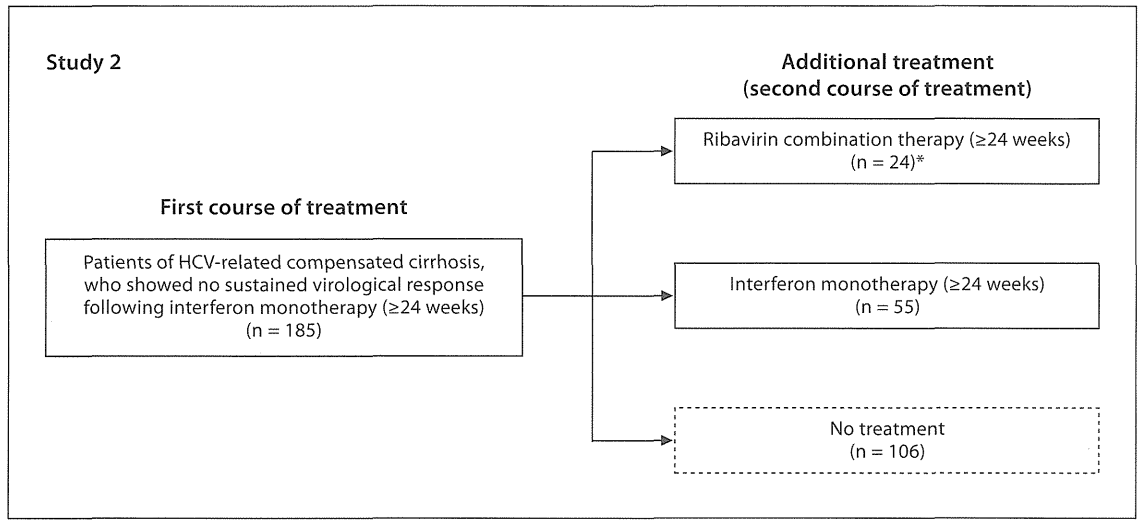

Fig. 1. For study 2, 185 patients with HCV-related compensated cirrhosis, who showed no sustained virological response following the first course of interferon monotherapy (≥24 weeks), were recruited. Hepatocarcinogenesis rates were evaluated according to the additional treatment (second course of treatment), and patients were classified into three groups: no treatment, interferon monotherapy (≥24 weeks), and ribavirin combination therapy (≥24 weeks). * 24 of 157 patients with HCV-related compensated cirrhosis in study 1 were also included in study 2.

diopulmonary disease during and after the first course of interferon monotherapy or the lower levels of serum alanine aminotransferase. The median follow-up time, from the end of the first course of interferon monotherapy until the last visit, was 6.4 years (range 0.0–21.0 years). 24 of the 157 patients in study 1 were also included in study 2; they showed no sustained virological response following the first course of interferon monotherapy (≥24 weeks) and were treated additionally with ribavirin combination therapy (≥24 weeks).

At the additional treatment of interferon monotherapy, 43 patients (78.2%) received IFNα alone, and the remaining 12 patients (21.8%) received IFNβ alone. They received interferon monotherapy including initial aggressive induction therapy (every day for 8 weeks followed by 3 times per week), with a median treatment duration of 44 weeks (range 24–382 weeks) at a median dose of 3 million units (range 3–10 million units) intramuscularly each day.

At the additional treatment of ribavirin combination therapy, 11 patients (45.8%) received PEG-IFNα-2b plus ribavirin, and the remaining 13 patients (54.2%) received IFNα-2b plus ribavirin. They received PEG-IFNα-2b at a median dose of 1.5 μg/kg (range 0.8–1.7 μg/kg) subcutaneously each week or IFNα-2b at a median dose of 6 million units (range 3–6 million units) intramuscularly each day (7 times per week for the initial 2 weeks followed by 3 times per week), with a median treatment duration of 26 weeks (range 24–48 weeks). They also received oral ribavirin at a median dose of 11.0 mg/kg (range 3.0–12.5 mg/kg) daily.

In the present studies, the patients were selected based on the following criteria. (1) Patients had compensated cirrhosis, but no decompensated cirrhosis or hepatocellular carcinoma. The diagnosis of compensated cirrhosis was based on clinical features (absence of signs for decompensation of ascites, encephalopathy, or gastrointestinal bleeding), laboratory tests, and peritoneoscopy or liver biopsy. (2) Patients were negative for hepatitis B surface antigen (radioimmunoassay, Dainabot, Tokyo, Japan), positive for anti-HCV (third-generation enzyme immunoassay, Chiron Corp., Emerville, Calif., USA), and positive for HCV-RNA by qualitative or quantitative analysis. (3) Patients were free of coinfection with human immunodeficiency virus. (4) Lifetime cumulative alcohol intake was <500 kg (mild to moderate alcohol intake). (5) Patients were free of other types of hepatitis, including hemochromatosis, Wilson disease, primary biliary cirrhosis, alcoholic liver disease, and autoimmune liver disease. (6) Each patient signed a consent form of the study protocol that had been approved by the human ethics review committee.

