Whether substitutions of aa 70 and/or 91 in the HCV-1b core region affect hepatocarcinogenesis in patients who do not achieve sustained virological response to IFN therapy and then receive another course of long-term IFN monotherapy remains to be investigated.

The present study included 494 consecutive patients who were infected with HCV genotype 1b and failed to achieve sustained virological response after the first course of IFN monotherapy for more than 24 weeks. The aims of the study were the following: (1) To evaluate the long-term efficacy of additional long-term IFN monotherapy on HCC in patients who showed no virological response to the first IFN therapy. (2) To analyze the predictive factors for HCC in patients on long-term IFN monotherapy. (3) To evaluate the clinical impact of aa substitutions in the HCV-1b core region on HCC.

PATIENTS AND METHODS

Patients

Among 2,716 consecutive HCV-1b infected Japanese adult patients, in whom IFN monotherapy was induced between February 1987 and May 2006 at Toranomon Hospital, 494 were selected in this retrospective study based on the following criteria: (1) Patients naive to IFN, (2) patients negative for hepatitis B surface antigen (by radioimmunoassay, Dainabot, Tokyo), positive for anti-HCV (by a third-generation enzyme immunoassay, Chiron Corp., Emerville, CA) and for HCV RNA by qualitative or quantitative analysis, before IFN therapy, (3) patients infected with a single genotype of HCV-1b, (4) patients with chronic liver disease, without HCC before and during IFN therapy, (5) patients treated with IFN alone for more than 24 weeks, and showed no sustained virological response, (6) patients who did or did not receive additional ≥48-week IFN monotherapy, (7) patients who had not been treated with IFN plus RBV or PEG-IFN alone during follow-up, (8) patients free of coinfection with human immunodeficiency virus, (9) patients who have not been treated with antiviral or immunosuppressants within 6 months before enrolment, (10) lifetime cumulative alcohol intake <500 kg (mild to moderate alcohol intake), and (11) patients free of other types of hepatitis, including hemochromatosis, Wilson disease, primary biliary cirrhosis, alcoholic liver disease, and autoimmune liver disease.

Figure 1 shows an overview of the study. All patients received the first treatment course of IFN alone (IFN-α and/or IFN-β) for more than 24 weeks, including initial aggressive induction therapy (every day within 8 weeks, followed by three times per week). Patients who did not achieve sustained virological response after the first course of IFN monotherapy were divided into two groups based on subsequent treatment with IFN alone; the additional-IFN group (representing patients who received another course of IFN monotherapy) and the no-additional-IFN group [representing patients who did not receive a subsequent course of IFN monotherapy based on concerns about adverse effects, lack of time for treatment, physician recommendation based on the appearance of depression, development of cardiopulmonary disease during or after the first course of IFN, and/or low levels of alanine aminotransferase (ALT)].

The study protocol was approved by the Human Ethics Review Committee of Toranomon Hospital and a signed consent form was obtained from each patient. Table I summarizes the clinical features of 494 patients at the start of the first course of IFN monotherapy. They included 292 men and 202 women, aged 21–75 (median, 53 years). The numbers of patients with fibrosis of the liver stages 1/2 and 3/4 were 384 and 45 patients, respectively. The median follow-up period was 10.5 years (range, 0.0–18.0 years).

Laboratory Investigations

Blood samples were frozen at −80°C within 4 hr of collection and thawed before testing. HCV genotype was determined by PCR using a mixed primer set derived from nucleotide sequences of the NS5 region [Chayama et al., 1993]. HCV RNA was quantitated by the branched DNA assay version 2.0 (Chiron Corp.), AMPLICOR GT HCV Monitor version 2.0 using the 10-fold dilution method (Roche Molecular Systems Inc., Pleasanton, CA), or COBAS TaqMan HCV test (Roche Diagnostics, Tokyo). A high viral load was defined as branched DNA assay ≥1.0 Meq/ml, AMPLICOR GT HCV Monitor ≥100 × 10^3 IU/ml, or COBAS TaqMan HCV test ≥5.0 log IU/ml. Low viral load was

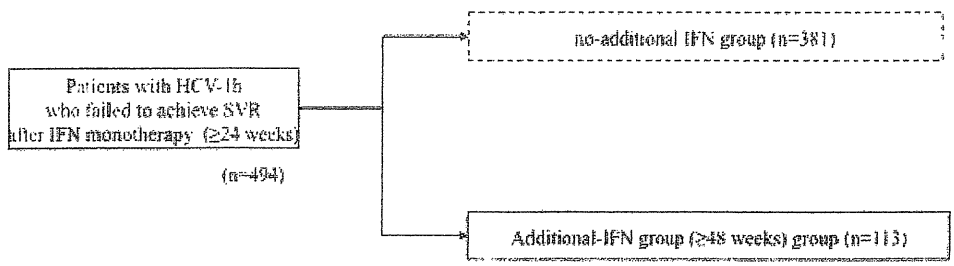

Fig. 1. Overview of the study.

TABLE I. Patient Characteristics at the Start of the First Course of IFN Treatment

Sex (male/female)	292/202
Age (year)	53 (21–75)
HCV genotype 1b	494
Fibrosis stage (F1/F2/F3/F4)	384/45
Aspartate aminotransferase (IU/l)	57 (18–348)
Alanine aminotransferase (IU/l)	84 (16–782)
Amino acid substitutions in core region of HCV genotype 1b [Gln70(His 70) and Met 91/Arg 70 and/or Leu 91)]	47/361
Treatment group (additional-IFN group)/ (no-additional-IFN group)	381/113
Total cholesterol (mg/dl)	166 (92–273)
Low-density lipoprotein cholesterol (mg/dl)	99 (28–280)
High-density lipoprotein cholesterol (mg/dl)	45 (18–102)
Triglyceride (mg/dl)	85 (28–437)
Body mass index (kg/m^2)	23 (16.7–35.2)

Data are number of patients or median values (range).

defined as branched DNA assay <1.0 Meq/ml, AMPLICOR GT HCV Monitor $<100 \times 10^3$ IU/ml, or COBAS TaqMan HCV test <5.0 log IU/ml. The lower limit of HCV RNA qualitative analysis (Amplicor, Roche Diagnostics, Manaheim, Germany) was 100 copies/ml, and that of COBAS TaqMan HCV test was 1.2 log IU/ml. Samples with undetectable HCV RNA by qualitative analysis or COBAS TaqMan HCV test were defined as negative HCV RNA.

Detection of Amino Acid (aa) Substitutions in the Core Regions of HCV-1b

Basically, aa substitutions of the HCV-1b core region were analyzed by direct sequencing. HCV RNA was extracted from serum samples at the start of antiviral therapy and reverse transcribed with random primer and MMLV reverse transcriptase (Takara Syuzo, Tokyo, Japan). Nucleic acids of the core region were amplified by nested PCR using the following primers: The first-round PCR was performed with CE1 (sense, 5′-GTC TGC GGA ACC GGT GAG TA-3′, nucleotides: 134–153) and CE2 (antisense, 5′-GAC GTG GCG TCG TAT TGT CG-3′, nucleotides: 1096–1115) primers, and the second-round PCR with CC9 (sense, 5′-ACT GCT AGC CGA GTA GTG TT-3′, nucleotides: 234–253) and CE6 (antisense, 5′-GGA GCA GTC GTT CGT GAC AT-3′, nucleotides: 934–953) primers. All samples were initially denatured at 95°C for 2 min. The 35 cycles of amplification were set as follow: Denaturation for 30 sec at 95°C, annealing of primers for 30 sec at 55°C, and extension for 1 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).

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 HCV-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].

Liver Histopathological Examination

Liver biopsy specimens from 429 patients 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 examination contained six or more portal areas. Histopathological diagnosis was made 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].

Diagnosis of Liver Cirrhosis

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 (CT) or ultrasonography (US). Ascites, edema and esophageal varicosities, facilitated the diagnosis when present. Furthermore, 19 patients with cirrhosis underwent peritoneoscopy and/or liver biopsy; the remaining 18 patients did not undergo histological assessment, and therefore their diagnosis was established morphologically based on imaging examination.

Follow-Up and Diagnosis of Hepatocellular Carcinoma

Clinical and laboratory assessments were performed at least once every month before, during, and after treatment. Adverse effects were monitored clinically by careful interviews and medical examination at least once every month. Blood samples were also obtained at least once every month before, during, and after treatment. Patients were examined for HCC by abdominal US every 3–6 months. If HCC was suspected, additional procedures, such as magnetic resonance imaging, abdominal angiography, and US-guided tumor biopsy if necessary, were used to confirm the diagnosis. Follow-up time represented the time from the end of the first course of IFN treatment until death or until the last visit or until the start of

IFN-based treatment including IFN plus ribavirin therapy.

