Journal of Medical Virology 81:452–458 (2009)

A Matched Case-Controlled Study of 48 and 72 Weeks of Peginterferon Plus Ribavirin Combination Therapy in Patients Infected With HCV Genotype 1b in Japan: Amino Acid Substitutions in HCV Core Region as Predictor of Sustained Virological Response

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Substitution of amino acid (aa) 70 and 91 in the core region of HCV genotype 1b is a useful pretreatment predictor of efficacy of 48-week peginterferon (PEG-IFN) plus ribavirin (RBV) therapy. Here, we determined the efficacy of 72-week PEG-IFN/RBV and the predictive factors to such therapy in a case–control study matched for sex, age, and periods from the start of treatment to initial point of HCV RNA-negative. We compared the treatment efficacy of 72-week regimen in 65 patients with that of 48-week in 130 patients, who were infected with HCV genotype 1b and treated with PEG-IFN/RBV. They consisted mainly of late virological responders (LVR) (HCV RNA-positive at 12 weeks and negative at 24 weeks after start of treatment). Sustained virological response (SVR) was achieved by 61.5% and 32.3% of patients of the 72- and 48-week groups, respectively, while non-virological response was noted in 9.2% and 29.2% of the respective groups. Multivariate analysis identified substitution of aa 70 and 91 (Arg70 and/or Leu91) and duration of treatment (72-week) as independent parameters that significantly influenced SVR. For Arg70 and/or Leu91 of core region, SVR rate was significantly higher in 72- (68.0%) than 48-week group (37.8%). For wild-type of ISDR, SVR rate was significantly higher in 72- (61.2%) than in 48-week group (29.3%). We conclude that 72-week PEG-IFN/RBV improves SVR rate for LVR, especially those with Arg70 and/or Leu91 of core region or wild-type of ISDR. Substitution of aa 70 and 91 is also a useful pretreatment predictor of response to 72-week PEG-IFN/RBV. ***J. Med. Virol. 81:452–458, 2009.*** © 2009 Wiley-Liss, Inc.

KEY WORDS: HCV; core region; NS5A-ISDR; peginterferon; ribavirin; 72-week; case–control study; LVR

INTRODUCTION

Hepatitis C virus (HCV) usually causes chronic infection that can result in chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma (HCC) [Dusheiko, 1998; Ikeda et al., 1998; Niederau et al., 1998; Kenny-Walsh, 1999; Akuta et al., 2001]. In patients with HCV-chronic hepatitis, treatment with interferon (IFN) can induce viral clearance and marked biochemical and histological improvement [Davis et al., 1989; Di Bisceglie et al., 1989]. Especially, peginterferon (PEG-IFN) plus ribavirin (RBV) combination therapy for 48 weeks can achieve a high sustained virological response (SVR) [Manns et al., 2001; Fried et al., 2002].

Although treatment of genotype 1-infected patients typically extends over 48 weeks, there has been interest in prolongation of therapy, particularly in late

Grant sponsor: Ministry of Health, Labor and Welfare, Japan (partial support).

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Accepted 18 October 2008

DOI 10.1002/jmv.21400

Published online in Wiley InterScience (www.interscience.wiley.com)

virological responders (LVR) (HCV RNA-positive at 12 weeks and negative at 24 weeks after the start of treatment), because high relapse rates in LVR may indicate that treatment was not administered for a sufficient duration [Ferenci et al., 2005]. Previous studies from Europe and United States have demonstrated that LVR improves SVR rates when treatment is extended to 72 weeks, compared with standard duration of therapy, largely as a result of reducing posttreatment relapse rates [Buti et al., 2003; Berg et al., 2006; Sánchez-Tapias et al., 2006; Pearlman et al., 2007]. Thus, prolongation of therapy in LVR may improve the virological response rate. However, it is not clear at present whether prolongation of treatment improves the SVR rate of treatment-resistant Japanese patients infected with HCV/genotype 1b [Akuta et al., 2007a,b,c].

Previous studies indicated that amino acid (aa) substitutions at position 70 and/or 91 in the HCV core region of genotype 1b were predictors of poor virological response to 48-week PEG-IFN plus RBV therapy [Akuta et al., 2005, 2006, 2007a,b,c; Donlin et al., 2007], and also risk factors for hepatocarcinogenesis [Akuta et al., 2007d, 2008a]. However, it is not clear at this stage whether aa substitutions in the core region can be used before therapy to predict the outcome of 72-week regimen.

The aims of the present study in HCV genotype 1b-infected Japanese adult patients, who received PEG-IFN plus RBV, were the following: (1) To conduct a case–control study matched for sex, age, and periods from the start of treatment to the initial point of HCV RNA-negative, to compare the treatment efficacy of 72-week regimen and 48-week regimen. (2) To identify the pretreatment factors that could predict treatment efficacy of the 72-week regimen, including pretreatment aa substitutions in the core region.

PATIENTS AND METHODS

Study Population

A total of 559 HCV genotype1b-infected Japanese adult patients were consecutively recruited into the study protocol of combination therapy with PEG-IFNα-2b plus RBV between 2001 and 2008 at Toranomon Hospital, Tokyo, Japan. They received PEG-IFNα-2b at a median dose of 1.4 μg/kg (range, 0.7–2.1 μg/kg) subcutaneously each week plus oral RBV at a median dose of 11.1 mg/kg (range, 3.4–16.0 mg/kg) daily. Among these, 383 patients, who could complete a total of 48 or 72 weeks of combination therapy, were enrolled in this retrospective study. The latter group consisted of 65 patients who extended combination therapy to 72-week (72-week group), and 318 patients who stopped combination therapy at the 48 weeks (48-week group). The decision to extend the combination therapy to 72 weeks was made by the patient. To compare the efficacy of the 72- and 48-week courses, all 65 patients of the 72-week group entered this study along with 130 patients of 48-week. The latter group was selected from among the 318 because they matched those patients of the 72-week group with respect to sex, age, and periods from the start of treatment to the initial point of HCV RNA-negative (matched case–control study). The treatment efficacy was evaluated by HCV-RNA positive based on qualitative PCR analysis at the end of treatment (non-virological response; NVR), and by HCV-RNA negative based on qualitative PCR analysis at 24 weeks after the completion of therapy (SVR). Furthermore, LVR was defined as HCV RNA-positive at 12 weeks and negative at 24 weeks after the start of treatment, based on qualitative PCR analysis. All patients fulfilled the following criteria: (1) Negativity for hepatitis B surface antigen (radioimmunoassay, Dainabot, Tokyo, Japan), positivity for anti-HCV (third-generation enzyme immunoassay, Chiron Corp., Emerville, CA), and positivity for HCV RNA qualitative analysis with PCR (Amplicor, Roche Diagnostics, Manheim, Germany). (2) Infection with HCV genotype 1b only. (3) A high viral load ($\geq 100 \times 10^3$ IU/ml) by quantitative analysis of HCV RNA with PCR (AMPLICOR GT HCV Monitor v2.0 using the 10-fold dilution method, Roche Molecular Systems Inc., Pleasanton, CA) within the preceding 2 months of enrolment. (4) No hepatocellular carcinoma. (5) Body weight > 40 kg. (6) Lack of coinfection with human immunodeficiency virus. (7) No previous treatment with antiviral or immunosuppressive agents within the preceding 3 months of enrolment. (8) None was an alcoholic; lifetime cumulative alcohol intake was <500 kg. (9) None had other forms of liver diseases, such as hemochromatosis, Wilson disease, primary biliary cirrhosis, alcoholic liver disease, or autoimmune liver disease. (10) None of the females was pregnant or a lactating mother. (11) All patients had completed a 24-week follow-up program after cessation of treatment, and SVR could be evaluated. (12) Each signed a consent form of the study protocol that had been approved by the human ethics review committee. The profile and laboratory data of 195 patients, who entered the matched case–control study, are summarized in Table I.

Laboratory Tests

Blood samples were obtained at least once every month before, during, and after treatment, and were analyzed for alanine aminotransferase (ALT) and HCV-RNA levels. The serum samples were frozen at −80°C within 4 hr of collection and thawed at the time of measurement. HCV genotype was determined by PCR using a mixed primer set derived from the nucleotide sequences of NS5 region [Chayama et al., 1993]. HCV-RNA levels were measured by quantitative PCR (AMPLICOR GT HCV Monitor v2.0 using the 10-fold dilution method, Roche Molecular Systems Inc.) at least once every month before, during, and after therapy. The dynamic range of the assay was 5.0×10^3 to 5.0×10^6 IU/ml. Samples collected during and after therapy that showed undetectable levels of HCV-RNA ($<5.0 \times 10^3$ IU/ml) were also checked by qualitative PCR (AMPLICOR HCV v2.0, Roche Molecular Systems Inc.),

TABLE I. Patient Profile and Laboratory Data at Commencement of 48- and 72-Week Combination Therapy of Peginterferon Plus Ribavirin in Patients Infected With HCV Genotype 1b (Matched Case–Control Study)

	72-week group	48-week group	
Matching data			
Number of patients	65	130	
Sex (M/F)	28/37	57/73	Matched
Age (years)*	57 (22–70)	56 (25–68)	Matched
Periods to the initial point of HCV RNA-negative (weeks)*	17.4 (5.9–72.0)	19.7 (6.0–48.0)	Matched
Demographic data			
History of blood transfusion	18 (27.7%)	42 (32.3%)	NS
Family history of liver disease	21 (32.3%)	31 (23.8%)	NS
Body mass index (kg/m^2)*	22.6 (16.6–38.0)	22.2 (17.0–32.4)	NS
Laboratory data*			
Serum aspartate aminotransferase (IU/L)	49 (23–213)	51 (21–217)	NS
Serum alanine aminotransferase (IU/L)	64 (25–430)	68 (20–391)	NS
Serum albumin (g/dl)	3.9 (3.2–4.5)	3.8 (3.2–4.6)	NS
Gamma-glutamyl transpeptidase (IU/L)	40 (14–171)	38 (15–581)	NS
Leukocytes ($/mm^3$)	4,400 (2,300–8,800)	4,600 (1,200–9,400)	NS
Hemoglobin (g/dl)	14.0(11.3–17.8)	13.9 (10.6–18.1)	NS
Platelet count ($\times 10^4/mm^3$)	16.2 (8.2–30.7)	15.8 (6.4–31.6)	NS
ICG R15 (%)	13 (2–73)	15 (2–45)	NS
Level of viremia (KIU/ml)	2,650 (52->5,000)	1,850 (49->5,000)	0.013
Alfa-fetoprotein (μg/L)	6 (2–47)	6(2–110)	NS
Total cholesterol (mg/dl)	174(111–276)	175 (104–274)	NS
High density lipoprotein cholesterol (mg/dl)	45 (27–86)	51 (24–78)	NS
Low density lipoprotein cholesterol (mg/dl)	104 (49–204)	107 (50–182)	NS
Triglycerides (mg/dl)	91 (35–259)	94 (35–315)	NS
Uric acid (mg/dl)	5.3 (2.6–7.7)	5.0 (2.3–8.7)	NS
Fasting blood sugar (mg/dl)	95 (79–218)	98 (76–157)	NS
Histological findings			
Stage of fibrosis (F1/F2/F3/ND)	20/12/11/1/21	44/27/22/0/37	NS
Hepatocyte steatosis (none to mild/moderate to severe/ND)	40/2/23	78/8/44	NS
Treatment			
PEG-IFN α-2b dose (μg/kg)*	1.4 (0.8–2.1)	1.4 (0.7–1.9)	NS
Ribavirin dose (mg/kg)*	10.9 (6.6–16.0)	10.8 (3.7–14.2)	NS
Amino acid substitutions in the HCV			
Core aa 70 (arginine/glutamine (histidine)/ND)	37/23/5	11 47/6	NS
Core aa 91 (leucine/methionine/ND)	42/18/5	66/57/7	0.038
ISDR of NS5A (wild-type/mutant-type/ND)	49/5/11	99/17/14	NS

Data are number and percentages of patients, except those denoted by *, which represent the median (range) values. ND: not determined.

which has a higher sensitivity than quantitative analysis, and the results were expressed as positive or negative. The lower limit of the assay was 50 IU/ml.