Laboratory Investigations

Blood samples were frozen at –80° within 4 h of collection and were not thawed until used for testing. HCV genotype was determined by PCR using a mixed primer set derived from nucleotide sequences of the NS5 region [12]. HCV-RNA quantitative analysis was measured by branched DNA assay version 2.0 (Chiron Corp., Emeryville, Calif., USA), AMPLICOR GT HCV Monitor version 2.0 using the 10-fold dilution method (Roche Molecular Systems Inc., Pleasanton, Calif., USA), or COBAS TaqMan HCV test (Roche Diagnostics, Tokyo, Japan). High viral load of viremia levels 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 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. The lower limit of HCV-RNA qualitative analysis (Amplicor, Roche Diagnostics, Mannheim, Germany) was 100 copies/ml, and that of

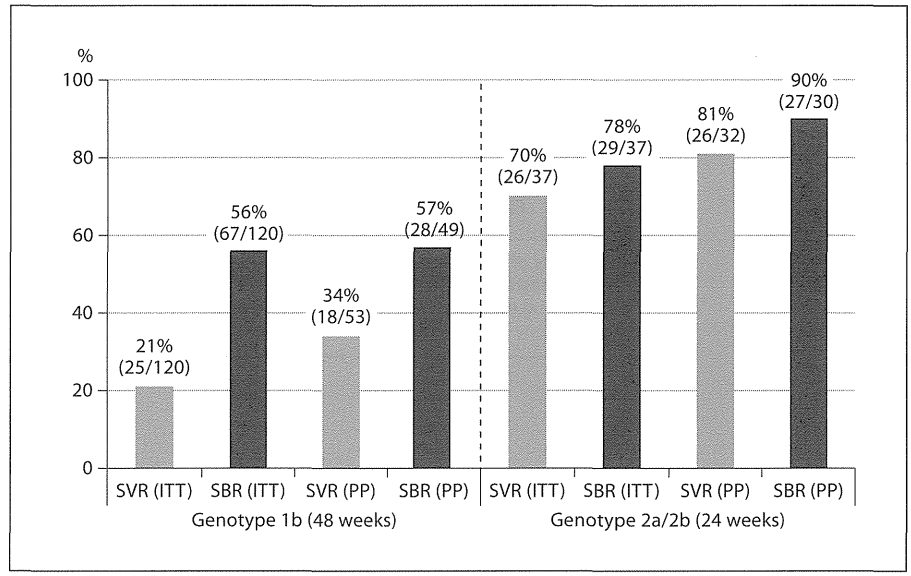

Fig. 2. In 157 patients with HCV-related compensated cirrhosis treatment efficacy with interferon plus ribavirin therapy was evaluated for 48 weeks of HCV genotype 1b or 24 weeks of genotype 2a/2b. In HCV genotype 1b, rates of sustained biochemical response (SBR) were significantly higher than those of sustained virological response (SVR; ITT analysis, $p < 0.001$, and PP analysis, $p = 0.028$).

COBAS TaqMan HCV test was 1.2 log IU/ml. The undetectable samples by HCV-RNA qualitative analysis or COBAS TaqMan HCV test were defined as negative HCV-RNA.

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. Patient compliance with treatment was evaluated with a questionnaire. Blood samples were also obtained at least once every month before, during, and after treatment, and were also analyzed for levels of serum alanine aminotransferase and HCV-RNA at various time points.

Patients were examined for hepatocellular carcinoma by abdominal ultrasonography every 3–6 months. If hepatocellular carcinoma was suspected based on ultrasonographic results, additional procedures, such as computed tomography, magnetic resonance imaging, abdominal angiography, and ultrasonography-guided tumor biopsy if necessary, were used to confirm the diagnosis.

Statistical Analysis

χ^2 test, Fisher's exact probability test, and Mann-Whitney's U test were used to compare the background characteristics between groups. Multiple comparisons were examined by the Bonferroni test. The cumulative hepatocarcinogenesis rates were calculated using the Kaplan-Meier technique, and differences between the curves were tested using the log-rank test. Statistical analysis of the hepatocarcinogenesis rates according to groups was calculated using the period from the end of the first course of interferon monotherapy until the appearance of hepatocellular carcinoma or until the last visit or until the start of the third course of interferon-based treatment. Stepwise Cox regression analysis was used to determine independent predictive factors that were associated with hepatocarcinogenesis. The hazard ratio (HR) and 95% confidence interval were also calculated. Potential predictive factors associated with hepatocarcinogenesis included the following 13 variables: age, sex, serum aspartate aminotransferase, serum alanine aminotransferase, platelet count, total cholesterol, high-density lipoprotein cholesterol, low-density lipoprotein cholesterol, triglycerides, HCV genotype, levels of viremia, total duration of additional treatment, and group of additional treatment. Each variable was transformed into categorical data consisting of two simple ordinal numbers for univariate and multivariate analyses. All p values <0.05 and <0.1 by the two-tailed test were considered significant ($p < 0.05$) and marginally significant ($p < 0.1$), respectively. Variables that achieved statistical significance ($p < 0.05$) on univariate analysis were tested by multivariate Cox proportional hazard model to identify significant independent factors. Statistical comparisons were performed using the SPSS software (SPSS Inc., Chicago, Ill., USA).

