70 and methionine (Met) at core 91, were analyzed. Genetic polymorphisms located near the IL-28B gene (rs8099917) and ITPA gene (rs1127354) were determined. As for the IL-28B gene, homozygosity for the major sequence (TT) was defined as having the IL-28B major allele, whereas homozygosity (GG) or heterozygosity (TG) of the minor sequence was defined as having

the IL-28B minor allele. As for the ITPA gene, homozygosity for the major sequence (CC) was defined as having the ITPA major allele, whereas homozygosity (AA) or heterozygosity (CA) of the minor sequence was defined as having the ITPA minor allele. The serum HCV RNA level was quantified using the COBAS AMPLICOR HCV MONITOR test ver. 2.0 (detection range, 6–5000 KIU/mL; Roche Diagnostics, Branchburg, NJ, USA) or COBAS TaqMan HCV test (detection range, 1.2–7.8 $\log_{10}$ IU/mL) and qualitatively analyzed using the COBAS AMPLICOR HCV test ver. 2.0 (lower limit of detection, 50 IU/mL). When the serum HCV RNA level quantified by the COBAS TaqMan HCV test was less than 1.7 $\log_{10}$ IU/mL, which was equivalent to 50 IU/mL of HCV RNA, that case was judged as HCV RNA negativiation against the lower limit of detection of the COBAS AMPLICOR HCV test.

Definition of virological response

A rapid virological response (RVR) was defined as undetectable serum HCV RNA level at week 4, partial early virological response (p-EVR) as a more than 2-log decrease in the HCV RNA level at week 12 compared with the baseline, complete EVR (c-EVR) as undetectable serum HCV RNA at week 12, late virological response (LVR) as detectable serum HCV RNA at week 12 and undetectable at week 24, and SVR as undetectable serum HCV RNA at 24 weeks after the treatment. Relapse was defined as undetectable serum HCV RNA at the EOT but a detectable amount after the treatment. Patients without p-EVR or without clearance of HCV RNA at week 24 were considered to be showing non-response (NR), and treatment was stopped in both the previous treatment and this re-treatment. A patient who attained HCV RNA negativiation during the re-treatment continued to be treated for 48 weeks or 72 weeks according to response-guided therapy or the decision of the investigator at the participating clinical center.

Statistical analysis

Baseline data of the patients are expressed as means ± standard deviation or median values. In order to analyze the difference between baseline data or the factors associated with SVR, univariate analysis using the Mann–Whitney *U*-test or χ^2-test and multivariate analysis using logistic regression analysis were performed. A two-tailed *P*-value of less than 0.05 was considered significant. The analysis was conducted with SPSS ver. 17.0J (IBM, Armonk, NY, USA).

RESULTS

THE PATIENT FLOW in this study is shown in Figure 1. Among the patients who had previously discontinued PEG IFN-α-2b plus ribavirin combination therapy, two patients underwent splenectomy to increase platelet count prior to re-treatment, 25 completed re-treatment of PEG IFN plus ribavirin combination therapy and 15 achieved SVR (genotype 1, $n = 11$; genotype 2, $n = 4$).

All of the patients who completed previous treatment also completed re-treatment and the baseline characteristics of those patients are shown in Table 1. Of the 86 genotype 1 patients, 54 were relapsers and 32 had shown NR to previous treatment. Of the 27 patients with genotype 2, 25 were relapsers and two had shown NR to previous treatment. Thirty-seven patients with genotype 1 and 14 patients with genotype 2 were assessed as IL-28B genotype, and 27 patients with genotype 1 and 10 patients with genotype 2 were assessed as ITPA genotype. There was no significant difference in the baseline characteristics between the previous treatment and the re-treatment with respect to peripheral blood cell counts, amino transaminase level and serum HCV RNA at the start of treatment (Table 1).

The baseline characteristics of patients with genotype 1 according to antiviral efficacy of the previous treatment are shown in Table 2. Among those with NR in the previous treatment, the rate of the minor allele of IL-28B was significantly higher than those with relapse in the previous treatment ($P < 0.01$). For genotype 1, the HCV RNA negative rate on re-treatment was 20% (17/86) at week 4, 61% (52/85) at week 12 and 76% (65/86) at week 24, and the SVR rate was 48% (41/86). The factors associated with SVR were assessed by univariate analysis and the factors of relapse after previous treatment and the serum HCV RNA level at the start of re-treatment were selected as being significant (Table 3). The SVR

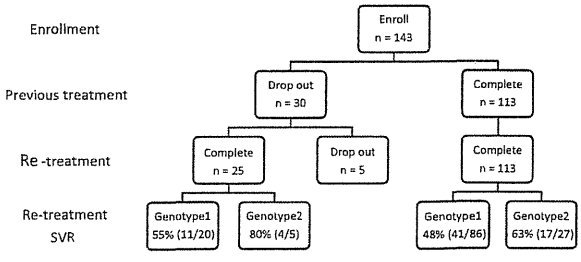

Figure 1 Patient flow for this study. SVR, sustained virological response.

Table 1 Baseline characteristics of patients and treatment factors in previous treatment and re-treatment

Factor	Genotype 1		Genotype 2	
No.	86		27	
Sex: male/female	46/40		15/12	
Effect of previous treatment: relapse/NR	54/32		25/2	
	Previous treatment	Re-treatment	Previous treatment	Re-treatment
PEG IFN type: α-2a/α-2b	0/86	41/45	0/27	6/21
Age (years)	58.1 ± 8.3	60.0 ± 8.5	58.9 ± 8.2	60.0 ± 8.1
White blood cells (/mm^3)	4779 ± 1383	4610 ± 1443	5195 ± 1473	4724 ± 1266
Neutrophils (/mm^3)	2478 ± 930	2355 ± 1071	2561 ± 827	2389 ± 941
Hemoglobin (g/dL)	13.7 ± 1.2	13.5 ± 1.7	14.4 ± 1.3	14.0 ± 1.2
Platelets (×10^4/mm^3)	16.0 ± 5.9	16.6 ± 6.2	18.0 ± 5.7	16.8 ± 5.2
ALT (IU/L)	75 ± 51	73 ± 72	57 ± 46	42 ± 32
Histology: activity, 0–1/2–3	29/29		11/7	
Fibrosis, 0–2/3–4	45/14		17/1	
Serum HCV RNA (KIU/mL)	1600	850	1500	700
IL-28B SNP: rs8099917; TT/TG	26/11		10/4	
ITPA SNP: rs1127354; CC/CA	20/7		9/1	
Core 70: wild/mutant	11/11			
Core 91: wild/mutant	15/7			
ISDR: 0–1/≥2	15/1			

ALT, alanine aminotransferase; HCV, hepatitis C virus; IFN, interferon; IL, interleukin; ISDR, IFN-sensitivity determining region; NR, non-response; PEG, pegylated; SNP, single nucleotide polymorphism.

rates of relapsers were significantly higher than those of patients with NR in the previous treatment (relapse, 67%, 36/54 vs NR, 16%, 5/32, $P < 0.0001$). As for the serum HCV RNA level at the start of re-treatment, although the SVR rate of those patients with 5 $\log_{10}$ IU/mL or more of HCV RNA was 38% (26/69), all patients with less than 5 $\log_{10}$ IU/mL of HCV RNA attained SVR (11/11) ($P = 0.0001$). As for the IL-28B genotype, among the patients with the major allele, the p-EVR rate was significantly higher and the EOT response rate showed marginal significance compared to that with the minor allele (p-EVR rate, 100%, 23/23 vs 30%, 3/10, $P < 0.0001$, EOT rate, 92%, 24/26 vs 64%, 7/11, $P = 0.05$). There was no significant difference of the SVR rate between major and minor alleles (major, 65%, 17/26 vs minor, 36%, 4/11, $P = 0.15$).

Figure 2(a) shows the result of stratified analysis according to the previous treatment response and HCV RNA at the start of re-treatment. The significant difference in SVR observed between high (≥5 $\log_{10}$ IU/mL) and low (<5 $\log_{10}$ IU/mL) baseline viral loads was still found in both previous relapsers ($P = 0.02$) and previous non-responders ($P = 0.02$). In patients with a high baseline viral load, previous relapsers achieved a higher SVR rate than previous non-responders ($P < 0.0001$). Next, the results of stratified analyses according to IL-28B genotype and previous treatment response or HCV RNA at the start of re-treatment showed no significant difference in SVR rates between the IL-28B genotype in patients with relapse after previous treatment ($P = 0.63$) (Fig. 2b). All patients with less than 5 $\log_{10}$ IU/mL of HCV RNA achieved SVR despite their IL-28B genotype and the SVR rates of patients with 5 $\log_{10}$ IU/mL or more of HCV RNA did not differ between IL-28B genotypes (Fig. 2c). Multivariate analysis among the factors of relapse to previous treatment response, HCV RNA at the start of re-treatment and IL-28B genotype showed that relapse after previous treatment response bore the most predictable relationship to SVR in re-treatment ($P = 0.074$).

