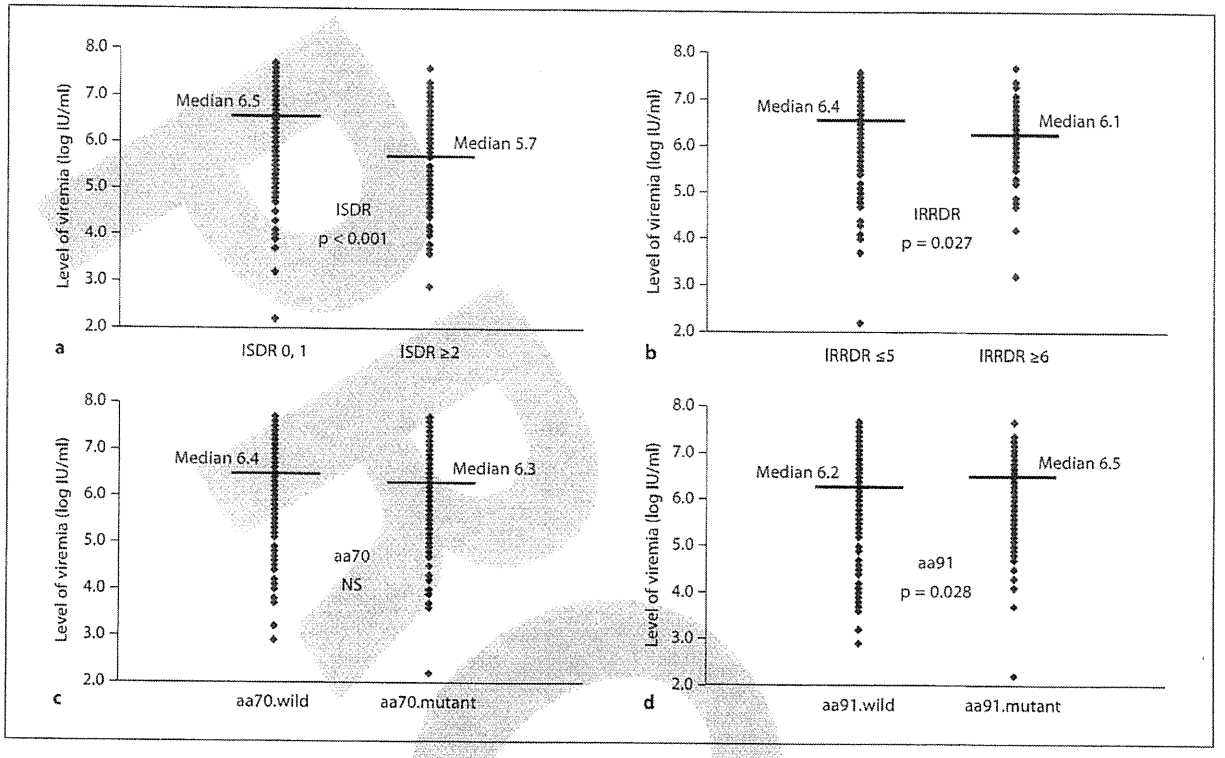

Fig. 4. Viremia level and aa substitutions in core region/ISDR/IRRDR. **a** Concerning the number of substitutions in ISDR, viremia levels of patients with WT were significantly higher than those of patients with non-WT ($p < 0.001$). **b** Concerning the number of substitutions in IRRDR, viremia levels of patients with ≤5 aa substitutions were significantly higher levels than those of patients with ≥6 ($p = 0.027$). **c** Concerning the substitution of core aa 70, viremia levels of patients with Arg70 were not significantly different from those of patients with Gln70 (His70). **d** Concerning the substitution of core aa 91, viremia levels of patients with Met91 were significantly higher than those of patients with Leu91 ($p = 0.028$). Thus, levels of viremia might be influenced by aa substitutions in core aa 91 and ISDR/IRRDR.

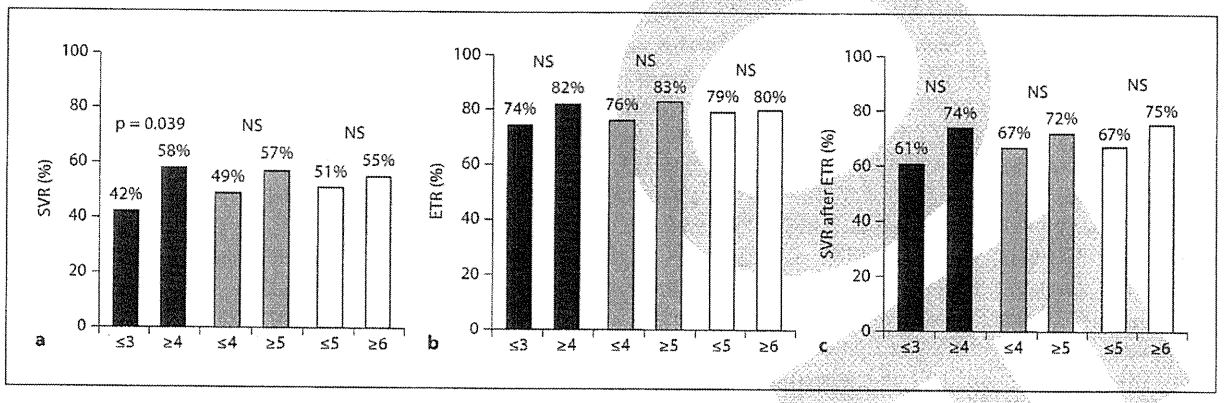

Fig. 5. Treatment response according to the number of aa substitutions in NS5A-IRRDR. **a** A significantly higher proportion of patients with ≥4 (58%) aa substitutions showed SVR compared to patients with ≤3 (42%) ($p = 0.039$), and it was useful as predictor of SVR to categorize into two groups of ≤4 and ≥5 aa substitutions by univariate analysis. **b**, **c** ETR and SVR after ETR rates were not significantly different according to the number of aa substitutions in IRRDR.

INT328327_NEU.indd 6 07.06.2011 10:26:58

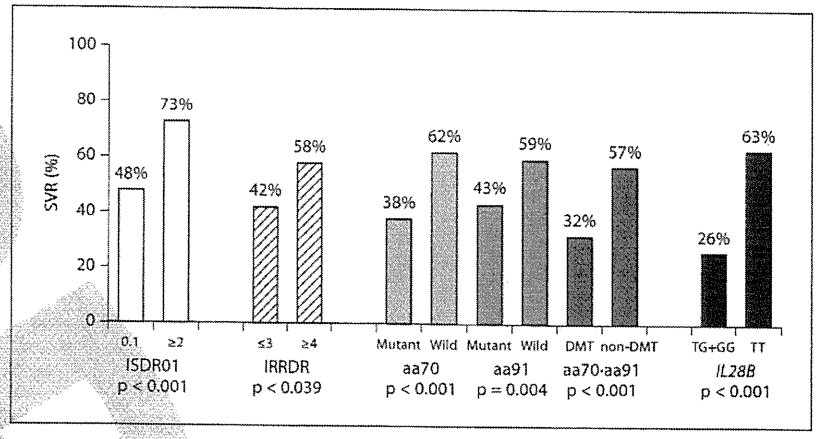

Fig. 6. SVR rate according to aa substitution in core/NS5A region and genetic variation near *IL28B* by univariate analysis.

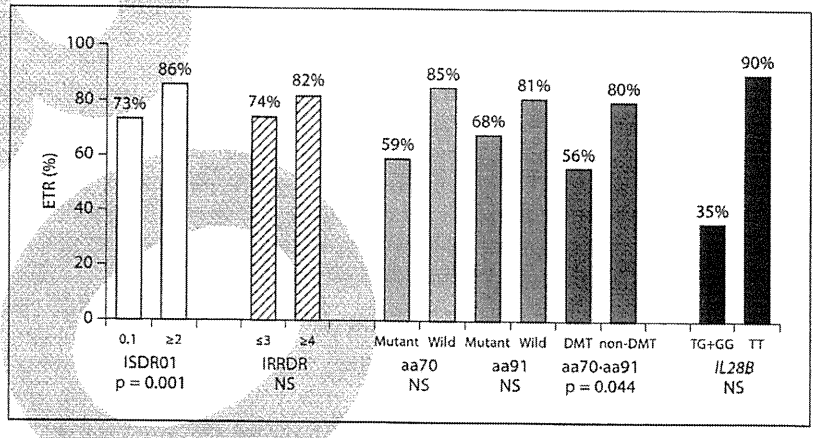

Fig. 7. ETR rate according to aa substitution in core/NS5A region and genetic variation near *IL28B* by univariate analysis.

Table 2. Factors associated with SVR to 48-week IFN + ribavirin combination therapy in patients infected with HCV-1b, identified by multivariate analysis

Factor	Category	OR (95% CI)	p
rs8099917 genotype	1: TG+GG 2: TT	1 16.7 (4.54–61.3)	<0.001
Gender	1: Female 2: Male	1 10.5 (3.47–32.3)	<0.001
ISDR of NS5A	1: WT 2: Non-WT	1 5.68 (1.22–26.3)	0.027

Only variables that achieved statistical significance ($p < 0.05$) on multivariate logistic regression are shown.

Table 3. Factors associated with ETR response to 48-week IFN + ribavirin combination therapy in patients infected with HCV-1b, identified by multivariate analysis

Factor	Category	OR (95% CI)	p
rs8099917 genotype	1: TG+GG 2: TT	1 18.2 (6.29–52.6)	<0.001
Level of viremia log IU/ml	1: ≥6.0 2: <6.0	1 9.20 (2.59–32.6)	0.001
Core aa 70	1: Gln70 (His70) 2: Arg70	1 4.68 (1.65–13.3)	0.004
Serum albumin g/dl	1: <3.9 2: ≥3.9	1 3.08 (1.11–8.47)	0.030

Only variables that achieved statistical significance ($p < 0.05$) on multivariate logistic regression are shown.

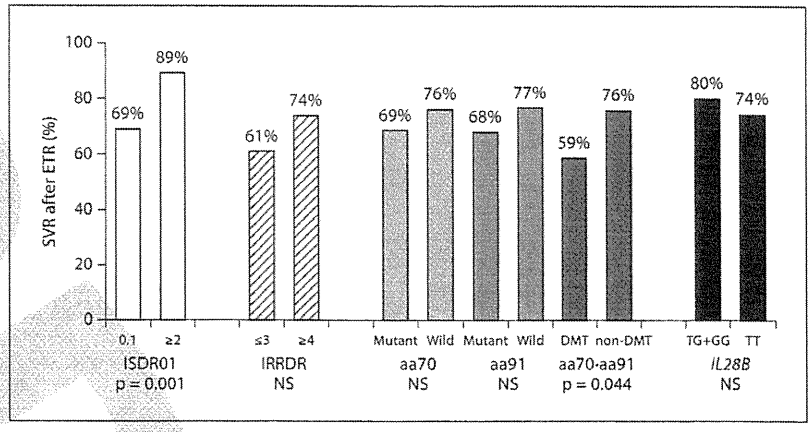

Fig. 8. SVR after ETR rate according to aa substitution in core/NS5A region and genetic variation near *IL28B* by univariate analysis.

substitution in the core/NS5A region and genetic variation near *IL28B* by univariate analysis.

Multivariate analysis that included the above variables identified 4 parameters that independently influenced ETR: genetic variation in rs8099917 (genotype TT; $p < 0.001$), level of viremia (<6.0 log IU/ml; $p = 0.001$), substitution of aa 70 (Arg70; $p = 0.004$), and albumin (≥3.9 g/dl; $p = 0.030$) (table 3).

Predictors of SVR after ETR as Determined by Uni- and Multivariate Analyses

Univariate analysis identified 11 parameters that influenced SVR after ETR: gender (male sex; $p < 0.001$), age (<55 years; $p < 0.001$), ribavirin dose (≥11.0 mg/kg; $p = 0.025$), leukocyte count (≥4,500/mm^3; $p = 0.033$), hemoglobin (≥14.0 g/dl; $p = 0.025$), platelet count (≥15.0 × 10^4/mm^3; $p = 0.001$), level of viremia (<6.0 log IU/ml; $p = 0.020$), total cholesterol (<170 mg/dl; $p = 0.017$), α-fetoprotein (<10 μg/l; $p = 0.004$), substitution of aa 70 and 91 (Arg70 and/or Leu91; $p = 0.044$), and the number of aa substitutions in ISDR (non-WT; $p = 0.001$). Figure 8 shows the SVR after ETR rate according to aa substitution in the core/NS5A region and genetic variation near *IL28B* by univariate analysis.