Histopathological Examination of Liver Biopsies

Liver biopsy specimens were obtained percutaneously or at peritoneoscopy using a modified Vim Silverman needle with an internal diameter of 2 mm (Tohoku University style, Kakinuma Factory, Tokyo), fixed in 10% formalin, and stained with hematoxylin and eosin, Masson's trichrome, silver impregnation, and periodic acid-Schiff after diastase digestion. All specimens for examinations contained six or more portal areas. Histopathological diagnosis was confirmed by an experienced liver pathologist (H.K.) who was blinded to the clinical data. Chronic hepatitis was diagnosed based on histopathological assessment according to the scoring system of Desmet et al. [1994]. Hepatocyte steatosis was graded as either none (absent), mild (less than 1/3 of hepatocytes involved), moderate (greater than 1/3 but less than 2/3 of hepatocytes involved), or severe (greater than 2/3 of hepatocytes involved) [D'Alessandro et al., 1991].

Detection of Amino Acid Substitutions in Core Region and NS5A Region

With the use of HCV-J (accession no. D90208) as a reference [Kato et al., 1990], the sequence of 1–191 aa in the core protein of genotype 1b was determined and then compared with the consensus sequence constructed on 50 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) [Akuta et al., 2005]. The sequence of 2209–2248 aa in the NS5A of genotype 1b (IFN-sensitivity determining region [ISDR]) reported by Enomoto et al. [1995, 1996] was also determined, and the numbers of aa substitutions in ISDR were defined as wild-type (≤ 1) or mutant-type (≥ 2).

In the present study, aa substitutions of the core region and NS5A-ISDR were analyzed by direct sequencing [Enomoto et al., 1995, 1996; Akuta et al., 2005]. HCV RNA was extracted from serum samples at

the start of treatment and reverse transcribed with random primer and MMLV reverse transcriptase (Takara Syuzo, Tokyo). Nucleic acids were amplified by PCR using the following primers: (a) Nucleotide sequences of the core region: The first-round PCR was performed with CC11 (sense, 5′-GCC ATA GTG GTC TGC GGA AC-3′) and e14 (antisense, 5′-GGA GCA GTC CTT CGT GAC ATG-3′) primers, and the second-round PCR with CC9 (sense, 5′-GCT AGC CGA GTA GTG TT-3′) and e14 (antisense) primers. (b) Nucleotide sequences of NS5A-ISDR: The first-round PCR was performed with ISDR1 (sense, 5′-ATG CCC ATG CCA GGT TCC AG-3′) and ISDR2 (antisense, 5′-AGC TCC GCC AAG GCA GAA GA-3′) primers, and the second-round PCR with ISDR3 (sense, 5′-ACC GGA TGT GGC AGT GCT CA-3′) and ISDR4 (antisense, 5′-GTA ATC CGG GCG TGC CCA TA-3′) primers ([a] hemi-nested PCR; [b] nested PCR). All samples were initially denatured at 95°C for 15 min. The 35 cycles of amplification were set as follows: denaturation for 1 min at 94°C, annealing of primers for 2 min at 55°C, and extension for 3 min at 72°C with an additional 7 min for extension. Then 1 μl of the first PCR product was transferred to the second PCR reaction. Other conditions for the second PCR were the same as the first PCR, except that the second PCR primers were used instead of the first PCR primers. The amplified PCR products were purified by the QIA quick PCR purification kit (Qiagen, Tokyo, Japan) after agarose gel electrophoresis and then used for direct sequencing. Dideoxynucleotide termination sequencing was performed with the Big Dye Deoxy Terminator Cycle Sequencing kit (Perkin-Elmer, Tokyo, Japan).

Statistical Analysis

Non-parametric tests (Mann–Whitney *U*-test, chi-squared 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 SVR. The odds ratios and 95% confidence intervals (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 multiple logistic regression analysis to identify significant independent factors. The potential pretreatment predictive factors associated with SVR included the following variables: sex, age, history of blood transfusion, familial history of liver disease, body mass index, aspartate aminotransferase (AST), ALT, albumin, gamma-glutamyl transpeptidase (γGTP), leukocyte count, hemoglobin, platelets, indocyanine green retention rate at 15 min (ICG R15), level of viremia, alfa-fetoprotein, total cholesterol, high-density lipoprotein cholesterol, low-density lipoprotein cholesterol, triglycerides, uric acid, fasting blood sugar, hepatocyte steatosis, stage of fibrosis, PEG-IFN dose/body weight, RBV dose/body weight, duration of treatment, and amino acid substitution in the core and ISDR of NS5A. Statistical analyses were performed using the SPSS software (SPSS Inc., Chicago, IL).

RESULTS

Comparison of Treatment Efficacy Between 48-Week Group and 72-Week Group

Figure 1 shows comparison of the treatment efficacy between 48- and 72-week groups. SVR was achieved by 42 of 130 patients (32.3%) and 40 of 65 (61.5%) in the 48- and 72-week groups, respectively. The proportion of SVR was significantly higher in 72-week group than in the 48-week group ($P < 0.001$). Furthermore, NVR was identified in 38 of 130 patients (29.2%) and 6 of 65 (9.2%) in the 48- and 72-week groups, respectively. The proportion of NVR was significantly lower in the 72-week group than in 48-week group ($P = 0.002$).

Predictive Factors Associated With SVR in Multivariate Analysis

Univariate analysis identified 13 parameters that influenced SVR either significantly or marginally: gender (female sex; $P = 0.002$), stage of fibrosis ($F_{1.2}$; $P = 0.008$), PEG-IFN dose/body weight (≥1.4 μg/kg; $P = 0.001$), RBV dose/body weight (≥11.0 mg/kg; $P = 0.029$), platelet count ($\geq 15.0 \times 10^4/mm^3$; $P = 0.002$), level of viremia (<1,000 KIU/ml; $P = 0.049$), γGTP (<50 IU/L; $P = 0.026$), ICG R15 (<15%; $P = 0.003$), triglycerides (<100 mg/dl; $P = 0.038$), high-density lipoprotein cholesterol (≥50 mg/dl; $P = 0.018$), α-fetoprotein (<20 μg/L; $P = 0.005$), substitution of aa 70 and 91 (Arg70 and/or Leu91; $P = 0.002$), and duration of treatment (72-week group; $P < 0.001$).

Multivariate analysis identified three independent parameters that either significantly influenced or tended to significantly influence SVR; substitution of aa 70 and 91 (Arg70 and/or Leu91; $P = 0.015$), duration of treatment (72-week group; $P = 0.014$), and high-density lipoprotein cholesterol (≥50 mg/dl; $P = 0.084$) (Table II).

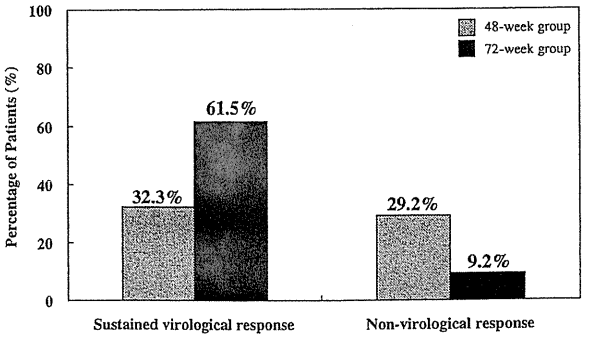

Fig. 1. Comparison of treatment efficacy between the 48-week group and 72-week group. The proportion of patients with sustained virological response in 72-week group was significantly higher than in 48-week group ($P < 0.001$). Furthermore, the proportion of patients with non-virological response in 72-week group was significantly lower than in 48-week group ($P = 0.002$).

TABLE II. Factors Associated With Sustained Virological Response to Combination Therapy of Peginterferon Plus Ribavirin in 195 Patients Infected With HCV Genotype1b, Identified by Multivariate Analysis

Factor	Category	Odds ratio (95% CI)	*P*
Substitution of aa 70 and 91	1: Gln70 (His70) and Met91	1	
	2: Arg70 and/or Leu91	5.46 (1.39–21.3)	0.015
Duration of treatment (weeks)	1: 48	1	
	2: 72	3.51 (1.28–9.62)	0.014
HDL-cholesterol (mg/dl)	1: <50	1	
	2: ≥50	2.42 (0.89–6.58)	0.084

*Only variables that achieved statistical significance ($P < 0.05$) or marginal significance ($P < 0.10$) on multivariate logistic regression are shown.

Treatment Efficacy According to Amino Acid Substitutions in Core Region

Figure 2 shows comparison of the treatment efficacy according to aa substitutions in the core region. In Gln70 (His70) and Met91, SVR was achieved by 4 of 26 patients (15.4%) and 3 of 10 (30.0%) in the 48- and 72-week groups, respectively. The proportion of SVR in 72-week group was not significantly different than in 48-week group. In Arg70 and/or Leu91, SVR was achieved by 37 of 98 patients (37.8%) and 34 of 50 (68.0%) in the 48- and 72-week groups, respectively. The proportion of SVR in 72-week group was significantly higher than in 48-week group ($P = 0.001$).