As for the efficacy of re-treatment according to treatment duration among patients with HCV RNA negativity during re-treatment, the SVR rate of 72-week treatment was significantly higher than that of 48-week treatment (72 weeks, 73%, 29/40, vs 48 weeks, 52%, 12/25, $P < 0.05$). This significant difference was especially found in patients who attained c-EVR but not RVR on re-treatment (72 weeks, 73%, 16/22, vs 48 weeks,

Table 2 Baseline characteristics of patients and treatment factors according to the virological response in previous treatment among patients with genotype 1

Factor	Relapser in previous treatment		NR in previous treatment	
No.	54		32	
Sex: male/female	28/26		18/14	
	Previous treatment	Re-treatment	Previous treatment	Re-treatment
PEG IFN type: α-2a/α-2b	0/54	29/25	0/32	12/20
Age (years)	58.1 ± 8.1	60.3 ± 8.4	57.9 ± 8.9	59.6 ± 8.8
White blood cells (/mm^3)	4917 ± 1290	4692 ± 1035	4546 ± 1520	4462 ± 1993
Neutrophils (/mm^3)	2618 ± 846	2479 ± 805	2225 ± 1033	2105 ± 1454
Hemoglobin (g/dL)	13.9 ± 1.2	13.7 ± 1.6	13.5 ± 1.3	13.1 ± 1.9
Platelets (×10^4/mm^3)	17.1 ± 6.3	17.7 ± 6.1	14.1 ± 4.7	14.7 ± 6.2
ALT (IU/L)	75 ± 57	70 ± 76	75 ± 39	78 ± 64
Histology: activity, 0–1/2–3	20/18		9/11	
Fibrosis, 0–2/3–4	31/8		14/6	
Serum HCV RNA (KIU/mL)	1600	980	1550	800
IL-28B SNP: rs8099917; TT/TG	24/5		2/6	
ITPA SNP: rs1127354; CC/CA	15/6		5/1	
Core 70: wild/mutant	6/6		5/5	
Core 91: wild/mutant	9/3		6/4	
ISDR: 0–1/≥2	9/0		6/1	

ALT, alanine aminotransferase; HCV, hepatitis C virus; IFN, interferon; IL, interleukin; ISDR, IFN-sensitivity determining region; NR, non-response; PEG, pegylated; SNP, single nucleotide polymorphism.

38%, 5/13, $P < 0.05$) but not in patients who attained RVR or LVR (Fig. 3).

In genotype 2, the HCV RNA negative rate on re-treatment was 59% (16/27) at week 4, 85% (23/27) at week 12 and 93% (25/27) at week 24, and the SVR rate was 63% (17/27). The two patients with NR in previous treatment did not attain SVR with re-treatment. The factors associated with SVR were assessed by univariate analysis and only the factor of younger age at the start of re-treatment showed marginal significance ($P = 0.06$) (Table 4). Among the patients with RVR on re-treatment, the SVR rates were similar at 75% (6/8) to those with 24-week and 48-week treatment.

DISCUSSION

PAST STUDIES HAVE revealed that the factors of age, sex, progression of liver fibrosis, value of HCV RNA, number of mutations in the ISDR, amino acid substitutions in the core region, drug adherence and treatment duration show association with HCV eradication in PEG IFN plus ribavirin combination for naïve patients with CH-C.[3–5,25–33] Recently, the IL-28B genotype has been reported to be the most powerful factor associated with the antiviral effect of this combination therapy.[21–25] While the predictive factors for SVR in PEG IFN plus ribavirin combination therapy for naïve patients have been actively analyzed, those factors for patients who had already experienced this therapy are still unclear. Especially needing assessment is the correlation between IL-28B SNP or the previous treatment response and the antiviral effect in re-treatment. In this study, we tried to determine which factors could most effectively predict the antiviral effect in re-treatment.

In the present study, patients with relapse after the previous treatment and patients with a low serum HCV RNA level at the start of re-treatment showed significantly different results in this study of re-treatment of CH-C patients who had previously failed to attain SVR with PEG IFN plus ribavirin therapy. This result was similar to those of the EPIC[3] study on relapse and NR[17] and the SYREN trial of NR.[18] On the other hand, there was no significant difference between the influence of the IL-28B genotype and SVR. More specifically, if the previous treatment response was the same, there was no difference regardless of the IL-28B genotype. Considering this result, in re-treatment, the previous treatment response was a more effective predictive factor than IL-28B genotype. However, further investigation is needed to clarify the association between IL-28B

Table 3 Factors associated with a sustained virological response in re-treatment with PEG IFN plus ribavirin in patients with genotype 1

Factor		SVR	Non-SVR	*P*-value
No. of patients		41	45	
Age (years)		60.2 ± 7.1	59.9 ± 9.6	0.71
Sex: male/female		24/17	22/23	0.40
Serum HCV RNA (log IU/mL)		5.8 ± 1.4	6.4 ± 0.6	0.11
Serum HCV RNA: <5 log/≥5 log		11/28	0/43	<0.001
White blood cells (/mm^3)		4656 ± 1029	4566 ± 1763	0.42
Neutrophils (/mm^3)		2443 ± 804	2259 ± 1301	0.16
Hemoglobin (g/dL)		13.5 ± 1.6	13.4 ± 1.8	0.80
Platelets (×10^4/mm^3)		16.9 ± 5.7	16.3 ± 6.7	0.36
ALT (IU/L)		68 ± 69	78 ± 75	0.43
IL-28B SNP: TT/TG		17/4	9/7	0.15
ITPA SNP: CC/CA		13/3	7/4	0.39
Core 70: wild/mutant		5/4	6/7	1.00
Core 91: wild/mutant		7/3	8/5	1.00
ISDR: 0–1/≥2		9/0	6/1	0.44
PEG IFN: α-2a/α-2b		16/25	25/20	0.14
PEG IFN dose (μg/kg per week)	α-2a	2.91 ± 0.77	2.74 ± 0.69	0.61
	α-2b	1.25 ± 0.39	1.20 ± 0.32	0.59
Ribavirin dose (mg/kg per day)		9.34 ± 2.72	9.64 ± 3.20	0.51
1st treatment virological response	Relapse/NR	36/5	18/27	<0.001

ALT, alanine aminotransferase; HCV, hepatitis C virus; IFN, interferon; IL, interleukin; ISDR, IFN-sensitivity determining region; NR, non-response; PEG, pegylated; SNP, single nucleotide polymorphism; SVR, sustained virological response.

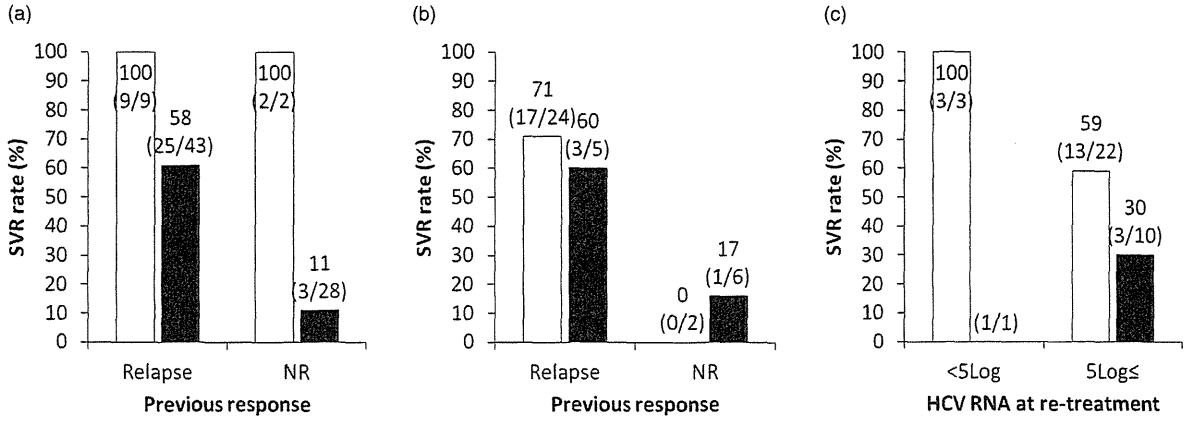

Figure 2 Sustained virological response (SVR) rates according to previous virological response, hepatitis C virus (HCV) RNA at start of re-treatment and genotype of interleukin (IL)-28B single nucleotide polymorphism (SNP) in patients with genotype 1. (a) Stratified analysis of previous virological response and HCV RNA at start of re-treatment. □, HCV RNA <5 log IU/mL at start of re-treatment; ■, HCV RNA ≥5 log IU/mL at start of re-treatment. (b) Stratified analysis of previous virological response and genotype of IL-28B SNP. □, Patients with major allele of IL-28B SNP; ■, patients with minor allele of IL-28B SNP. (c) Stratified analysis of HCV RNA at start of re-treatment and genotype of IL-28B SNP. □, Patients with major allele of IL28B SNP; ■, patients with minor allele of IL-28B SNP.