Multivariate analysis that included the above variables identified 6 parameters that independently influenced the SVR after ETR: gender (male sex; $p < 0.001$), ribavirin dose (≥11.0 mg/kg; $p = 0.002$), the number of aa substitutions in ISDR (non-WT; $p = 0.012$), substitution of aa 70 and 91 (Arg70 and/or Leu91; $p = 0.023$), platelet count (≥15.0 × 10^4/mm^3; $p = 0.033$), and α-fetoprotein (<10 μg/l; $p = 0.042$) (table 4).

Comparison of Factors Associated with Treatment Efficacy Identified by Multivariate Analysis

Table 5 shows the variables that achieved statistical significance on multivariate logistic regression for each evaluation of treatment efficacy. Rs8099917 genotype was an important predictor of ETR and SVR. With regard to viral factors, core region was an important predictor of ETR, and SVR after ETR. ISDR was an important predictor of SVR, and SVR after ETR. Level of viremia was an important predictor of ETR. Thus, genetic variation near *IL28B* and viral factors (core region, ISDR, and level of viremia) were important predictors of treatment efficacy. Furthermore, gender, α-fetoprotein, albumin, and platelet count were also identified as other important predictors of treatment efficacy, in addition to genetic variation near *IL28B* and viral factors.

Discussion

Using multivariate analysis, the present study identified viral- (aa substitutions in core aa 70/91 and NS5A-ISDR/IRRDR) and host-related factors (genetic variation near *IL28B* gene) that influenced treatment efficacy to 48-week IFN/ribavirin combination therapy, which is in agreement with recent findings [22, 23]. Identification of these viral and host factors before the start of IFN/ribavirin combination therapy should help to select better therapeutic regimens, including triple therapy of telaprevir/PEG-IFN/ribavirin [24–26], for those patients who are less likely to achieve SVR.

According to the number of substitutions in ISDR, a previous report showed that levels of viremia were sig-

INT328327_NEU.indd 8 07.06.2011 10:26:59

Table 4. Factors associated with SVR in patients who achieved ETR response to 48-week IFN + ribavirin combination therapy in patients infected with HCV-1b, identified by multivariate analysis

Factor	Category	OR (95% CI)	p
Gender	1: Female 2: Male	1 4.27 (2.15–8.55)	<0.001
Ribavirin dose, mg/kg	1: <11.0 2: ≥11.0	1 2.95 (1.48–5.86)	0.002
ISDR of NS5A	1: WT 2: Non-WT	1 4.00 (1.35–11.8)	0.012
Core aa 70 and 91	1: Gln70 (His70) and Met91 2: Arg70 and/or Leu91	1 2.96 (1.16–7.52)	0.023
Platelet count $\times 10^4/mm^3$	1: <15.0 2: ≥15.0	1 2.19 (1.07–4.50)	0.033
α-Fetoprotein μg/l	1: ≥10 2: <10	1 2.66 (1.04–6.80)	0.042

Only variables that achieved statistical significance ($p < 0.05$) on multivariate logistic regression are shown.

Table 5. Comparison of factors associated with efficacy of 48-week IFN + ribavirin combination therapy in patients infected with HCV-1b, identified by multivariate analysis

Factor	ETR response (at 48 weeks)	SVR after ETR response	SVR
IL28B	rs8099917 p < 0.001, 18.2 (6.29–52.6)[a]		rs8099917 p < 0.001, 16.7 (4.54–61.3)[a]
Virus	Core aa 70 p = 0.004, 4.68 (1.65–13.3)[a]	Core aa 70 and 91 p = 0.023, 2.96 (1.16–7.52)[a]	
	Level of viremia p = 0.001, 9.20 (2.59–32.6)[a]	ISDR p = 0.012, 4.00 (1.35–11.8)[a]	ISDR p = 0.027, 5.68 (1.22–26.3)[a]
Others	Albumin p = 0.030, 3.08 (1.11–8.47)[a]	α-Fetoprotein p = 0.042, 2.66 (1.04–6.80)[a]	
		Platelet count p = 0.033, 2.19 (1.07–4.50)[a]	
		Gender p < 0.001, 4.27 (2.15–8.55)[a]	Gender p < 0.001, 10.5 (3.47–32.3)[a]
		Ribavirin dose p = 0.002, 2.95 (1.48–5.86)[a]	

Only variables that achieved statistical significance ($p < 0.05$) on multivariate logistic regression are shown.
[a] OR (95% CI).

nificantly lower in patients with non-WT of ISDR than in those with WT [8]. The present study indicated that substitution of IRRDR and core aa 91, in addition to substitution of ISDR, also significantly influenced levels of viremia. Furthermore, there was a significant positive correlation between the number of aa substitutions in ISDR and those in IRRDR, and the number of aa substitutions in ISDR/IRRDR of patients with Leu91 was significantly higher than that of patients with Met91. To our knowledge, this is the first report of the relationship between viremia levels and aa substitutions in core region/ISDR/IRRDR. This result might be interpreted to mean

that core aa 91/ISDR/IRRDR might be associated with viremia levels involved in resistance to combination therapy. Further studies that examine the functional impact of aa substitutions to combination therapy should be conducted to confirm the above finding.

The present results showed that α-fetoprotein, albumin, platelet count, and gender were predictors of virological response to IFN/ribavirin combination therapy. Previous data indicated that absence of advanced liver fibrosis was a positive predictor of SVR to IFN monotherapy and IFN/ribavirin combination therapy [2, 3, 13, 27–29], and that advanced liver fibrosis was usually associated with higher levels of α-fetoprotein, and lower levels of albumin and platelet count [1, 3, 30–32]. Furthermore, gender is also a predictor of treatment response to IFN/ribavirin combination therapy [2, 3, 14]. In the present study based on a large number of patients, histopathological changes in the liver and gender were identified as independent predictors of virological response, in addition to genetic variation near *IL28B* and viral factors (core region, ISDR, and level of viremia).

In a previous study, multivariate analysis identified core region, gender, and stage of liver fibrosis as parameters that independently influenced the SVR of patients who achieved early virological response, but ISDR was not entered into uni- and multivariate analysis [3]. To our knowledge, the present study based on multivariate analysis is the first report to identify ISDR as pretreatment predictor of SVR after ETR to combination therapy. Interestingly, ISDR was not a predictor of ETR, but was a significant predictor of SVR to combination therapy. Thus, the underlying mechanisms of failure to develop SVR in those patients who achieve HCV-RNA negativity remain unclear. Further studies that examine the impact of aa substitutions of ISDR to combination therapy should be conducted to confirm the above finding.

One limitation of the present study was that aa substitutions in areas other than the core region and NS5A-ISDR/IRRDR of the HCV genome were not examined. Other limitations were differences in host factors including race [24, 33, 34] and differences in viral factors, such as the distribution of HCV-1a or -1b, and geographic diversities of HCV-1b [35]. Further large-scale prospective studies are necessary to investigate whether the present results relate to the efficacy of 48-week IFN/ribavirin combination therapy, and further understanding of the complex interaction between virus- and host- related factors should facilitate the development of more effective therapeutic regimens.

Acknowledgement

This study was supported in part by a Grant-in-Aid from the Ministry of Health, Labor and Welfare, Japan.

References

1 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: 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;48:372–380.

2 Akuta N, Suzuki F, Kawamura Y, Yatsuji H, Sezaki H, Suzuki Y, Hosaka T, Kobayashi M, Kobayashi M, Arase Y, Ikeda K, Kumada H: 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;46:403–410.

3 Akuta N, Suzuki F, Kawamura Y, Yatsuji H, Sezaki H, Suzuki Y, Hosaka T, Kobayashi M, Kobayashi M, Arase Y, Ikeda K, Kumada H: Predictors of viral kinetics to peginterferon plus ribavirin combination therapy in Japanese patients infected with hepatitis C virus genotype 1b. J Med Virol 2007;79:1686–1695.

4 Donlin MJ, Cannon NA, Yao E, Li J, Wahed A, Taylor MW, Belle SH, Di Bisceglie AM, Aurora R, Tavis JE: Pretreatment sequence diversity differences in the full-length hepatitis C virus open reading frame correlate with early response to therapy. J Virol 2007;81:8211–8224.

5 Akuta N, Suzuki F, Kawamura Y, Yatsuji H, Sezaki H, Suzuki Y, Hosaka T, Kobayashi M, Kobayashi M, Arase Y, Ikeda K, Kumada H: Amino acid substitutions in the hepatitis C virus core region are the important predictor of hepatocarcinogenesis. Hepatology 2007;46:1357–1364.

6 Fishman SL, Factor SH, Balestrieri C, Fan X, Dibisceglie AM, Desai SM, Benson G, Branch AD: Mutations in the hepatitis C virus core gene are associated with advanced liver disease and hepatocellular carcinoma. Clin Cancer Res 2009;15:3205–3213.

7 Enomoto N, Sakuma I, Asahina Y, Kurosaki M, Murakami T, Yamamoto C, Izumi N, Marumo F, Sato C: Comparison of full-length sequences of interferon sensitive and resistant hepatitis C virus 1b. Sensitivity to interferon is conferred by amino acid substitutions in the NS5A region. J Clin Invest 1995;96:224–230.

8 Enomoto N, Sakuma I, Asahina Y, Kurosaki M, Murakami T, Yamamoto C, Ogura Y, Izumi N, Marumo F, Sato C: 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.

9 El-Shamy A, Sasayama M, Nagano-Fujii M, Sasase N, Imoto S, Kim SR, Hotta H: Prediction of efficient virological response to pegylated interferon/ribavirin combination therapy by NS5A sequences of hepatitis C virus and anti-NS5A antibodies in pre-treatment sera. Microbiol Immunol 2007;51:471–482.