Results

Efficacy of Ribavirin Combination Therapy (Study 1)

Treatment efficacy of a 48-week regimen of interferon plus ribavirin combination therapy in 120 patients infected with HCV-1b was evaluated. In ITT analysis, rates of sustained virological response and sustained biochemical response were 21% (25 of 120 patients) and 56% (67 of 120 patients), respectively. In the PP analysis, rates of sustained virological response and sustained biochemical response were 34% (18 of 53 patients) and 57% (28 of 49 patients), respectively (fig. 2). In both analyses, rates of sustained biochemical response were significantly higher than those of sustained virological response (ITT analysis, $p < 0.001$, and PP analysis, $p = 0.028$).

Table 2. Profile and laboratory data of 185 patients with HCV-related compensated cirrhosis according to additional treatment groups (study 2)

	No treatment	Interferon monotherapy (≥24 weeks)	Ribavirin combination therapy[1] (≥24 weeks)
Demographic data			
Patients, n	106	55	24
Sex (male/female), n	64/42	37/18	20/4
Age, years	56 (30–75)[a]	56 (35–76)[b]	51 (34–68)
Laboratory data			
Serum aspartate aminotransferase, IU/l	75 (26–285)	83 (35–213)	62 (30–160)
Serum alanine aminotransferase, IU/l	92 (17–400)	104 (30–316)	93 (36–250)
Platelet count, $\times 10^4/mm^3$	10.7 (2.5–18.2)[c]	10.8 (5.7–19.8)[d]	13.0 (5.2–23.5)
Total cholesterol, mg/dl	165 (103–273)[h]	152 (101–220)	160 (111–211)
High-density lipoprotein cholesterol, mg/dl	46 (25–93)	43 (21–65)	47 (28–56)
Low-density lipoprotein cholesterol, mg/dl	93 (38–168)	87 (45–139)	100 (34–135)
Triglycerides, mg/dl	96 (36–437)	80 (51–215)	108 (52–206)
HCV genotype (1b/2a or 2b), n	70/36	39/16	17/7
Levels of viremia (high viral load/low viral load), n	84/16	37/15[e]	24/0
Additional treatment			
Duration of additional treatment, weeks	–	44 (24–382)[f]	26 (24–48)
Sustained virological response (ITT), n	–	11 (20%)	7 (29%)
Sustained biochemical response (ITT), n	–	25 (45%)[g]	16 (67%)

Unless otherwise indicated, values represent median (range).

Demographic data and laboratory data, at the start of the first course of interferon monotherapy, are shown.

[a] $p = 0.013$, [b] $p = 0.030$, [c] $p = 0.002$, [d] $p = 0.015$, [e] $p = 0.006$, [f] $p = 0.044$, [g] $p = 0.083$ compared with ribavirin combination therapy by Bonferroni test, Mann-Whitney U test, or χ^2 test. [h] $p = 0.039$ compared with interferon monotherapy by Bonferroni test.

[1] 24 of 157 patients with HCV-related compensated cirrhosis in study 1 were also included in study 2. They showed no sustained virological response following the first course of interferon monotherapy (≥24 weeks), and were additionally treated with ribavirin combination therapy (≥24 weeks).

Treatment efficacy of a 24-week regimen of interferon plus ribavirin combination therapy in 37 patients infected with HCV-2a or 2b was evaluated. In the ITT analysis, rates of sustained virological response and sustained biochemical response were 70% (26 of 37 patients) and 78% (29 of 37 patients), respectively. In the PP analysis, rates of sustained virological response and sustained biochemical response were 81% (26 of 32 patients) and 90% (27 of 30 patients), respectively (fig. 2). In both analyses, rates of the sustained biochemical response were not significantly higher than those of the sustained virological response.

Profile, Laboratory Data, and Efficacy according to Additional Treatment Groups (Study 2)

Profile and laboratory data, at the start of the first course of interferon monotherapy of 185 patients with HCV-related compensated cirrhosis, are summarized in table 2. The age of patients with ribavirin combination therapy was significantly lower than that of patients with no treatment ($p = 0.013$; Bonferroni test) and interferon monotherapy ($p = 0.030$; Bonferroni test). The platelet count of patients of ribavirin combination therapy was significantly higher than that of patients without treatment ($p = 0.002$; Bonferroni test) and interferon monotherapy ($p = 0.015$; Bonferroni test). The total cholesterol level of patients with interferon monotherapy was significantly lower than that of patients without treatment ($p = 0.039$; Bonferroni test). Low viral load rates of patients with interferon monotherapy were significantly higher than those of patients with ribavirin combination therapy ($p = 0.006$; Bonferroni test). There were no other significant differences in clinical features at the start of the first course of interferon monotherapy among the three groups.