Statistical Analysis

Non-parametric tests were used to compare variables between groups, including the Mann–Whitney U-test. The cumulative rate of HCC was calculated using the Kaplan–Meier technique and differences between the HCC rate curves were tested using the log-rank test. Statistical analysis of the HCC rate according to groups was calculated using the period between the end of first IFN monotherapy and appearance of HCC. Stepwise Cox regression analysis was used to determine independent predictive factors associated with hepatocarcinogenesis. The hazard ratio (HR) and 95% confidence interval (95% CI) were also calculated. Potential predictive factors associated with hepatocarcinogenesis were sex, age, body mass index, aspartate aminotransferase (AST), ALT, total cholesterol, low-density lipoprotein cholesterol (LDL-cholesterol), high density lipoprotein cholesterol (HDL-cholesterol), triglyceride, treatment group, levels of viremia, stage of fibrosis, and aa substitutions in the core region. Variables that achieved statistical significance ($P < 0.05$) or marginal significance ($P < 0.10$) on univariate analysis were entered into multivariate Cox proportional hazard model to identify significant independent factors that correlated with the HCC rate. Statistical comparisons were performed using the SPSS software (SPSS Inc., Chicago, IL). All P-values less than 0.05 by the two-tailed test were considered significant.

RESULTS

Background of Treatment Groups

Of the 494 patients who did not achieve sustained virological response after the first course of IFN of more than 24 weeks, 381 (77.1%) did not receive another course of IFN monotherapy (no-additional-IFN group), while the remaining 113 (22.9%) received another course of IFN monotherapy for more than 48 weeks (additional-IFN group); 24 patients of the latter group achieved sustained virological response following long-term IFN monotherapy.

Of the 494 patients, 418 (84.6%) received IFN-α alone; 55 patients (11.1%) received IFN-β alone; while the remaining 21 patients (4.3%) received a combination of IFN-α and IFN-β. In the no-additional-IFN group, the median duration of the first course of IFN was 24.9 weeks (range, 29–553.6 weeks). The median total dose of the first course of IFN was 630 MU (range, 16–7,873 MU). In the additional-IFN group, for the first and second courses of IFN monotherapy, the median duration of IFN was 24.4 weeks (range, 20–180.4 weeks) and 134.9 weeks (range, 63–752.4), respectively. In the additional-IFN group, for the first and second courses of IFN monotherapy, the median total dose of IFN was 624 MU (range, 210–2,777 MU) and 2,113 MU (range, 444–1,1373), respectively. There were no significant differences between the duration and total dose of the first course of IFN in the no-additional-IFN treatment group and those of the additional-IFN group (Mann–Whitney U-test). The median cumulative total duration and cumulative total dose for the additional-IFN group were 143.4 weeks (range, 23.7–867.7 weeks) and 2,756 MU (range, 1,002–12,020 MU), respectively.

Cumulative HCC Rates According to Treatment Group

During follow-up after the first course of IFN monotherapy, HCC was diagnosed in 62 (16.3%) patients of the no-additional-IFN group and 13 (11.5%) of the additional-IFN group, with cumulative HCC rates of 9.9, 5.2% at the end of 5 years; 18.7, 13.3% at the end of 10 years; and 34.6, 17.4% at the end of 15 years, for the no-additional-IFN and additional-IFN groups, respectively. The rates were significantly different between the two groups ($P = 0.035$; Log-rank test) (Fig. 2A).

HCC Rates According to aa Substitutions in the Core Region of HCV-1b

During follow-up after the first course of IFN monotherapy, 14 of 47 patients (29.8%) with Gln70(His 70) and Met 91, and 56 of 361 (15.5%) with Arg70 and/or Leu 91 developed HCC. In patients with Arg70 and/or Leu 91, the cumulative HCC rate was 4.7% at the end of 5 years; 16.4% at the end of 10 years; 27.9% at the end of 15 years. In patients with Gln70(His 70) and Met 91, the respective rates were 15.3, 27.3, and 46.6%. The rates for patients with Gln70(His 70) and Met 91 were significantly higher than those with Arg70 and/or Leu 91 ($P = 0.031$; Log-rank test) (Fig. 2B).

The subset data of 252 patients with Gln70(His 70) and/or Met 91 was also analyzed separately. HCC was diagnosed in 34 patients of the no-additional-IFN group and five patients in additional-IFN group, with cumulative HCC rates of 10.3, 3.8% at the end of 5 years; 22.9, 11.6% at the end of 10 years; 38.1, and 11.6% at the end of 15 years, for the no-additional-IFN and additional-IFN groups, respectively. The cumulative HCC rates were significantly different between the additional-IFN group and the no-additional-IFN group ($P = 0.022$; Log-rank test) (Fig. 2C).

Determinants of HCC by Multivariate Analysis

The entire data set was also analyzed to determine those factors that could predict HCC. Univariate analysis showed significant relationship between HCC and the following parameters: Stage of fibrosis of the liver (F3/4, $P < 0.001$), AST level (≥58 IU/L, $P < 0.001$), ALT level (≥100 IU/L, $P < 0.001$), age (≥55 years, $P = 0.001$), LDL-cholesterol level

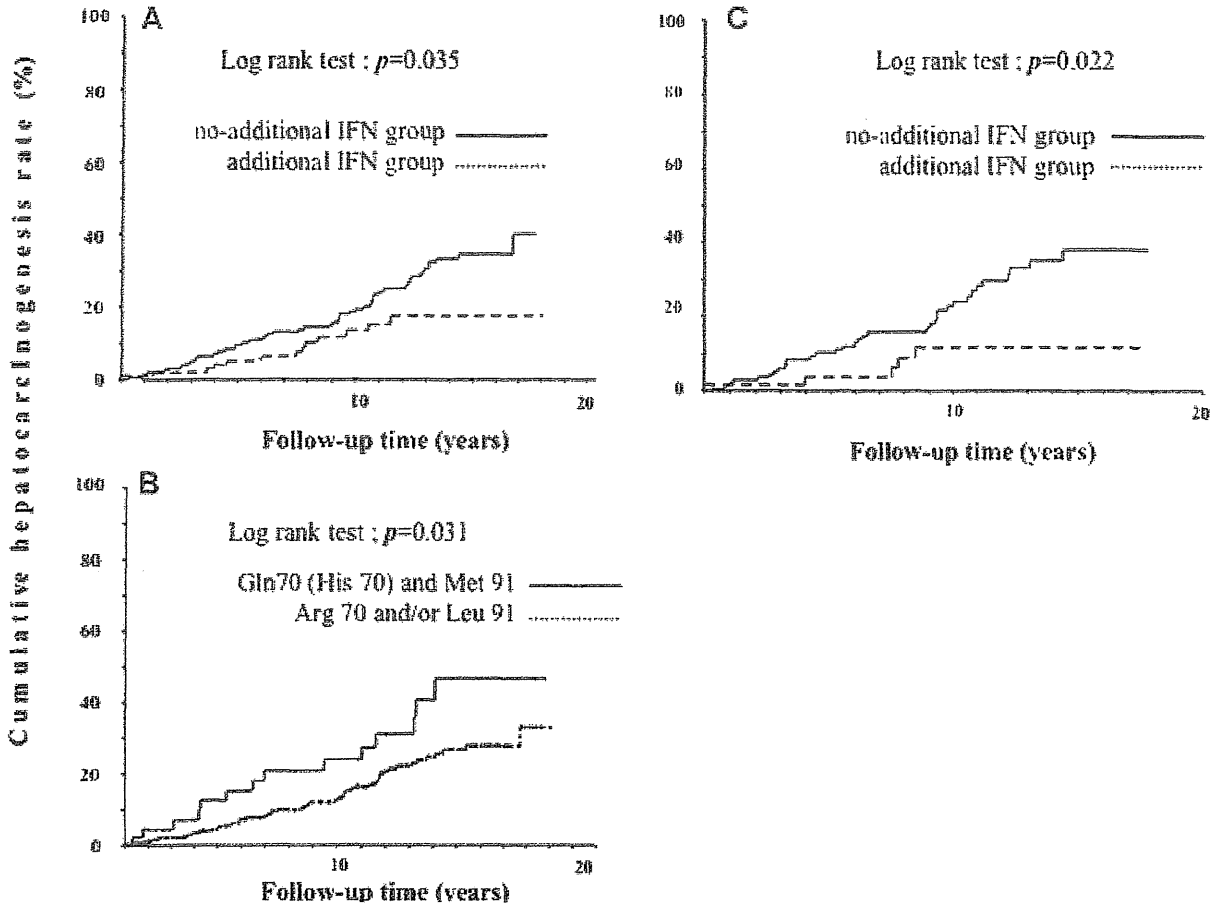

Fig. 2. Cumulative HCC rates according to study group (**A**), aa substitutions of core region (**B**), and patients with Gln70(His 70) and/or Met 91 substitutions (**C**). *P*-values by Log-rank test.