10 El-Shamy A, Nagano-Fujii M, Sasase N, Imoto S, Kim SR, Hotta H: Sequence variation in hepatitis C virus nonstructural protein 5A predicts clinical outcome of pegylated interferon/ribavirin combination therapy. Hepatology 2008;48:38–47.
11 Shirakawa H, Matsumoto A, Joshita S, Komatsu M, Tanaka N, Umemura T, Ichijo T, Yoshizawa K, Kiyosawa K, Tanaka E: Nagano Interferon Treatment Research Group: Pretreatment prediction of virological response to peginterferon plus ribavirin therapy in chronic hepatitis C patients using viral and host factors. Hepatology 2008;48:1753–1760.
12 Mori N, Imamura M, Kawakami Y, Saneto H, Kawaoka T, Takaki S, Aikata H, Takahashi S, Chayama K: Hiroshima Liver Study Group: Randomized trial of high-dose interferon-α-2b combined with ribavirin in patients with chronic hepatitis C: Correlation between amino acid substitutions in the core/NS5A region and virological response to interferon therapy. J Med Virol 2009;81:640–649.
13 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: Genetic variation in *IL28B* predicts hepatitis C treatment-induced viral clearance. Nature 2009;461:399–401.
14 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: Genome-wide association of *IL28B* with response to pegylated interferon-α and ribavirin therapy for chronic hepatitis C. Nat Genet 2009;41:1105–1109.
15 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: *IL28B* is associated with response to chronic hepatitis C interferon-α and ribavirin therapy. Nat Genet 2009;41:1100–1104.
16 Rauch A, Kutalik Z, Descombes P, Cai T, Di Iulio J, Mueller T, Bochud M, Battegay M, Bernasconi E, Borovicka J, Colombo S, Cerny A, Dufour JF, Furrer H, Günthard HF, Heim M, Hirschel B, Malinverni R, Moradpour D, Müllhaupt B, Witteck A, Beckmann JS, Berg T, Bergmann S, Negro F, Telenti A, Bochud PY: Swiss Hepatitis C Cohort Study; Swiss HIV Cohort Study: Genetic variation in *IL28B* is associated with chronic hepatitis C and treatment failure: a genome-wide association study. Gastroenterology 2010;138:1338–1345.
17 Thomas DL, Thio CL, Martin MP, Qi Y, Ge D, O'Huigin C, Kidd J, Kidd K, Khakoo SI, Alexander G, Goedert JJ, Kirk GD, Donfield SM, Rosen HR, Tobler LH, Busch MP, McHutchison JG, Goldstein DB, Carrington M: Genetic variation in *IL28B* and spontaneous clearance of hepatitis C virus. Nature 2009;461:798–801.
18 Akuta N, Suzuki F, Hirakawa M, Kawamura Y, Yatsuji H, Sezaki H, Suzuki Y, Hosaka T, Kobayashi M, Kobayashi M, Saitoh S, Arase Y, Ikeda K, Chayama K, Nakamura Y, Kumada H: Amino acid substitution in HCV core region and genetic variation near the interleukin-28B gene predict viral response to telaprevir with peginterferon and ribavirin. Hepatology 2010;52:421–429.
19 Kato N, Hijikata M, Ootsuyama Y, Nakagawa M, Ohkoshi S, Sugimura T, Shimotohno K: Molecular cloning of the human hepatitis C virus genome from Japanese patients with non-A, non-B hepatitis. Proc Natl Acad Sci USA 1990;87:9524–9528.
20 Ohnishi Y, Tanaka T, Ozaki K, Yamada R, Suzuki H, Nakamura Y: A high-throughput SNP typing system for genome-wide association studies. J Hum Genet 2001;46:471–477.
21 Suzuki A, Yamada R, Chang X, Tokuhiro S, Sawada T, Suzuki M, Nagasaki M, Nakayama-Hamada M, Kawaida R, Ono M, Ohtsuki M, Furukawa H, Yoshino S, Yukioka M, Tohma S, Matsubara T, Wakitani S, Teshima R, Nishioka Y, Sekine A, Iida A, Takahashi A, Tsunoda T, Nakamura Y, Yamamoto K: Functional haplotypes of PADI4, encoding citrullinating enzyme peptidylarginine deiminase 4, are associated with rheumatoid arthritis. Nat Genet 2003;34:395–402.
22 Kurosaki M, Tanaka Y, Nishida N, Sakamoto N, Enomoto N, Honda M, Sugiyama M, Matsuura K, Sugauchi F, Asahina Y, Nakagawa M, Watanabe M, Sakamoto M, Maekawa S, Sakai A, Kaneko S, Ito K, Masaki N, Tokunaga K, Izumi N, Mizokami M: 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;54:439–448.
23 Hayes CN, Kobayashi M, Akuta N, Suzuki F, Kumada H, Abe H, Miki D, Imamura M, Ochi H, Kamatani N, Nakamura Y, Chayama K: HCV substitutions and *IL28B* polymorphisms on outcome of peg-interferon plus ribavirin combination therapy. Gut 2011;60:261–267.
24 McHutchison JG, Everson GT, Gordon SC, Jacobson IM, Sulkowski M, Kauffman R, McNair L, Alam J, Muir AJ: PROVE1 Study Team: Telaprevir with peginterferon and ribavirin for chronic HCV genotype 1 infection. N Engl J Med 2009;360:1827–1838.
25 McHutchison JG, Manns MP, Muir AJ, Terrault NA, Jacobson IM, Afdhal NH, Heathcote EJ, Zeuzem S, Reesink HW, Garg J, Bsharat M, George S, Kauffman RS, Adda N, Di Bisceglie AM: PROVE3 Study Team: Telaprevir for previously treated chronic HCV infection. N Engl J Med 2010;362:1292–1303.
26 Hézode C, Forestier N, Dusheiko G, Ferenci P, Pol S, Goeser T, Bronowicki JP, Bourlière M, Gharakhanian S, Bengtsson L, McNair L, George S, Kieffer T, Kwong A, Kauffman RS, Alam J, Pawlotsky JM, Zeuzem S: PROVE2 Study Team: Telaprevir and peginterferon with or without ribavirin for chronic HCV infection. N Engl J Med 2009;360:1839–1850.
27 Jouet P, Roudot-Thoraval F, Dhumeaux D, Metreau JM: Comparative efficacy of interferon alfa in cirrhotic and noncirrhotic patients with non-A, non-B, C hepatitis. Gastroenterology 1994;106:686–690.
28 Poynard T, McHutchinson J, Goodman Z, Ling MH, Albrecht J: Is an 'a la carte' combination interferon alfa-2b plus ribavirin regimen possible for the first-line treatment in patients with chronic hepatitis C? The ALGOVIRC Group. Hepatology 2000;31:211–218.
29 Bruno S, Camma C, Di Marco V, Rumi M, Vinci M, Camozzi M, Rebucci C, Di Bona D, Colombo M, Craxi A, Mondelli MU, Pinzello G: Peginterferon alfa-2b plus ribavirin for naïve patients with genotype 1 chronic hepatitis C: a randomized controlled trial. J Hepatol 2004;41:474–481.
30 Bayati N, Silverman AL, Gordon SC: Serum α-fetoprotein levels and liver histology in patients with chronic hepatitis C. Am J Gastroenterol 1998;93:2452–2456.
31 Chu CW, Hwang SJ, Luo JC, Lai CR, Tsay SH, Li CP, Wu JC, Chang FY, Lee SD: Clinical, virological, and pathologic significance of elevated serum α-fetoprotein levels in patients with chronic hepatitis C. J Clin Gastroenterol 2001;32:240–244.
32 Hu KQ, Kyulo NL, Lim N, Elhazin B, Hillebrand DJ, Bock T: Clinical significance of elevated α-fetoprotein in patients with chronic hepatitis C, but not hepatocellular carcinoma. Am J Gastroenterol 2004;99:860–865.
33 McHutchison JG, Poynard T, Pianko S, Gordon SC, Reid AE, Dienstag J, Morgan T, Yao R, Albrecht J: The impact of interferon plus ribavirin on response to therapy in black patients with chronic hepatitis C. The International Hepatitis Interventional Therapy Group. Gastroenterology 2000;119:1317–1323.
34 Kaplan DE, Sugimoto K, Ikeda F, Stadanlick J, Valiga M, Shetty K, Reddy KR, Chang KM: T-cell response relative to genotype and ethnicity during antiviral therapy for chronic hepatitis C. Hepatology 2005;41:1365–1375.
35 Nakano I, Fukuda Y, Katano Y, Nakano S, Kumada T, Hayakawa T: Why is the interferon sensitivity-determining region (ISDR) system useful in Japan? J Hepatol 1999;30:1014–1022.

Original Paper

Intervirology

Intervirology
DOI: 10.1159/000323526

Received: August 11, 2010
Accepted after revision: November 18, 2010
Published online: February 16, 2011

Amino Acid Substitution in HCV Core Region and Genetic Variation near the *IL28B* Gene Affect Viral Dynamics during Telaprevir, Peginterferon and Ribavirin Treatment

Norio Akuta[a] Fumitaka Suzuki[a] Miharu Hirakawa[a] Yusuke Kawamura[a] Hiromi Yatsuji[a] Hitomi Sezaki[a] Yoshiyuki Suzuki[a] Tetsuya Hosaka[a] Masahiro Kobayashi[a] Mariko Kobayashi[b] Satoshi Saitoh[a] Yasuji Arase[a] Kenji Ikeda[a] Kazuaki Chayama[d] Yusuke Nakamura[c] Hiromitsu Kumada[a]

[a]Department of Hepatology, and [b]Liver Research Laboratory, Toranomon Hospital, [c]Laboratory of Molecular Medicine, Human Genome Center, Institute of Medical Science, University of Tokyo, Tokyo, and [d]Department of Medical and Molecular Science, Division of Frontier Medical Science, Programs for Biomedical Research, Graduate School of Biomedical Science, Hiroshima University, Hiroshima, Japan

Key Words
Hepatitis C virus · Core region · *IL28B* · Telaprevir · Peginterferon · Ribavirin · Viral dynamics

Abstract
Objectives: Genetic variation near the *IL28B* gene and substitution of aa 70 and 91 in the core region of HCV-1b are useful as predictors of treatment efficacy to telaprevir/pegylated interferon (PEG-IFN)/ribavirin, but its impact on viral dynamics is not clear. ***Methods:*** This study investigated predictive factors of viral dynamics during 12- or 24-week regimen of triple therapy in 80 Japanese adults infected with HCV-1b. ***Results:*** After 24 h of commencement of treatment, the proportion of patients with Arg70 and Leu91 substitutions in the core region who showed ≥3.0 log drop in HCV RNA level was significantly higher than that of patients with Gln70 (His70) and/or Met91. At 8 and 12 weeks, HCV RNA loss rate of patients with rs8099917 genotype TT near *IL28B* gene was significantly higher than that of patients with non-TT. Multivariate analysis identified substitution of aa 70 and 91 as a predictor of ≥3.0 log fall in HCV RNA level at 24 h (Arg70 and Leu91) and SVR (Arg70), and rs8099917 (TT) as a predictor of HCV RNA loss at 12 weeks and SVR. ***Conclusions:*** This study identified genetic variation near *IL28B* gene and aa substitution of the core region as predictors of viral dynamics during triple therapy. Copyright © 2011 S. Karger AG, Basel

Introduction

Hepatitis C virus (HCV) usually causes chronic infection that can result in chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma (HCC) [1, 2]. At present, treatments based on interferon (IFN), in combination with ribavirin, are mainstay for combating HCV infection. In Japan, HCV genotype 1b (HCV-1b) in high viral loads (>100 kIU/ml) accounts for more than 70% of HCV infections, making it difficult to treat patients with chronic hepatitis

KARGER

Fax +41 61 306 12 34
E-Mail karger@karger.ch
www.karger.com

© 2011 S. Karger AG, Basel
0300–5526/11/0000–0000$38.00/0

Accessible online at:
www.karger.com/int

Norio Akuta, MD
Department of Hepatology, Toranomon Hospital
2-2-2 Toranomon, Minato-ku
Tokyo 105-0001 (Japan)
Tel. +81 44 877 5111, E-Mail akuta-gi@umin.ac.jp

C [3]. Such a background calls for efficient treatments of Japanese patients with chronic HCV infection.

Even with pegylated IFN (PEG-IFN) combined with ribavirin, a sustained virological response lasting over 24 weeks after the withdrawal of treatment is achieved in at most 50% of the patients infected with HCV-1b and high viral loads [4, 5]. Recently, a new strategy was introduced in the treatment of chronic HCV infection by means of inhibiting protease in the NS3/NS4 of the HCV polyprotein. Of these, telaprevir (VX-950) was selected as a candidate agent for treatment of chronic HCV infection [6]. Later, it was found that telaprevir, when combined with PEG-IFN and ribavirin, gains a robust antiviral activity [7, 8]. Two previous studies (PROVE1 and PROVE2) showed that the 12- and 24-week regimen of telaprevir/PEG-IFN/ribavirin could achieve sustained virological response rates of 35–60 and 61–69% in patients infected with HCV-1, respectively [9, 10]. Furthermore, a recent study (PROVE3) also showed that the 24- and 48-week regimen of triple therapy could achieve sustained virological response rates of 51 and 53% in HCV-1 infected patients in whom initial PEG-IFN/ribavirin treatment failed, respectively [11].

Amino acid (aa) substitutions at positions 70 and/or 91 in the HCV core region of patients infected with HCV-1b and high viral loads are pretreatment predictors of poor virological response to PEG-IFN plus ribavirin combination therapy [12–14], and also affect clinical outcome, including hepatocarcinogenesis [15, 16]. Furthermore, genetic variations near the *IL28B* gene (rs8099917, rs12979860) on chromosome 19 as host-related factor, which encodes IFN-λ-3, are pretreatment predictors of virological response to 48-week PEG-IFN plus ribavirin combination therapy in individuals infected with HCV-1 [17–20], and also affect clinical outcome, including spontaneous clearance of HCV [21]. A recent report identified genetic variation near *IL28B* gene and aa substitution of the core region as predictors of sustained virological response to triple therapy of telaprevir/PEG-IFN/ribavirin in Japanese patients infected with HCV-1b [22]. However, it is not clear at this stage whether genetic variation near the *IL28B* gene and aa substitution of the core region can be used before therapy to predict viral dynamics during triple therapy.