Treatment Efficacy According to Amino Acid Substitutions in NS5A-ISDR

Figure 3 shows comparison of the treatment efficacy according to aa substitutions in NS5A-ISDR. In mutant-type, SVR was achieved by 9 of 17 patients (52.9%) and 3 of 5 (60.0%) in the 48- and 72-week groups, respectively. The proportion of SVR in 72-week group was not significantly different from that in 48-week group. In wild-type, SVR was achieved by 29 of 99 patients (29.3%) and 30 of 49 (61.2%) in the 48- and 72-week groups, respectively. The proportion of SVR in 72-week group was significantly higher than that in 48-week group ($P < 0.001$).

DISCUSSION

This matched case-controlled study of PEG-IFN plus RBV for LVR infected with HCV genotype 1b, showed that treatment extension to 72 weeks seems to improve SVR rates in Japanese patients. To our knowledge, the present study is the first to report that 72-week regimen of PEG-IFN plus RBV might be also useful in Asians. Especially, the 72-week regimen significantly improved the SVR rates in LVR with Arg70 and/or Leu91 of core or wild-type of ISDR. The present study based on patients, who could complete a total of 48 or 72 weeks of combination therapy, did not show the frequencies of patients, who could not complete by side effects. Patients, who dropped out by side effects between 48 and 72 week for therapy prolonged to 72 weeks, were only 3 of 559 HCV genotype1b-infected Japanese adult patients (data not shown), so the frequencies of side effects with 72-week regimen might be nearly equal to those with 48-week regimen. Large-scale prospective study based on the intention to treat analysis should be conducted to confirm the above finding in future.

NS5A-ISDR, reported as predictor of treatment efficacy with IFN monotherapy by Enomoto et al. [1995, 1996], is also useful as predictor of 48-week PEG-IFN plus RBV combination therapy [Murayama et al., 2007; Shirakawa et al., in press; Yen et al., 2008]. Furthermore, the present study also indicated that

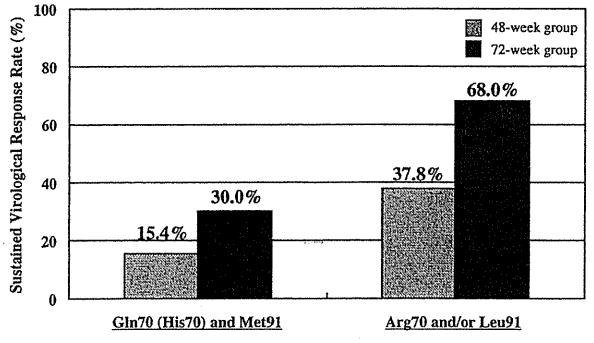

Fig. 2. Comparison of treatment efficacy according to amino acid substitutions in the core region. In Gln70 (His70) and Met91, the proportion of patients with sustained virological response in 72-week group was not significantly different from that in 48-week group. However, in Arg70 and/or Leu91, the proportion of patients with sustained virological response in 72-week group was significantly higher than in 48-week group ($P = 0.001$).

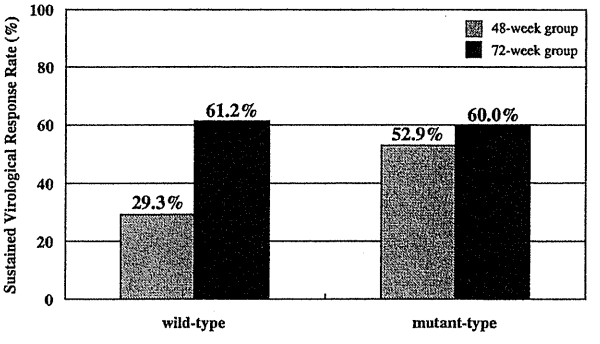

Fig. 3. Comparison of treatment efficacy according to amino acid substitutions in NS5A-ISDR. In mutant-type, the proportion of patients with sustained virological response in 72-week group was not significantly different from that in 48-week group. However, in wild-type, the proportion of patients with sustained virological response in 72-week group was significantly higher than in 48-week group ($P < 0.001$).

72-week regimen of PEG-IFN plus RBV significantly improved the SVR rate in LVR with wild-type of ISDR. Unfortunately, the 72-week regimen of PEG-IFN plus RBV did not improve the SVR rate in LVR with Gln70 (His70) and Met91 of the core region. Multivariate analysis also identified Gln70 (His70) and Met91 of the core region as independent parameter that significantly influenced non-SVR. PEG-IFN plus RBV carries potential serious side effects and is costly especially when used long enough to achieve higher SVR rates. For these reasons, we need to identify those patients who do not achieve SVR, to free them of unnecessary side effects and reduce costs, preferably before the start of the combination therapy. For patients unsuitable for PEG-IFN plus RBV, including LVR with Gln70 (His70) and Met91 of the core region, low-dose intermittent IFN monotherapy might be an efficacious therapeutic regimen, because it can lead to ALT normalization and thus reduce the risk of hepatocarcinogenesis [Akuta et al., 2008b].

One limitation of this study is that LVR could not be evaluated by the COBAS AmpliPrep/COBAS TaqMan HCV Test (the lower limit of this assay; 15 IU/ml), which has a higher sensitivity than AMPLICOR HCV v2.0 (the lower limit of this assay; 50 IU/ml) [Sizmann et al., 2007]. Rapid virological response (HCV RNA-negative at 4 weeks after the start of treatment) and early virological response (HCV RNA-positive at 4 weeks and negative at 12 weeks after the start of treatment) by AMPLICOR HCV v2.0 might be diagnosed as LVR by the COBAS AmpliPrep/COBAS TaqMan HCV Test. Further studies using highly sensitive real-time PCR assay should be performed to facilitate the development of more effective therapeutic regimens in future.

We previously reported that viral factors (e.g., aa substitutions in core region) and host factors (e.g., lipid metabolic factors, sex, and AFP) might be important predictors of treatment response to 48-week PEG-IFN plus RBV in Japanese patients infected with HCV genotype 1b, in addition to treatment-related factors (e.g., RBV dose) [Akuta et al., 2005, 2006, 2007a,b,c]. The present study also identified viral (aa substitutions in the core region), host (HDL-cholesterol), and treatment-related factors (duration of treatment) that can be useful as independent and significant pretreatment predictors of SVR. Thus, substitution of aa 70 and 91 is also useful as a pretreatment predictor of 72-week regimen. Further studies that examine the structural and functional impact of aa substitutions during combination therapy should be conducted to confirm the above finding.

Another limitation of our study was that we did not examine aa substitutions in areas other than the core region and NS5A-ISDR of HCV genome, such as the interferon/ribavirin resistance determining region (IRRDR), including V3 of NS5A region, although they should be investigated in future studies [El-Shamy et al., 2008; Muñoz de Rueda et al., 2008].

We conclude that treatment efficacy of 72-week PEG-IFN plus RBV seems to be based on a dynamic tripartite interaction of viral-, host-, and treatment-related factors. Further understanding of the complex interaction between these factors should facilitate the development of more effective therapeutic regimens.

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Journal of Medical Virology 81:1032–1039 (2009)

Amino Acid Substitutions in the Hepatitis C Virus Core Region of Genotype 1b Are the Important Predictor of Severe Insulin Resistance in Patients Without Cirrhosis and Diabetes Mellitus

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Previous studies provided a direct experimental evidence for the contribution of HCV core protein in the development of insulin resistance (IR), but the clinical impact of HCV core region on IR is still not clear. The present study evaluated the impact of Amino acid (aa) substitutions of HCV-1b core region on IR in 123 Japanese patients infected with HCV-1b without cirrhosis and diabetes mellitus, and investigated the treatment efficacy of 48-week pegylated interferon (PEG-IFN) plus ribavirin (RBV) according to HOMA-IR values. Patients with IR (HOMA-IR $\geq$2.5) and severe IR (HOMA-IR $\geq$3.5) were present in 51.2% and 27.6%, respectively. Multivariate analysis identified body mass index ($\geq$25 kg/m^2) and hepatocyte steatosis ($\geq$5%) as significant determinants of IR. Furthermore, multivariate analysis identified hepatocyte steatosis ($\geq$5%), aa substitutions of the core region (Gln70 (His70) and/or Met91), and age ($\geq$55 years) as significant determinants of severe IR. Especially, significantly lower proportions of patients with Gln70 (His70) and/or Met91 were noted among those without severe IR (59.6%) than those with severe IR (82.4%). The rates of sustained virological response in patients with IR (50.0%) were not significantly different from those without IR (52.9%). Furthermore, the rates of non-virological response in patients with IR (28.9%) were not significantly also different from those without IR (20.6%). In conclusion, the present study indicated that substitutions of HCV-1b core region were the important predictor of severe IR in patients without cirrhosis and diabetes mellitus, but HOMA-IR values might be not useful as predictors of 48-week PEG-IFN plus RBV therapy. ***J. Med. Virol. 81:1032–1039, 2009.*** © 2009 Wiley-Liss, Inc.

KEY WORDS: HCV; core region; genotype; HOMA-IR; hepatocyte steatosis; cirrhosis; diabetes mellitus

INTRODUCTION

Hepatitis C virus (HCV) usually causes chronic infection that can result in chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma (HCC) [Dusheiko, 1998; Ikeda et al., 1998; Niederau et al., 1998; Kenny-Walsh, 1999; Akuta et al., 2001]. Furthermore, HCV infection also affects an increased risk of diabetes mellitus [Allison et al., 1994; Caronia et al., 1999; Mason et al., 1999; Mehta et al., 2000, 2003; Zein et al., 2000, 2005; Antonelli et al., 2005] or insulin resistance (IR) [Hickman et al., 2003; Hui et al., 2003; Lecube et al., 2004, 2006]. IR and glucose metabolism impairment are associated with liver necroinflammation [Hui et al., 2003], hepatocyte steatosis [Fartoux et al., 2005; Cammà et al., 2006; Conjeevaram et al., 2007], cirrhosis [Petrides et al., 1994], and HCC [El-Serag et al., 2001; Lai et al., 2006; Veldt et al., 2008]. Especially, in patients infected with HCV genotype 1 (HCV-1), significant fibrosis is associated with IR independent from hepatocyte steatosis [Moucari et al., 2008; Petta et al., 2008].

Previous studies reported that HCV core protein induced HCC and IR in transgenic mice, and provided

Grant sponsor: Ministry of Health, Labor and Welfare, Japan (partly supported).