Additional treatment duration of only 1 patient, who was diagnosed with hepatocellular carcinoma during additional treatment, was evaluated using the period from the start of the second course of interferon monotherapy

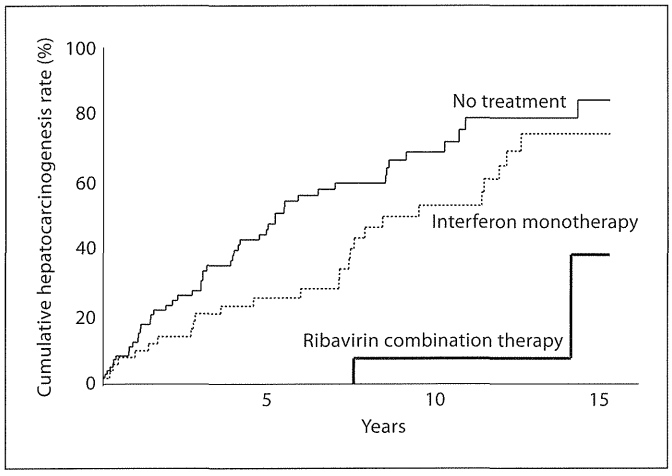

Fig. 3. Cumulative hepatocarcinogenesis rates in the three groups of additional treatment. The rates in no treatment were significantly higher than those in interferon monotherapy ($p = 0.047$; log-rank test) and ribavirin combination therapy ($p < 0.001$; log-rank test), and the rates in interferon monotherapy were significantly higher than those in ribavirin combination therapy ($p < 0.001$; log-rank test).

Table 3. Factors associated with hepatocarcinogenesis in 185 patients of HCV-related compensated cirrhosis identified by multivariate analysis (study 2): Cox proportional hazard model

Factors/category	Hazard ratio (95% confidence interval)	p
Additional treatment		
Ribavirin combination therapy	1	
Interferon monotherapy	4.47 (1.04–19.3)	0.045
No treatment	9.14 (2.19–38.2)	0.002
Age		
<55 years	1	
≥55 years	2.87 (1.76–4.67)	<0.001
Aspartate aminotransferase		
<58 IU/l	1	
≥58 IU/l	2.11 (1.20–3.74)	0.010

until the appearance of hepatocellular carcinoma. During additional treatment, the total duration of interferon monotherapy was significantly longer than that of ribavirin combination therapy ($p = 0.044$; Mann-Whitney U test). In ITT analysis, sustained virological response rates of ribavirin combination therapy (29%) were not different from those of interferon monotherapy (20%), but sustained biochemical response rates of ribavirin combination therapy (67%) tended to be higher than those of interferon monotherapy (45%; $p = 0.083$; χ^2 test) (table 2).

Predictive Factors Associated with Hepatocarcinogenesis by Multivariate Analysis

The data for the whole population sample were analyzed to determine those factors that could predict hepatocarcinogenesis. Hepatocarcinogenesis rates in older patients (≥55 years), in patients with higher levels of aspartate aminotransferase (≥58 IU/l), and lower levels of platelet count (<15.0 × 10^4/mm^3) were significantly higher than those in younger patients (<55 years), in patients with lower levels of aspartate aminotransferase (<58 IU/l), and higher levels of platelet count (≥15.0 × 10^4/mm^3), respectively ($p < 0.001$, $p = 0.006$, and $p = 0.017$; log-rank test). Furthermore, the rates in no treatment were significantly higher than those in interferon monotherapy ($p = 0.047$; log-rank test) and ribavirin combination therapy ($p < 0.001$; log-rank test), and the rates in interferon monotherapy were significantly higher than those in ribavirin combination therapy ($p < 0.001$; log-rank test) (fig. 3). Thus, univariate analysis identified four parameters that significantly correlated with hepatocarcinogenesis. These factors were entered into multivariate analysis, which then identified three parameters that significantly influenced hepatocarcinogenesis independently: additional treatment (no treatment; HR 9.14, $p = 0.002$), age (≥55 years; HR 2.87, $p < 0.001$), and levels of aspartate aminotransferase (≥58 IU/l; HR 2.11, $p = 0.010$) (table 3).

The data for 167 patients, except for 18 patients who showed a sustained virological response following additional treatment, were also analyzed to determine those factors that could predict hepatocarcinogenesis. Hepatocarcinogenesis rates in older age (≥55 years) and higher levels of aspartate aminotransferase (≥58 IU/l) were significantly higher than those in younger age (<55 years) and lower levels of aspartate aminotransferase (<58 IU/l), respectively ($p < 0.001$ and $p = 0.007$; log-rank test). Furthermore, the rates in ribavirin combination therapy were significantly lower than those in interferon monotherapy ($p < 0.001$; log-rank test) and no treatment ($p < 0.001$; log-rank test) (fig. 4). Thus, univariate analysis identified three parameters that significantly correlated with hepatocarcinogenesis. These factors were entered into multivariate analysis, which then identified three parameters that significantly influenced hepatocarcinogenesis independently: additional treatment (no treatment; HR 7.87, $p = 0.005$), age (≥55 years; HR 2.52, $p < 0.001$), and levels of aspartate aminotransferase (≥58 IU/l; HR 2.13, $p = 0.010$) (table 4).