The present study included 80 patients with HCV-1b and high viral loads, who received the triple therapy of telaprevir with PEG-IFN plus ribavirin. The aims of the study were to identify the pretreatment factors that could predict viral dynamics during treatment, including viral- (aa substitutions in the HCV core and NS5A regions) and host-related factors (genetic variation near *IL28B* gene).

Patients and Methods

Study Population

Between May 2008 and September 2009, 81 patients infected with HCV were recruited to this study at the Department of Hepatology in Toranomon Hospital in metropolitan Tokyo. The study protocol was in compliance with the Good Clinical Practice Guidelines and the 1975 Declaration of Helsinki, and was approved by the institutional review board. Each patient gave an informed consent before participating in this trial. Patients were divided into two groups: 20 (25%) patients were allocated to a 12-week regimen of triple therapy [telaprevir (MP-424), PEG-IFN and ribavirin] (the T12PR12 group), and 61 patients (75%) were assigned to a 24-week regimen of the same triple therapy for 12 weeks followed by dual therapy of PEG-IFN and ribavirin for 12 weeks (the T12PR24 group).

Eighty of the 81 patients met the following inclusion and exclusion criteria: (1) Diagnosis of chronic hepatitis C. (2) HCV-1b confirmed by sequence analysis. (3) HCV RNA levels of ≥5.0 log IU/ml determined by the COBAS TaqMan HCV test (Roche Diagnostics, Tokyo, Japan). (4) Japanese (Mongoloid) ethnicity. (5) Age at study entry of 20–65 years. (6) Body weight ≥35 kg and ≤120 kg at the time of registration. (7) Lack of decompensated liver cirrhosis. (8) Negativity for hepatitis B surface antigen (HBsAg) in serum. (9) Negative history of HCC. (10) No previous treatment for malignancy. (11) Negative history of autoimmune hepatitis, alcohol liver disease, hemochromatosis, and chronic liver disease other than chronic hepatitis C. (12) Negative history of depression, schizophrenia or suicide attempts, hemoglobinopathies, angina pectoris, cardiac insufficiency, myocardial infarction or severe arrhythmia, uncontrollable hypertension, chronic renal dysfunction or creatinine clearance of ≤50 ml/min at baseline, diabetes requiring treatment or fasting glucose level of ≥110 mg/dl, autoimmune disease, cerebrovascular disorders, thyroidal dysfunction uncontrollable by medical treatment, chronic pulmonary disease, allergy to medication or anaphylaxis at baseline. (13) Hemoglobin level of ≥12 g/dl, neutrophil count ≥1,500/mm^3, and platelet count of ≥100,000/mm^3 at baseline. Pregnant or breast-feeding women or those willing to become pregnant during the study and men with a pregnant partner were excluded from the study. In this study, all of the 80 patients were evaluated for the pretreatment predictors for viral dynamics during triple therapy, and 77 of the 80 patients were followed up for at least 24 weeks after the completion of treatment. The treatment efficacy was evaluated by 24 weeks after the completion of therapy (sustained virological response), based on the COBAS TaqMan HCV test (Roche Diagnostics).

Telaprevir (MP-424; Mitsubishi Tanabe Pharma, Osaka, Japan) was administered at 750 or 500 mg three times a day at an 8-hour (q8) interval after the meal. PEG-IFNα-2b (PEG-Intron; Schering Plough, Kenklworth, N.J., USA) was injected subcutaneously at a median dose of 1.5 μg/kg (range 1.3–2.0 μg/kg) once a week. Ribavirin (Rebetol; Schering Plough) was administered at 200–600 mg twice a day after breakfast and dinner (daily dose 600–1,000 mg).

PEG-IFN and ribavirin were discontinued or their doses reduced, as required, upon reduction of hemoglobin level, leukocyte count, neutrophil count or platelet count, or the development of adverse events. Thus, the dose of PEG-IFN was reduced by 50% when the leukocyte count decreased below 1,500/mm^3, neutro-

Table 1. Profile and laboratory data at commencement of telaprevir, peginterferon and ribavirin triple therapy in Japanese patients infected with HCV-1b

Demographic data	
Number of patients	80
Sex, M/F	43/37
Age, years*	55 (23–65)
History of blood transfusion	24 (20.0%)
Family history of liver disease	13 (16.3%)
Body mass index*	22.5 (13.2–32.4)
*Laboratory data**	
Level of viremia, log IU/ml	6.8 (5.1–7.6)
Serum aspartate aminotransferase, IU/l	34 (15–118)
Serum alanine aminotransferase, IU/l	42 (12–175)
Serum albumin, g/dl	3.9 (3.3–4.6)
Gamma-glutamyl transpeptidase, IU/l	36 (9–229)
Leukocyte count, per mm^3	4,800 (2,800–8,100)
Hemoglobin, g/dl	14.3 (11.7–16.8)
Platelet count, $\times 10^4/mm^3$	17.3 (9.5–33.8)
α-Fetoprotein, μg/l	4 (2–39)
Total cholesterol, mg/dl	180 (112–276)
Fasting plasma glucose, mg/dl	92 (64–125)
Treatment	
PEG-IFNα-2b dose, μg/kg*	1.5 (1.3–2.0)
Ribavirin dose, mg/kg*	11.5 (7.2–18.4)
Telaprevir dose, 1,500/2,250 mg/day	10/70
Treatment regimen (T12PR12 group/T12PR24 group)	20/60
Amino acid substitutions in the HCV-1b	
Core aa 70, arginine/glutamine (histidine)	47/33
Core aa 91, leucine/methionine	43/37
ISDR of NS5A, wild-type/non-wild-type	76/4
Genetic variation near IL28B *gene*	
rs8099917 genotype, TT/TG/GG/ND	46/30/2/2
rs12979860 genotype, CC/CT/TT/ND	43/31/2/4
Past history of IFN therapy	
Treatment naive	27
Relapsers to previous treatment	33
Nonresponders to previous treatment	20

Data are numbers and percentages of patients, except those denoted by *, which represent the median (range) values.
ND = Not determined.

phil count below 750/mm^3 or platelet count below 80,000/mm^3; PEG-IFN was discontinued when these counts decreased below 1,000/mm^3, 500/mm^3 or 50,000/mm^3, respectively. When hemoglobin decreased to <10 g/dl, the daily dose of ribavirin was reduced from 600 to 400, 800 to 600 and 1,000 to 600 mg, depending on the initial dose. Ribavirin was withdrawn when hemoglobin decreased to <8.5 g/dl. However, the dose of telaprevir (MP-424) remained the same, and its administration was stopped when the discontinuation was appropriate for the development of adverse events. In those patients who discontinued telaprevir, treatment with PEG-IFNα-2b and ribavirin was also terminated.

Table 1 summarizes the profiles and laboratory data of the 80 patients at the commencement of treatment. They included 43 males and 37 females, aged 23–65 years (median 55 years).

Measurement of HCV RNA

The antiviral effects of the triple therapy on HCV were assessed by measuring plasma HCV RNA levels. In this study, HCV RNA levels during treatment were evaluated at least once every month before, during, and after therapy. Furthermore, to investigate the pretreatment predictors for viral dynamics, HCV RNA levels during treatment were evaluated at 7 time points; 24 h, 1, 2, 4, 6, 8 and 12 weeks after the commencement of treatment. HCV RNA levels during treatment were evaluated in 80 (100%), 80 (100%), 80 (100%), 79 (98.8%), 75 (93.8%), 74 (92.5%), and 69 (86.3%) of the 80 patients, at the above time intervals, respectively. HCV RNA concentrations were determined using the COBAS TaqMan HCV test (Roche Diagnostics). The linear dynamic range of the assay was 1.2–7.8 log IU/ml, and the undetectable samples were defined as loss of HCV RNA. Especially, falls in HCV RNA levels at 24 h relative to baseline were investigated as very early dynamics.

Detection of Amino Acid Substitutions in Core and NS5A Regions of HCV-1b

With the use of HCV-J (accession No. D90208) as a reference [23], the sequence of 1–191 aa in the core protein of HCV-1b was determined and then compared with the consensus sequence constructed on 80 clinical samples to detect substitutions at aa 70 of arginine (Arg70) or glutamine/histidine (Gln70/His70) and aa 91 of leucine (Leu91) or methionine (Met91) [12]. The sequence of 2209–2248 aa in the NS5A of HCV-1b (IFN sensitivity-determining region; ISDR) reported by Enomoto et al. [24] was determined, and the numbers of aa substitutions in ISDR were defined as wild-type (0, 1) or non-wild-type (≥2). In the present study, aa substitutions of the core region and NS5A-ISDR of HCV-1b were analyzed by direct sequencing [22].

Genetic Variation near IL28B Gene

Samples for genomewide association survey were genotyped using the Illumina HumanHap610-Quad Genotyping BeadChip. Genotyping data were subjected to quality control before the data analysis. Genotyping for replication and fine mapping was performed by use of the Invader assay, TaqMan assay, or direct sequencing as described previously [25, 26].

In this study, genetic variations near *IL28B* gene (rs8099917, rs12979860), reported as the pretreatment predictors of treatment efficacy and clinical outcome [17–22], were investigated.

Statistical Analysis

Nonparametric tests (χ^2 test and Fisher's exact probability test) were used to compare the characteristics of the groups. Univariate and multivariate logistic regression analyses were used to determine those factors that significantly contributed to viral dynamics and sustained virological response. The ORs and 95%CI were also calculated. All p values less than 0.05 by the two-tailed test were considered significant. Variables that achieved statistical significance ($p < 0.05$) on univariate analysis were entered into

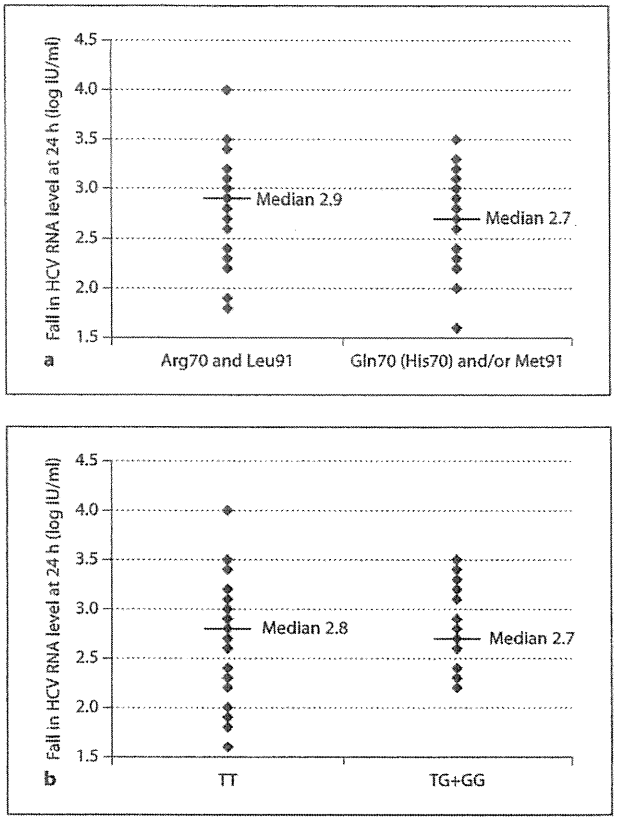

Fig. 1. a Very early dynamics according to amino acid substitutions in core region. After 24 h of commencement of the triple therapy, patients with Arg70 and Leu91 (median 2.9 log IU/ml; range 1.8–4.0 log IU/ml) significantly showed the steeper decline of HCV RNA level than those with Gln70 (His70) and/or Met91 (median 2.7 log IU/ml; range 1.6–3.5 log IU/ml). **b** Very early dynamics according to genetic variation near the *IL28B* gene. After 24 h of commencement of the triple therapy, the decline of HCV RNA level of patients with rs8099917 genotype TT (median 2.8 log IU/ml; range 1.6–4.0 log IU/ml) was not significantly different from that of patients with genotype TG and GG (median 2.7 log IU/ml; range 2.2–3.5 log IU/ml).

multiple logistic regression analysis to identify significant independent predictive factors. Each variable was transformed into categorical data consisting of two simple ordinal numbers for univariate and multivariate analyses. The potential pretreatment factors associated with treatment efficacy included the following variables: sex, age, history of blood transfusion, familial history of liver disease, body mass index, aspartate aminotransferase (AST), alanine aminotransferase (ALT), albumin, gamma-glutamyl transpeptidase (γGTP), leukocyte count, hemoglobin, platelet count, HCV RNA level, α-fetoprotein, total cholesterol, fasting blood sugar, PEG-IFN dose/body weight, ribavirin dose/body weight, telaprevir dose/day, treatment regimen of triple therapy, past history of IFN therapy, genetic variation near the *IL28B* gene, and amino acid substitution in the core region, and NS5A-ISDR. Statistical analyses were performed using the SPSS software (SPSS Inc., Chicago, Ill., USA).