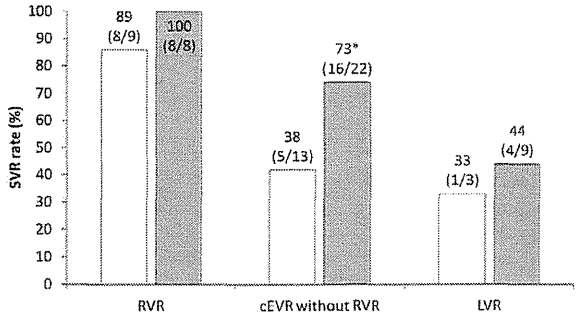

Figure 3 Sustained virological response (SVR) rates according to virological response in re-treatment and treatment duration in patients with genotype 1. □, Patients treated for 48 weeks; ▨, patients treated for 72 weeks. RVR, rapid virological response; cEVR, complete early virological response; LVR, late virological response. *$P < 0.05$; compared to 48 weeks of treatment.

genotype and antiviral effect of re-treatment because of their small number in this study. In this study, only one patient with the minor allele of IL-28B and NR in previous treatment could start and continue with the increased dose of PEG IFN (from 1.37 μg/kg in the previous treatment to 1.79 μg/kg in re-treatment) and ribavirin (from 10.3 mg/kg per day in the previous treatment to 11.1 mg/kg per day in re-treatment) and attained SVR by extended treatment. If the drug adherence does not improve, patients with the minor allele of IL-28B who show NR in the previous treatment should be treated with new drugs.

The next question is how the patients should be re-treated in order to attain SVR on re-treatment. In this study, the patients with a low serum HCV RNA level (<5 $\log_{10}$ IU/mL) at the start of re-treatment showed a significant rate of cure on re-treatment, and this is almost the same result as that previously reported.[16,17] In this study, the two patients with NR in the previous treatment and with less than 5 $\log_{10}$ IU/mL of HCV RNA level (20 KIU/mL and 52 KIU/mL of HCV RNA) at the start of re-treatment attained SVR. On the other hand, even if the previous treatment response was a relapse, the SVR rates were 58% (25/43) among the patients with 5 $\log_{10}$ IU/mL or more of HCV RNA. Because the HCV RNA level changed after the antiviral treatment, it is important to not miss the timing of when the HCV RNA level is low.

With respect to treatment duration among patients with HCV RNA negativiation during re-treatment, 72 weeks of treatment significantly increased the SVR rate compared to 48 weeks. This result was almost the same as that of the REPEAT study.[16] In our present study, the SVR rate among the patients with c-EVR but not RVR in re-treatment was significantly high by 72 weeks of treatment. On the other hand, the SVR rates among the

Table 4 Factors associated with a sustained virological response in re-treatment with PEG IFN plus ribavirin in patients with genotype 2

Factor		SVR	Non-SVR	*P*-value
No. of patients		17	10	
Age (years)		57.7 ± 8.8	63.7 ± 5.1	0.06
Sex: male/female		7/10	8/2	0.11
Serum HCV RNA (log IU/mL)		5.4 ± 1.4	6.1 ± 0.8	0.15
Serum HCV RNA: <5 log/≥5 log		5/11	1/9	0.35
White blood cells (/mm^3)		5049 ± 1355	4171 ± 910	0.10
Neutrophils (/mm^3)		2556 ± 1064	1999 ± 404	0.24
Hemoglobin (g/dL)		14.1 ± 1.3	13.8 ± 1.6	0.51
Platelets (×10^4/mm^3)		17.9 ± 5.4	14.8 ± 4.3	0.17
ALT (IU/L)		38 ± 19	48 ± 47	0.71
IL-28B SNP: TT/TG		6/2	4/2	1.00
ITPA SNP: CC/CA		5/1	4/0	1.00
PEG IFN: α-2a/α-2b		4/13	2/8	1.00
PEG IFN dose (μg/kg per week)	α-2a	3.23 ± 0.34	2.24 ± 2.25	1.00
	α-2b	1.32 ± 0.28	1.18 ± 0.23	0.21
Ribavirin dose (mg/kg per day)		10.4 ± 2.21	10.1 ± 1.31	0.44
1st treatment virological response	RVR/non-RVR	4/13	3/7	1.00

ALT, alanine aminotransferase; HCV, hepatitis C virus; IFN, interferon; IL, interleukin; ISDR, IFN-sensitivity determining region; PEG, pegylated; RVR, rapid virological response; SNP, single nucleotide polymorphism; SVR, sustained virological response.

patients with RVR in re-treatment were similar between the patients with 48 weeks and 72 weeks of treatment. Thus, patients with c-EVR but not RVR in re-treatment should be re-treated for a longer period. In order to attain better SVR, extended treatment duration is generally recommended for patients with on-treatment LVR, whereas standard treatment duration is considered to be sufficient for patients with on-treatment c-EVR. However, the present study revealed that, even if patients achieved c-EVR on re-treatment, 72 weeks of treatment seems to be better than 48 weeks for treatment-experienced patients. The majority of naïve patients showing on-treatment c-EVR could eradicate HCV with 48 weeks of treatment while some could not. In a treatment-experienced setting, patients who are able to respond early but not eradicate HCV would be selected, and therefore extended treatment may be needed.

With genotype 2, the SVR rate was relatively high (63%). The patients who could not attain SVR in re-treatment (two patients) showed NR in the previous treatment. Thus, the patients with genotype 2 and showing NR in previous treatment seemed to be difficult to treat and could be treated with other drugs. Among the patients with RVR in re-treatment, the SVR rates were similar among those with RVR in re-treatment between 24 weeks and 48 weeks of treatment. The effectiveness of extended treatment for the patients with genotype 2 in re-treatment could not be demonstrated because of their small number in this study. Further investigation is needed to clarify this.

In conclusion, this study shows that the efficacy of re-treatment for genotype 1 patients who failed to show SVR to previous treatment with PEG IFN plus ribavirin could be predicted from the previous treatment response and a low HCV RNA level at the start of re-treatment. Re-treatment for 72 weeks led to clinical improvement for genotype 1 patients with c-EVR and without RVR on re-treatment.

ACKNOWLEDGMENT

THIS WORK WAS supported by a Grant-in-Aid for Research on Hepatitis from Ministry of Health Labor and Welfare of Japan, and Scientific Research from the Ministry of Education, Science, and Culture of Japan.

REFERENCES

1 Ghany MG, Strader DB, Thomas DL, Seeff LB. Diagnosis, management, and treatment of hepatitis C: an update. *Hepatology* 2009; 49: 1335–74.
2 Hayashi N, Takehara T. Antiviral therapy for chronic hepatitis C: past, present, and future. *J Gastroenterol* 2006; 41: 17–27.
3 Manns MP, McHutchison JG, Gordon SC *et al.* Peginterferon alfa-2b plus ribavirin compared with interferon alfa-2b plus ribavirin for initial treatment of chronic hepatitis C: a randomised trial. *Lancet* 2001; 358: 958–65.
4 Fried MW, Shiffman ML, Reddy KR *et al.* Peginterferon alfa-2a plus ribavirin for chronic hepatitis C virus infection. *N Engl J Med* 2002; 347: 975–82.
5 Hadziyannis SJ, Sette H, Jr, Morgan TR *et al.* Peginterferon-alpha2a and ribavirin combination therapy in chronic hepatitis C: a randomized study of treatment duration and ribavirin dose. *Ann Intern Med* 2004; 140: 346–55.
6 Zeuzem S, Hultcrantz R, Bourliere M *et al.* Peginterferon alfa-2b plus ribavirin for treatment of chronic hepatitis C in previously untreated patients infected with HCV genotypes 2 or 3. *J Hepatol* 2004; 40: 993–9.
7 McHutchison JG, Everson GT, Gordon SC *et al.* Telaprevir with peginterferon and ribavirin for chronic HCV genotype 1 infection. *N Engl J Med* 2009; 360: 1827–38.
8 Hezode C, Forestier N, Dusheiko G *et al.* Telaprevir and peginterferon with or without ribavirin for chronic HCV infection. *N Engl J Med* 2009; 360: 1839–50.
9 McHutchison JG, Manns MP, Muir AJ *et al.* Telaprevir for previously treated chronic HCV infection. *N Engl J Med* 2010; 362: 1292–303.
10 Kumada H, Toyota J, Okanoue T, Chayama K, Tsubouchi H, Hayashi N. Telaprevir with peginterferon and ribavirin for treatment-naive patients chronically infected with HCV of genotype 1 in Japan. *J Hepatol* 2012; 56: 78–84.
11 Hayashi N, Okanoue T, Tsubouchi H, Toyota J, Chayama K, Kumada H. Efficacy and safety of telaprevir, a new protease inhibitor, for difficult-to-treat patients with genotype 1 chronic hepatitis C. *J Viral Hepat* 2012; 19: 134–42.
12 Reesink HW, Fanning GC, Farha KA *et al.* Rapid HCV-RNA decline with once daily TMC435: a phase I study in healthy volunteers and hepatitis C patients. *Gastroenterology* 2010; 138: 913–21.
13 Lok AS, Gardiner DF, Lawitz E *et al.* Preliminary study of two antiviral agents for hepatitis C genotype 1. *N Engl J Med* 2012; 366: 216–24.
14 Chayama K, Takahashi S, Toyota J *et al.* Dual therapy with the NS5A inhibitor BMS-790052 and the NS3 protease inhibitor BMS-650032 in HCV genotype 1b-infected null responders. *Hepatology* 2012; 55: 742–8.
15 Bacon BR, Shiffman ML, Mendes F *et al.* Retreating chronic hepatitis C with daily interferon alfacon-1/ribavirin after nonresponse to pegylated interferon/ribavirin: DIRECT results. *Hepatology* 2009; 49: 1838–46.
16 Jensen DM, Marcellin P, Freilich B *et al.* Re-treatment of patients with chronic hepatitis C who do not respond to peginterferon-alpha2b: a randomized trial. *Ann Intern Med* 2009; 150: 528–40.