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Accepted 5 February 2009
DOI 10.1002/jmv.21473
Published online in Wiley InterScience (www.interscience.wiley.com)

a direct experimental evidence for the contribution of HCV core protein in the development of HCC and IR in human HCV infection [Moriya et al., 1998; Shintani et al., 2004]. Amino acid (aa) substitutions at position 70 and/or 91 in the HCV core region of genotype 1b (HCV-1b core region) were predictors of poor virological response to 48-week pegylated interferon (PEG-IFN) plus ribavirin (RBV) combination therapy [Akuta et al., 2005, 2006, 2007a,b,c; Donlin et al., 2007; Okanoue et al., 2008], and also risk factors for hepatocarcinogenesis [Akuta et al., 2007d, 2008]. Thus, previous reports supported the oncogenic potential of the HCV core region and clinically linked substitutions of aa 70 and/or 91 in HCV-1b core region to HCC [Akuta et al., 2007d, 2008], but the clinical impact of HCV-1b core region on IR is still not clear. IR develops type 2 diabetes mellitus as its major late feature, and is also associated with advanced fibrosis [Petrides et al., 1994; Petta et al., 2008]. Hence, the biological mechanisms underlying the association between HCV core region and IR are probably multifactorial, and study based on patients without diabetes mellitus and cirrhosis, that might affect IR, should be performed to investigate whether HCV core region might affect IR clinically.

Previous reports showed that IR might be predictors of poor virological response to PEG-IFN plus RBV combination therapy [D'Souza et al., 2005; Romero-Gómez et al., 2005]. Chu et al. [2008] reported that IR was a major determinant of sustained virological response (SVR) in HCV-1 patients receiving 24-week PEG-IFN plus RBV. However, to our knowledge, there is little evidence that IR affects treatment efficacy of HCV-1b patients receiving 48-week PEG-IFN plus RBV combination therapy.

The aims of the present study conducted in Japanese patients infected with HCV-1b without cirrhosis and diabetes mellitus, were the following. (1) To evaluate the HOMA-IR values of patients infected with HCV-1b. (2) To identify the impact of aa substitutions in the core region on IR in such patients, and determine the factors associated with IR, and (3) to investigate the treatment efficacy of 48-week PEG-IFN plus RBV combination therapy according to HOMA-IR values.

PATIENTS AND METHODS

Study Population

At Toranomon Hospital, Tokyo, Japan, 221 HCV-infected Japanese patients were consecutively recruited into the study protocol of the combination therapy with PEG-IFNα-2b plus RBV between December of 2001 and June of 2005. Among these, 123 patients were selected in the present retrospective study based on the following criteria. (1) Negativity for hepatitis B surface antigen (radioimmunoassay, Dainabot, Tokyo, Japan), positivity for anti-HCV (third-generation enzyme immunoassay, Chiron Corp, Emerville, CA), and positivity for HCV RNA qualitative analysis with PCR (Amplicor, Roche Diagnostics, Mannheim, Germany). (2) They were infected with HCV-1b alone. (3) HOMA-IR values and substitutions of aa 70 and 91 in the HCV core region were determined at the commencement of treatment. (4) They were free of cirrhosis and hepatocellular carcinoma, based on biopsy examination, laboratory tests, and imaging studies at baseline. (5) None had diabetes mellitus. (6) None was an alcoholic; lifetime cumulative alcohol intake was <500 kg (mild to moderate alcohol intake). (7) All were free of coinfection with human immunodeficiency virus. (8) None had other forms of hepatitis, such as hemochromatosis, Wilson disease, primary biliary cirrhosis, alcoholic liver disease, and autoimmune liver disease. (9) Each signed a consent form of the study protocol that had been approved by the human ethics review committee. Table I summarizes the profiles and laboratory data of the 123 patients at the commencement of treatment. They included 71 males and 52 females, aged 20–70 years (median, 55 years). The treatment efficacy was evaluated by HCV-RNA positive based on qualitative PCR analysis at the end of treatment (non-virological response; NVR), and by HCV-RNA negative based on qualitative PCR analysis at 24 weeks after the completion of therapy (SVR).

Laboratory Tests

Blood samples were obtained at least once every month before, during, and after treatment, and were analyzed for alanine aminotransferase (ALT) and HCV-RNA levels. The serum samples were frozen at −80°C within 4 hr of collection and thawed at the time of measurement. HCV genotype was determined by PCR using a mixed primer set derived from the nucleotide sequences of NS5 region [Chayama et al., 1993]. HCV-RNA levels were measured by quantitative PCR (AMPLICOR GT HCV Monitor v2.0 using the 10-fold dilution method, Roche Molecular Systems, Inc.) at least once every month before, during, and after therapy. The dynamic range of the assay was 5.0×10^3 to 5.0×10^6 IU/ml. Samples collected during and after therapy that showed undetectable levels of HCV-RNA ($<5.0 \times 10^3$ IU/ml) were also checked by qualitative PCR (AMPLICOR HCV v2.0, Roche Molecular Systems, Inc.), which has a higher sensitivity than quantitative analysis, and the results were expressed as positive or negative. The lower limit of the assay was 50 IU/ml.

Histopathological Examination of Liver Biopsies

Liver biopsy specimens were obtained percutaneously or at peritoneoscopy using a modified Vim Silverman needle with an internal diameter of 2 mm (Tohoku University style, Kakinuma Factory, Tokyo, Japan). The biopsy material was fixed in 10% formalin, and stained with hematoxylin and eosin, Masson's trichrome, silver impregnation, and periodic acid-Schiff after diastase digestion. All specimens for examination contained six or more portal areas. Histopathological diagnosis was confirmed by an experienced liver pathologist (H.K.) who was blinded to the clinical data. Chronic hepatitis and liver cirrhosis were diagnosed

TABLE I. Profile and Laboratory Data of 123 Patients Infected With HCV Genotype 1b

Demographic data	
Number of patients	123
Sex (M/F)	71/52
Age (years)*	55 (20–70)
History of blood transfusion	41 (33.3%)
Family history of liver disease	37 (30.1%)
Body mass index (kg/m^2)*	23.6 (17.6–32.0)
Laboratory data*	
Serum aspartate aminotransferase (IU/L)	59 (17–266)
Serum alanine aminotransferase (IU/L)	81 (25–504)
Serum albumin (g/dl)	3.8 (3.1–4.5)
Gamma-glutamyl transpeptidase (IU/L)	50 (15–393)
Leukocytes ($/mm^3$)	4,800 (2,300–8,800)
Hemoglobin (g/dl)	14.4 (10.6–17.6)
Platelet count ($\times 10^4/mm^3$)	16.8 (7.5–27.7)
Indocyanine green retention rate at 15 min (%)	15 (4–41)
Serum iron (μg/dl)	138 (18–290)
Serum ferritin (μg/L)	130 (<10–711)
Creatinine clearance (ml/min)	99 (46–146)
Level of viremia (KIU/ml)	1,900 (23–>5,000)
Alpha-fetoprotein (μg/L)	6 (2–161)
Total cholesterol (mg/dl)	166 (96–294)
High-density lipoprotein cholesterol (mg/dl)	46 (10–83)
Low-density lipoprotein cholesterol (mg/dl)	101 (53–207)
Triglycerides (mg/dl)	95 (33–362)
Uric acid (mg/dl)	5.5 (2.3–9.4)
Fasting plasma glucose (mg/dl)	94 (62–120)
Fasting insulin (μU/ml)	10.5 (0.4–55.5)
HOMA-IR	2.6 (0.1–12.5)
Treatment*	
PEG-IFNα-2b dose (μg/kg)	1.4 (0.7–1.9)
Ribavirin dose (mg/kg)	11.0 (3.7–14.2)
Histological findings	
Stage of fibrosis (F1/F2/F3/ND)	53/32/20/18
Hepatocyte steatosis (<5% (Absent)/≥5% (Present)/ND)	38/64/21
Amino acid substitutions in the HCV	
Core aa 70 (arginine/glutamine (histidine))	69/54
Core aa 91 (leucine/methionine)	71/52
ISDR of NS5A (wild-type/mutant-type/ND)	94/22/7

HOMA-IR, homeostasis model for assessment of insulin resistance; ND, not determined.
Data are number and percentages of patients, except those denoted by *, which represent the median (range) values.

based on histological assessment according to the scoring system of Desmet et al. [1994]. Hepatocyte steatosis was assessed as the percentage of hepatocytes containing fat droplet, and subjects were considered to have steatosis in the presence of fat droplets in ≥5% of hepatocytes.

Diagnosis of Liver Cirrhosis, Insulin Resistance, and Diabetes Mellitus

Liver cirrhosis was diagnosed based on the presence of markedly irregular surface with nodular formation in the liver, evident on peritoneoscopy, histological assessment according to the scoring system of Desmet et al. [1994], or on computed tomography or ultrasonography. Ascites, edema, and esophageal varicosities, facilitated the diagnosis when present.

The diagnosis of type 2 diabetes was based on the revised criteria of the American Diabetes Association using a value of fasting plasma glucose of ≥126 mg/dl on at least two occasions [American Diabetes Association, 2000]. IR was assessed by the Homeostasis Model for Assessment of Insulin Resistance (HOMA-IR) method [Matthews et al., 1985], using the following equation: HOMA-IR = fasting plasma glucose (mg/dl) × fasting insulin (μU/ml)/405. HOMA-IR values of ≥2.5, and ≥3.5 were evaluated as IR, and severe IR, respectively.

Detection of Amino Acid Substitutions in Core Region and NS5A Region

With the use of HCV-J (accession no. D90208) as a reference [Kato et al., 1990], the sequence of 1–191 aa in the core protein of genotype 1b was determined and then compared with the consensus sequence constructed on 50 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) [Akuta et al., 2005]. The sequence of 2209–2248 aa in the NS5A of genotype 1b (IFN-sensitivity determining region [ISDR]) reported by Enomoto et al. [1995, 1996] was also determined, and the numbers of aa substitutions in ISDR were defined as wild-type (≤1) or mutant-type (≥2).

In the present study, aa substitutions of the core region and NS5A-ISDR were analyzed by direct

sequencing [Enomoto et al., 1995, 1996; Akuta et al., 2005]. HCV RNA was extracted from serum samples at the start of treatment and reverse transcribed with random primer and MMLV reverse transcriptase (Takara Syuzo, Tokyo, Japan). Nucleic acids were amplified by PCR using the following primers: (a) Nucleotide sequences of the core region: The first-round PCR was performed with CC11 (sense, 5′-GCC ATA GTG GTC TGC GGA AC-3′) and e14 (antisense, 5′-GGA GCA GTC CTT CGT GAC ATG-3′) primers, and the second-round PCR with CC9 (sense, 5′-GCT AGC CGA GTA GTG TT-3′) and e14 (antisense) primers. (b) Nucleotide sequences of NS5A-ISDR: The first-round PCR was performed with ISDR1 (sense, 5′-ATG CCC ATG CCA GGT TCC AG-3′) and ISDR2 (antisense, 5′-AGC TCC GCC AAG GCA GAA GA-3′) primers, and the second-round PCR with ISDR3 (sense, 5′-ACC GGA TGT GGC AGT GCT CA-3′) and ISDR4 (antisense, 5′-GTA ATC CGG GCG TGC CCA TA-3′) primers. ([a]; hemi-nested PCR. [b]; nested PCR). All samples were initially denatured at 95°C for 15 min. The 35 cycles of amplification were set as follows: denaturation for 1 min at 94°C, annealing of primers for 2 min at 55°C, and extension for 3 min at 72°C with an additional 7 min for extension. Then 1 μl of the first PCR product was transferred to the second PCR reaction. Other conditions for the second PCR were the same as the first PCR, except that the second PCR primers were used instead of the first PCR primers. The amplified PCR products were purified by the QIA quick PCR purification kit (Qiagen, Tokyo, Japan) after agarose gel electrophoresis and then used for direct sequencing. Dideoxynucleotide termination sequencing was performed with the Big Dye Deoxy Terminator Cycle Sequencing kit (Perkin-Elmer, Tokyo, Japan).