Results

Virological Response to Therapy and Loss of HCV RNA during Treatment

Sustained virological response was achieved by 63.6% (49 of 77 patients). The disappearance rate of HCV RNA during treatment was 0% (0 of 80), 1.3% (1 of 80), 33.8% (27 of 80), 81.0% (64 of 79), 90.7% (68 of 75), 94.6% (70 of 74), and 89.9% (62 of 69) at 24 hours, 1, 2, 4, 6, 8, and 12 weeks, respectively.

Very Early Dynamics according to Amino Acid Substitutions in Core Region and Genetic Variation near the IL28B Gene

After 24 h of commencement of the triple therapy, the proportion of patients with Arg70 and Leu91 substitutions who showed ≥3.0 log drop in HCV RNA level (45.2%; 14 of 31 patients) was significantly higher than that of patients with Gln70 (His70) and/or Met91 (14.3%; 7 of 49) ($p = 0.004$). Thus, patients with Arg70 and Leu91 (median 2.9 log IU/ml; range 1.8–4.0 log IU/ml) significantly showed the steeper decline of HCV RNA level than those with Gln70 (His70) and/or Met91 (median 2.7 log IU/ml; range 1.6–3.5 log IU/ml) (fig. 1a).

After 24 h of commencement of treatment, the proportion of patients with rs8099917 genotype TT who showed ≥3.0 log drop in HCV RNA level (30.4%; 14 of 46 patients) was not significantly different from that of patients with genotype TG and GG (21.9%; 7 of 32). Thus, the decline of HCV RNA level of patients with genotype TT (median 2.8 log IU/ml; range 1.6–4.0 log IU/ml) was not significantly different from that of patients with genotype TG and GG (median 2.7 log IU/ml; range 2.2–3.5 log IU/ml) (fig. 1b).

Hence, the fall in HCV RNA level at 24 h was influenced by aa substitution patterns in the core region, but was independent of genetic variation near *IL28B* gene.

Rates of Loss of HCV RNA according to Amino Acid Substitutions in Core Region and Genetic Variation near the IL28B Gene

According to the substitution of core aa 70 and 91, the rate of HCV RNA loss of patients with Arg70 and Leu91 was not significantly different from that of patients with

Gln70 (His70) and/or Met91 at each time point (1, 2, 4, 6, 8 and 12 weeks).

According to genetic variation near the *IL28B* gene, the rate of HCV RNA loss at 1, 2, 4 and 6 weeks was not significantly different between rs8099917 genotype TT and non-TT (TG and GG). However, at 8 and 12 weeks, the rate of HCV RNA loss of patients with genotype TT was significantly higher than that of patients with genotype non-TT (fig. 2).

Predictive Factors Associated with ≥3.0 log Fall in HCV RNA Level at 24 Hours

Univariate analysis identified two parameters that correlated with ≥3.0 log fall in HCV RNA level at 24 h significantly: substitution of aa 70 and 91 (Arg70 and Leu91; OR 4.94, p = 0.003) and body mass index (≥25.0; OR 3.92, p = 0.022). Two factors were identified by multivariate analysis as independent parameters that either significantly ($p < 0.05$) or marginally ($p < 0.10$) influenced ≥3.0 log fall in HCV RNA level at 24 h [Arg70 and Leu91 (OR 3.99, p = 0.015) and body mass index ≥25.0 (OR 3.24, p = 0.061)] (table 2).

Predictive Factors Associated with Loss of HCV RNA at 2, 4 and 12 Weeks

Univariate analysis identified two parameters that correlated with loss of HCV RNA at 2 weeks significantly: platelet count (≥15.0 × 10^4/mm^3; OR 6.99, p = 0.014) and level of viremia (<7.0 log IU/ml; OR 3.13, p = 0.045). One factor was identified by multivariate analysis as independent parameter that either significantly or marginally influenced loss of HCV RNA at 2 weeks (platelet count ≥15.0 × 10^4/mm^3; OR 6.99, p = 0.014) (table 2).

Univariate analysis identified two parameters that correlated with loss of HCV RNA at 4 weeks significantly: history of blood transfusion (absence; OR 5.71, p = 0.006) and body mass index (≥20.0; OR 4.29, p = 0.019). Two factors were identified by multivariate analysis as independent parameters that either significantly or marginally influenced loss of HCV RNA at 4 weeks (history of blood transfusion: absence; OR 4.29, p = 0.026, and body mass index ≥20.0; OR 3.47, p = 0.069) (table 2).

Univariate analysis identified two parameters that correlated with loss of HCV RNA at 12 weeks significantly: sex (male; OR 9.52, p = 0.043) and genetic variation in rs8099917 (genotype TT; OR 9.00, p = 0.048). Two factors were identified by multivariate analysis as independent parameters that either significantly or marginally influenced loss of HCV RNA at 12 weeks (male sex; OR 11.0, p = 0.036, and rs8099917 genotype TT; OR 10.3, p = 0.042) (table 2).

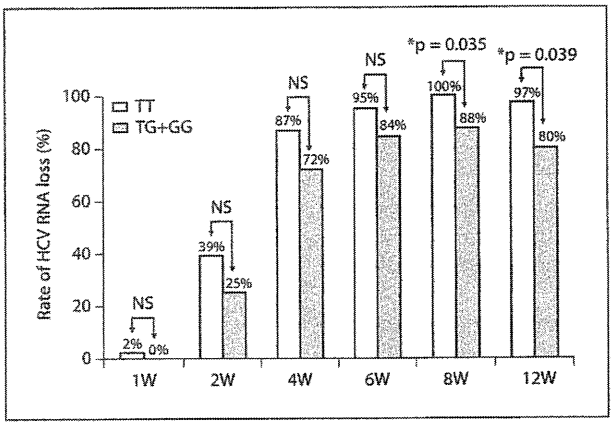

Fig. 2. Rates of loss of HCV RNA according to genetic variation near the *IL28B* gene. According to genetic variation near the *IL28B* gene, the rate of HCV RNA loss at 1, 2, 4 and 6 weeks was not significantly different between rs8099917 genotype TT and non-TT (TG and GG). However, at 8 and 12 weeks, the rate of HCV RNA loss of patients with genotype TT was significantly higher than that of patients with genotype non-TT.

Predictive Factors Associated with Sustained Virological Response

Univariate analysis identified three parameters that correlated with sustained virological response significantly: substitution of aa 70 (Arg70; OR 3.51, p = 0.011), and genetic variation in rs8099917 (genotype TT; OR 11.1, $p < 0.001$) and rs12979860 (genotype CC; OR 10.2, $p < 0.001$). Two factors were identified by multivariate analysis as independent parameters that either significantly or marginally influenced sustained virological response (rs8099917 genotype TT; OR 9.94, $p<0.001$, and Arg70; OR 3.15, p = 0.055) (table 2).

Comparison of Factors Associated with Each Treatment Efficacy Identified by Multivariate Analysis

Table 3 shows independent parameters that either significantly or marginally influenced multivariate logistic regression for each evaluation of treatment efficacy. Multivariate analysis identified substitution of aa 70 and 91 as a predictor of ≥3.0 log fall in HCV RNA level at 24 h (Arg70 and Leu91) and sustained virological response (Arg70), and rs8099917 (TT) as a predictor of HCV RNA loss at 12 weeks and sustained virological response. Thus, genetic variation near *IL28B* gene and aa substitution of the core region affect viral dynamics of different phases during triple therapy.

Table 2. Factors associated with treatment efficacy of telaprevir, peginterferon and ribavirin triple therapy in Japanese patients infected with HCV-1b, identified by univariate and multivariate analysis

	Factor	Category	Univariate logistic regression		Multivariate logistic regression	
			OR (95% CI)	p	OR (95% CI)	p
A	≥3.0 log fall in HCV RNA at 24 h					
	Substitution of aa 70 and 91	1: Gln70 (His70) and/or Met91	1		1	
		2: Arg70 and Leu91	4.94 (1.70–14.4)	0.003	3.99 (1.31–12.2)	0.015
	Body mass index	1: <25.0	1		1	
		2: ≥25.0	3.92 (1.22–12.6)	0.022	3.24 (0.95–11.1)	0.061
B	HCV RNA loss at 2 weeks					
	Platelet count, $\times 10^4/mm^3$	1: <15.0	1		1	
		2: ≥15.0	6.99 (1.49–32.8)	0.014	6.99 (1.49–32.8)	0.014
	Level of viremia, log IU/ml	1: ≥7.0	1		–	–
		2: <7.0	3.13 (1.02–9.52)	0.045	–	–
C	HCV RNA loss at 4 weeks					
	History of blood transfusion	1: presence	1		1	
		2: absence	5.71 (1.66–19.6)	0.006	4.29 (1.86–15.6)	0.026
	Body mass index	1: <20.0	1		1	
		2: ≥20.0	4.29 (1.26–14.5)	0.019	3.47 (0.91–13.3)	0.069
D	HCV RNA loss at 12 weeks					
	Sex	1: female	1		1	
		2: male	9.52 (1.08–83.3)	0.043	11.0 (1.16–100)	0.036
	rs8099917 genotype	1: TG+GG	1		1	
		2: TT	9.00 (1.02–79.5)	0.048	10.3 (1.08–98.0)	0.042
E	Sustained virological response					
	rs8099917 genotype	1: TG+GG	1		1	
		2: TT	11.1 (3.68–33.5)	<0.001	9.94 (3.05–32.4)	<0.001
	Substitution of aa 70	1: Gln70 (His70)	1		1	
		2: Arg70	3.51 (1.33–9.26)	0.011	3.15 (0.97–10.2)	0.055
	rs12979860 genotype	1: CT+TT	1		–	–
		2: CC	10.2 (3.33–3.13)	<0.001	–	–

Variables that achieved statistical significance ($p < 0.05$) on univariate analysis were entered into multiple logistic regression analysis to identify significant independent predictive factors.

The other significant predictors of HCV RNA loss were platelet count ($\geq 15.0 \times 10^4/mm^3$) at 2 weeks, history of blood transfusion (absence) at 4 weeks, and sex (male) at 12 weeks.

Discussion

Thompson et al. [27] reported that genetic variation near *IL28B* gene was also associated with increased on-treatment and sustained virological response and effectively predicted treatment outcome in treatment-naive HCV-1 patients treated with PEG-IFN plus ribavirin. However, HCV RNA loss at 4 weeks (rapid virological response) was a strong predictor of sustained virological response regardless of genetic variation near the *IL28B* gene. This phenomenon probably explains why it might be important to identify the pretreatment factors that could predict viral dynamics during treatment. The present study is the first to identify the pretreatment factors that could predict viral dynamics during triple therapy in patients infected with HCV-1. These results should be interpreted with caution since races other than Japanese and the patients infected with HCV-1a were not included. Any generalization of the results should await confirmation by studies including patients of other races and with HCV-1a to explore whether genetic variation near *IL28B* gene and aa substitution

Table 3. Comparison of factors associated with treatment efficacy of telaprevir, peginterferon and ribavirin triple therapy in Japanese patients infected with HCV-1b identified by multivariate analysis

Factor	≥3.0 log fall in HCV RNA (at 24 h)	HCV RNA loss (at 2 weeks)	HCV RNA loss (at 4 weeks)	HCV RNA loss (at 12 weeks)	Sustained virological response
Core aa 70 and 91	Arg70 and Leu91 p = 0.015 3.99 (1.31–12.2)*				Arg70 p = 0.055 3.15 (0.97–10.2)*
IL28B rs8099917				genotype TT p = 0.042 10.3 (1.08–98.0)*	genotype TT p < 0.001 9.94 (3.05–32.4)*
Others	body mass index p = 0.061 3.24 (0.95–11.1)*	platelet count p = 0.014 6.99 (1.49–32.8)*	body mass index p = 0.069 3.47 (0.91–13.3)* history of blood transfusion p = 0.026 4.29 (1.86–15.6)*	sex p = 0.036 11.0 (1.16–100)*	

Only variables that achieved statistical significance ($p < 0.05$) or marginal significance ($p < 0.10$) on multivariate logistic regression are shown. * OR (95% CI).

of core region also affect viral dynamics during triple therapy.