17 Poynard T, Colombo M, Bruix J *et al.* Peginterferon alfa-2b and ribavirin: effective in patients with hepatitis C who failed interferon alfa/ribavirin therapy. *Gastroenterology* 2009; **136**: 1618–28.
18 Chevaliez S, Hezode C, Soulier A *et al.* High-dose pegylated interferon-alpha and ribavirin in nonresponder hepatitis C patients and relationship with IL-28B genotype (SYREN trial). *Gastroenterology* 2011; **141**: 119–27.
19 Berg C, Goncales FL, Jr, Bernstein DE *et al.* Re-treatment of chronic hepatitis C patients after relapse: efficacy of peginterferon-alpha-2a (40 kDa) and ribavirin. *J Viral Hepat* 2006; **13**: 435–40.
20 Oze T, Hiramatsu N, Yakushijin T *et al.* Efficacy of re-treatment with pegylated interferon plus ribavirin combination therapy for patients with chronic hepatitis C in Japan. *J Gastroenterol* 2011; **46**: 1031–7.
21 Thomas DL, Thio CL, Martin MP *et al.* Genetic variation in IL28B and spontaneous clearance of hepatitis C virus. *Nature* 2009; **461**: 798–801.
22 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.
23 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.
24 Thompson AJ, Muir AJ, Sulkowski MS *et al.* Interleukin-28B polymorphism improves viral kinetics and is the strongest pretreatment predictor of sustained virologic response in hepatitis C virus-1 patients. *Gastroenterology* 2010; **139**: 120–9.
25 Kurosaki M, Tanaka Y, Nishida N *et al.* Pre-treatment prediction of response to pegylated-interferon plus ribavirin for chronic hepatitis C using genetic polymorphism in IL28B and viral factors. *J Hepatol* 2011; **54**: 439–48.
26 Akuta N, Suzuki F, Kawamura Y *et al.* Predictive factors of early and sustained responses to peginterferon plus ribavirin combination therapy in Japanese patients infected with hepatitis C virus genotype 1b: amino acid substitutions in the core region and low-density lipoprotein cholesterol levels. *J Hepatol* 2007; **46**: 403–10.
27 Berg T, von Wagner M, Nasser S *et al.* Extended treatment duration for hepatitis C virus type 1: comparing 48 versus 72 weeks of peginterferon-alfa-2a plus ribavirin. *Gastroenterology* 2006; **130**: 1086–97.
28 Mangia A, Minerva N, Bacca D *et al.* Individualized treatment duration for hepatitis C genotype 1 patients: a randomized controlled trial. *Hepatology* 2008; **47**: 43–50.
29 Oze T, Hiramatsu N, Yakushijin T *et al.* The efficacy of extended treatment with pegylated interferon plus ribavirin in patients with HCV genotype 1 and slow virologic response in Japan. *J Gastroenterol* 2011; **46**: 944–52.
30 Oze T, Hiramatsu N, Yakushijin T *et al.* Indications and limitations for aged patients with chronic hepatitis C in pegylated interferon alfa-2b plus ribavirin combination therapy. *J Hepatol* 2011; **54**: 604–11.
31 McHutchison JG, Manns M, Patel K *et al.* Adherence to combination therapy enhances sustained response in genotype-1-infected patients with chronic hepatitis C. *Gastroenterology* 2002; **123**: 1061–9.
32 Oze T, Hiramatsu N, Yakushijin T *et al.* Pegylated interferon alpha-2b (Peg-IFN alpha-2b) affects early virologic response dose-dependently in patients with chronic hepatitis C genotype 1 during treatment with Peg-IFN alpha-2b plus ribavirin. *J Viral Hepat* 2009; **16**: 578–85.
33 Hiramatsu N, Oze T, Yakushijin T *et al.* Ribavirin dose reduction raises relapse rate dose-dependently in genotype 1 patients with hepatitis C responding to pegylated interferon alpha-2b plus ribavirin. *J Viral Hepat* 2009; **16**: 586–94.

Journal of Medical Virology 85:449–458 (2013)

Model Incorporating the *ITPA* Genotype Identifies Patients at High Risk of Anemia and Treatment Failure With Pegylated-Interferon Plus Ribavirin Therapy for Chronic Hepatitis C

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This study aimed to develop a model for predicting anemia using the inosine triphosphatase (*ITPA*) genotype and to evaluate its relationship with treatment outcome. Patients with genotype 1b chronic hepatitis C (n = 446) treated with peg-interferon alpha and ribavirin (RBV) for 48 weeks were genotyped for the *ITPA* (rs1127354) and *IL28B* (rs8099917) genes. Data mining analysis generated a predictive model for anemia (hemoglobin (Hb) concentration <10 g/dl); the CC genotype of *ITPA*, baseline Hb <14.0 g/dl, and low creatinine clearance (CLcr) were predictors of anemia. The incidence of anemia was highest in patients with Hb <14.0 g/dl and CLcr <90 ml/min (76%), followed by Hb <14.0 g/dl and *ITPA* CC (57%). Patients with Hb ≥14.0 g/dl and *ITPA* AA/CA had the lowest incidence of anemia (17%). Patients with two predictors (high-risk) had a higher incidence of anemia than the others (64% vs. 28%, $P < 0.0001$). At baseline, the *IL28B* genotype was a predictor of a sustained virological response [adjusted odds ratio 9.88 (95% confidence interval 5.01–19.48), $P < 0.0001$]. In patients who achieved an early virological response, the *IL28B* genotype was not associated with a sustained virological response, while a high risk of anemia was a significant negative predictor of a sustained virological response [0.47 (0.24–0.91), $P = 0.026$]. For high-risk patients with an early virological response, giving >80% of the planned RBV dose increased sustained virological responses by 24%. In conclusion, a predictive model incorporating the *ITPA* genotype could identify patients with a high risk of anemia and reduced probability of sustained virological response. ***J. Med. Virol. 85:449–458, 2013.***

KEY WORDS: hemolytic anemia; ribavirin; creatinine clearance; antiviral therapy

INTRODUCTION

Hepatitis C virus (HCV) infection is a leading cause of cirrhosis and hepatocellular carcinoma worldwide [Kim, 2002]. The rate of eradication of HCV by pegylated interferon (PEG-IFN) plus ribavirin (RBV), defined as a sustained virological response, is around 50% in patients with HCV genotype 1 [Manns et al., 2001; Fried et al., 2002]. Failure of treatment is attributable to the lack of a virological response or relapse after completion of therapy. Genome-wide association studies and subsequent cohort studies

Grant sponsor: Ministry of Health, Labor and Welfare, Japan.
Conflicts of interest and financial disclosures: None reported.

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Accepted 19 November 2012
DOI 10.1002/jmv.23497
Published online 7 January 2013 in Wiley Online Library (wileyonlinelibrary.com).

have shown that single nucleotide polymorphisms (SNPs) located near the *IL28B* gene are the most important determinant of virological response to PEG-IFN/RBV therapy [Ge et al., 2009; Suppiah et al., 2009; Tanaka et al., 2009; Rauch et al., 2010]. On the other hand, among patients with a virological response, the probability of a sustained virological response decreases when the patients become intolerant to therapy because of RBV-induced hemolytic anemia and receive a reduced dose of RBV [McHutchison et al., 2002; Kurosaki et al., 2012]. Genome-wide association studies have shown that variants of the inosine triphosphatase (*ITPA*) gene protect against hemolytic anemia [Fellay et al., 2010; Tanaka et al., 2011]. These variants are associated with a reduced requirement for an anemia-related dose reduction of RBV [Sakamoto et al., 2010; Thompson et al., 2010a; Kurosaki et al., 2011d; Seto et al., 2011]. However, factors other than the *ITPA* gene also contribute to the risk of severe anemia or RBV dose reduction [Ochi et al., 2010; Kurosaki et al., 2011d] and the results of studies on the impact of the *ITPA* genotype on treatment outcome are inconsistent [Ochi et al., 2010; Sakamoto et al., 2010; Thompson et al., 2010a, 2011; Kurosaki et al., 2011d].