Statistical Analysis

Non-parametric tests were used to compare variables between groups, including the Mann–Whitney U test, chi-squared test, and Fisher's exact probability test. Univariate and multivariate logistic regression analyses were used to determine the independent predictive factors of IR. The odds ratios and 95% confidence intervals (95%CI) were also calculated. All P-values <0.05 by the two-tailed test were considered significant. Variables that achieved statistical significance ($P<0.05$) or marginal significance ($P<0.10$) on univariate analysis were entered into multiple logistic regression analysis to identify significant independent factors. Potential predictive factors of IR included the following pretreatment variables: sex, age, history of blood transfusion, familial history of liver disease, body mass index, aspartate aminotransferase (AST), ALT, albumin, gamma-glutamyl transpeptidase (γGTP), leukocyte count, hemoglobin, platelet, count, indocyanine green retention rate at 15 min (ICG R15), serum iron, serum ferritin, creatinine clearance, level of viremia, alfa-fetoprotein, total cholesterol, high-density lipoprotein cholesterol, low-density lipoprotein cholesterol, triglycerides, uric acid, fasting plasma glucose, fasting insulin, HOMA-IR values, stage of fibrosis, hepatocyte steatosis, PEG-IFN dose/body weight, RBV dose/body weight, and aa substitution in the core and ISDR of NS5A. Statistical analyses were performed using the SPSS software (SPSS, Inc., Chicago, IL).

RESULTS

HOMA-IR Values of Patients Infected With HCV-1b Without Cirrhosis and Diabetes Mellitus

As a whole, 16.3% (20 of 123 patients), 32.5% (40 of 123), 23.6% (29 of 123), and 27.6% (34 of 123) indicated HOMA-IR values of ≤1.4, 1.5–2.4, 2.5–3.4, and ≥3.5, respectively (Fig. 1). Thus, patients with IR (HOMA-IR ≥2.5) were present in 51.2% (63 of 123), and exceeded 50%. Furthermore, patients with severe IR (HOMA-IR ≥3.5) were present in 27.6%. These results show that patients, infected with HCV-1b without cirrhosis and diabetes mellitus, might indicate IR frequently.

Factors Associated With Insulin Resistance in Univariate and Multivariate Analyses

The whole population sample of 123 patients were analyzed to determine factors that could be associated with IR. IR (HOMA-IR ≥2.5) was detected in 63 of 123 (51.2%) patients. Univariate analysis identified six parameters that tended to or significantly influenced IR. These included age (≥55 years, $P=0.072$), body mass index (≥25 kg/m^2, $P<0.001$), serum ferritin (≥200 μg/L, $P=0.071$), family history of liver disease (Absent, $P=0.077$), hepatocyte steatosis (Present (≥5%), $P=0.002$), and aa substitutions of the core region (Gln70 (His70) and/or Met91, $P=0.092$). Multivariate analysis identified two parameters that independently influenced IR, including body mass index (≥25 kg/m^2, $P=0.001$) and hepatocyte steatosis (Present (≥5%), $P=0.028$).

Severe IR (HOMA-IR ≥3.5) was detected in 34 of 123 (27.6%) patients. Univariate analysis identified five

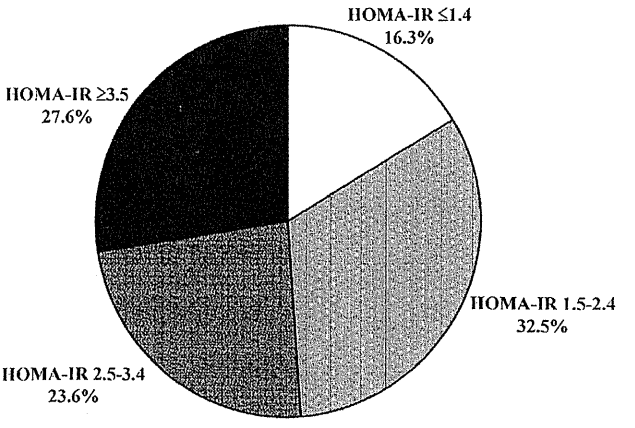

Fig. 1. HOMA-IR values of patients infected with HCV genotype 1b without cirrhosis and diabetes mellitus. As a whole, 16.3%, 32.5%, 23.6%, and 27.6% indicated HOMA-IR values of ≤1.4, 1.5–2.4, 2.5–3.4, and ≥3.5, respectively. These results show that patients, infected with HCV genotype 1b without cirrhosis and diabetes mellitus, might indicate IR frequently.

TABLE II. Factors Associated With Severe IR (HOMA-IR ≥3.5) in Patients Infected With HCV Genotype 1b, Identified by Multivariate Analysis

Factor	Category	Odds ratio (95% CI)	*P*
Hepatocyte steatosis	1: Absent (<5%)	1	
	2: Present (≥5%)	4.170 (1.235–14.08)	0.021
Substitution of aa 70 and 91	1: Arg70 and Leu91	1	
	2: Gln70 (His70) and/or Met91	3.654 (1.215–10.99)	0.021
Age (years)	1: <55	1	
	2: ≥55	3.015 (1.071–8.488)	0.037

parameters that tended to or significantly influenced severe IR. These included age (≥55 years, $P=0.015$), body mass index (≥25 kg/m^2, $P=0.025$), hepatocyte steatosis (Present (≥5%), $P=0.003$), triglycerides (≥100 mg/dl, $P=0.060$), and aa substitutions of the core region (Gln70 (His70) and/or Met91, $P=0.020$). Multivariate analysis identified three parameters that independently influenced severe IR, including hepatocyte steatosis (Present (≥5%), $P=0.021$), aa substitutions of the core region (Gln70 (His70) and/or Met91, $P=0.021$), and age (≥55 years, $P=0.037$) (Table II).

aa Substitutions of Core Region and HOMA-IR Values

The entire population sample was also analyzed to determine the relationship between aa substitutions of the core region and HOMA-IR values. HOMA-IR values of 81 patients with Gln70 (His70) and/or Met91 (median; 2.9) indicated the higher levels than those of 42 patients with Arg70 and Leu91 (median; 2.3), significantly ($P=0.022$) (Fig. 2).

Furthermore, the proportions of patients with Gln70 (His70) and/or Met91 among those with HOMA-IR values of ≤1.4, 1.5–2.4, 2.5–3.4, 3.5–3.9, and ≥4.0 were 60.0% (12 of 20 patients), 57.5% (23 of 40), 62.1% (18 of 29), 83.3% (5 of 6), and 82.1% (23 of 28), respectively (Fig. 3). Thus, the higher the proportion of patients with Gln70 (His70) and/or Met91, the higher HOMA-IR values, and significantly lower proportions of patients with Gln70 (His70) and/or Met91 were noted among those without severe IR (59.6% (53 of 89)) than those with severe IR (82.4% (28 of 34)) ($P=0.020$).

Treatment Efficacy of PEG-IFN Plus RBV Combination Therapy According to HOMA-IR Values

Of the 123 patients, 72 could be evaluated as 48-week regimen of PEG-IFN plus RBV combination therapy. Seventy-two patients received PEG-IFNα-2b combination therapy at a median dose of 1.5 μg/kg (range, 0.8–1.8 μg/kg) subcutaneously each week plus oral RBV at a median dose of 11.3 mg/kg (range, 9.7–14.2 mg/kg) daily for 48 weeks. 51.4% (37 of 72 patients) could achieve SVR, and 25.0% (18 of 72) had NVR.

In each groups with IR or without IR, SVR was achieved by 19 of 38 patients (50.0%) and 18 of 34 (52.9%), respectively. The proportions of SVR in group with IR was not significantly different from those in group without IR. Furthermore, in each groups with IR or without IR, NVR was identified in 11 of 38 patients (28.9%) and 7 of 34 (20.6%), respectively. The proportions of NVR in group with IR was not significantly different from those in group without IR.

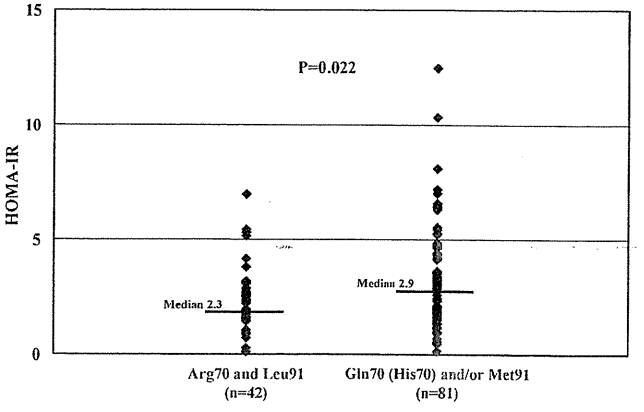

Fig. 2. aa substitutions of HCV core region and HOMA-IR values. HOMA-IR values of 81 patients with Gln70 (His70) and/or Met91 indicated the higher levels than those of 42 patients with Arg70 and Leu91, significantly ($P=0.022$).

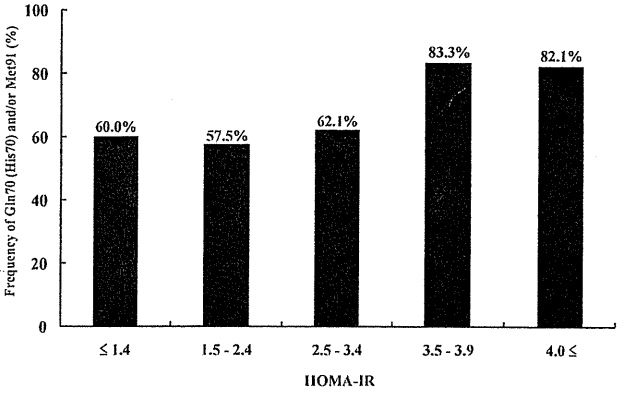

Fig. 3. The proportions of patients with Gln70 (His70) and/or Met91 and HOMA-IR values. Higher frequencies of Gln70 (His70) and/or Met91 correlated with higher HOMA-IR values. Significantly lower proportions of patients with Gln70 (His70) and/or Met91 were noted among those without severe IR (HOMA-IR <3.5) (59.6%) than those with severe IR (HOMA-IR ≥3.5) (82.4%) ($P=0.020$).