(<100 mg/dl, $P = 0.003$), sex (male, $P = 0.025$), aa substitutions in the core region (Gln70(His 70) and Met 91, $P = 0.031$), triglyceride level (≥100 IU/L, $P = 0.031$), and treatment group (no-additional-IFN group, $P = 0.035$). Next, these factors were entered into multivariate analysis, which then identified seven significant and independent determinants of HCC: Stage of fibrosis of the liver (F3/4; HR 9.98, $P < 0.001$), AST (≥58 IU/L; HR 3.27, $P = 0.001$), age (≥55 years; HR 2.71, $P = 0.002$), treatment group (no-additional-IFN group; HR 2.28, $P = 0.034$), substitution of aa 70 and/or 91 in the core region (Gln70(His 70) and Met 91); HR 2.21, $P = 0.024$), LDL-cholesterol (<100 mg/dl; HR 2.10, $P = 0.017$), and sex (male; HR 2.02, $P = 0.027$) (Table II).

Determinants of HCC in Patients With Cirrhosis by Multivariate Analysis

Data of the 37 patients with cirrhosis were also analyzed separately to determine those factors that could predict HCC in this subset of patients. Univariate analysis showed that sex (male) and IFN treatment tended to correlate ($P = 0.074$) and correlated significantly ($P = 0.032$) with HCC, respectively. Multivariate analysis identified treatment group (no-additional-IFN group. HR 3.04, $P = 0.040$) as the only independent parameter that significantly correlated with HCC (Table III).

DISCUSSION

Previous studies showed that IFN monotherapy can reduce the risk of hepatocarcinogenesis [Nishiguchi et al., 1995; Ikeda et al., 1999; Yoshida et al., 1999; Arase et al., 2007; Nomura et al., 2007; McHutchison et al., 2008; Akuta et al., 2008]. Furthermore, a recent large-scale cohort study showed that patients with cirrhosis who received IFN alone had a lower risk of HCC than those who did not receive such therapy after a median follow up of 6.7 years [Lok et al., 2011]. However, there is no report of follow up of IFN monotherapy for more than 10 years, especially in patients who had failed to achieve sustained virological

TABLE II. Results of Univariate and Multivariate Analyses for Factors Associated With HCC in Patients Infected With HCV Genotype 1b, Who Showed no Sustained Virological Response Following IFN Monotherapy

Factors	Category	*P*-value (univariate analysis)	*P*-value (multivariate analysis)	Hazard ratio (95%CI)
Fibrosis stage	1:<F2	<0.001	<0.001	1
	2:≥F3			9.98 (4.35–22.90)
Aspartate aminotransferase (IU/L)	1: <58	<0.001	0.001	1
	2: ≥58			3.27 (1.60–6.67)
Age (year)	1: <55	0.001	0.002	1
	2: ≥55			2.71 (1.45–5.04)
Treatment group	1: Additional-IFN group	0.035	0.034	1
	2: No-additional-IFN group			2.49 (1.06–4.88)
Amino acid substitutions (Core aa 70 and 91)	1:Arg 70 and/or Leu 91	0.031	0.024	1
	2: Gln70(His 70) and Met 91			2.21 (1.12–4.41)
LDL-cholesterol (mg/dl)	1: ≥100	0.003	0.017	1
	2:<100			2.10 (1.14–3.85)
Sex	1: Female	0.025	0.027	1
	2: Male			2.02 (1.08–3.78)

CI, confidence interval.

response to IFN monotherapy, and thus the long-term effect of IFN monotherapy on the risk of HCC is unknown. To our knowledge, this study is the first to report the HCC rates in patients infected with HCV-1b, who failed to achieve a sustained virological response after the first IFN monotherapy for more than 24 weeks, in whom more than 10 years had elapsed since the end of the first IFN monotherapy. The main finding of this study is that long-term IFN monotherapy significantly reduced the risk of HCC in the whole population sample (HR = 2.28), as well as in patients with cirrhosis (HR = 3.04).

Various factors have been reported to correlate with HCV-related HCC, such as old age, male sex, advanced histological stage of liver damage, alcohol intake, HCV genotype, and hepatocyte steatosis [Ikeda et al., 1999; Freeman et al., 2001; Ohata et al., 2003; Bruno et al., 2007; Kurosaki et al., 2010; Koike et al., 2010]. Other studies also identified various host-related predictors of HCC, including mutations in a region spanning aas 2209 to 2248 within the NS5A protein, the so-called IFN sensitivity determining region (ISDR) [Enomoto et al., 1996; Giménez-Barcons et al., 2001] and aa 70/91 substitution in the core region of HCV-1b [Akuta et al., 2007b], as viral-related factors, in addition to genetic variation near interleukin-28B (IL28B) [Fabris et al., 2011]. In present study, analysis of data of the entire group identified age, male sex, progressive fibrosis stage, high AST level, and low LDL-cholesterol level as host factors, Gln70(His 70) and Met 91 as viral factor, and additional IFN treatment as determinants of HCC rates. These results suggest that HCC seems to develop from a dynamic tripartite interaction of various viral, host, and treatment factors. Further understanding of the complex interaction between these factors should facilitate the development of more effective therapeutic strategies.

Despite numerous lines of epidemiologic evidence linking HCV infection and HCC, it is not clear whether HCV itself plays a direct or indirect role in the pathogenesis of HCC [Koike, 2005]. While studies on transgenic mice suggest that the HCV core region is probably oncogenic [Moriya et al., 1998], the clinical impact of the core region on HCC remains unclear. Previous reports indicated that aa substitutions in the core region of HCV-1b are predictors of poor virological response to antiviral therapy [Akuta et al., 2005, 2007a, 2010] and HCC [Akuta et al., 2007b; Fishman et al., 2009; Hu et al., 2009; Nakamoto et al., 2010]. The present study indicated that aa substitution in the core region of HCV-1b at the start of antiviral therapy also affected the HCC rate in those patients who showed no sustained virological response after the first IFN monotherapy after more than 10 years of follow-up. Previous reports identified PA28γ-dependent pathway as one of the mechanisms of HCV-associated HCC. Moriishi et al. [2003; 2007]

TABLE III. Results of Univariate and Multivariate Analyses for Factors Associated With HCC in Patients With Cirrhosis Infected With HCV Genotype 1b, Who Showed no Sustained Virological Response Following IFN Monotherapy

Factors	Category	*P*-value (univariate analysis)	*P*-value (multivariate analysis)	Hazard ratio (95% CI)
Treatment group	1: Additional-IFN group	0.032	0.040	1
	2: No-additional-IFN group			3.04 (1.05–8.85)

reported that knockout of the PA28γ gene induced HCV core protein accumulation in nuclei of hepatocytes of HCV-core gene transgenic mice and disrupted the development of both hepatic steatosis and HCC. Furthermore, HCV core protein also enhanced the binding of liver X receptor α (LXRα)/retinoid X receptor α (RXRα) to LXR-response element in the presence of PA28γ [Moriishi et al., 2007]. Thus, PA28γ seems to play a crucial role in the development of HCV-associated steatogenesis and HCC. Further studies should be performed to link the findings of animal studies to the clinical impact of aa substitutions in the HCV-1b core region on HCC.

The association between metabolic factors and the risk of HCC is still not clear. Previous studies reported that hepatic steatosis is a significant factor in the development of HCV-related HCC, independent of age, sex, body mass index, stage of fibrosis of the liver, and response to antiviral therapy [Ohata et al., 2003, Kurosaki et al., 2010]. Other reports indicated that obesity and diabetes mellitus are risk factors for HCC [Polesel et al., 2009, Kawamura et al., 2010, Sumida et al., 2011]. Evidence suggests that HCV core protein causes mitochondrial electron transfer system dysfunction and activation of peroxisome proliferator activated receptor-α (PPARα). In the presence of mitochondrial dysfunction, PPARα exacerbates steatosis and the persistent activation of PPARα contributes to hepatocarcinogenesis by inducing oxidative stress and cell-growth signal activation [Koike et al., 2010]. In the present study, multivariate analysis identified aa substitution in the core region and low levels of LDL-cholesterol as determinants of HCC. This is the first study to report lipid metabolism as a factor associated with HCC. This result is not inconsistent with those of basic research. Further studies of larger number of patients of different races are required to confirm these findings.