Data mining is a novel statistical method used to extract relevant factors from a plethora of factors and combine them to predict the incidence of the outcome of interest [Breiman et al., 1980]. Decision tree analysis, a primary component of data mining analysis, has found medical applications recently [Averbook et al., 2002; Miyaki et al., 2002; Baquerizo et al., 2003; Leiter et al., 2004; Garzotto et al., 2005; Zlobec et al., 2005; Valera et al., 2007] and has proven to be a useful tool for predicting therapeutic efficacy [Kurosaki et al., 2010, 2011a,b,c, 2012] and adverse events [Hiramatsu et al., 2011] in patients with chronic hepatitis C treated with PEG-IFN/RBV therapy. Because the results of data mining analysis are presented as a flowchart [LeBlanc and Crowley, 1995], they are easily understandable and usable by clinicians lacking a detailed knowledge of statistics.

For the general application of this genetic information in clinical practice, this study aimed to construct a predictive model of severe anemia using the *ITPA* genotype, together with other relevant factors. This study also aimed to analyze the impact of the risk of anemia on treatment outcome, after adjustment for the *IL28B* genotype. These analyses were carried out at baseline and during therapy, when the early virological response became evident.

MATERIALS AND METHODS

Patients

Data were collected from a total of 446 genotype 1b chronic hepatitis C patients who were treated with PEG-IFN alpha and RBV at five hospitals and universities throughout Japan. The inclusion criteria were: (1) infection by hepatitis C genotype 1b; (2) no co-infection with hepatitis B virus or human immunodeficiency virus; (3) no other causes of liver disease such as autoimmune hepatitis and primary biliary cirrhosis; and (4) availability of DNA for the analysis of the genetic polymorphisms of *IL28B* and *ITPA*. Patients received PEG-IFN alpha-2a (180 μg) and 2b (1.5 μg/kg) subcutaneously every week and a daily weight-adjusted dose of RBV (600 mg for patients weighing <60 kg, 800 mg for patients weighing 60–80 kg, and 1,000 mg for patients weighing >80 kg) for 48 weeks. Dose reduction or discontinuation of PEG-IFN and RBV was primarily based on the recommendations on the package inserts and the discretion of the physicians at each university and hospital. The standard duration of therapy was set at 48 weeks. No patient received erythropoietin or other growth factors for the treatment of anemia. Written informed consent was obtained from each patient, and the study protocol conformed to the ethical guidelines of the Declaration of Helsinki and was approved by the institutional ethics review committees.

Laboratory Tests

Blood samples obtained before therapy were analyzed for hematologic data, blood chemistry, and HCV RNA. Genetic polymorphisms in SNPs of the *ITPA* gene (rs1127354) and the *IL28B* gene (rs8099917) were determined using ABI TaqMan Probes (Applied Biosystems, Carlsbad, CA) and the DigiTag2 assay, respectively. Baseline creatinine clearance (CLcr) levels were calculated using the formula of Cockcroft and Gault [1976]: for males, CLcr = [(140 − age in years) × body weight in kg] ÷ (72 × serum creatinine in mg/dl) and for females, CLcr = 0.85 × [(140 − age in years) × body weight in kg] ÷ (72 × serum creatinine in mg/dl). The stage of liver fibrosis was scored according to the METAVIR scoring system: F0 (no fibrosis), F1 (mild fibrosis: portal fibrosis without septa), F2 (moderate fibrosis: few septa), F3 (severe fibrosis: numerous septa without cirrhosis), and F4 (cirrhosis). A rapid virological response was defined as undetectable HCV RNA by qualitative PCR with a lower detection limit of 50 IU/ml (Amplicor, Roche Diagnostic Systems, Pleasanton, CA) at week 4 of therapy and a complete early virological response was defined as undetectable HCV RNA at week 12. A sustained virological response was defined as undetectable HCV RNA at 24 weeks after completion of therapy. Severe anemia was defined as hemoglobin (Hb) <10 g/dl.

Statistical Analysis

Database for analysis included the following variables: age, sex, body mass index, serum aspartate aminotransferase (AST) levels, alanine aminotransferase (ALT) levels, gamma-glutamyltransferase (GGT) levels, creatinine levels, CLcr, Hb, platelet count, serum levels of HCV RNA, and the stage of liver fibrosis

TABLE I. Patients' Baseline Characteristics

Age (years)	58.6	(9.6)
Gender: male (n, %)	185	(42%)
Body mass index (kg/m^2)	23.1	(3.7)
AST (IU/L)	59.9	(53.8)
ALT (IU/L)	69.8	(53.8)
GGT (IU/L)	48.5	(41.6)
Creatinine (mg/dl)	0.7	(0.2)
Creatinine clearance (ml/min)	89.5	(23.0)
Hemoglobin (g/dl)	14	(1.4)
Platelet count (10^9/L)	154.5	(52.1)
HCV RNA > 600,000 IU/ml (n, %)	354	(79%)
Liver fibrosis: F3-4 (n, %)	108	(24%)
Initial ribavirin dose (n, %)		
600 mg/day	300	(67%)
800 mg/day	138	(31%)
1,000 mg/day	9	(2%)
Pegylated interferon (n, %)		
alpha2a 180 mcg	58	(13%)
alpha2b 1.5 mcg/kg	388	(87%)
ITPA rs1127354: CC (n, %)	317	(71%)
IL28B rs809917: TT (n, %)	311	(70%)

AST, aspartate aminotransferase; ALT, alanine aminotransferase; GGT, gamma-glutamyltransferase.
Data expressed as mean (standard deviation) unless otherwise mentioned.

(Table I). Based on these data set, a model for predicting the risk of developing severe anemia was constructed by data mining analysis using the IBM-SPSS Modeler 13 as described previously [Kurosaki et al., 2010, 2011a,b,c; Hiramatsu et al., 2011]. Briefly, the software was used to explore the database automatically to search for optimal predictors that discriminated most efficiently patients with severe anemia from those without. The software also determined the optimal cutoff values of each predictor. Patients were divided into two groups according to the predictor and each of the two groups was repeatedly divided in the same way until no significant factor remained or 20 or fewer patients were in a group.

The incidence of severe anemia, the total dose of RBV, and treatment outcome were compared between groups with high and low risks of anemia. On univariate analysis, Student's *t*-test was used for continuous variables, and Fisher's exact test was used for categorical data. Logistic regression was used for multivariate analysis. *P* values of <0.05 were considered significant. SPSS Statistics 18 was used for these analyses.

RESULTS

Predictive Model of Severe Anemia

The incidence of severe anemia in the whole cohort was 49% (Fig. 1). The best predictor of severe anemia was the baseline Hb concentration. Patients with a low baseline Hb concentration (<14 g/dl) were more likely to develop severe anemia (67%) than those with a higher Hb (>14 g/dl) (34%). The second best predictor for those patients with a baseline Hb <14.0 g/dl was CLcr. Patients with a CLcr below 90 ml/min had the highest incidence of severe anemia (76%). In those with a CLcr above >90 ml/min the incidence of severe anemia was 57% in patients with the CC allele of the *ITPA* gene while it was 37% in patients with the CA or AA allele. On the other hand, the second best predictor for those patients with a baseline Hb concentration above 14 g/dl was the *ITPA* genotype. Patients with the AA or AC allele had the lowest incidence of anemia (17%). For those with the *ITPA* CC allele, CLcr was the third best predictor; the optimal cutoff value was 85 ml/min for this group. The incidence of severe anemia was 49% in patients with a CLcr below 85 ml/min while it was 32% in those with a CLcr above 85 ml/min.

Following this analysis, the patients were divided into six groups, with the incidence of severe anemia ranging from 17% to 76%. Three groups with two predictors, having an incidence of anemia >40%, were defined as the high-risk group and the remainder were defined as the low-risk group. The incidence of severe anemia was higher in the high-risk group than the low-risk group (65% vs. 28%, $P = 0.029$) (Fig. 2). Comparison of the *ITPA* genotype and the predictive model showed that the sensitivity for the prediction of severe anemia was similar (75.9% vs. 76.4%) but the specificity of the predictive model was greater (33.6% vs. 59.3%).