Discussion

One of our previous studies indicated that the cancer-suppressive activity of interferon monotherapy in patients with HCV-RNA eradication was similar to that in patients with alanine aminotransferase normalization without HCV-RNA elimination [9]. Other studies also indicated a higher incidence and more rapid development of hepatocellular carcinoma in HCV patients with high levels of alanine aminotransferase [13, 14]. Collectively, these results suggest that the carcinogenic process in patients with chronic HCV infection is enhanced by high levels and fluctuations of alanine aminotransferase, and indicate a close relationship between suppression of inflammatory necrosis of hepatocytes and a lower incidence of hepatocellular carcinoma in patients with HCV-associated chronic liver disease. Recent studies based on interferon plus ribavirin combination therapy also showed that the attainment of sustained virological response or lower levels of alanine aminotransferase after ribavirin combination therapy could reduce the rates of hepatocellular carcinoma [15, 16], but the small numbers of patients with compensated cirrhosis (5% or less of all patients) were recruited. The present study 1 based on the patients with compensated cirrhosis showed that rates of sustained virological response and sustained biochemical response in HCV-2a/2b were high rates of 70 and 78%, and that rates of sustained biochemical response (57%) were significantly higher than those of sustained virological response (34%) in HCV-1b. Furthermore, the present study 2 based on the patients with compensated cirrhosis, who showed no sustained virological response following the first course of interferon monotherapy, also showed that sustained biochemical response rates of ribavirin combination therapy (67%) tended to be higher than those of interferon monotherapy (45%). Thus, in ribavirin combination therapy for compensated cirrhosis, higher rates of sustained biochemical response might be associated with lower rates of hepatocarcinogenesis. One limitation is that the present study was performed based on the small numbers of patients who showed no sustained virological response with interferon monotherapy. In further prospective studies a larger number of patients need to be investigated to confirm this finding.

Previous studies have shown that gender, age, fibrosis stage, alanine aminotransferase, and interferon regimen are important pretreatment predictors of hepatocarcinogenesis [9, 10, 17]. In the present study 2 based on the patients with compensated cirrhosis, higher age and aspartate aminotransferase were associated with higher hepatocarcinogenesis rates in the whole population sample and in the sample which excluded patients who showed sustained virological response following additional treatment. Furthermore, as treatment-related factors, the hepatocarcinogenesis rates in ribavirin combination therapy were significantly lower than those in interferon monotherapy. Thus, in patients with compensated cirrhosis representing a high-risk group of hepatocarcino-

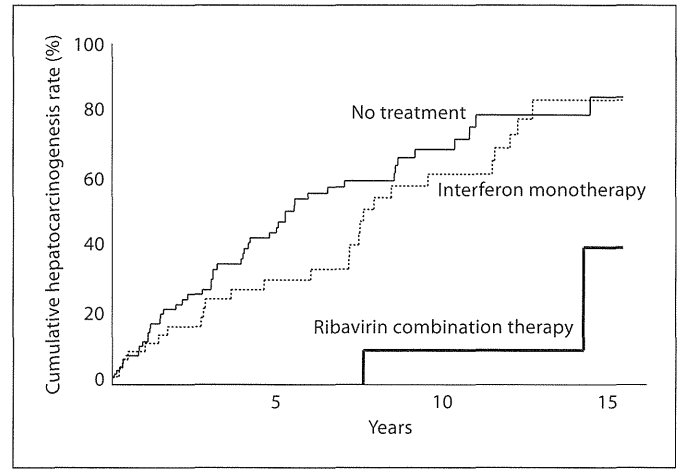

Fig. 4. Cumulative hepatocarcinogenesis rates in the three groups of additional treatment, except for patients who showed sustained virological response following additional treatment. The rates in ribavirin combination therapy were significantly lower than those in interferon monotherapy ($p < 0.001$; log-rank test) and no treatment ($p < 0.001$; log-rank test).

Table 4. Factors associated with hepatocarcinogenesis in 167 patients of HCV-related compensated cirrhosis, except for 18 patients who showed sustained virological response following additional treatment identified by multivariate analysis (study 2): Cox proportional hazard model

Factors/category	Hazard ratio (95% confidence interval)	p
Additional treatment		
Ribavirin combination therapy	1	
Interferon monotherapy	4.68 (1.08–20.3)	0.039
No treatment	7.87 (1.89–32.9)	0.005
Age		
<55 years	1	
≥55 years	2.52 (1.54–4.11)	<0.001
Aspartate aminotransferase		
<58 IU/l	1	
≥58 IU/l	2.13 (1.20–3.79)	0.010

genesis, ribavirin combination therapy might reduce the risk of hepatocellular carcinoma in comparison with interferon monotherapy. One reason for the higher anticarcinogenic activity by ribavirin combination therapy might be due to higher rates of sustained biochemical response. The other reason might be due to the difference in the background (lower age and higher levels of platelet count as an indicator of fibrosis stage) of patients with ribavirin combination therapy. Further studies of a larger number of patients matched for background, including age, sex, genotype, and platelet count, are required to investigate the rates of hepatocarcinogenesis and the mechanism of anticarcinogenic activity by ribavirin combination therapy for HCV-related compensated cirrhosis.