Genetic variations near the IL28B gene are predictors of poor virological response to PEG-IFN/ribavirin combination therapy and triple therapy of telaprevir/PEG-IFN/ribavirin [Ge et al., 2009; Suppiah et al., 2009; Tanaka et al., 2009; Akuta et al., 2010; Rauch et al., 2010] and are reported to be associated with HCC, although their impact on HCC is controversial [Bochud et al., 2011; Fabris et al., 2011]. In this study, the effect of genetic variation in rs8099917 on HCC was assessed in 159 of 494 patients. Interestingly, the HCC rate in genotype TT of the treatment-sensitive type was not significantly lower than that in genotype non-TT of the treatment-resistant type ($P = 0.54$). Further studies based on larger patient sample should be performed to investigate the relationship between genetic variations near the IL28B gene and HCC.

The present study has certain limitations. The study did not investigate all the viral factors associated with HCC. Previous studies reported the association of HCV-1b strains based on the secondary structure of the aminoterminal portion of the HCV NS3 protein and HCC [Ogata et al., 2003], and mutations in a region spanning aas 2209 to 2248 within the NS5A protein (ISDR) and HCC [Enomoto et al., 1996; Giménez-Barcons et al., 2001]. In this study, viral factors other than the HCV core region were not investigated. Furthermore, the clinical impact of life-style related diseases (e.g., diabetes, insulin resistance, non-alcoholic steatohepatitis, and smoking) on the HCC rate were not investigated, with the exception of body mass index and lipid profile [Mason et al., 1999, El-Serag et al., 2004, Kawamura et al., 2010, Sumida et al., 2011]. Finally, all patients enrolled in this study were Japanese and none was infected with HCV-1a. Further studies should be performed to investigate the clinical impact of viral factors and life style-related diseases on HCC.

In conclusion, long-term IFN monotherapy reduced the risk of HCC, even in patients with cirrhosis. Substitution of aa 70 and/or 91 in the core region and lipid metabolism are important predictors of HCC after long-term IFN monotherapy.

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Determinants of Response to Triple Therapy of Telaprevir, Peginterferon, and Ribavirin in Previous Non-Responders Infected With HCV Genotype 1

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Patients who do not achieve sustained virological response to telaprevir/peginterferon (PEG-IFN)/ribavirin need to be identified. Predictive factors of virological response to the triple therapy in non-responders to previous PEG-IFN/ribavirin therapy are not clear. The aims of this study were to determine the predictive factors of virological response to a 24-week regimen of triple therapy in 15 non-responders to previous PEG-IFN/ribavirin therapy among 61 Japanese adults infected with HCV genotype 1. Overall, sustained virological response and end-of-treatment response were achieved by 27% and 60%, respectively. Telaprevir-resistant variants (by direct sequencing) appeared during or after treatment in 82% of patients who did not show sustained virological response, but disappeared at the end of study, except for one patient with resistant variant at baseline. Substitution at aa 70 (Arg70) and type of previous response to PEG-IFN/ribavirin (partial response) were identified as significant determinants of sustained virological response. In addition, alpha-fetoprotein level (<10 μg/L) and type of previous response (partial response) were identified as significant determinants of end-of-treatment response. Prediction of response to therapy based on the combination of these factors had high sensitivity, specificity, positive, and negative predictive values. In conclusion, this study identified amino acid substitution of the core region, alpha-fetoprotein level, and type of previous response as predictors of virological response to telaprevir/PEG-IFN/ribavirin in patients infected with HCV genotype 1b who had not responded to previous PEG-IFN/ribavirin therapy. ***J. Med. Virol. 84:1097–1105, 2012.*** © 2012 Wiley Periodicals, Inc.

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KEY WORDS: HCV; core region; *IL28B*; telaprevir; peginterferon; ribavirin; partial response; null response; alpha-fetoprotein

INTRODUCTION

For chronic hepatitis C virus (HCV) infection, even when treated with the combination of peginterferon (PEG-IFN) and ribavirin, a sustained virological response lasting more than 24 weeks after withdrawal of treatment is achieved at most in 50% of patients with high viral load and infected with HCV genotype 1b (HCV-1b) [Manns et al., 2001; Fried et al., 2002]. Recently, new strategies were introduced for the treatment of chronic HCV infection based on inhibition of 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 [Lin et al., 2006]. Subsequent studies found that telaprevir, when combined with PEG-IFN and ribavirin, exhibited a robust antiviral activity [Modi and Hoofnagle, 2007; Zeuzem, 2008]. Two previous studies (PROVE1 and PROVE2) showed that the 12- and 24-week regimens of telaprevir/PEG-IFN/ribavirin achieved sustained virological response rates of 35–60% and 61–69%, respectively, in patients infected

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with HCV-1 [Hézode et al., 2009; McHutchison et al., 2009]. However, a recent study (PROVE3) also showed that the sustained virological response rates after 24- and 48-week regimens of the above triple therapy were lower (39% and 38%, respectively) in non-responders to previous PEG-IFN/ribavirin therapy infected with HCV-1, who did not achieve HCV-RNA negativity during or at the end of the initial combination therapy [McHutchison et al., 2010]. Furthermore, telaprevir-based regimen is reported to induce resistant variants [Lin et al., 2005; Kieffer et al., 2007], and side effects such as anemia and rash [Hézode et al., 2009; McHutchison et al., 2009, 2010]. Hence, prior non-responders, who do not achieve sustained virological response by triple therapy, need to be identified to avoid unnecessary side effects and appearance of telaprevir-resistant variants.

Amino acid (aa) substitutions at position 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 [Akuta et al., 2005, 2007a; Donlin et al., 2007], and also affect the clinical outcome, including hepatocarcinogenesis [Akuta et al., 2007b; Fishman et al., 2009]. Furthermore, *IL28B* genotype (rs8099917, rs12979860) on chromosome 19 as a host-related factor, which encodes IFN-λ-3, is a pretreatment predictor of virological response to 48-week PEG-IFN plus ribavirin combination therapy in individuals infected with HCV-1 [Ge et al., 2009; Suppiah et al., 2009; Tanaka et al., 2009], and also affect the clinical outcome, such as spontaneous clearance of HCV [Thomas et al., 2009]. Recent reports identified *IL28B* genotype and aa substitution of the core region as predictors of sustained virological response to telaprevir/PEG-IFN/ribavirin triple therapy in Japanese patients infected with HCV-1b [Akuta et al., 2010, 2011; Chayama et al., 2011]. However, it is not clear at this stage whether *IL28B* genotype and aa substitution of the core region can be used to predict the virological response to triple therapy in previous non-responders.

The aim of this study was to investigate the predictive factors of virological response to 24-week regimen of triple therapy in Japanese adult patients infected with HCV-1 who did not respond to previous dual PEG-IFN/ribavirin therapy.

PATIENTS AND METHODS

Study Patients

Between May 2008 and September 2009, 61 patients infected with HCV were recruited in this study at the Department of Hepatology, Toranomon Hospital, which is located 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 participation in this trial. Patients were assigned to a 24-week regimen of triple therapy [telaprevir (MP-424), PEG-IFN, and ribavirin] for 12 weeks followed by dual therapy of PEG-IFN and ribavirin for 12 weeks (the T12PR24 group).

Fifteen of 61 patients met the following inclusion and exclusion criteria: (i) diagnosis of chronic hepatitis C. (ii) HCV-1 confirmed by sequence analysis. (iii) HCV-RNA levels of $\geq$5.0 log IU/ml determined by the COBAS TaqMan HCV test (Roche Diagnostics, Tokyo, Japan). (iv) Japanese (Mongoloid) ethnicity. (v) Age at study entry of 20–65 years. (vi) Body weight $\geq$35 and $\leq$120 kg at the time of registration. (vii) Absence of decompensated liver cirrhosis. (viii) No detectable hepatitis B surface antigen (HBsAg) in serum. (ix) No history of hepato cellular carcinoma. (x) No previous treatment for malignancy. (xi) No history of autoimmune hepatitis, alcohol liver disease, hemochromatosis, and chronic liver disease other than chronic hepatitis C. (xii) No 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 $\leq$50 ml/min at baseline, diabetes requiring treatment or fasting glucose level of $\geq$110 mg/dl, autoimmune disease, cerebrovascular disorders, thyroid dysfunction uncontrollable by medical treatment, chronic pulmonary disease, allergy to medication or anaphylaxis at baseline. (xiii) Hemoglobin level of $\geq$12 g/dl, neutrophil count $\geq$1,500/mm^3, and platelet count of $\geq$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. (xiv) Previous non-responders, who did not achieve HCV-RNA negativity during or at the end of 24- to 48-week PEG-IFN plus ribavirin combination therapy. Previous non-response was defined as null response (a reduction of <2 $\log_{10}$ in HCV-RNA during treatment) or partial response (a reduction of 2 $\log_{10}$ or more in HCV-RNA during treatment).