Two studies showed that aa substitution of the core region and genetic variation near *IL28B* gene affected viral dynamics during treatment, and sustained virological response to 48-week PEG-IFN plus ribavirin therapy in patients infected with HCV-1 [27, 28]. Furthermore, a recent report also showed that aa substitutions of core region might be used to predict very early dynamics (within 48 h) after the start of triple therapy of telaprevir with PEG-IFN and ribavirin [29]. In the present study, multivariate analysis identified substitution of aa 70 and 91 as a predictor of ≥3.0 log fall in HCV RNA level at 24 hours (i.e. viral dynamics of very early phase) and sustained virological response, and rs8099917 as a predictor of HCV RNA loss at 12 weeks (i.e. viral dynamics of later phase) and sustained virological response. This study is the first to report that genetic variation near *IL28B* gene and aa substitution of the core region affect viral dynamics of different phases during triple therapy, and probably explains why the combination of these independent factors is very useful as pretreatment predictors of sustained virological response by triple therapy [22]. The underlying mechanisms of the different viral dynamics to treatment are still unclear, and further studies based on a larger number of patients are necessary to investigate the present results.

Previous data indicated that absence of advanced liver fibrosis and male gender were positive predictors of virological response to 48-week PEG-IFN plus ribavirin therapy [13, 28]. The present study also showed that higher levels of platelet count at 2 weeks, as a surrogate marker of milder liver fibrosis, and male gender at 12 weeks were significant positive predictors of HCV RNA loss during triple therapy. The other positive predictors were absence of history of blood transfusion at 4 weeks and higher levels of body mass index at 24 h and 4 weeks, but the underlying mechanisms are still unclear. Thus, this report identified the pretreatment factors that could predict viral dynamics during triple therapy, but this study, based on a small number of patients, might provide misleading results (e.g. possible type error). Further studies of a larger number of patients are required to explore predictors, including viral- and host-related factors.

The limitations of the present study were that aa substitutions in areas other than the core region and NS5A-ISDR of the HCV genome, such as the interferon/ribavirin resistance determining region (IRRDR) [30], were not examined. Furthermore, HCV mutants with aa conversions for resistance to telaprevir during triple therapy, such as the 156S mutation [31], were also not investigated. In this regard, telaprevir-resistant HCV mutants were reported to be susceptible to IFN in both in vivo and in vitro studies [32, 33]. Thus, viral factors before and during triple therapy should be investigated in

future studies, and identification of these factors should facilitate the development of more effective therapeutic regimens.

In conclusion, this study identified genetic variation near *IL28B* gene and aa substitution of the core region as predictors of viral dynamics during triple therapy of telaprevir/PEG-IFN/ribavirin in Japanese patients infected with HCV-1b. Further large-scale prospective studies are necessary to investigate whether the present results relate to the efficacy of the triple therapy, and further understanding of the complex interaction between virus- and host-related factors should facilitate the development of more effective therapeutic regimens.

Acknowledgment

This study was supported in part by a Grant-in-Aid from the Ministry of Health, Labor and Welfare, Japan.

References

1 Niederau C, Lange S, Heintges T, Erhardt A, Buschkamp M, Hürter D, Nawrocki M, Kruska L, Hensel F, Petry W, Häussinger D: Progress of chronic hepatitis C: results of a large, prospective cohort study. Hepatology 1998;28:1687–1695.

2 Kenny-Walsh E: Clinical outcomes after hepatitis C infection from contaminated anti-D immune globulin: Irish Hepatology Research Group. N Engl J Med 1999;340:1228–1233.

3 Tsubota A, Arase Y, Someya T, Suzuki Y, Suzuki F, Saitoh S, Ikeda K, Akuta N, Hosaka T, Kobayashi M, Kumada H: Early viral kinetics and treatment outcome in combination of high-dose interferon induction vs. pegylated interferon plus ribavirin for naive patients infected with hepatitis C virus of genotype 1b and high viral load. J Med Virol 2005;75:27–34.

4 Manns MP, McHutchison JG, Gordon SC, Rustgi VK, Shiffman M, Reindollar R, Goodman ZD, Koury K, Ling MH, Albrecht JK: Peginterferon alfa-2b plus ribavirin compared with interferon alfa-2b plus ribavirin for initial treatment of chronic hepatitis C: a randomized trial. Lancet 2001;358:958–965.

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

6 Lin C, Kwong AD, Perni RB: Discovery and development of VX-950, a novel, covalent, and reversible inhibitor of hepatitis C virus NS3.4A serine protease. Infect Disord Drug Targets 2006;6:3–16.

7 Modi AA, Hoofnagle JH: New therapies for hepatitis C. Hepatology 2007;46:615–617.

8 Zeuzem S: Telaprevir, peginterferon alfa-2a, and ribavirin for 28 days in chronic hepatitis C patients. J Hepatol 2008;49:157–159.

9 McHutchison JG, Everson GT, Gordon SC, Jacobson IM, Sulkowski M, Kauffman R, McNair L, Alam J, Muir AJ, PROVE1 Study Team: Telaprevir with peginterferon and ribavirin for chronic HCV genotype 1 infection. N Engl J Med 2009;360:1827–1838.

10 Hézode C, Forestier N, Dusheiko G, Ferenci P, Pol S, Goeser T, Bronowicki JP, Bourlière M, Gharakhanian S, Bengtsson L, McNair L, George S, Kieffer T, Kwong A, Kauffman RS, Alam J, Pawlotsky JM, Zeuzem S, PROVE2 Study Team: Telaprevir and peginterferon with or without ribavirin for chronic HCV infection. N Engl J Med 2009;360:1839–1850.

11 McHutchison JG, Manns MP, Muir AJ, Terrault NA, Jacobson IM, Afdhal NH, Heathcote EJ, Zeuzem S, Reesink HW, Garg J, Bsharat M, George S, Kauffman RS, Adda N, Di Bisceglie AM, PROVE3 Study Team: Telaprevir for previously treated chronic HCV infection. N Engl J Med 2010;362:1292–1303.

12 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: Association of amino acid substitution pattern in core protein of hepatitis C virus genotype1b high viral load and non-virological response to interferon-ribavirin combination therapy. Intervirology 2005;48:372–380.

13 Akuta N, Suzuki F, Kawamura Y, Yatsuji H, Sezaki H, Suzuki Y, Hosaka T, Kobayashi M, Kobayashi M, Arase Y, Ikeda K, Kumada H: 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;46:403–410.

14 Donlin MJ, Cannon NA, Yao E, Li J, Wahed A, Taylor MW, Belle SH, Di Bisceglie AM, Aurora R, Tavis JE: Pretreatment sequence diversity differences in the full-length hepatitis C virus open reading frame correlate with early response to therapy. J Virol 2007;81:8211–8224.

15 Akuta N, Suzuki F, Kawamura Y, Yatsuji H, Sezaki H, Suzuki Y, Hosaka T, Kobayashi M, Kobayashi M, Arase Y, Ikeda K, Kumada H: Amino acid substitutions in the hepatitis C virus core region are the important predictor of hepatocarcinogenesis. Hepatology 2007;46:1357–1364.

16 Fishman SL, Factor SH, Balestrieri C, Fan X, Dibisceglie AM, Desai SM, Benson G, Branch AD: Mutations in the hepatitis C virus core gene are associated with advanced liver disease and hepatocellular carcinoma. Clin Cancer Res 2009;15:3205–3213.

17 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: Genetic variation in IL28B predicts hepatitis C treatment-induced viral clearance. Nature 2009;461:399–401.

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

19 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: IL28B is associated with response to chronic hepatitis C interferon-alpha and ribavirin therapy. Nat Genet 2009;41:1100–1104.

20 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;138:1338–1345.

21 Thomas DL, Thio CL, Martin MP, Qi Y, Ge D, O'Huigin C, Kidd J, Kidd K, Khakoo SI, Alexander G, Goedert JJ, Kirk GD, Donfield SM, Rosen HR, Tobler LH, Busch MP, McHutchison JG, Goldstein DB, Carrington M: Genetic variation in IL28B and spontaneous clearance of hepatitis C virus. Nature 2009;461:798–801.

22 Akuta N, Suzuki F, Hirakawa M, Kawamura Y, Yatsuji H, Sezaki H, Suzuki Y, Hosaka T, Kobayashi M, Kobayashi M, Saitoh S, Arase Y, Ikeda K, Chayama K, Nakamura Y, Kumada H: Amino acid substitution in HCV core region and genetic variation near *IL28B* gene predict viral response to telaprevir with peginterferon and ribavirin. Hepatology 2010;52:421–429.
23 Kato N, Hijikata M, Ootsuyama Y, Nakagawa M, Ohkoshi S, Sugimura T, Shimotohno K: Molecular cloning of the human hepatitis C virus genome from Japanese patients with non-A, non-B hepatitis. Proc Natl Acad Sci USA 1990;87:9524–9528.
24 Enomoto N, Sakuma I, Asahina Y, Kurosaki M, Murakami T, Yamamoto C, Ogura Y, Izumi N, Marumo F, Sato C: 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.
25 Ohnishi Y, Tanaka T, Ozaki K, Yamada R, Suzuki H, Nakamura Y: A high-throughput SNP typing system for genome-wide association studies. J Hum Genet 2001;46:471–477.
26 Suzuki A, Yamada R, Chang X, et al: Functional haplotypes of PADI4, encoding citrullinating enzyme peptidylarginie deminase 4, are associated with rheumatoid arthritis. Nat Genet 2003;34:395–402.
27 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;139:120–129.
28 Akuta N, Suzuki F, Kawamura Y, Yatsuji H, Sezaki H, Suzuki Y, Hosaka T, Kobayashi M, Kobayashi M, Arase Y, Ikeda K, Kumada H: Predictors of viral kinetics to peginterferon plus ribavirin combination therapy in Japanese patients infected with hepatitis C virus genotype 1b. J Med Virol 2007;79:1686–1695.
29 Akuta N, Suzuki F, Hirakawa M, Kawamura Y, Yatsuji H, Sezaki H, Suzuki Y, Hosaka T, Kobayashi M, Kobayashi M, Saitoh S, Arase Y, Ikeda K, Kumada H: Amino acid substitutions in the hepatitis C virus core region of genotype 1b affect very early viral dynamics during treatment with telaprevir, peginterferon, and ribavirin. J Med Virol 2010;82: 575–582.
30 El-Shamy A, Nagano-Fujii M, Sasase N, Imoto S, Kim SR, Hotta H: Sequence variation in hepatitis C virus nonstructural protein 5A predicts clinical outcome of pegylated interferon/ribavirin combination therapy. Hepatology 2008;48:38–47.
31 Lin C, Gates CA, Rao BG, Brennan DL, Fulghum JR, Luong YP, Frantz JD, Lin K, Ma S, Wei YY, Perni RB, Kwong AD: In vitro studies of cross-resistance mutations against two hepatitis C virus serine protease inhibitors, VX-950 and BILN 2061. J Biol Chem 2005;280:36784–36791.
32 Forestier N, Reesink HW, Weegink CJ, McNair L, Kieffer TL, Chu HM, Purdy S, Jansen PL, Zeuzem S: Antiviral activity of telaprevir (VX-950) and peginterferon alfa-2a in patients with hepatitis C. Hepatology 2007;46: 640–648.
33 Zhou Y, Müh U, Hanzelka BL, Bartels DJ, Wei Y, Rao BG, Brennan DL, Tigges AM, Swenson L, Kwong AD, Lin C: Phenotypic and structural analyses of hepatitis C virus NS3 protease Arg155 variants: sensitivity to telaprevir (VX-950) and interferon alpha. J Biol Chem 2007;282:22619–22628.