Two previous studies (PROVE1 and PROVE2) showed that the 12- and 24-week regimen of telaprevir/PEG-IFN/ribavirin could achieve sustained virological response rates of 35–60 and 61–69% in patients infected with HCV-1, respectively [18, 19]. However, a recent study (PROVE3) also showed that the sustained virological response rates were the lower rates of 39 and 38% with the 24- and 48-week regimen of triple therapy in previously nonresponding patients infected with HCV-1, who do not become HCV-RNA negative during or at the end of the initial PEG-IFN/ribavirin treatment, respectively [20]. Furthermore, the telaprevir-based regimen induces resistant variants [21–23] and has side effects including anemia and rash [18–20, 24]. Hence, patients, who do not achieve sustained virological response by triple therapy, need to be identified, in order to avoid unnecessary side effects and telaprevir-resistant variants. Recent studies identified amino acid substitutions at position 70 and/or 91 in the HCV-1b core region, advanced fibrosis stage, and higher levels of α-fetoprotein as pretreatment predictors of poor virological response to PEG-IFN/ribavirin combination therapy or triple therapy of telaprevir/PEG-IFN/ribavirin [23, 25–28], and these factors are also risk factors and surrogate markers of hepatocarcinogenesis [29–34]. Hence, ribavirin combination therapy for these patients might be an efficacious therapeutic regimen for sustained biochemical response and thus a reduction of the risk of hepatocarcinogenesis. Large-scale prospective studies should be conducted in the future to confirm this finding.

In conclusion, the present retrospective study indicated that ribavirin combination therapy for HCV-related compensated cirrhosis could reduce the risk of hepatocarcinogenesis in comparison with interferon monotherapy. Large-scale prospective studies need to be conducted in the future to confirm these findings.

Acknowledgments

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Amino Acid Substitutions in the Hepatitis C Virus Core Region and Lipid Metabolism Are Associated with Hepatocarcinogenesis in Nonresponders to Interferon plus Ribavirin Combination Therapy

Yuya Seko[a] Norio Akuta[a] Fumitaka Suzuki[a] Yusuke Kawamura[a] Hitomi Sezaki[a] Yoshiyuki Suzuki[a] Tetsuya Hosaka[a] Masahiro Kobayashi[a] Mariko Kobayashi[b] Satoshi Saitoh[a] Yasuji Arase[a] Kenji Ikeda[a] Hiromitsu Kumada[a]

[a]Department of Hepatology, and [b]Liver Research Laboratory, Toranomon Hospital, Tokyo, Japan

Key Words
Hepatitis C virus · Genotype · Ribavirin · Interferon · Hepatocellular carcinoma · Core region · High-density lipoprotein cholesterol · IL28B

Abstract
Background: Substitution of amino acid 70 and/or 91 in the core region of hepatitis C virus (HCV) genotype 1b (HCV-1b) is an important predictor of hepatocellular carcinoma (HCC), but its impact on HCC in nonresponders to interferon (IFN) and ribavirin (RIB) combination therapy is not clear. ***Methods:*** A total of 292 patients with HCV-1b-related chronic liver disease who did not achieve a sustained virological response to 24–48 weeks of IFN+RIB combination therapy were included in a follow-up study to investigate the risk factors for HCC. ***Results:*** Sixteen patients developed HCC during the follow-up. The cumulative HCC rates were 5.0, 13.1 and 16.9% at the end of 3, 5 and 7 years, respectively. Multivariate analysis identified substitution of core amino acid 70 (Gln70/His70; hazard ratio 4.64, p = 0.018) and low serum levels of high-density lipoprotein cholesterol (<50 mg/dl; hazard ratio 9.35, p = 0.041) as determinants of HCC. Gender, stage of fibrosis and interleukin-28B showed no such relationship. ***Conclusions:*** Amino acid substitution in the core region of HCV-1b and low serum levels of high-density lipoprotein cholesterol are significant and independent predictors of HCC in nonresponders to IFN+RIB combination therapy. These results emphasize the importance of viral and lipid metabolic factors in the development of HCC after combination therapy.

Introduction

Infection with hepatitis C virus (HCV) is often chronic and can progress to cirrhosis and hepatocellular carcinoma (HCC) [1, 2]. At present, interferon (IFN), in combination with ribavirin (RIB), is the mainstay for treatment of HCV infection. In Japan, more than 70% of HCV infections are caused by HCV genotype 1b (HCV-1b) and are associated with a high viral load, making their treatment difficult [3].