In each groups with severe IR or without severe IR, SVR was achieved by 11 of 20 patients (55.0%) and 26 of 52 (50.0%), respectively. The proportions of SVR in group with severe IR was not significantly different from those in group without severe IR. Furthermore, in each groups with severe IR or without severe IR, NVR was identified in 6 of 20 patients (30.0%) and 12 of 52 (23.1%), respectively. The proportions of NVR in group with severe IR was not significantly different from those in group without severe IR.

In this study, HOMA-IR values were not useful as pretreatment predictors of 48-week PEG-IFN plus RBV combination therapy in HCV-1b patients without cirrhosis and diabetes mellitus.

DISCUSSION

Shintani et al. [2004] reported that HCV core protein induced IR in transgenic mice, and provided a direct experimental evidence for the contribution of HCV core protein in the development of IR in human HCV infection. The results of the present study showed that higher frequencies of Gln70 (His70) and/or Met91 in HCV-1b core region might correlated with higher HOMA-IR values. Thus, the present results supported the potential of core region in the development of IR, and clinically linked substitutions of aa 70 and/or 91 in HCV-1b core region to IR. Especially, these findings without diabetes mellitus and cirrhosis suggest that the real connection between IR and HCV-1b infection is initiated at early stages of liver disease. The limitations of the present study were that it could not investigate an improvement of IR in patients who developed the viral eradication after antiviral treatment [Kawaguchi et al., 2007; Arase et al., 2008], as a direct evidence for the contribution of aa substitutions in HCV-1b core region. Further studies that examine the structural and functional impact of aa substitutions should be conducted to confirm the above finding.

To our knowledge, the present study is first report to identify the factors associated with IR of patients without diabetes mellitus and cirrhosis infected with HCV-1b. Especially, multivariate analysis identified age ($\geq$55 years), body mass index ($\geq$25 kg/m^2), hepatocyte steatosis (Present ($\geq$5%)), and aa substitutions of the core region (Gln70 (His70) and/or Met91) as significant determinants of IR (HOMA-IR $\geq$2.5) and/or severe IR (HOMA-IR $\geq$3.5). However, this study identified aa substitutions of the core region as significant determinants of severe IR, and did not identify as determinants of IR. The discrepant results may be due to one or more factors. The first reason for this is probably the small number of patients in the present study (e.g., possible type II error). Univariate analysis really identified aa substitutions of the core region that tended to influence IR. Furthermore, even if HOMA-IR values were also divided into two groups of $\geq$3.0 and $\leq$2.9, multivariate analysis identified aa substitutions of the core region as significant determinants of $\geq$3.0 (data not shown). Hence, further studies based on the large number of patients should be performed in the future. The second reason is probably the difference of objects, based on HCV-1b patients without diabetes mellitus and cirrhosis. Previous report indicated that HCV-related diabetes mellitus might occur in association with IR, hepatocyte steatosis, and high levels of both tumor-necrosis factor and CXCL10 [Antonelli et al., 2009], so patients with severe IR do not always have diabetes mellitus. However, IR develops type 2 diabetes mellitus as its major late feature, and is also associated with advanced fibrosis [Petrides et al., 1994; Petta et al., 2008]. Hence, the biological mechanisms underlying the association between HCV core region and IR are probably multifactorial, and the present study based on patients without diabetes mellitus and cirrhosis as confounding factors, that might affect IR, are very important for estimating the true relationship between HCV core region and IR. The present study is first report to identified aa substitutions of the core region (Gln70 (His70) and/or Met91) as significant determinants of severe IR, in HCV-1b patients without cirrhosis and diabetes mellitus.

Moriya et al. [1998] reported that HCV core protein induced HCC in transgenic mice, and provided a direct experimental evidence for the contribution of HCV core protein in the development of HCC in human HCV infection. Previous reports supported the oncogenic potential of the HCV core region and clinically linked substitutions of aa 70 and/or 91 in HCV-1b core region to HCC [Akuta et al., 2007d, 2008]. IR and glucose metabolism impairment are associated with HCC [El-Serag et al., 2001; Lai et al., 2006; Veldt et al., 2008]. The present study suggested the presence of IR-dependent pathway as a mechanism of HCV-1b core region-associated hepatocarcinogenesis, and the importance of eradication of the virus with Gln70 (His70) and/or Met91 in reducing the development of HCC through this pathway.

Treatment efficacy of 48-week PEG-IFN plus RBV combination therapy according to HOMA-IR values is controversial. Chu et al. [2008] reported that IR was a major determinant of SVR in HCV-1 patients receiving PEG-IFN plus RBV, but treatment duration was 24 weeks. Georgescu et al. [2008] reported that high HOMA-IR values could not affect treatment efficacy of 48-week PEG-IFN plus RBV therapy in HCV-1 patients, after excluding the patients of metabolic syndrome criteria. The present study based on HCV-1b patients without cirrhosis and diabetes mellitus also showed that HOMA-IR values might be not useful as predictors of 48-week PEG-IFN plus RBV therapy. This reason is probably related to exclude patients of diabetes mellitus as one of metabolic syndrome criteria, and the results might support the previous report of Georgescu et al. [2008]. To our knowledge, the present study is first report to investigate the relation between HOMA-IR values and treatment efficacy of HCV-1b patients, especially without cirrhosis and diabetes mellitus, receiving 48-week PEG-IFN plus RBV combination

therapy. Further studies based on the large number of patients should be performed in the future.

In conclusion, the results of the present study indicated that substitutions of HCV-1b core region were the important predictor of severe IR in patients without cirrhosis and diabetes mellitus. This finding highlights the importance of eradication of the virus with Gln70 (His70) and/or Met91 in reducing the development of severe IR. The limitations of the present study were that it did not investigate other genotypes apart from HCV-1b, the geographic diversities of HCV-1b core region (distribution of Arg70 or Gln70 (His70), and Leu91 or Met91), and the study of other races apart from Asians in Japan. Further prospective studies, matched for HCV genotype, aa substitutions of the core region, and race, of a large group of patients are required to determine the meaning of higher HOMA-IR values in HCV infection.

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Sustained Virological Response Reduces Incidence of Onset of Type 2 Diabetes in Chronic Hepatitis C

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Diabetes is present in patients with chronic hepatitis C virus infection. The aim of this retrospective cohort study was to assess the cumulative development incidence and predictive factors for type 2 diabetes after the termination of interferon therapy in Japanese patients positive for hepatitis C virus (HCV). A total of 2,842 HCV-positive patients treated with interferon (IFN) monotherapy or combination therapy with IFN and ribavirin were enrolled. The mean observation period was 6.4 years. An overnight (12-hour) fasting blood sample or a casual blood sample was taken for routine analyses during follow-up. The primary goal was the onset of type 2 diabetes. Evaluation was performed by using the Kaplan-Meier method and Cox proportional hazard analysis. Of 2,842 HCV patients, 143 patients developed type 2 diabetes. The cumulative development rate of type 2 diabetes was 3.6% at 5 years, 8.0% at 10 years, and 17.0% at 15 years. Multivariate Cox proportional hazard analysis revealed that type 2 diabetes development after the termination of IFN therapy occurred when histological staging was advanced (hazard ratio 3.30; 95% confidence interval [CI] 2.06-5.28; $P < 0.001$), sustained virological response was not achieved (hazard ratio 2.73; 95% CI 1.77-4.20; $P < 0.001$), the patient had pre-diabetes (hazard ratio 2.19; 95% CI 1.43-3.37; $P < 0.001$), and age was ≥50 years (hazard ratio 2.10; 95% CI 1.38-3.18; $P < 0.001$). *Conclusion:* Our results indicate sustained virological response causes a two-thirds reduction in the risk of type 2 diabetes development in HCV-positive patients treated with IFN. (HEPATOLOGY 2009;49:739-744.)

Hepatitis C virus (HCV) is one of the more common causes of chronic liver disease in world. Chronic hepatitis C is an insidiously progressive form of liver disease that relentlessly but silently progresses to cirrhosis in 20% to 50% of cases over a period of 10 to 30 years.[1-3] In addition, HCV is a major risk for hepatocellular carcinoma (HCC).[4-8] Moreover, chronic HCV infection has been associated with a variety of extrahepatic complications such as essential mixed cryoglobulinemia, porphyria cutanea tarda, membranoproliferative glomerulonephritis, autoimmune thyroiditis, sialadenitis, and cardiomyopathy.[9-13] Lately, data supporting a link between type 2 diabetes mellitus (T2DM) and chronic hepatitis C infection have been reported.[14,15]

Although there is growing evidence to support the concept that HCV infection is a risk factor for developing T2DM, there have been a few interventional studies confirming this issue. This issue needs to be confirmed with a long-term follow-up of patients with high risk of developing diabetes. Thus, prospective studies including metabolic evaluations are clearly needed to clarify these issues.

With this background in mind, the cohort study was initiated to investigate the cumulative incidence and risk factors of T2DM after prolonged follow-up in HCV-infected patients treated with interferon (IFN) monotherapy or combination therapy with IFN and ribavirin. The strengths of the current study are the large numbers of patients included and the long-term follow-up of patients.

Patients and Methods

Patients. There were 5,890 patients diagnosed with chronic HCV infection and treated with IFN mono-

Abbreviations: CI, confidence interval; FPG, fasting plasma glucose; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; IFN, interferon; SVR, sustained virological response; T2DM, type 2 diabetes mellitus.

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Received September 25, 2008; accepted October 13, 2008.

Supported in part by grants-in-aid from Okinaka Memorial Institute for Medical Research and the Japanese Ministry of Health, Labor, and Welfare.

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Published online in Wiley InterScience (www.interscience.wiley.com).

DOI 10.1002/hep.22703

Potential conflict of interest: Nothing to report.

therapy or combination IFN + ribavirin therapy between September 1990 and March 2007 in the Department of Hepatology, Toranomon Hospital, Tokyo, Japan. Of these, 2,842 patients satisfied the following criteria: (1) no evidence of diabetes mellitus for 3 months after the termination of IFN (plasma glucose concentration <126 mg/dL [6.9 mmol/L] in the fasting state, <200 mg/dL [11.0 mmol/L] in casual state and/or 2 hours after a 75-g oral glucose load); (2) features of chronic hepatitis or cirrhosis diagnosed via laparoscopy and/or liver biopsy before the initiation of IFN therapy; (3) positivity for serum HCV RNA before the initiation of IFN therapy; (4) period of ≤1 year of IFN therapy; (5) negativity for hepatitis B surface antigen (HBsAg), antinuclear antibodies, or antimitochondrial antibodies in serum, as determined via radioimmunoassay or spot hybridization; (6) no evidence of HCC nodules as shown on ultrasonography and/or computed tomography; and (7) no underlying systemic disease, such as systemic lupus erythmatosus or rheumatic arthritis.