Large-Scale Long-Term Follow-Up Study of Japanese Patients With Non-Alcoholic Fatty Liver Disease for the Onset of Hepatocellular Carcinoma

Yusuke Kawamura, MD[1,3], Yasuji Arase, MD[1-3], Kenji Ikeda, MD[1,3], Yuya Seko, MD[1,3], Norihiro Imai, MD[1,3], Tetsuya Hosaka, MD[1,3], Masahiro Kobayashi, MD[1,3], Satoshi Saitoh, MD[1,3], Hitomi Sezaki, MD[1,3], Norio Akuta, MD[1,3], Fumitaka Suzuki, MD[1,3], Yoshiyuki Suzuki, MD[1,3], Yuki Ohmoto, MD[2,3], Kazuhisa Amakawa, MD[2,3], Hiroshi Tsuji, MD[2,3] and Hiromitsu Kumada, MD[1,3]

OBJECTIVES: **The aim of this study was to determine the incidence and risk factors of hepatocellular carcinoma (HCC), and to elucidate the utility of two non-invasive predictive procedures for liver fibrosis: the aspartate aminotransferase (AST) to platelet ratio index (APRI) and the BARD score (which includes the following three variables: body mass index, AST/alanine aminotransferase ratio, and diabetes) in the prediction of HCC in a large population of Japanese patients with non-alcoholic fatty liver disease (NAFLD).**

METHODS: **This was a retrospective cohort study conducted at a public hospital. Study subjects included 6,508 patients with NAFLD diagnosed by ultrasonography. The median follow-up period was 5.6 years. The primary end point was the onset of HCC. Evaluation was performed using Kaplan–Meier methodology and Cox's proportional hazards analysis.**

RESULTS: **In all, 16 (0.25%) new cases with HCC were diagnosed during the study. The cumulative rates of NAFLD-related HCC were 0.02% at year 4, 0.19% at year 8, and 0.51% at year 12. The annual rate of new HCC was 0.043%. Multivariate analysis identified serum AST level ≥40 IU/L (hazard ratio (HR): 8.20; 95% confidence interval (95% CI): 2.56–26.26; $P<0.001$), platelet count $<150\times10^3/\mu l$ (HR: 7.19; 95% CI: 2.26–23.26; $P=0.001$), age ≥60 years (HR: 4.27; 95% CI: 1.30–14.01; $P=0.017$), and diabetes (HR: 3.21; 95% CI: 1.09–9.50; $P=0.035$) as independent risk factors for HCC. With regard to the APRI, 184 patients (2.83%) were considered to have significant fibrosis (equivalent to non-alcoholic steatohepatitis (NASH) stage 3–4). The cumulative rate of HCC was significantly higher in this group (HR: 25.03; 95% CI: 9.02–69.52; $P<0.001$). In contrast, regarding the BARD score, 3,841 (59%) patients were considered to have advanced fibrosis (NASH stage 3–4). However, no significant associations between the BARD score and the incidence of HCC were observed (HR: 1.16; 95% CI: 0.40–3.37; $P=0.780$).**

CONCLUSIONS: **This retrospective study indicates that the annual incidence rate of HCC among Japanese NAFLD patients is low. Elderly NAFLD patients with diabetes, elevated serum AST, and especially thrombocytopenia (suggested to be associated with advanced liver fibrosis) should be monitored carefully during follow-up that includes using the APRI to ensure early diagnosis and treatment of HCC.**

Am J Gastroenterol advance online publication, 18 October 2011; doi:10.1038/ajg.2011.327

INTRODUCTION

Hepatocellular carcinoma (HCC) is a common malignancy worldwide, and its incidence is increasing in Asia and in the United States (1–3). Chronic viral hepatitis and liver cirrhosis after infection with hepatitis B and C viruses have important roles in the development of HCC (4–5). However, a substantial proportion (5–10%) of Japanese patients with HCC are negative for markers of hepatitis B and C viruses (6–8). In addition to viral infection, non-alcoholic fatty liver disease (NAFLD) is a common cause of chronic liver disease in western countries (9–12),

[1]Department of Hepatology, Toranomon Hospital, Tokyo, Japan; [2]Health Management Center, Toranomon Hospital, Tokyo, Japan; [3]Okinaka Memorial Institute for Medical Research, Toranomon Hospital, Tokyo, Japan. **Correspondence:** Yusuke Kawamura, MD, Department of Hepatology, Toranomon Hospital, 2-2-2, Toranomon, Minato-ku, Tokyo 105-8470, Japan. E-mail: k-yusuke@toranomon.gr.jp
Received 5 April 2011; accepted 23 August 2011

© 2011 by the American College of Gastroenterology

and more recently in many Asian nations (13,14). NAFLD is sometimes considered to be the liver component of metabolic syndrome (15–17). It is associated with obesity, dyslipidemia, pituitary dysfunction, hypertension, sleep apnea, and type 2 diabetes mellitus (18–24). In particular, patients with non-alcoholic steatohepatitis (NASH), a subcategory of NAFLD, are at a higher risk for the incidence of HCC (25). At this stage, NASH can only be diagnosed by histopathology. Despite being common and potentially serious, the natural history of NAFLD remains poorly defined. Most of the studies reported to date included limited numbers of highly selected patients, i.e., patients with histopathologically confirmed NAFLD who were referred to specialized tertiary care centers (26–31). However, in reality, larger numbers of NAFLD patients are diagnosed by ultrasonography (US) alone. To our knowledge, no information about the incidence and risk factors of HCC in Japanese individuals with NAFLD diagnosed by US has been published.

The number of patients with NAFLD is predicted to increase in the future, and it is unlikely that all NAFLD patients will be diagnosed by histopathological examination of liver biopsy due to the potential risks associated with fat deposition and fibrosis in the liver (e.g., risk of bleeding, allergy to local anesthetics, and patient refusal). Therefore, there is a need to define the clinical impact of NAFLD and risk factors for the incidence of HCC. One aim of this retrospective study was to determine the incidence and risk factors of HCC in patients with US-diagnosed NAFLD. The aspartate aminotransferase (AST) to platelet ratio index (APRI), a non-invasive index for prediction of significant fibrosis in patients with chronic hepatitis C, has been previously reported (32), and its utility in NAFLD has also been reported (33). More recently, the BARD score (which includes the three following variables: body mass index (**B**MI), AST/alanine aminotransferase (**A**LT) **R**atio, and **D**iabetes), a non-invasive estimation formula for predicting advanced fibrosis in patients with NALFD, has also been reported (34). The other purpose of this study was to elucidate the utility of these non-invasive predictive procedures for liver fibrosis in the prediction for incidence of HCC in NAFLD patients.

METHODS

Study population

In this retrospective cohort study, we obtained the medical records of all patients in our database who were diagnosed with NAFLD by US (35) between January 1997 and December 2010 at the Department of Hepatology and the Health Management Center (Toranomon Hospital, Tokyo, Japan). Of these, 6,508 patients satisfied the following criteria: (i) past daily alcohol intake of <20 g/day; (ii) negativity for hepatitis C virus antibodies, hepatitis B surface antigen, antinuclear antibodies, and anti-mitochondrial antibodies in serum, as determined by radioimmunoassay or spot hybridization; (iii) no underlying systemic disease, such as systemic lupus erythematosus and rheumatic arthritis; (iv) no underlying metabolic disease, such as hemochromatosis, α-1-antitrypsin deficiency, and Wilson's disease; (v) no evidence of HCC on US and/or computed tomography; and (vi) follow-up period of ≥48 weeks. Clinical and laboratory data were collected from the medical records of all 6,508 patients and analyzed. The study was approved by the Institutional Review Board of our hospital.

Clinical background and laboratory data

Table 1 summarizes the clinical profile and laboratory data of NAFLD patients. The male:female ratio was 7.15:1, and the median BMI was 24.8 kg/m^2. Of the total population, 841 (12.9%) patients were hypertensive, and 536 (8.2%) patients had diabetes at the time of diagnosis of NAFLD. Hypertension was defined as seated systolic/diastolic blood pressure of $>140/>90$ mm Hg measured after 5 minutes of rest (36). Diabetes was diagnosed based on the 2003 criteria of the American Diabetes Association (37). These criteria include: (i) casual plasma glucose ≥200 mg/dl; (ii) fasting plasma glucose ≥126 mg/dl; and (iii) 2-h post-glucose (oral glucose tolerance test) ≥200 mg/dl.

Hepatitis C virus antibodies and hepatitis B surface antigen were examined at study entry. Hepatitis C virus antibodies were detected using a third-generation enzyme-linked immunosorbent assay (Abbott Laboratories, North Chicago, IL). Hepatitis B surface antigen was tested by radioimmunoassay (Abbott Laboratories).

Table 1. **Characteristics of 6,508 patients with non-alcoholic fatty liver disease**

Gender, M:F	5,709:799
Age, years[a]	49 (23–86)
Body mass index, kg/m^2 [a]	24.8 (15.9–45.1)
Hypertension, yes/no	841:5,667
Albumin, g/dl[a]	4.2 (2.9–5.1)
Total bilirubin, mg/dl[a]	0.8 (0.2–4.3)
AST, IU/L[a]	26 (11–516)
ALT, IU/L[a]	30 (7–803)
LDH, IU/L[a]	145 (49–392)
γ-GTP, IU/L[a]	53 (8–2,376)
Platelet count, $\times10^3/\mu l$[a]	226 (27–554)
Fasting plasma glucose, mg/dl[a]	99 (71–377)
Diabetes mellitus, yes/no	536:5,972
Uric acid, mg/dl[a]	6.3 (0.7–11.5)
Total cholesterol, mg/dl[a]	210 (100–521)
Triglyceride, mg/dl[a]	138 (22–1,758)
LDL cholesterol, mg/dl[a]	131 (29–270)
HDL cholesterol, mg/dl[a]	46 (5–106)
Follow-up period, days[a]	2,051 (366–11,190)

ALT, alanine aminotransferase; AST, aspartate aminotransferase; F, female; γ-GTP, gamma-glutamyl transpeptidase; HDL, high-density lipoprotein; LDH, lactate dehydrogenase; LDL, low-density lipoprotein; M, male.
[a]These are expressed as median (minimum, maximum).

Medical evaluation

The diagnosis of NAFLD was based on the US finding of bright liver with stronger echoes in the hepatic parenchyma than in the renal or spleen parenchyma. US was performed using a high-resolution, real-time scanner (model SSD-2000; Aloka, Tokyo, Japan, or Mode Logic-700 MR; GE-Yokokawa Medical Systems, Tokyo, Japan). Body weight was measured in light clothing and without shoes to the nearest 0.1 kg. Height was measured to the nearest 0.1 cm. Height and weight were recorded at baseline and BMI was calculated as weight (in kg)/height (in m^2). All patients were interviewed at the Toranomon Hospital using a questionnaire that collected information on demographic characteristics, medical history, and health-related habits, including questions about alcohol intake at the time of diagnosis of NAFLD.

Follow-up and diagnosis of HCC

The observation starting point (study entry) was the time of diagnosis of NAFLD by US. After that, patients were followed up monthly to every 6 months at the Toranomon Hospital. In this cohort, 5,657 (86.9%) patients underwent US every 6 months. A blood sample was taken for routine analysis. Overall, 585 patients were lost to follow-up; these were considered as censored data in statistical analysis as the appearance of HCC was not identified in these 585 patients (38).

Histopathological examination of the liver

In patients who underwent histological examination of the liver, specimens were fixed in 10% formalin and stained with hematoxylin–eosin, Masson's trichrome, silver impregnation, and periodic acid-Schiff after diastase digestion. Fibrosis was scored using a five-grade scale proposed by Brunt *et al.* (39): stage 0, normal connective tissue; stage 1, pericellular or perivenular fibrosis in zone 3 (pericentral vein area); stage 2, pericellular or perivenular fibrosis confined to zones 2 and 3 with or without periportal fibrosis; stage 3, bridging or septal fibrosis; and stage 4, cirrhosis.

A total of 104 patients underwent histological examination, and 10 (9.6%) patients received a histological diagnosis at the time of treatment of HCC. As a result of histological diagnosis, 73 (70.2%) patients were diagnosed with NASH, 30 (28.8%) patients were diagnosed with fatty liver without fibrosis, and 1 (1.0%) patient was diagnosed with liver cirrhosis without steatosis.