The Risk of Anemia Impacts on Sustained Virological Responses by Patients Who Achieved an Early Virological Response

The impact of *IL28B* genotype, *ITPA* genotype, and risk group of anemia on the rate of sustained virological response was studied at baseline and week 12. At baseline, patients with the TT allele of the *IL28B* gene had a significantly higher rate of sustained virological response than those with the TG or GG allele (43% vs. 10%, $P < 0.0001$), the high-risk group for anemia had a significantly lower rate of sustained virological response than the low-risk group (28% vs. 40%, $P = 0.011$), and the *ITPA* genotype was not associated with a sustained virological response (Fig. 3A–C). At week 4, patients with rapid virological response had a high rate of sustained virological response, irrespective of the *IL28B* genotype (TT vs. TG/GG; 97% vs. 100%, $P = 1.000$), the *ITPA* genotype (CC vs. CA/AA; 95% vs. 100%, $P = 1.000$), and the risk of anemia (high vs. low; 95% vs. 100%, $P = 1.000$). Among the patients who did not achieve a rapid virological response, those with the *IL28B* TT allele had a significantly higher rate of sustained virological response than those with the TG or GG allele (38% vs. 8%, $P < 0.0001$), and the high-risk group for anemia had a significantly lower rate of sustained virological response than the low-risk group (24% vs. 35%, $P = 0.015$). At week 12, in patients who achieved a complete early virological response, the *IL28B* genotype was not associated with a sustained virological response, while the high-risk group for anemia had a

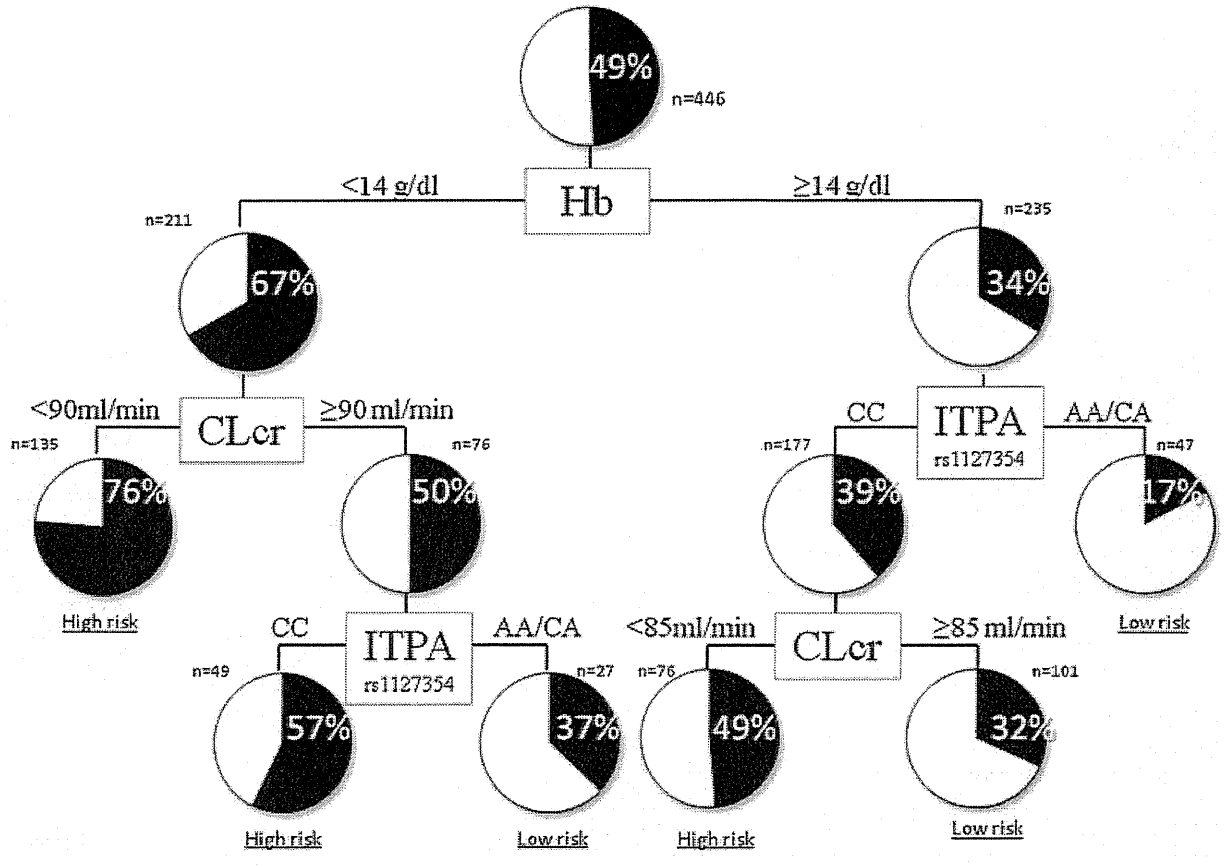

Fig. 1. The predictive model for severe anemia. The boxes indicate the factors used to differentiate patients and the cutoff values for the different groups. The pie charts indicate the rate of severe anemia (Hb <10.0 g/dl) for each group of patients, after differentiation. Terminal groups of patients differentiated by analysis are classified as at high risk if the rate is >40% and low risk if the rate is <40%. *ITPA*, inosine triphosphatase; CLcr, creatinine clearance; Hb, hemoglobin.

significantly lower rate of sustained virological response than the low-risk group (59% vs. 76%, $P = 0.013$) (Fig. 3D–F). In patients who did not achieve a complete early virological response, the *IL28B* genotype was a significant predictor of a sustained virological response (TT vs. TG/GG; 14% vs. 2%, $P < 0.0001$) but a high risk for anemia was not (high vs. low; 10% vs. 6%, $P = 0.361$).

From multivariate analysis (Table II), the *IL28B* genotype was the most important predictor of a sustained virological response at baseline [adjusted odds ratio 9.88 (95% confidence interval 5.01–19.48), $P < 0.0001$], along with female sex [0.42 (0.26–0.68), $P < 0.0001$], platelet count [1.09 (1.04–1.15), $P < 0.0001$], advanced fibrosis [0.49 (0.27–0.91), $P = 0.024$], and baseline HCV RNA load [4.14 (2.27–7.55), $P < 0.0001$]. At week 4, in patients without a rapid virological response, the *IL28B* genotype remained the most important predictor of a sustained virological response [7.16 (3.60–14.25), $P < 0.0001$], along with female sex and platelet count. At week 12, in patients with a complete early virological response, the risk of anemia was an independent and significant predictor of a sustained virological response [0.47 (0.24–0.91), $P = 0.026$], together with the platelet count and HCV RNA load, but the *IL28B* genotype was not associated with a sustained virological response. In patients without a complete early virological response, the *IL28B* genotype was a predictor of a sustained virological response [9.13 (2.02–41.3), $P = 0.004$] along with the platelet count. Thus, *IL28B* was a significant predictor of a sustained virological response at baseline and among virological non-responders at weeks 4 and 12. On the other hand, once a complete early virological response was achieved, the *IL28B* genotype was no longer associated with a sustained virological response but the risk of anemia was an independent predictor of a sustained virological response.

The Risk of Anemia, RBV Dose, and Treatment Outcome in Patients With a Complete Early Virological Response

Patients who achieved a complete early virological response were stratified according to adherence to

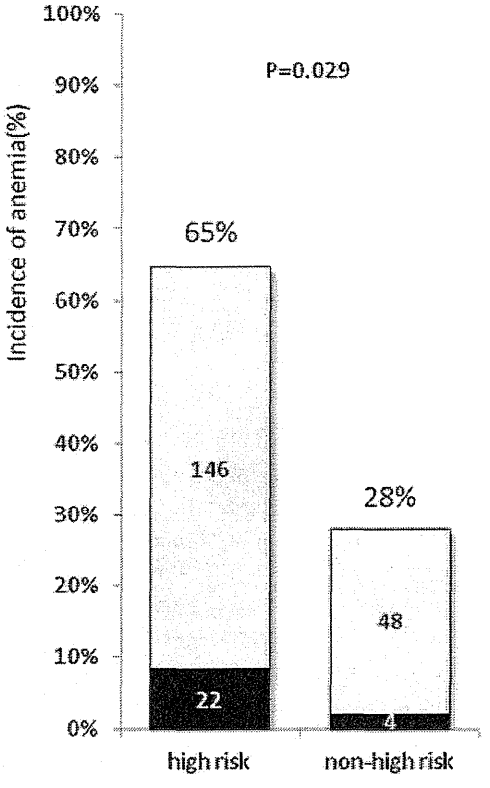

Fig. 2. The incidence of severe anemia stratified by risk of anemia. The incidence of anemia during therapy is shown for each group of patients at high and low risk of anemia. The black and white bars represent the percentages of patients with Hb concentrations below 8.5 g/dl and above 10 g/dl, respectively.

RBV (≤40%, 41–60%, 61–80%, and >80%), which showed that patients with a high risk of anemia were predominantly in subgroups with a lower adherence to RBV (≤40%, 41–60%, and 61–80%), whereas patients with a low risk of anemia were predominantly in subgroups with a higher adherence to RBV (>80%) (Fig. 4, upper panel). The percentage of patients who received >80% of the planned dose of RBV was significantly higher in the low-risk group for anemia than in the high-risk group (74% vs. 55%, $P < 0.0001$).

Within the groups with high and low risks of anemia, there was a stepwise increase in the rate of sustained virological response according to the increase in adherence to RBV (Fig. 4, lower panel). The rate of sustained virological response was higher in patients who received >80% of the planned dose of RBV than those who received less, for both high-risk patients (71% vs. 47%, $P = 0.016$) and low-risk patients (81% vs. 60%, $P = 0.072$). Within the same subgroup of RBV adherence, however, the rate of sustained virological response did not differ between patients with a high risk and a low risk of anemia. Taken together, these results suggest that patients with a high risk of anemia have a disadvantage because they are likely to be intolerant to RBV, leading to reduced adherence to RBV throughout the 48 weeks of therapy and a reduced rate of sustained virological response. However, if >80% adherence to RBV could be obtained, the rate of sustained virological response would increase by 24%.