In this study, all of 15 patients were followed-up for at least 24 weeks after the completion of treatment. The treatment efficacy was evaluated by HCV-RNA negativity at the end of treatment (end-of-treatment response) and 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 mg three times a day at an 8-hr (q8) interval after the meal. PEG-IFNα-2b (PEG-Intron; Schering Plough, Kenklworth, NJ) was injected subcutaneously at a median dose of 1.5 μg/kg (range: 1.3–1.7 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, neutrophil 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 mg, 800 to 600 mg 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 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 I. Profile and Laboratory Data at Commencement of Telaprevir, Peginterferon, and Ribavirin Triple Therapy of 15 Japanese Patients Infected With HCV Genotype 1, Who had been Non-Responders to Peginterferon Plus Ribavirin Combination Therapy

Demographic data	
n	15
Sex (M/F)	8/7
Age (years)*	56 (40–65)
History of blood transfusion	3 (20.0%)
Family history of liver disease	2 (13.3%)
Body mass index (kg/m^2)*	22.7 (18.1–26.5)
Laboratory data*	
HCV genotype (la/lb)	1/14
Level of viremia (log IU/ml)	6.6 (5.8–7.4)
Serum aspartate aminotransferase (IU/L)	36 (20–137)
Serum alanine aminotransferase (IU/L)	48 (17–136)
Serum albumin (g/dl)	3.9 (3.2–4.5)
Gamma-glutamyl transpeptidase (IU/L)	52 (20–154)
Leukocyte count (/mm^3)	4,700 (3,300–6,500)
Hemoglobin (g/dl)	14.4 (12.6–16.6)
Platelet count (×10^4/mm^3)	16.0 (9.1–23.9)
Alpha-fetoprotein (μg/L)	7 (2–38)
Total cholesterol (mg/dl)	178 (110–228)
Fasting plasma glucose (mg/dl)	89(81–111)
Treatment	
PEG-IFNα-2b dose (μg/kg)*	1.5 (1.3–1.7)
Ribavirin dose (mg/kg)*	11.8 (8.1–14.5)
Amino acid substitutions in the HCV genotype lb	
Core aa 70 (arginine/glutamine (histidine)/ND)	6/8/1
Core aa 91 (leucine/methionine/ND)	6/8/1
ISDR of NS5A (wild-type/non wild-type/ND)	13/1/1
IRRDR of NS5A (≤5/≥6/ND)	12/2/1
IL28B genotype	
rs8099917 genotype (TT/TG/GG)	1/12/2
rsl2979860 genotype (CC/CT/TT)	1/12/2
ITPA genotype	
rsl12735 genotype (CC/CA/AA)	14/1/0
Type of previous response to peginterferon/ribavirin	
Partial response/Null response	8/7

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

Table I summarizes the profiles and laboratory data of the 15 patients at the commencement of treatment. They included eight males and seven females, aged 40–65 years (median, 56 years). The present study was performed based on the Japanese patients infected with HCV-1b, except for one patient infected with HCV-1a.

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. 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 negative.

Assessments of Telaprevir-Resistant Variants

To analyze for resistant variants before, during, and after triple therapy, HCV-RNA was isolated from plasma, and the NS3/4A protease domains were amplified by reverse-transcriptase polymerase chain reaction assay and sequenced. Analyses were performed on baseline samples and in non-responders (HCV-RNA detectable during or at the end of treatment), viral breakthrough (re-elevation of viral loads before the end of treatment, even when HCV-RNA was temporarily negative during treatment), and relapse (re-elevation of viral loads after the end of treatment, even when HCV-RNA was negative at the end of treatment) by triple therapy. Telaprevir-resistant variants included V36A/M, T54A/S, R155I/K/M/T, and A156S/T/V [Kieffer et al., 2007]. In the present study, aa substitutions of NS3/4A were analyzed by direct sequencing.

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 [Kato et al., 1990], the sequence of 1–191 aa in the core protein of HCV-1b was determined and then compared with the consensus sequence constructed in a previous study 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 HCV-1b (ISDR) reported by Enomoto et al. [1996] was determined, and the numbers of aa substitutions in ISDR were defined as wild-type (0, 1) or non wild-type (≥2) in comparison with HCV-J. Furthermore, the sequence of 2334-2379 aa in the NS5A of HCV-1b (IRRDR) reported by El-Shamy et al. [2008] was determined and then compared with the consensus sequence constructed in a previous study. In the present study, aa substitutions

of the core region, and NS5A-ISDR/IRRDR of HCV-1b were analyzed by direct sequencing.

Determination of IL28B and ITPA Genotype

IL28B (rs8099917 and rs12979860) and *ITPA* (rs1127354) were genotyped by the Invader assay, TaqMan assay, or direct sequencing, as described previously [Ohnishi et al., 2001; Suzuki et al., 2003, 2011].

Statistical Analysis

Non-parametric tests (chi-squared test and Fisher's exact probability test) were used to determine those factors that significantly contributed to sustained virological response and end-of-treatment response. 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 determined. Each variable was transformed into categorical data consisting of two simple ordinal numbers for analyses. The potential pretreatment factors associated with sustained virological response and end-of-treatment response included the following variables: sex, age, history of blood transfusion, familial history of liver disease, body mass index, aspartate aminotransferase, alanine aminotransferase, albumin, gamma-glutamyl transpeptidase, leukocyte count, hemoglobin, platelet count, HCV genotype, HCV-RNA level, alpha-fetoprotein, total cholesterol, fasting blood sugar, PEG-IFN dose/body weight, ribavirin dose/body weight, type of previous response to PEG-IFN/ribavirin, *IL28B* and *ITPA* genotype, and amino acid substitution in the core region, and NS5A-ISDR/IRRDR. Statistical analyses were performed using the SPSS software (SPSS Inc., Chicago, IL). Sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) were also calculated to determine the reliability of predictors of the response to therapy.

RESULTS

Virological Response to Therapy

Figure 1 shows the profile at commencement of triple therapy, virological course, and efficacy of treatment. The sustained virological response rates were 26.7% [four patients (Cases 1–4)], and the end-of-treatment response rates were 60.0% [nine patients (Case 1–9)]. Of the 11 patients (Cases 5–15) who did not show sustained virological response, the relapse, breakthrough, and non-response rates were 45.5% [five patients (Cases 5–9)], 36.4% [4 (Cases 10–13)], and 18.2% [2 (Cases 14, 15)], respectively. Three patients (Cases 10, 13, 15) stopped telaprevir before the completion of 12-week treatment (PEG-IFN and ribavirin continued), and one patient (9 weeks, Case 9) stopped the triple therapy before the completion of the 24-week regimen, due to a fall in Hb concentration.

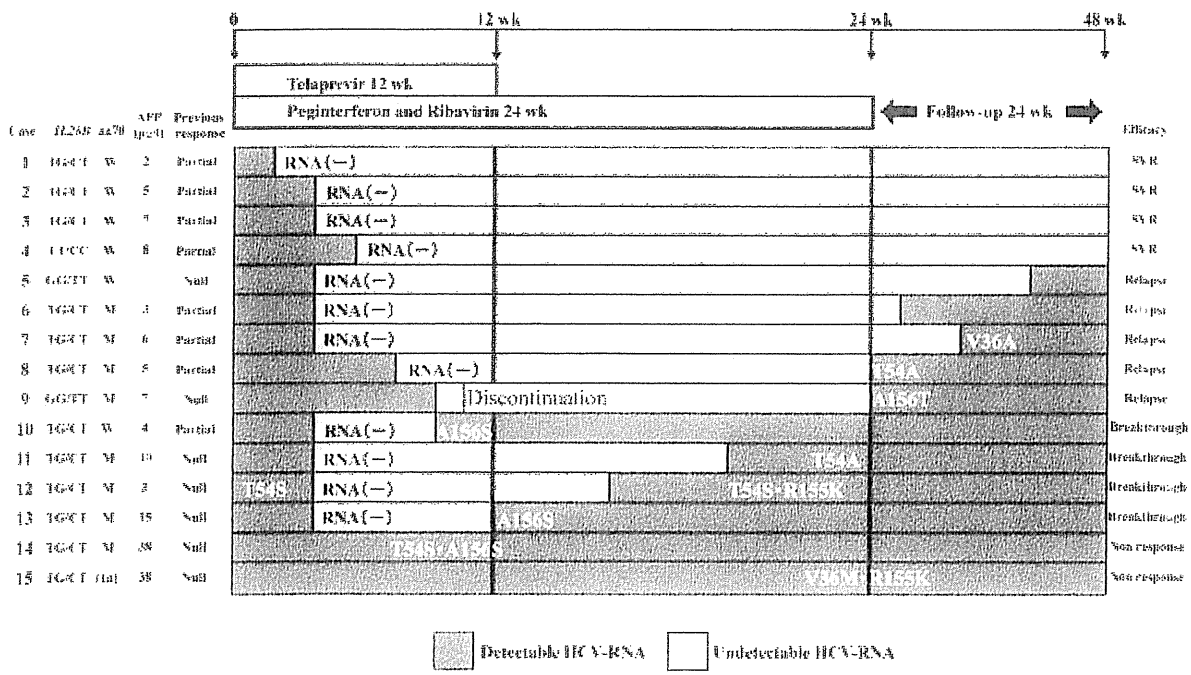

Fig. 1. Profiles at commencement of triple therapy, virological course, and treatment efficacy. The sustained virological response rates were 27%, and the end-of-treatment response rates were 60%. rs8099917/rs12979860 genotypes: *IL28B*, W: wild type (Arg70 substitution at core aa 70), M: mutant type (Gln70/His70). SVR: sustained virological response.