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Yuya Seko, MD
Department of Hepatology, Toranomon Hospital
2-2-2 Toranomon
Minato-ku, Tokyo 105-0001 (Japan)
Tel. +81 33 588 1111, E-Mail yseko523@toranomon.gr.jp

IFN monotherapy slightly reduces the rates of HCC and normalization of alanine transaminase [4–6]. Furthermore, IFN plus RIB combination therapy also minimizes the risk of HCC, especially among patients who achieve a sustained virological response (SVR) [7]. However, there are currently no suitable factors that could be used to predict HCC in patients who receive the combination therapy but do not achieve SVR.

Several factors have been found to correlate with HCV-related HCC, such as old age, male sex, advanced histopathological stage of liver damage, alcohol intake, HCV genotype and hepatic steatosis [6, 8–12]. Furthermore, mutations in a region spanning amino acids (aa) 2209–2248 within the NS5A protein, the so-called IFN sensitivity-determining region (ISDR) [13], and substitution of aa 70/91 in the core region of HCV-1b [14] as viral-related factors, and genetic variation near the interleukin-28B (IL28B) gene as a host-related factor [15] are also used to predict HCC. The aim of the present study was to identify the viral- and host-related predictive factors for HCC in patients on IFN plus RIB combination therapy (IFN+RIB) who did not achieve SVR. For this purpose, we recruited 292 patients with HCV-related chronic liver disease who did not achieve SVR after 24–48 weeks of IFN+RIB.

Table 1. Profile and laboratory data at the start of IFN+RIB combination therapy of 292 patients infected with HCV-1b who did not achieve SVR

Demographic data	
Number of patients	292
Males/females	144/148
Age, years	56 (20–74)
BMI	22.5 (16.5–40.8)
Laboratory data	
Serum aspartate aminotransferase, IU/l	54 (19–273)
Serum alanine aminotransferase, IU/l	66 (17–504)
Total cholesterol, mg/dl	167 (107–255)
HDL-Chol, mg/dl	48 (24–94)
Low-density lipoprotein cholesterol, mg/dl	95 (35–169)
Triglyceride, mg/dl	93 (28–325)
Platelet count, $\times 10^4/mm^3$	15.0 (6.4–33.1)
Histological findings	
Fibrosis stage F1/F2/F3/F4	77/53/39/1
Amino acid substitutions in HCV-1b	
Core aa 70, arginine/glutamine (histidine)	147/129
Core aa 91, leucine/methionine	139/138
ISDR of NS5A, wild type/non-wild type	217/31
Genetic variation near IL28B gene	
rs8099917 genotype, TT/TG/GG	113/87/4

Data represent numbers of patients or medians (range), as appropriate.

Materials and Methods

Patients

A total of 1,540 HCV-1b-infected adult Japanese patients were consecutively recruited into a study of combination therapy with IFN [IFN or pegylated (PEG)-IFN] plus RIB between March 1999 and October 2010 at Toranomon Hospital, Tokyo, Japan. Among them, 292 were enrolled in this retrospective study. These patients fulfilled the following criteria: (1) positive for anti-HCV (by a third-generation enzyme immunoassay, Chiron Corp., Emeryville, Calif., USA) and HCV RNA by qualitative or quantitative analysis before combination therapy; (2) treated with IFNα-2b or PEG-IFNα-2b plus RIB combination therapy for 24–48 weeks; (3) did not achieve SVR, defined as negative HCV RNA 24 weeks after cessation of antiviral therapy, based on the COBAS TaqMan HCV test (Roche Diagnostics, Tokyo, Japan); (4) free of HCC, both before and during IFN therapy; (5) infected with a single genotype of HCV-1b; (6) negative for hepatitis B surface antigen (by radioimmunoassay, Dainabot, Tokyo, Japan); (7) free of co-infection with the human immunodeficiency virus; (8) lifetime cumulative alcohol intake <500 kg (mild to moderate alcohol intake); (9) free of other types of hepatitis and without hemochromatosis, Wilson disease, primary biliary cirrhosis, alcoholic liver disease and autoimmune liver disease, and (10) had signed a consent form for the study protocol, which had been approved by the human ethics review committee.

Of the total 292 patients, 226 (77%) received PEG-IFNα-2b at a median dose of 1.4 μg/kg (range 1.3–1.9 μg/kg) subcutaneously each week for a median duration of 47 weeks (range 28–48 weeks). The remaining 66 patients (23%) received 6 million units of IFNα-2b intramuscularly for a median duration of 27 weeks (range 24–48 weeks), daily for the initial 2 weeks and then 3 times per week until the last week. The dose of RIB was adjusted according to body weight (600 mg for weight ≤60 kg, 800 mg for weight 60–80 kg and 1,000 mg for weight ≥80 kg).

Table 1 summarizes the profile and laboratory data of the participating patients at the start of combination therapy. The group included 144 males and 148 females aged 20–74 years (median 56 years). The median follow-up period, from the end of antiviral therapy until the last visit, was 1.3 years (range 0.0–8.2 years).