Patients who were taking medications known to alter glucose tolerance or had illnesses that could seriously reduce their life expectancy or their ability to participate in the trial were excluded from the study. Patients were classified as having normal glucose or pre-diabetes based on fasting plasma glucose (FPG), casual plasma glucose, or 2-hour plasma glucose. The normal glucose group was regarded as having an FPG of <100 mg/dL, casual plasma glucose of <140 mg/dL, and/or 2-hour plasma glucose of <140 mg/dL. The pre-diabetes group was regarded as having an FPG of 100-125 mg/dL, casual plasma glucose of 140-200 mg/dL, and/or 2-hour plasma glucose of 140-200 mg/dL.[16]

Next, we assessed predictive factors for T2DM in chronic hepatitis C patients treated with IFN. The physicians in charge explained the purpose and method of this clinical trial to each patient and/or the patient's family. Informed consent was obtained from all living patients included in the present cohort study. The study was approved by the Institutional Review Board of our hospital.

Outcome Measures. The primary outcome was T2DM, diagnosed by the use of the 2003 criteria of the American Diabetes Association.[16] These criteria include (1) casual plasma glucose ≥200 mg/dL; (2) FPG ≥126 mg/dL; (3) 2-hour post-glucose (oral glucose tolerance test) ≥200 mg/dL.

Laboratory Investigation. Anti-HCV was detected using a second-generation enzyme-linked immunosorbent assay (ELISA II; Abbott Laboratories, North Chicago, IL). HCV-RNA was determined by the Amplicor method (Cobas Amplicor HCV Monitor Test, version 2.0; Roche, Tokyo, Japan). Hepatitis B surface antigen was tested via radioimmunoassay (Abbott Laboratories, Detroit, MI). The used serum samples were stored at −80°C at the first consultation. Diagnosis of HCV infection was based on detection of serum HCV antibody and positive RNA. Height and weight were recorded at baseline, and the body mass index was calculated as weight (in kg)/height (in m^2).

Evaluation of Liver Cirrhosis. Liver status of the 2,842 patients was mainly determined via peritoneoscopy and/or liver biopsy. Liver biopsy specimens were obtained using a modified Vim Silverman needle with an internal diameter of 2 mm (Tohoku University, Kakinuma Factory, Tokyo, Japan), fixed in 10% formalin, and stained with hematoxylin-eosin, Masson's trichrome, silver impregnation, and periodic acid-Schiff after diastase digestion. The size of specimens for examination was more than six portal areas.[17]

Follow-up. The starting time of follow-up was 3 months after the termination of IFN therapy. After that, patients were followed up monthly to tri-monthly in our hospital. Physical examination and biochemical tests were conducted at each examination together with regular check-up. An overnight (12-hour) fasting blood sample or a casual blood sample was taken for routine analyses. These included aminotransferase activities, total cholesterol, platelet counts, and serum HCV RNA level. Three hundred twenty-four patients were lost to follow-up; because the appearance of T2DM and death was not identified in these patients, they were considered as censored data in the statistical analysis.[18] Moreover, patients retreated with antiviral agents were regarded as withdrawals at the time of starting the retreatment of antiviral agents.

Statistical Analysis. The cumulative appearance rate of T2DM was calculated from 3 months after the termination of IFN treatment to the appearance of T2DM using the Kaplan-Meier method. Differences in the development of T2DM were tested using the log rank test. Independent factors associated with the incidence rate of T2DM were analyzed by the Cox proportional hazard model. The following 11 variables were analyzed for potential covariates for incidence of T2DM at the time of termination of IFN therapy at our hospital: age, sex, state of liver disease (chronic hepatitis or liver cirrhosis), body mass index, glucose level, aspartate aminotransferase level, alanine aminotransferase level, type of IFN, total dose of IFN, efficacy of IFN therapy, hypertension, triglyceride level, and total cholesterol level. A P value of less than 0.05 was considered significant. Data analysis was performed using SPSS 11.5 for Windows (SPSS, Chicago, IL).

Results

Patient Characteristics. Table 1 shows the characteristics of the 2,842 HCV-positive patients treated with

Table 1. Patient Characteristics

N	2,842
Sex (male/female)	1,778/1,064
Age (years)	51.8 ± 9.0
Height (cm)	163.8 ± 9.1
Body weight (kg)	62.7 ± 11.7
Body mass index	23.3 ± 3.2
Blood pressure (systolic/diastolic, mm Hg)	128 ± 18/77 ± 12
HCV genotype (1b/2a/2b/other)	744/752/290/56
HCV RNA level (KIU/mL)	593 ± 540
Staging (non-LC/LC)	2,649/193
Blood glucose level (normal/prediabetes)	2,601/241
Fasting plasma glucose (mg/dL)	87 ± 24
Triglyceride (mg/dL)	166 ± 31
Total bilirubin (g/dL)	102 ± 56
AST (IU/L)	74 ± 63
ALT (IU/L)	116 ± 102
IFN monotherapy*/combination therapy†	2,417/425
Efficacy of treatment (SVR/non-SVR)	1,175/1,667
Follow-up period (years)	6.4 ± 5.0

Data are expressed as the number of patients or mean ± standard deviation.

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; LC, liver cirrhosis; SVR, sustained virological response.

*Outbreak of IFN monotherapy: recombinant IFN-α2a, 304 cases; recombinant IFN-α2b, 235 cases; natural IFN-bα, 1,355 cases; natural IFN-β, 522 cases; total dose of IFN = 598 ± 170 MU.

†Outbreak of combination therapy: recombinant IFN-α2b + ribavirin, 175 cases; total dose of IFN = 537 ± 196 MU; total dose of ribavirin = 182 ± 69 g; pegylated IFN-α2b + ribavirin, 250 cases; total dose of pegylated IFN = 4.28 ± 1.17 mg; total dose of ribavirin = 232 ± 60 g.

IFN monotherapy or combination therapy with IFN and ribavirin. The sustained virological response (SVR) rate was 36.7% (886/2417) in IFN monotherapy and 68% (289/425) in IFN + ribavirin therapy. Thus, the number of patients with SVR was 1,175. The mean period after the termination of antiviral drugs was 6.4 years.

Incidence of T2DM in Patients with HCV. A total of 143 patients (102 men and 41 women) developed T2DM during a mean observation period of 6.4 years. Of these, 26 were SVR and 117 were non-SVR. The cumulative development rate of T2DM was determined to be 3.6% at 5 years, 8.0% at 10 years, and 17.0% at 15 years using the Kaplan-Meier method (Fig. 1). The factors associated with the incidence of T2DM in all 2,842 patients treated with IFN therapy are shown in Table 2.

Multivariate Cox proportional hazard analysis revealed that type 2 diabetes development after the termination of IFN therapy occurred when histological staging was advanced (hazard ratio 3.30; 95% confidence interval [CI] 2.06-5.28; $P < 0.001$), sustained virological response was not achieved (hazard ratio 2.73; 95% CI 1.77-4.20; $P < 0.001$), patient had pre-diabetes (hazard ratio 2.19; 95% CI 1.43-3.37; $P < 0.001$), and age was >50 years (hazard ratio 2.10; 95% CI 1.38-3.18; $P < 0.001$). SVR causes a two-thirds reduction of development of T2DM in patients treated with IFN. In addition to SVR, age ≥50 years, liver cirrhosis, and pre-diabetes contribute to a high risk of developing diabetes. The cumulative development rates of T2DM based on difference of age, efficacy of the IFN therapy, histological diagnosis, and glucose level at the starting time of follow-up are shown in Fig. 2.

Fig. 3 shows the impact of reduction due to SVR on the incidence of T2DM in patients with ≥50 years, liver cirrhosis, or pre-diabetes. When patients with age ≥50 years, liver cirrhosis, and pre-diabetes have SVR after IFN therapy, SVR could statistically reduce the onset of T2DM compared with those without SVR.

Discussion

We have described the development incidence of diabetes after the termination of antiviral therapy in HCV-positive patients treated with IFN therapy in the present study. Diabetes has been reported in less than 0.08% of patients treated with IFN[19,20]; thus, to exclude diabetes originating from IFN-related side effects, patients without diabetes for 3 months after the termination of IFN were enrolled in the present study. The present study indicates that the annual incidence of T2DM for a prolonged follow-up after the termination of IFN therapy among HCV patients is 0.8% to 1.0%. The present study was limited by a retrospective cohort trial. We started the present study in 1991 based on the diabetes mellitus criteria published by Fajans.[21] However, after that, diabetes mellitus criteria were revised. We thus rechecked the diagnosis of T2DM based on the diabetes mellitus criteria of 2003 in patients seen prior to 2003.[16] Because of rechecking the diagnosis of T2DM on the basis of diabetes mellitus criteria in 2003, the present study was regarded as a retrospective cohort study. However, the patients were

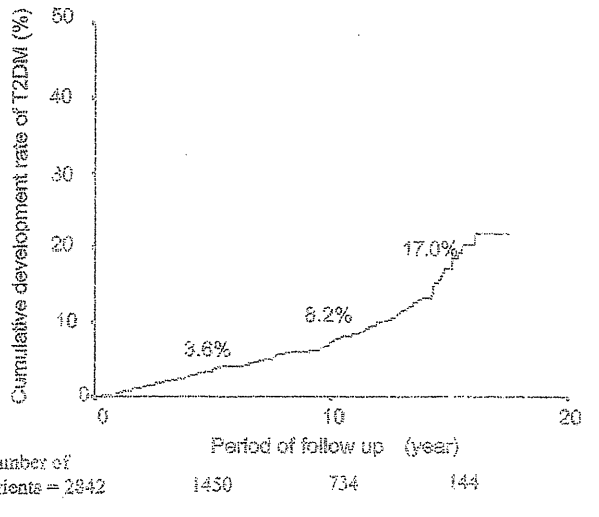

Fig. 1. Cumulative development rate of T2DM in patients treated with IFN.