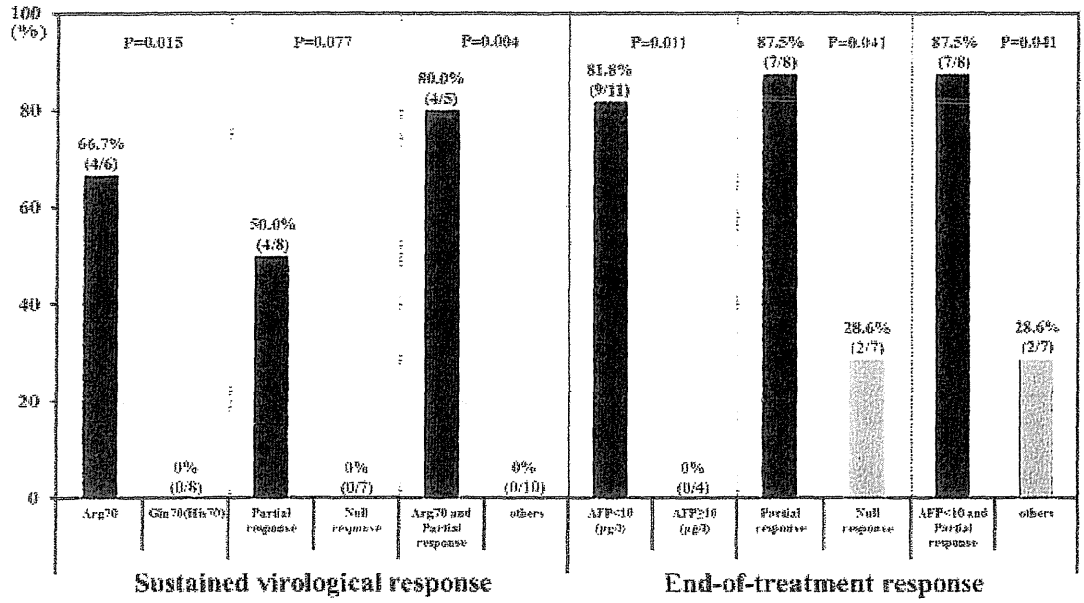

Fig. 2. Predictive factors associated with sustained virological response and end-of-treatment response to triple therapy. Arg70 and partial response are significant predictors of high-sustained virological response rate. Low level of alpha-fetoprotein and partial response are significant predictors of high end-of-treatment response rate.

Telaprevir-resistant variants were detected at baseline by direct sequencing in 6.7% [one patient (Case 12 with T54S)]. Of 11 patients who did not show a sustained virological response to triple therapy, telaprevir-resistant variants were detected during or after treatment in 81.8% [nine patients (Cases 7–15)], and not detected in 18.2% [two patients (Cases 5, 6)]. Resistant variants were consistent with those that have been reported previously [two patients with V36A/M (Cases 7, 15), four with T54A/S (Cases 8, 11, 12, 14), two with R155K (Cases 12, 15), and four with A156S/T (Cases 9, 10, 13, 14)] [Kieffer et al., 2007]. They were no longer detected by direct sequencing at 24 weeks after the completion of treatment, except for one patient with baseline-resistant variant (Case 12 with T54S).

Predictive Factors Associated With Sustained Virological Response

Fourteen of 15 patients showed *IL28B* rs8099917 non TT and rs12979860 non CC, whereas the other one patient (Case 4) had rs8099917 TT and rs12979860 CC. Thus, in non-responders to previous treatment, *IL28B* genotype did not play a role in sustained virological response.

The sustained virological response rate was significantly higher in patients with Arg70 [66.7% (four of six patients)] than in those with Gln70(His70) [0% (0 of 8)] ($P = 0.015$). Furthermore, the rate tended to be higher in patients with partial response to previous treatment [50.0% (four of eight patients)] than those with null response [0% (0 of 7)] ($P = 0.077$). Especially, the sustained virological response rate was significantly higher in patients with Arg70 plus partial response [80.0% (four of five patients)] than in other patients [0% (0 of 10)] ($P = 0.004$; Fig. 2). Thus, all four patients (100%) who achieved sustained virological response had Arg70 and showed partial response.

Predictive Factors Associated With End-of-Treatment Response

The end-of-treatment response rate was significantly higher in patients with low levels of alpha-fetoprotein [81.8% [9 of 11 patients)] than those with high levels of alpha-fetoprotein [0% (0 of 4)] ($P = 0.011$). Furthermore, the same rate was significantly higher in patients with partial response to previous treatment [87.5% (seven of eight patients)] than in those with null response [28.6% (two of seven patients)] ($P = 0.041$). The end-of-treatment response rate was also significantly higher in patients with low levels of alpha-fetoprotein plus partial response [87.5% (seven of eight patients)] than in others [28.6% (two of seven patients)] ($P = 0.041$; Fig. 2). Thus, seven of nine patients (77.8%) who achieved end-of-treatment response had low levels of alpha-fetoprotein and showed partial response. Inversely, all four patients (100%) with high levels of alpha-fetoprotein and null response did not achieve end-of-treatment response.

Assessment of Amino Acid Substitutions in Core Region and Type of Previous Response as Predictors of Sustained Virological Response

Next, the importance of substitution of core aa 70 and type of previous response to PEG-IFN/ribavirin in predicting sustained virological response were evaluated. The sustained virological response rate in patients with a combination of Arg70 or partial response was defined as PPV (prediction of sustained virological response), whereas the non-sustained virological response rate in patients with a combination of Gln70(His70) or null response was defined as NPV (prediction of non-sustained virological response).

In patients with Arg70, the sensitivity, specificity, PPV, and NPV for sustained virological response were 100%, 80.0%, 66.7%, and 100%, respectively. Therefore, Arg70 has high sensitivity, specificity, and NPV for prediction of sustained virological response. In patients with partial response, the sensitivity, specificity, PPV, and NPV were 100%, 63.6%, 50.0%, and 100%, respectively. Thus, partial response has high sensitivity and NPV in predicting sustained virological response. Furthermore, when both predictors were used, the sensitivity, specificity, PPV, and NPV were 100%, 90.9%, 80.0%, and 100%, respectively. These results indicate that the use of the combination of the above two predictors has high sensitivity, specificity, PPV, and NPV for prediction of a sustained virological response (Table II).

Assessment of Alpha-fetoprotein and Type of Previous Response as Predictors of End-of-Treatment Response

The ability to predict end-of-treatment response by alpha-fetoprotein and type of previous response to PEG-IFN/ribavirin was evaluated. The end-of-treatment response rate in patients with a combination of low levels of alpha-fetoprotein (<10 μg/L) or partial response was defined as PPV (prediction of end-of-treatment response). The non end-of-treatment response rate of patients with a combination of high levels of alpha-fetoprotein (≥10 μg/L) or null response was defined as NPV (prediction of non end-of-treatment response).

In patients with low levels of alpha-fetoprotein, the sensitivity, specificity, PPV, and NPV for end-of-treatment response were 100%, 66.7%, 81.8%, and 100%, respectively. Thus, low level of alpha-fetoprotein has high sensitivity, PPV, and NPV for prediction of end-of-treatment response. In patients with partial response, the sensitivity, specificity, PPV, and NPV were 77.8%, 83.3%, 87.5%, and 71.4%, respectively. Thus, partial response has high sensitivity, specificity, and PPV in predicting end-of-treatment response. Furthermore, when both predictors were used, the sensitivity, specificity, PPV, and NPV were 80.0%, 100%, 100%, and 71.4%, respectively. These results indicate that the use of the combination of the above two predictors has high sensitivity, specificity, PPV, and NPV for prediction of end-of-treatment response (Table III).