DISCUSSION

This study confirmed previous reports that the *IL28B* genotype is the most significant predictor of a sustained virological response to PEG-IFN plus RBV therapy in chronic hepatitis C patients at baseline [Ge et al., 2009; Suppiah et al., 2009; Tanaka et al., 2009; Rauch et al., 2010; Kurosaki et al., 2011c] and at week 4 [Thompson et al., 2010b], but it had no impact on the rate of sustained virological response among those patients who achieved a complete early virological response [Thompson et al., 2010b; Kurosaki et al., 2011c]. In contrast, the risk of anemia, assessed by the combination of the *ITPA* genotype, baseline Hb concentration, and baseline CLcr, was found to be associated with a sustained virological response in patients who achieved a complete early virological response. Generally, a complete early virological response is the hallmark of a high probability of a sustained virological response, but the rate of sustained virological responses in patients who achieved a complete early virological response and had a high risk of anemia was as low as 59%. This reduced rate of sustained virological response in these patients was attributable to poor adherence to RBV throughout the 48 weeks of therapy. Because administration of >80% of the planned RBV dose increased the rate of sustained virological response by 24%, it may be postulated that personalizing the treatment schedule to achieve a sufficient dose of RBV, such as extension of treatment duration, may improve sustained virological response rates in these patients. Clearly, this postulate needs to be confirmed in future study. Thus, the findings presented here may have the potential to support selection of the optimum, personalized treatment strategy for an individual patient, based on the risk of anemia.

The degree of hemolytic anemia caused by RBV varies among individuals. A reduction of the Hb concentration early during therapy predicts the likely development of severe anemia [Hiramatsu et al., 2008, 2011] but there are no reliable predictors at baseline. A breakthrough came from the results of a genome-wide association study that revealed that variants of the *ITPA* gene are protective against hemolytic anemia [Fellay et al., 2010]. The *ITPA* genotype has been shown repeatedly to be associated with the degree of hemolytic anemia and dose reduction of RBV [Fellay et al., 2010; Sakamoto et al., 2010; Thompson et al., 2010a; Seto et al., 2011; Tanaka et al., 2011; Kurosaki et al., 2011d]. However, factors other than the *ITPA* gene, such as baseline Hb concentrations [Ochi et al., 2010; Kurosaki et al., 2011d], platelet counts [Ochi

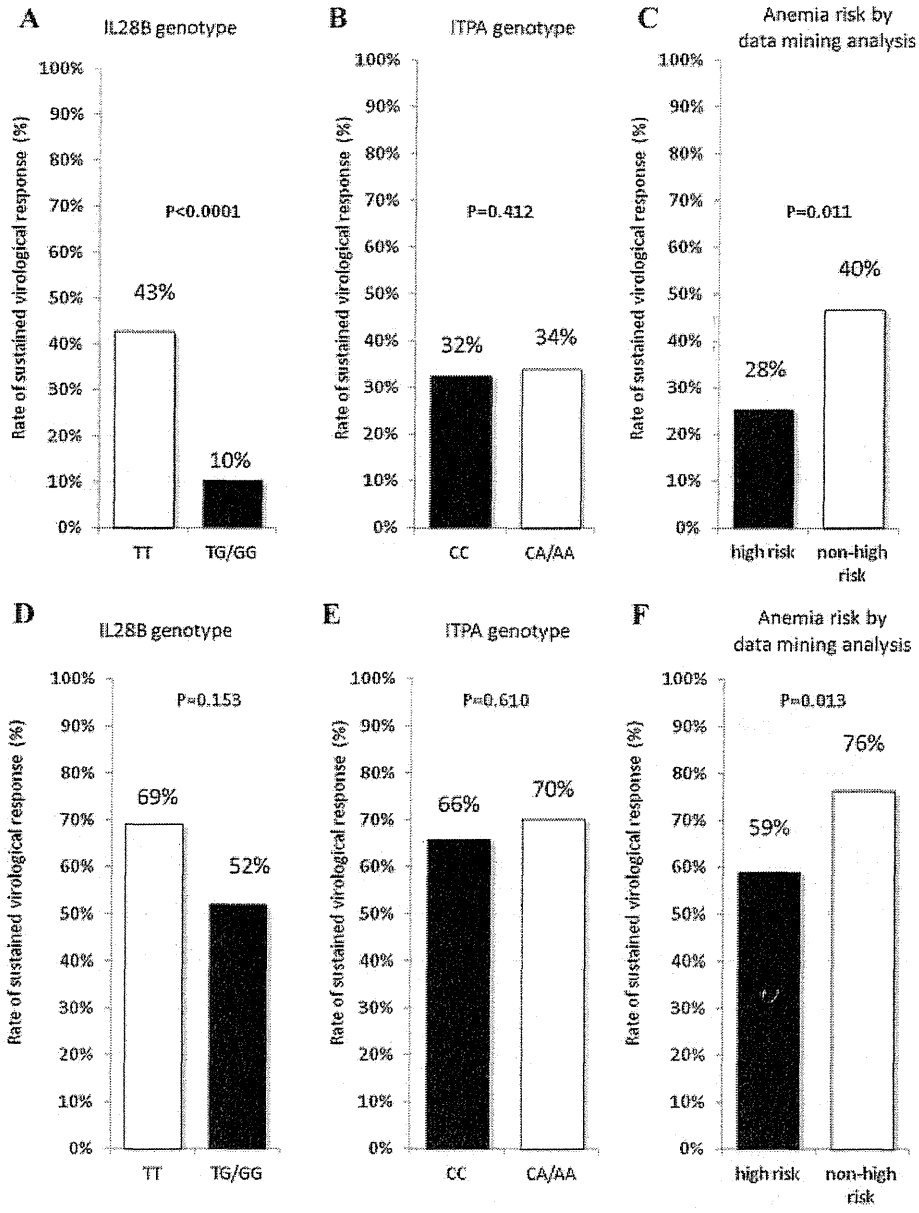

Fig. 3. Rates of sustained virological responses at baseline and among those with a virological response at week 12. The impacts of *IL28B* genotype, *ITPA* genotype, and risk group of anemia on the rate of sustained virological response were studied at baseline (**A–C**) and among those with complete early virological responses (defined as undetectable HCV RNA at week 12) (**D–F**). At baseline, those with the TT allele of the *IL28B* gene had a significantly higher rate of sustained virological response than those with the TG or GG allele and the group at high-risk of anemia had a significantly lower rate of sustained virological response than the low-risk group. Among patients with complete early virological responses, the *IL28B* genotype was not associated with a sustained virological response, while the group at high-risk of anemia had a significantly lower rate of sustained virological response than the low-risk group.

et al., 2010], and CLcr [Kurosaki et al., 2011d], also contribute to the risk of severe anemia or RBV dose reduction. In the present study, the predictive model of anemia based on the data mining analysis selected the *ITPA* genotype, baseline Hb concentration, and baseline CLcr as predictive factors and identified six subgroups of patients with a variable rate of severe anemia, ranging from 17% to 76%. The specificity of the prediction of severe anemia was improved by 25.7% in the predictive model, compared to *ITPA*

TABLE II. Logistic Regression Analysis for Factors Associated With Sustained Virological Response at Baseline, Week 4 and Week 12

	Multi-variable		
	Odds	95% CI	*P*-value
Pre-treatment			
Sex: female	0.42	0.26–0.68	<0.0001
Platelet (10^9/L)	1.09	1.04–1.15	<0.0001
Fibrosis: F3-4	0.49	0.27–0.91	0.024
HCV RNA: <600,000 IU/L	4.14	2.27–7.55	<0.0001
IL28B rs8099917: TT	9.88	5.01–19.48	<0.0001
At week 4			
Non-RVR patients			
Sex: female	0.45	0.28–0.72	0.001
Platelet (10^9/L)	1.10	1.05–1.16	0.000
IL28B rs8099917: TT	7.16	3.60–14.25	<0.0001
At week 12			
cEVR patients			
Platelet (10^9/L)	1.09	1.02–1.17	0.015
HCV RNA: <600,000 IU/L	3.21	1.39–7.55	0.007
High-risk of anemia[a]	0.47	0.24–0.91	0.026
At week 12			
Non-cEVR patients			
Platelet (10^9/L)	1.11	1.02–1.21	0.017
IL28B rs8099917: TT	9.13	2.02–41.3	0.004

RVR: rapid virological response, defined as undetectable HCV RNA at week 4.
cEVR: complete early virological response, defined as undetectable HCV RNA at week 12.
[a]High-risk of anemia defined by decision tree analysis includes the following groups: (1) baseline hemoglobin <14.0 g/dl and creatinine clearance <90 ml/min, (2) baseline hemoglobin <14.0 g/dl, creatinine clearance ≥90 ml/min and ITPA rs1127354 genotype CC, and (3) baseline hemoglobin ≥14.0 g/dl, ITPA rs1127354 genotype CC, and creatinine clearance <85 ml/min.

genotyping alone. Because hemolytic anemia induced by RBV is one of the major adverse events leading to premature termination of therapy [Fried et al., 2002], a method to predict the risk of severe anemia before treatment is important clinically. A predictive model of anemia may have the potential to support individualized treatment strategies; patients at high risk of anemia may be tested intensively for anemia or may be candidates for erythropoietin therapy, whereas those with a low risk of anemia may be treated with a higher dose of RBV. Prediction of anemia will remain important in the era of direct antiviral agents for chronic hepatitis C, because these newer therapies still require RBV and PEG-IFN in combination, and the degree of anemia complicating these therapies may be even greater than with the current combination therapy [McHutchison et al., 2009; Kwo et al., 2010].