Table 2. Predictive Factors for T2DM Development

Variables	Univariate Analysis		Cox Regression	
	HR (95% CI)	*P* Value	HR (95% CI)	*P* Value
Age, years (≥50/<50)	2.55 (1.74-3.73)	<0.001	2.10 (1.38-3.18)	<0.001
Sex (female/male)	0.84 (0.59-1.19)	0.318		
Body mass index (≥25/<25)	1.44 (0.98-2.08)	0.057		
HCV load (KIU/mL, ≥1,000/<1,000)	0.67 (0.43-1.03)	0.069		
Genotype (1/2)	0.73 (0.50-1.06)	0.098		
ALT (IU/L, ≥50/<50)	1.83 (1.14-2.94)	0.012		
Glucose level (prediabetes/normal)	2.25 (1.53-3.33)	<0.0001	2.19 (1.43-3.37)	<0.001
Triglyceride (mg/dL, ≥150/<150)	1.66 (0.93-2.98)	0.088		
Cholesterol (mg/dL, ≥220/<220)	1.56 (0.62-3.95)	0.346		
Histological diagnosis (LC/non-LC)	4.03 (2.55-6.36)	<0.0001	3.30 (2.06-5.28)	<0.001
Combination of ribavirin (−/+)	1.53 (0.99-2.38)	0.058		
Type of IFN (α/β)	0.88 (0.57-1.35)	0.882		
Total dose of IFN (MU, ≥500/<500)	0.91 (0.59-1.40)	0.672		
Efficacy (non-SVR/SVR)	2.73 (1.77-4.20)	<0.0001	2.78 (1.75-4.41)	<0.001

Data are expressed as the median (range).
Abbreviations: ALT, alanine aminotransferase; HR, hazard ratio; LC, liver cirrhosis.

prospectively followed. Another limitation of the study was that patients were treated with different types of antiviral therapy (IFN monotherapy or combination IFN + ribavirin therapy) for different duration (4 to 52 weeks). This heterogeneity makes it difficult to interpret the results of the study. On the other hand, the strength of the present study is the long-term follow-up in the large numbers of patients included.

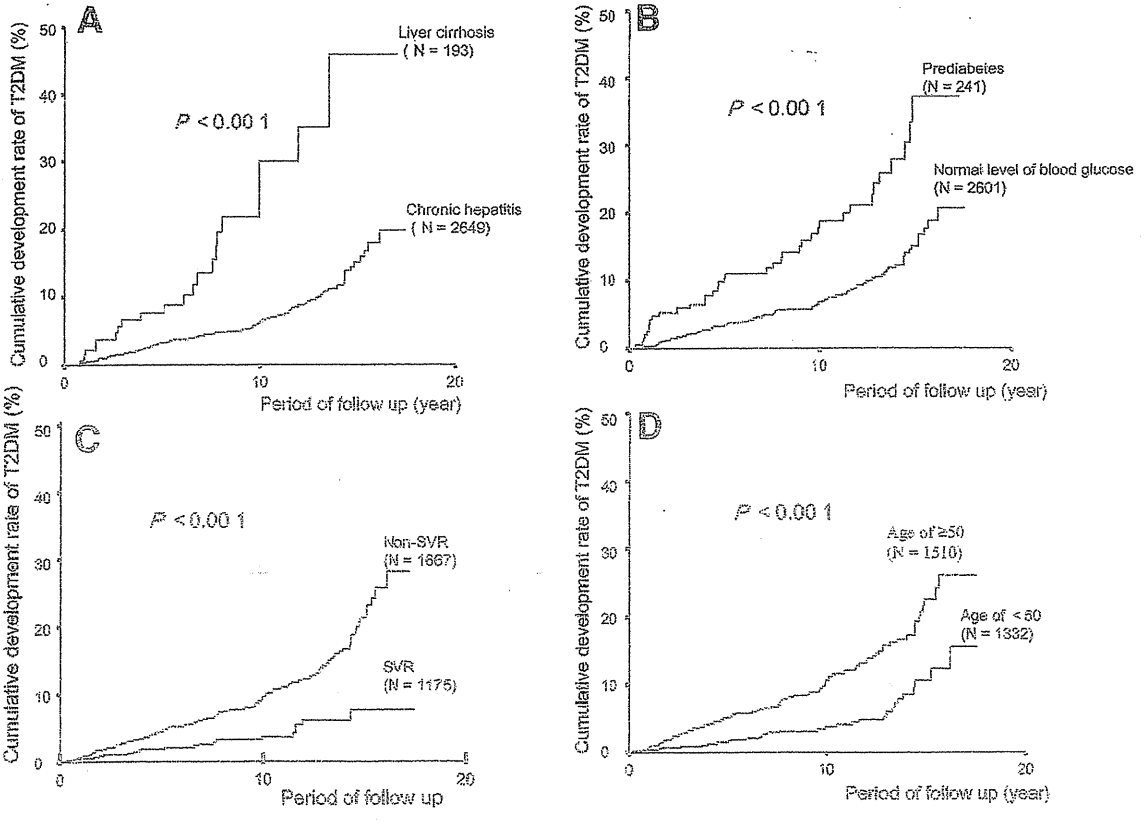

Fig. 2. Cumulative development rate of T2DM in patients treated with IFN. (A) Cumulative development rate of T2DM based on difference of hepatic fibrosis. (B) Cumulative development rate of T2DM based on the difference of glucose level. (C) Cumulative development rate of T2DM based on the difference of efficacy. (D) Cumulative development rate of T2DM based on the difference of age.

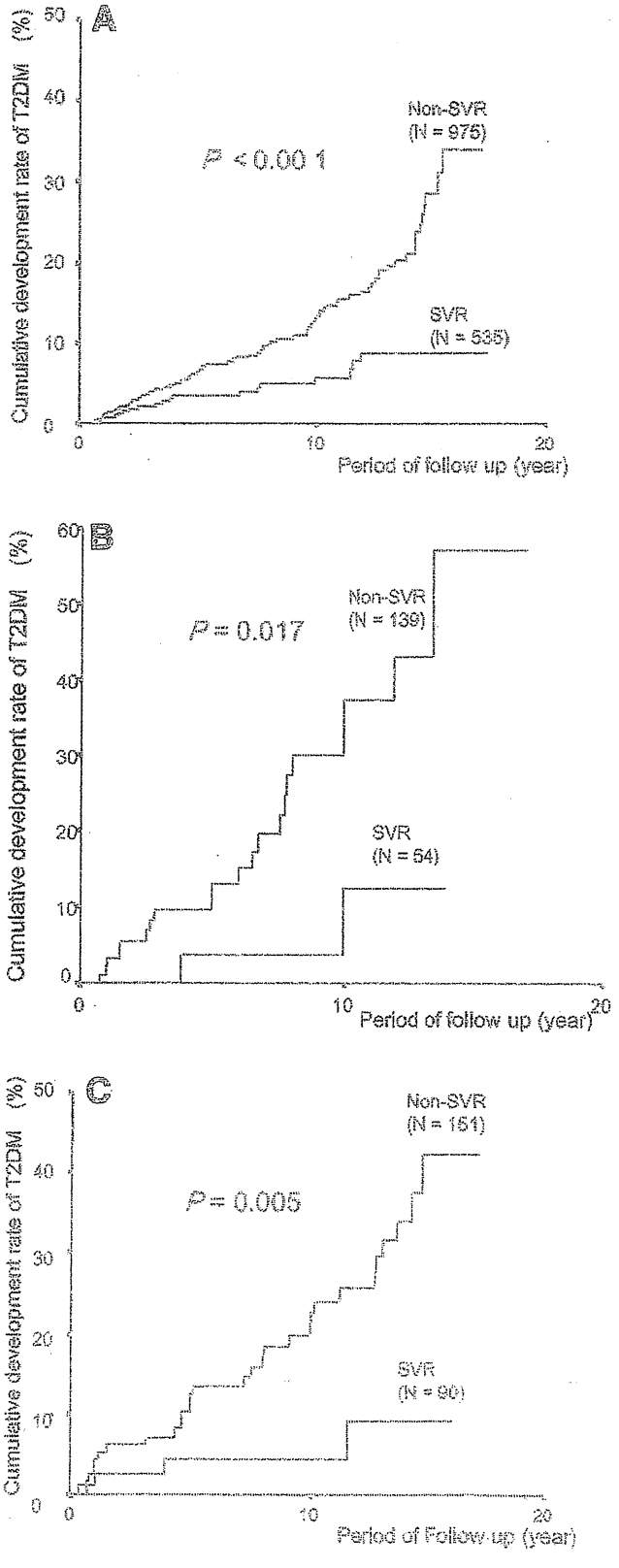

Fig. 3. Cumulative development rate of T2DM in patients with SVR or without SVR after IFN therapy. (A) Cumulative development rate of T2DM based on SVR or non-SVR in patients with age ≥50 years. (B) Cumulative development rate of T2DM based on SVR or non-SVR in patients with liver cirrhosis. (C) Cumulative development rate of T2DM based on the difference of SVR or non-SVR in patients with pre-diabetes.

The present study shows several findings with regard to development of T2DM after the termination of antivirus agents for HCV positive patients. First, the T2DM development rate in the non-SVR group was higher than that in the SVR group. The SVR caused a two-thirds reduction in the onset of T2DM in the course of posttreatment follow-up. That SVR reduced the onset of diabetes mellitus in HCV patients is in accordance with the data reported by Simó et al.[22] and Romero-Gómez et al.[23] Though the role of HCV in the pathogenesis of diabetes mellitus remains speculative, the following possible mechanisms have been reported: (1) patients with HCV have a tendency to attain insulin resistance[24]; (2) in transgenic mice, the expression of HCV core protein is associated with insulin resistance and T2DM development[25]; and (3) SVR in HCV patients reduces insulin resistance and onset of the incidence of abnormal glucose value.[26] Thus, it is accepted that clearance of HCV reduces the onset of T2DM.

Second, in addition to persistence of HCV, the present study suggests that aging, histological progression, and pre-diabetes enhanced the onset of T2DM in patients with HCV infection. However, when HCV was eradicated even in patients with age ≥50 years, pre-diabetes, or liver cirrhosis, the cumulative development rate of T2DM decreased.

T2DM is increasing dramatically in many Asian nations, including Japan, over the past decades.[27] It is widely accepted that 7 to 8 million people are affected by diabetes mellitus in Japan. Approximately 8% to 10% of adults in Japan have T2DM. In general, T2DM is associated with a genetic predisposition, but it is also strongly influenced by lifestyle-related factors, such as eating habits and/or physical activity.[28-33] The risk factors associated with T2DM include family history, age, sex, obesity, smoking, and physical activity. T2DM occurred in elderly patients compared to young patients. Life expectancies are long in Japan; thus, in the near future, a large number of patients with HCV will be >60 years of age. Therefore, it is apparent that the incidence of T2DM will increase in HCV-positive patients.

T2DM is a serious, costly disease. Treatment for T2DM may prevent some of its devastating complications, but does not usually restore normoglycemia or eliminate all the adverse consequences.[28,29] Moreover, HCV patients with T2DM are at major risk for HCC.[34] On the efficacy of IFN therapy, it has been reported that T2DM reduces HCV eradication via combination IFN + ribavirin therapy.[26] Thus, it should be considered whether HCV-positive patients should be treated with antiviral drugs in the histological nonprogression stage and at a non-elderly age for prevention of T2DM onset. If