DISCUSSION

A recent study (PROVE3) reported low-sustained virological response rates (39% and 38%) for 24- and 48-week regimens of triple therapy, respectively, in previous non-responders infected with HCV-1 [McHutchison et al., 2010]. In the present study, the sustained virological response rate was also low (27%) in the T12PR24 group, similar to the above study. Four differences were evident between the present study and the above recent study: (i) the present study was based on a small number of non-responders. (ii) PEG-IFN was used in the above study at a fixed dose of PEG-IFNα-2a, whereas PEG-IFNα-2b was used at a body weight-adjusted dose in the present study. (iii) Body mass index of our patients (median; 23 kg/m^2) was lower than that of the participants of the recent study (median; >25 kg/m^2); and (iv) the present study included Japanese patients infected with HCV-1b, with the exception of one patient infected with HCV-1a. In another previous study (PROVE1), the viral breakthrough rate in HCV-1a subjects was higher than in HCV-1b, and this was due, at least in part, to the low genetic barrier to the emergence of the R155K variant in HCV-1a [Kieffer et al., 2007; McHutchison et al., 2009]. Further studies of larger number of patients matched for background, including genotype, race, and body mass index, as well as treatment regimen are required to determine the sustained virological response rate to triple therapy.

TABLE II. Sensitivity, Specificity, Positive Predictive Value (PPV), and Negative Predictive Value (NPV) for Sustained Virological Response, According to Substitution of Core aa 70 and Type of Previous Response

	% (Number)			
	Sensitivity	Specificity	PPV	NPV
(A) Substitution at aa 70 of arginine (Arg70)	100 (4/4)	80.0 (8/10)	66.7 (4/6)	100 (8/8)
(B) Type of previous response (partial response)	100 (4/4)	63.6 (7/11)	50.0 (4/8)	100 (7/7)
(A) and (B)	100 (4/4)	90.9 (10/11)	80.0 (4/5)	100 (10/10)

PPV, sustained virological response rate for patients with a combination of Arg70 and partial response (prediction of sustained virological response). NPV, non-sustained virological response rates for patients with a combination of Gln70(His70) and null response (prediction of non-sustained virological response).

TABLE III. Sensitivity, Specificity, Positive Predictive Value (PPV), and Negative Predictive Value (NPV) for End-of-Treatment Response, According to Alpha-Fetoprotein and Type of Previous Response to Therapy

	% (Number)			
	Sensitivity	Specificity	PPV	NPV
(A) Alpha-fetoprotein (<10 μg/1)	100 (9/9)	66.7 (4/6)	81.8 (9/11)	100 (4/4)
(B) Type of previous response (partial response)	77.8 (7/9)	83.3 (5/6)	87.5 (7/8)	71.4 (5/7)
(A) and (B)	80.0 (8/10)	100 (5/5)	100 (8/8)	71.4 (5/7)

PPV, end-of-treatment response rates for patients with a combination of low levels of alpha-fetoprotein (<10 μg/L) and partial response (prediction of end-of-treatment response). NPV, non end-of-treatment response rates for patients with a combination of high levels of alpha-fetoprotein (≥10 μg/L) and null response (prediction of non end-of-treatment response).

The present study is the first to identify the pretreatment factors that can predict virological response to triple therapy in prior non-responders infected with HCV-1. The study identified substitution of aa70 (Arg70) and type of previous response (partial response) as predictors of sustained virological response in prior non-responders. The use of the combination of the above two predictors resulted in high sensitivity, specificity, PPV, and NPV for prediction of sustained virological response. Especially, all four patients (100%) who achieved sustained virological response had the combination of Arg70 and partial response. Hence, the T12PR24 regimen might achieve a higher-sustained virological response rate in prior non-responders with the combination of Arg70 and partial response.

A recent study (REALIZE Study) showed that 59% of prior partial responders infected with HCV-1 achieved sustained virological response following 48-week regimen of triple therapy [Zeuzem et al., 2011]. In this regard, predictors of end-of-treatment response might be useful in selecting prior non-responders who could achieve sustained virological response following extension of the combination therapy to 48 weeks (T12PR48). The present study identified alpha-fetoprotein level (<10 μg/L) and type of previous response (partial response) as predictors of end-of-treatment response in previous non-responders. The combination of the above two predictors had high sensitivity, specificity, PPV, and NPV for prediction of end-of-treatment response. Especially, seven of nine patients (77.8%), who achieved end-of-treatment response were patients with low levels of alpha-fetoprotein and showed partial response. Hence, the T12PR48 regimen might achieve high-sustained virological response rates in prior non-responders who have low levels of alpha-fetoprotein and experienced partial response to prior therapy. All four patients (100%) who had high levels of alpha-fetoprotein and null response could not achieve end-of-treatment response. Thus, triple therapy might not achieve sustained virological response in prior non-responders with high levels of alpha-fetoprotein and history of null response, and the development of more effective therapeutic regimens is desirable for these patients in the future. This result should be interpreted with caution, since the present study was performed in Japanese patients infected with HCV-1b (with the exception of one patient infected with HCV-1a). Furthermore, the present study, based on a small number of patients, could not identify independent predictors by multivariate analysis. Any generalization of the results should await confirmation by a multicenter-randomized trial based on a larger number of prior non-responders, including patients of other races and those infected with HCV-1a.

The present study showed that high level of alpha-fetoprotein is a pretreatment predictor of poor virological response to triple therapy. Advanced liver fibrosis is usually associated with high levels of alpha-fetoprotein [Bayati et al., 1998; Chu et al., 2001; Hu et al., 2004]. Previous studies showed that high indocyanine green retention rates at 15 min (ICG R15) or low-serum albumin levels were also associated with advanced liver fibrosis, and that they were independent and significant predictors of poor virological response to PEG-IFN plus ribavirin combination therapy [Akuta et al., 2005, 2007a]. Further studies of large number of patients are required to explore the importance of various histopathological changes in the liver (including stage of fibrosis, platelet count, serum albumin, ICG R15, and alpha-fetoprotein), and to investigate the relationship between the severity of histopathological changes and the response to triple therapy.

The present study based on the direct sequencing identified the appearance of telaprevir-resistant variants during or after treatment in 82% of patients who did not show sustained virological response to triple therapy, but such variants were no longer detected at the end of the study except for one patient with baseline-resistant variant. The limitation of the present study was that the existence of minor clones of telaprevir-resistant variants could not be investigated. Further large-scale studies should be performed to investigate the effects of telaprevir-resistant variants on the response to treatment using the new drugs, including direct-acting antiviral therapy agents.

In conclusion, this study identified aa substitution of the core region, alpha-fetoprotein level, and type of previous response as predictors of virological response

to treatment with telaprevir/PEG-IFN/ribavirin in previous non-responders infected with HCV-1b. Further large-scale prospective studies are necessary to confirm these findings, and to help in the design of more effective therapeutic regimens.

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Original Article

Difference in malignancies of chronic liver disease due to non-alcoholic fatty liver disease or hepatitis C in Japanese elderly patients

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Aim: Malignancies that include hepatocellular carcinoma often occurred in patients with chronic liver disease. The aim of this retrospective match control study was to assess the cumulative development incidence and predictive factors for total malignancies in elderly Japanese patients with non-alcoholic hepatic diseases (NAFLD) or hepatitis C virus (HCV).

Methods: A total of 1600 NAFLD patients with age of ≥60 years were enrolled, and 1600 HCV patients with age of ≥60 years were selected as control by matching 1:1 with NAFLD group for age, sex, and follow-up period. The primary goal is the first development of malignancies. Evaluation was performed by the use of the Wilcoxon rank sum test, the Kaplan–Meier method, and Cox proportional hazard model. The mean observation period is 8.2 years in both NAFLD and HCV group, respectively.

Results: The number of patients with the development of malignancies was 167 in the NAFLD group and 395 in the HCV group. The 10th development rate of malignancies was 13.9% in the NAFLD group and 28.2% in the HCV group (risk ratio 2.27; $P < 0.001$). The incident rates of hepatocellular carcinoma in all the malignancies were 6.0% (10/167) in the NAFLD group and 67.6% (267/395) in the HCV group ($P < 0.001$). The malignancies in the NAFLD group were observed in the following order: gastric cancer 34 cases (20.4%) > colon cancer 31 cases (18.6%) > prostate cancer 21 cases (12.6%).

Conclusions: The incident rates of hepatocellular carcinoma in all the malignancies were approximately 6% in the NAFLD group and two-thirds in the HCV group.