APRI calculation method and prevalence of significant fibrosis

The APRI was calculated according to the following formula:

$$\text{APRI} = \frac{\text{AST level (/ULN*)}}{\text{Platelet count } (10^9/\text{l})} \times 100$$

*ULN, AST upper level of normal (33 IU/l)

As previously reported, an APRI > 1.50 is predictive of significant fibrosis (positive predictive value, 88%; negative predictive value, 64%). In association with the APRI, hepatic fibrosis was assessed using the Ishak fibrosis score (40). Significant fibrosis was defined as an Ishak score of ≥3 (presence of occasional bridging fibrosis) (32). In this study, 184 of 6,508 patients (2.83%) had an APRI > 1.50 and were therefore considered to have significant fibrosis.

BARD score calculation method and prevalence of advanced fibrosis

The BARD score consists of three variables: BMI ≥28 kg/m^2, AST/ALT ratio ≥0.8, and diabetes. The following points are given to each variable: BMI, 1 point; AST/ALT ratio, 2 points; and presence of diabetes, 1 point; thus, scores range from 0 to 4. As previously reported, a BARD score of 2–4 is associated with an odds ratio for advanced fibrosis of 17 (positive predictive value, 43%; negative predictive value, 96%) (34). In association with the BARD score, advanced fibrosis was defined as NASH stage 3–4, and in this study, 3,841 of 6,508 (59.0%) patients had a BARD score of ≥2 points, and were therefore considered to have advanced fibrosis.

Statistical analysis

The cumulative incidence rate of HCC (new cases of HCC) was calculated from study entry to diagnosis of HCC using the Kaplan–Meier method. Differences in the development of HCC between groups were tested using the log-rank test. Independent factors associated with the incidence of HCC were analyzed by Cox's proportional hazards model. The following 17 variables were analyzed as potential covariates for incidence of HCC at the time of study entry: sex, age, BMI, hypertension, diabetes, serum concentration of albumin, total bilirubin, AST, ALT, lactate dehydrogenase, γ-glutamyl transpeptidase, uric acid, total cholesterol, triglyceride, low-density lipoprotein cholesterol, high-density lipoprotein cholesterol, and platelet count. Several variables were transformed into categorical data consisting of two simple ordinal numbers for univariate and multivariate analyses. All factors found to be at least marginally associated with the incidence of HCC ($P<0.15$) in univariate analysis were entered into a multivariate Cox's proportional hazards model. A P value <0.05 in a two-tailed test was considered significant. Data analysis was performed using The Statistical Package for Social Sciences version 16.0 for Windows (SPSS, Chicago, IL).

RESULTS

Incidence of HCC in patients with NAFLD

The follow-up period for all patients ranged from 366 to 11,190 days (median, 2,051 days). Of the 6,508 NAFLD patients, 16 (0.25%) patients developed HCC. The cumulative rate of HCC was 0.02% at the end of the 4th year, 0.19% at the end of the 8th year, and 0.51% at the end of the 12th year (**Figure 1**). The annual incidence of HCC in patients with NAFLD was 0.043%.

Effect of diabetes mellitus on the incidence of HCC in NAFLD patients

During the follow-up period, 9 of the 5,972 (0.15%) non-diabetic patients developed HCC, whereas 7 of the 536 (1.31%) diabetic patients developed HCC. The cumulative rate of HCC in non-diabetic patients was 0.0% at the end of the 4th year, 0.10% at the end of the 8th year, and 0.10% at the end of the 12th year. For diabetic patients, these rates were 0.22, 0.83, and 3.42%,

© 2011 by the American College of Gastroenterology

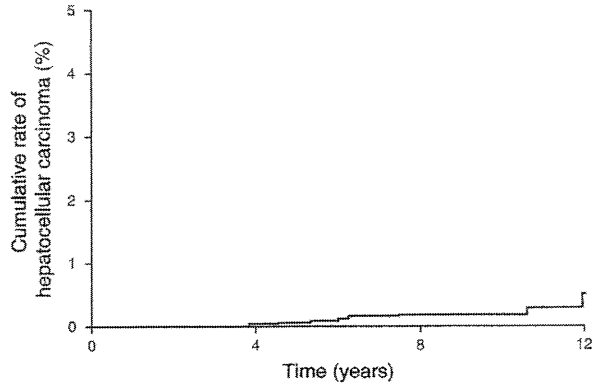

Figure 1. Cumulative rate of development of hepatocellular carcinoma in Japanese patients with non-alcoholic fatty liver disease diagnosed by ultrasonography.

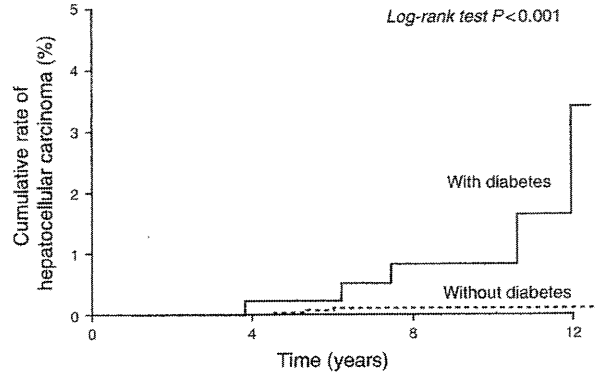

Figure 2. Cumulative rate of development of hepatocellular carcinoma in Japanese patients with or without diabetes mellitus diagnosed with non-alcoholic fatty liver disease by ultrasonography.

respectively (**Figure 2**). The cumulative rate of HCC was significantly higher in patients with diabetes than in non-diabetic patients ($P<0.001$).

Factors associated with the incidence of HCC

Multivariate Cox's proportional hazards analysis identified AST level ≥40 IU/L (hazard ratio (HR): 8.20; 95% confidence interval (95% CI): 2.56–26.26; $P<0.001$), platelet count $<150\times10^3/\mu l$ (HR: 7.19; 95% CI: 2.26–23.26; $P=0.001$), age ≥60 years (HR: 4.27; 95% CI: 1.30–14.01; $P=0.017$), and diabetes (HR: 3.21; 95% CI: 1.09–9.50; $P=0.035$) to be independent factors for development of HCC in Japanese NAFLD patients diagnosed by US (**Table 2**).

Incidence of HCC in patients with APRI-estimated significant fibrosis

On the basis of APRI estimation, 10 of the 6,324 (0.16%) non-significant fibrotic patients developed HCC during the follow-up period, whereas 6 of the 184 (3.26%) significant fibrotic patients developed HCC. The cumulative rate of HCC in non-significant fibrotic patients was 0.02% at the end of the 4th year, 0.06% at the end of the 8th year, and 0.39% at the end of the 12th year. For significant fibrotic patients, these rates were 0, 4.03, and 4.03%, respectively (**Figure 3**). The cumulative rate of HCC was significantly higher in patients with significant fibrosis than in patients without significant fibrosis (HR: 25.03; 95% CI: 9.02–69.52; $P<0.001$).

Incidence of HCC in patients with BARD score-estimated advanced fibrosis

On the basis of BARD score estimation, 5 of the 2,667 (0.19%) non-advanced fibrotic patients developed HCC during the follow-up period, whereas 11 of the 3,841 (0.29%) advanced fibrotic patients developed HCC. The cumulative rate of HCC in non-advanced fibrotic patients was 0% at the end of the 4th year, 0.06% at the end of the 8th year, and 0.06% at the end of the 12th year. For advanced fibrotic patients, these rates were 0.04, 0.27, and 0.76%, respectively (**Figure 4**). However, no significant associations between the BARD score and the incidence of HCC were observed (HR: 1.16; 95% CI: 0.40–3.37; $P=0.780$).

Clinicopathological features of NAFLD patients with HCC

Table 3 summarizes the characteristics and clinical features of the 16 patients with NAFLD-related HCC. In these patients, the median period from study entry to diagnosis of HCC was 12.5 years. In 12 of these 16 (75.0%) patients, platelet count decreased from study entry to diagnosis of HCC. Furthermore, the pathological diagnosis of background liver disease, which was performed in 11 of the 16 (68.8%) patients at the time of treatment of HCC, was NASH stage 4 (cirrhosis) in 3 (27.3%) patients, NASH stage 3 (pre-cirrhosis) in 2 (18.2%) patients, NASH stage 1–2 (slight-to-moderate fibrosis) in 3 (27.3%) patients, liver cirrhosis without fatty deposition in 1 (9.1%) patient, and fatty liver without fibrosis in 2 (18.2%) patients. Thus, 8 (72.7%) of the 11 patients had NASH. In case 4 (**Table 3**), splenectomy was performed because of associated thrombocytopenia, although the platelet count was increased at the time of diagnosis of HCC.

DISCUSSION

Previous retrospective studies have reported that the incidence of HCC from NASH ranges from 4 to 27% after development of cirrhosis, although the development of HCC in the setting of NAFLD remains a rare complication (41,42). The incidence of HCC in patients with NAFLD reported in several longitudinal follow-up studies ranged from 0 to 0.5%, whereas that in patients with NASH ranged from 0 to 2.8% over a follow-up period of 19.5 years (25,43–45). According to Japanese annual health check reports, 9–30% of Japanese adults demonstrate evidence of NAFLD by US (46–48). As it is known that almost 10–20% of individuals with NAFLD have NASH, the prevalence of NASH is estimated to be 1–3% of the adult Japanese population, which represents an extremely large number of potential patients. To our knowledge, no information about the incidence of HCC after

Table 2. **Predictors of hepatocellular carcinoma in patients with non-alcoholic fatty liver disease**

Variables	Category	Univariate analysis		Multivariate analysis	
		HR (95% CI)	*P* value	HR (95% CI)	*P* value
Gender	1: Female	1			
	2: Male	2.02 (0.69–5.93)	0.198		
Age	1: <60	1		1	
	2: ≥60	9.98 (2.73–36.49)	0.001	4.27 (1.30–14.01)	0.017
Body mass index (kg/m^2)	1: <25	1			
	2: ≥25	1.69 (0.63–4.55)	0.300		
Hypertension	1: No	1			
	2: Yes	10.26 (3.78–27.83)	<0.001		
Albumin (g/dl)	1: ≥4.0	1			
	2: <4.0	2.18 (0.78–6.17)	0.139		
Total bilirubin (mg/dl)	1: ≥1.0	1			
	2: <1.0	1.06 (0.37–3.70)	0.907		
AST (IU/L)	1: <40	1		1	
	2: ≥40	16.28 (5.65–46.96)	<0.001	8.20 (2.56–26.26)	<0.001
ALT (IU/L)	1: <50	1			
	2: ≥50	12.31 (4.24–35.70)	<0.001		
LDH (IU/L)	1: <160	1			
	2: ≥160	3.35 (1.25–8.99)	0.017		
γ-GTP (IU/L)	1: <70	1			
	2: ≥70	2.10 (0.79–5.60)	0.140		
Platelet count (×10^3/μl)	1: ≥150	1		1	
	2: <150	18.18 (6.49–50.00)	<0.001	7.19 (2.26–23.26)	0.001
Diabetes	1: No	1		1	
	2: Yes	6.08 (2.26–16.36)	<0.001	3.21 (1.09–9.50)	0.035
Uric acid (mg/dl)	1: <6.0	1			
	2: ≥6.0	1.55 (0.56–4.30)	0.397		
Total cholesterol level (mg/dl)	1: ≥220	1			
	2: <220	1.04 (0.38–2.87)	0.936		
Triglyceride level (mg/dl)	1: ≥150	1			
	2: <150	4.31 (0.98–19.23)	0.054		
LDL cholesterol level (mg/dl)	1: <140	1			
	2: ≥140	1.07 (0.40–2.89)	0.889		
HDL cholesterol level (mg/dl)	1: <40	1			
	2: ≥40	1.34 (0.38–4.75)	0.648		

ALT, alanine aminotransferase; AST, aspartate aminotransferase; CI, confidence interval; F, female; γ-GTP, gamma-glutamyl transpeptidase; HDL, high-density lipoprotein; HR, hazard ratio; LDH, lactate dehydrogenase; LDL, low-density lipoprotein; M, male.

long-term follow-up in a large number of Japanese patients with NAFLD has been previously published.

This study revealed several findings about the development of HCC in Japanese NAFLD patients. This is the first study to determine the annual rate and risk factors of newly developed HCC in a large number of Japanese patients with NAFLD diagnosed by US. In this study, the incidence of HCC calculated after long-term follow-up in NAFLD patients was 0.25%, with an annual rate of 0.043%. These low rates are similar to those reported by other groups in other countries (25,43–45). However, a total of

© 2011 by the American College of Gastroenterology