Studies of the impact of the *ITPA* genotype on treatment outcome have produced conflicting results. Previous studies of American [Thompson et al., 2010a] and Italian [Thompson et al., 2011] cohorts did not find any association between the *ITPA* genotype and treatment outcome, whereas a marginal difference was observed in a report from Japan [Ochi et al., 2010]. Moreover, with a subgroup analysis of Japanese patients, the variant of the *ITPA* gene was associated with a sustained virological response in patients with the *IL28B* major genotype [Kurosaki et al., 2011d], in patients infected with HCV other than genotype 1[Sakamoto et al., 2010], and in patients with pre-treatment Hb concentrations between 13.5 and 15 g/dl [Azakami et al., 2011]. These inconsistent results may be because the impact of anemia may be greater on a cohort of aged patients, such as in Japan. Another reason may be that the *ITPA* genotype is not the sole determinant of anemia; the *ITPA* genotype alone was not associated with treatment outcome in the present study but a high-risk of anemia, defined by the combination of the *ITPA* genotype, baseline Hb concentration, and baseline CLcr, was associated with sustained virological responses by patients with complete early virological responses, even after adjustment for the *IL28B* genotype and other relevant factors. This is in contrast to the finding that the *IL28B* genotype is an independent and significant predictor at baseline of a sustained virological response by patients without a rapid virological response and those without a complete early virological response, but not those with a complete early virological response. These results indicate that the *IL28B* genotype could be used to predict a sustained virological response at baseline or during therapy in patients in whom HCV RNA has not yet become undetectable, but it has no predictive value in patients in whom HCV RNA has become undetectable. The risk of anemia may be used to predict sustained virological responses in a selected subgroup of patients who achieve a complete early virological response.

Patients who received more than 80% of the planned dose of PEG-IFN or RBV had a higher rate of sustained virological responses than those who received a lower cumulative dose [McHutchison et al., 2002; Davis et al., 2003]. Patients who achieve a complete early virological response usually have a good chance of a sustained virological response and the treatment duration is not extended beyond 48 weeks. However, reduced adherence to drugs in these patients was related to relapse after the completion of 48 weeks of therapy [Hiramatsu et al., 2009; Kurosaki et al., 2012]. In the present study, the rate of sustained virological response was 59% in patients who achieved a complete early virological response but had a high risk of anemia, 17% lower than in patients with a low risk of anemia. However, there was a stepwise increase in the rate of sustained virological response according to the increase in adherence to RBV, and the rate of sustained virological response was higher in high-risk patients who received >80% of the planned dose of RBV (71% vs. 47%). This 24% increase in sustained virological response was observed among the patients in the present study who received 48 weeks of treatment. These findings suggest that receiving a sufficient RBV dose is essential for patients with a complete early virological response to attain a sustained virological response and that the treatment strategy should be personalized for patients with a

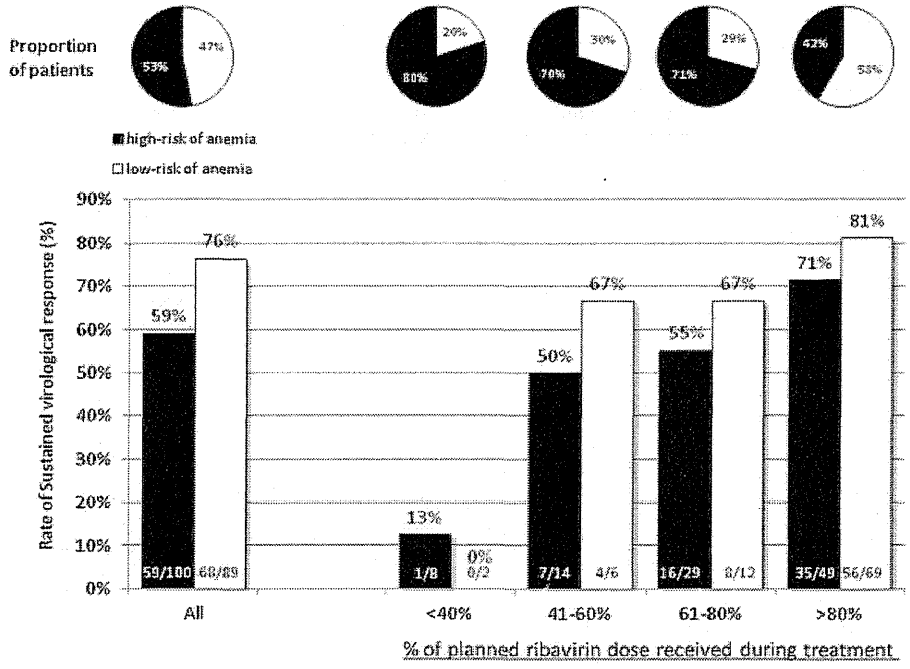

Fig. 4. The impact of risk of anemia and RBV dose on treatment outcome after a complete early virological response. Patients with complete early virological responses were divided into subgroups according to their adherence to RBV: ≤40%, 41–60%, 61–80%, and >80%. For each subgroup, the proportion of patients with a high risk and a low risk of anemia is shown in the upper panel by pie charts, and the rates of sustained virological responses, stratified by high risk and low risk of anemia, are shown in the lower panel by bar graphs. The black and white bars or charts represent patients with high and low risks of anemia, respectively.

high risk of anemia to extend the duration of treatment, even those patients with a complete early virological response, to obtain >80% adherence to RBV.

In conclusion, the combination of the *ITPA* genotype, baseline Hb concentration, and baseline CLcr could be used as a pre-treatment predictor of anemia. The risk of anemia thus identified is associated with adherence to RBV and impacts on the treatment outcome of patients who achieve a complete early virological response. This is in contrast to the major role of the *IL28B* genotype in the prediction of sustained virological responses at baseline and among non-responders at weeks 4 and 12. Patients who achieve a complete early virological response generally have a high probability of a sustained virological response but those who have a high risk of anemia have a high rate of relapse because of reduced adherence to RBV. To improve the rate of sustained virological responses in these patients, it may be postulated that the treatment schedule may be personalized to obtain >80% adherence to RBV. Clearly, this postulate needs to be confirmed in a future study.

REFERENCES

Averbook BJ, Fu P, Rao JS, Mansour EG. 2002. A long-term analysis of 1018 patients with melanoma by classic Cox regression and tree-structured survival analysis at a major referral center: Implications on the future of cancer staging. Surgery 132:589–602.

Azakami T, Hayes CN, Sezaki H, Kobayashi M, Akuta N, Suzuki F, Kumada H, Abe H, Miki D, Tsuge M, Imamura M, Kawakami Y, Takahashi S, Ochi H, Nakamura Y, Kamatani N, Chayama K. 2011. Common genetic polymorphism of ITPA gene affects ribavirin-induced anemia and effect of peg-interferon plus ribavirin therapy. J Med Virol 83:1048–1057.

Baquerizo A, Anselmo D, Shackleton C, Chen TW, Cao C, Weaver M, Gornbein J, Geevarghese S, Nissen N, Farmer D, Demetriou A, Busuttil RW. 2003. Phosphorus ans an early predictive factor in patients with acute liver failure. Transplantation 75:2007–2014.

Breiman LJH, Friedman RA, Olshen CJ, Stone CM. 1980. Classification and regression trees. Calif: Wadsworth.

Cockcroft DW, Gault MH. 1976. Prediction of creatinine clearance from serum creatinine. Nephron 16:31–41.

Davis GL, Wong JB, McHutchison JG, Manns MP, Harvey J, Albrecht J. 2003. Early virologic response to treatment with peginterferon alfa-2b plus ribavirin in patients with chronic hepatitis C. Hepatology 38:645–652.

Fellay J, Thompson AJ, Ge D, Gumbs CE, Urban TJ, Shianna KV, Little LD, Qiu P, Bertelsen AH, Watson M, Warner A, Muir AJ, Brass C, Albrecht J, Sulkowski M, McHutchison JG, Goldstein DB. 2010. ITPA gene variants protect against anaemia in patients treated for chronic hepatitis C. Nature 464: 405–408.

Fried MW, Shiffman ML, Reddy KR, Smith C, Marinos G, Goncales FL, Jr., Haussinger D, Diago M, Carosi G, Dhumeaux D, Craxi A, Lin A, Hoffman J, Yu J. 2002. Peginterferon alfa-2a plus ribavirin for chronic hepatitis C virus infection. N Engl J Med 347:975–982.

Garzotto M, Park Y, Mongoue-Tchokote S, Bledsoe J, Peters L, Blank BH, Austin D, Beer TM, Mori M. 2005. Recursive partitioning for