Key words: carcinogenesis, hepatitis C virus, non-alcoholic fatty liver disease

INTRODUCTION

NON-ALCOHOLIC FATTY LIVER disease (NAFLD) is one of the more common causes of chronic liver disease worldwide.[1–6] NAFLD is considered to be the liver component of metabolic syndrome.[7,8] It is associated with obesity, dyslipidemia, pituitary dysfunction, hypertension, sleep apnea, and diabetes mellitus type 2

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(T2DM).[9–13] In addition, NAFLD sometimes progressed to non-alcoholic steatohepatitis (NASH). In patients with cirrhotic NASH, liver-related events such as hepatocellular carcinoma (HCC) and liver failure are one of the main causes of morbidity and mortality.[14] However, studies on prolonged prognosis of NAFLD are few in Japan. Thus, the true prevalence and natural history of NAFLD in Japanese patients are still unclear.

On the other hand, hepatitis C virus (HCV) often causes liver cirrhosis and HCC.[15–18] The majority of HCC is ascribed to hepatitis viruses, of which 70–80% corresponding to approximately 35 000 per year is attributed to the persistent infection with HCV in Japan. However, studies on malignancies other than HCC are few in the HCV patients.

With this background in mind, the present study was initiated to investigate the cumulative incidence and risk factors of malignancies that includes HCC after prolonged follow-up in elderly Japanese patients with NAFLD or HCV. The strengths of the current study are the large numbers of patients included and the long-term follow-up of patients.

METHODS

Patients

THE NUMBER OF patients who were diagnosed with fatty liver by the ultrasonography (US) between January 1994 and December 2007 in the Health Management Center and/or Department of Hepatology, Toranomon Hospital, Tokyo, Japan was 10 810. Of these, 1600 Japanese patients satisfied the following enrolled criteria; (i) age of ≥60 years; (ii) daily alcohol intake of <20 g/day; (iii) negativity for hepatitis B surface antigens (HBsAg), hepatitis C virus antibodies, antinuclear antibodies, or antimitochondrial antibodies in serum, as determined by radioimmunoassay, enzyme-linked immunosorbent assay or indirect immunofluorescence assay; (iv) the absence of malignancies by gastrofiberscope, abdominal US, chest X-ray, and/or chest computed tomography (CT); (v) annual examination for health screening; and (vi) no underlying systemic disease, such as systemic lupus erythmatosus, rheumatic arthritis. Patients with either of the following criteria were excluded from the study: (i) they had illnesses that could seriously reduce their life expectancy; and (ii) they had history of carcinogenesis. In the same period, 7189 HCV patients without fatty liver determined by US were followed in the same hospital. Seven inclusion criteria and two exclusion criteria described in NAFLD group were applied to 2575 of these 7189 HCV patients without fatty liver. Thus, a total of 1600 NAFLD patients with age of ≥60 years were enrolled, and 1600 HCV patients with age of ≥60 years were selected as controls by matching 1:1 with the NAFLD group for age, sex, and follow-up period.

Patients were classified into three groups according to fasting plasma glucose (FPG): (i) those with FPG level of <109 mg/dL (normal glucose group); (ii) those with FPG level of 109–125 mg/dL (pre-diabetes group); and (iii) those with FPG level of ≥126 mg/dL (diabetes group).[19] Patients were regarded as hypertensive by the confirmation of blood pressure ≥140 mmHg systolic and/or ≥90 mmHg diastolic on at least three visits. We considered persons smokers if they had smoked a cigarette at the initiation of follow-up.

The primary goal is the development of malignancies. The diagnosis of malignancies was made due to tumor marker, imaging (US, CT or magnetic resonance imaging [MRI]), and/or histological examination.[20–27] All of the studies were performed retrospectively by collecting and analyzing data from the patient records. This study had been approved by the Institutional Review Board of our hospital.

Medical evaluation

Diagnosis of fatty liver was based on the presence of an ultrasonographic pattern consistent with bright liver with stronger echoes in the hepatic parenchyma than in the renal parenchyma.[28] US test was performed with a high-resolution, real-time scanner (model SSD-2000; Aloka Co., Ltd, Tokyo Japan. 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 the body mass index (BMI) was calculated as weight (in kg)/height (in m^2). All the patients were interviewed by physicians or nurse staff in the Toranomon Hospital using a questionnaire that gathered information on demographic characteristics, medical history, and heath-related habits including questions on alcohol intake and smoking history.

Laboratory investigation

Anti-HCV was detected using a second-generation enzyme-linked immunosorbent assay (ELISA II) (Abbott Laboratories, North Chicago, IL, USA). HCV-RNA was determined by the Amplicor method (Cobas Amplicor HCV Monitor Test, v2.0, Roche, Tokyo,

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Japan). HBsAg was tested by radioimmunoassay (Abbott Laboratories, Detroit, MI, USA). 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.

Follow-up

We used 60 years of age as the starting point for observations in 1417 patients (NAFLD, 694 patients; HCV, 723 patients) who came to our hospital before the age of 60. In 1783 patients (NAFLD, 906 patients; HCV, 877 patients) who came after the age of 60, the day of first visit was used as the start of observations. All patients were followed up at least twice a year by monitoring hematological and biochemical data. Imaging examinations were done approximately once a year for each patient, using abdominal-US and Chest X-ray. Moreover, the patients were checked for tumor marker (carcinoembryonic antigen [CEA], α-fetoprotein [AFP], and prostate-specific antigen [PSA]), gastrofiberscope (or gastrography), and occult blood test of feces at least one year. Two hundred and eighty-two patients were lost to follow-up. Because the appearance of malignancy was not identified in these 282 patients, they were considered as censored data in statistical analysis.[29] Patients treated with antiviral agents were regarded as withdrawals at the time of having the negativity of HCV RNA level by the Amplicor method.

Statistical analysis

Clinical differences between the NAFLD group and HCV group were evaluated by Wilcoxon rank sum test or Fisher's exact test. The cumulative development rates of malignancies were calculated by using the Kaplan–Meier technique, and differences in the curves were tested using the log-rank test.[30] Independent risk factors associated with malignancies were studied using the stepwise Cox regression analysis.[31] The following 15 variables were analyzed for potential covariates for incidence of primary goals in NAFLD group and HCV group: age, gender, body mass index, hypertension, current smoking, albumin, triglyceride, total cholesterol, aspartate aminotransferase (AST), alanine aminotransferase (ALT), gamma-glutamyltransferase (GGT), fasting plasma glucose, platelet, and AFP at the initiation time of follow-up. A *P*-value of less than 0.05 was considered significant. Data analysis was performed using the computer program SPSS package (SPSS 11.5 for Windows, SPSS, Chicago, IL, USA).

RESULTS

Characteristics of the patients enrolled

TABLE 1 SHOWS the baseline characteristics of the 1600 patients in NAFLD group and the 1600 patients in the HCV group at the initiation of follow-up. There are significant differences in several baseline characteristics such as body mass index, AST, ALT, triglyceride, total cholesterol, fasting plasma glucose, platelet, AFP between the HCV group and NAFLD group as shown in Table 1.

Development of malignancy

A total of 562 subjects (167 in NAFLD group and 395 in HCV group) developed malignancy during follow-up. The cumulative development rate of carcinogenesis at the 10th year was determined to be 13.9% in the NAFLD group and 28.2% in the HCV group by the use of the Kaplan–Meier method (Fig. 1). The development rate of each malignancy in both groups is shown in Table 2. The malignancies in the NAFLD group were observed in the following order: gastric cancer 34 cases (20.4%) > colon cancer 31 cases (18.6%) > prostate cancer 21 cases (12.6%). On the other hand, HCC in the HCV group accounted for two-thirds of malignancy. The development rates per 1000 person years in HCC and malignant lymphoma in the HCV group was statistically higher than those in the NAFLD group. However, there were no significant differences in gastric cancer, colon cancer, prostate cancer, and lung cancer between both groups. The incidence rates of HCC in all of the malignancies were 6.0% (10/167) in the NAFLD group and 67.6% (267/395) in the HCV group ($P < 0.001$). Seven of 10 NAFLD patients with development of HCC were evaluated as having histological liver condition at the time of development of HCC. One patient had simple steatosis, and another six patients had non-alcoholic steatohepatitis (NASH). The grade of liver fibrosis in six NASH patients with development of HCC was as follows: grade 1, one patient; grade 2, two patients; grade 3, two patients; grade 4, one patient.

The development rates of each malignancy between the NAFLD group and the HCV group based on the difference of gender are shown in Table 3. The development rates of HCC expressed by 1000 person years in the HCV group were two orders of magnitude higher than those in the NAFLD group in both males and females. There were no significant differences in other malignancies except for HCC between the

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