Laboratory Investigations

Blood samples were frozen at –80° within 4 h of collection until used for testing. HCV genotype was determined by PCR using a mixed primer set derived from nucleotide sequences of the NS5 region [16]. Quantitative measurement of HCV RNA was analyzed by the COBAS TaqMan HCV test (Roche Diagnostics). The lower limit of the COBAS TaqMan HCV test is 1.2 log IU/ml, and samples with undetectable levels were defined as negative.

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

Amino acid substitutions in the core region and NS5A-ISDR of HCV-1b were analyzed by direct sequencing. HCV RNA was

extracted from serum samples at the start of treatment and reverse transcribed with random primer and Moloney murine leukemia virus reverse transcriptase (Takara Syuzo). Nucleic acids were amplified by PCR. For nucleotide sequences of the core region, the first-round PCR was performed with primers 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) and the second-round PCR with primers 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). For nucleotide sequences of NS5A-ISDR, the first-round PCR was performed with primers ISDR1 (sense, 5′-ATG CCC ATG CCA GGT TCC AG-3′, nucleotides 6662–6681) and ISDR2 (antisense, 5′-AGC TCC GCC AAG GCA GAA GA-3′, nucleotides 7350–7369) and the second-round PCR with primers ISDR3 (sense, 5′-ACC GGA TGT GGC AGT GCT CA-3′, nucleotides 6824–6843) and ISDR4 (antisense, 5′-GTA ATC CGG GCG TGC CCA TA-3′, nucleotides 7189–7208). Nested PCR was used for both the core region and NS5A-ISDR. All samples were initially denatured at 95° for 2 min. The 35 cycles of amplification were set as follows: denaturation for 30 s at 95°, annealing of primers for 30 s at 55° and extension for 1 min at 72° 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) 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).

Using HCV-J (accession No. D90208) as a reference [17], the sequence of aa 1–191 in the core protein of HCV-1b was determined and then compared with the consensus sequence constructed on 279 clinical samples to detect substitutions at aa 70 of arginine (Arg70) or glutamine/histidine (Gln70/His70) and at aa 91 of leucine (Leu91) or methionine (Met91) [18]. The sequence of aa 2209–2248 in the NS5A of HCV-1b (ISDR) reported by Enomoto et al. [19] was determined, and the numbers of amino acid substitutions in ISDR were defined as wild type (0) or non-wild type (≥1).

Genetic Variation near the IL28B Gene

Samples for a genome-wide association survey were genotyped using the Illumina HumanHap610-Quad Genotyping BeadChip. Genotyping data were subjected to quality control before data analysis. Genotyping for replication and fine mapping was performed using the Invader assay, TaqMan assay or direct sequencing as described previously [20, 21]. In this study, genetic variations near the IL28B gene (rs8099917), reported as the pretreatment predictors of treatment efficacy and clinical outcome [22–26], were investigated.

Histopathological Examination of the Liver

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), 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 6 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. [27].

Follow-Up and Diagnosis of HCC

Hematological, biochemical and virological tests were performed at least once every month. Imaging studies were conducted every 3 or 4 months in the majority of patients (except those patients who were lost to follow-up); these included computed tomography, magnetic resonance imaging and ultrasonography. If HCC was suspected, additional procedures, such as abdominal angiography and ultrasonography-guided tumor biopsy, if necessary, were used to confirm the diagnosis.

Statistical Analysis

The cumulative rate of HCC was calculated using the Kaplan-Meier technique, and differences in the rates were examined by the log-rank test. Differences in the HCC rate among groups were calculated using the period between the end of combination therapy and appearance of HCC. Stepwise Cox regression analysis was used to determine independent predictive factors associated with HCC. Hazard ratios (HRs) and 95% confidence intervals were also calculated. Potential predictive factors associated with HCC included the following variables: sex, age, type of IFN received, body mass index, platelet count, aspartate aminotransferase, alanine aminotransferase, total cholesterol, high-density lipoprotein cholesterol (HDL-Chol), low-density lipoprotein cholesterol, triglyceride, stage of fibrosis, genetic variation near the IL28B gene and amino acid substitution in the core region and NS5A-ISDR of HCV. Variables that achieved statistical significance ($p < 0.05$) or marginal significance ($p < 0.10$) on univariate analysis were entered into a multivariate Cox proportional hazard model to identify significant independent factors. Statistical comparisons were performed using the SPSS software (SPSS Inc., Chicago, Ill., USA). All p values of less than 0.05 by the two-tailed test were considered significant.

Results

Rate of Hepatocarcinogenesis

During the follow-up, 16 patients (5.4%) developed HCC. The median interval between the end of combination therapy and detection of HCC was 2.0 years (range 0.0–7.6 years). The cumulative rates of HCC were 5.0, 13.2 and 16.9% at the end of 3, 5 and 7 years, respectively.

Predictive Factors Associated with Hepatocarcinogenesis

Data of the entire population sample were analyzed to determine those factors that could predict HCC. Univariate analysis identified 4 parameters that tended to or were significantly correlated with carcinogenesis.

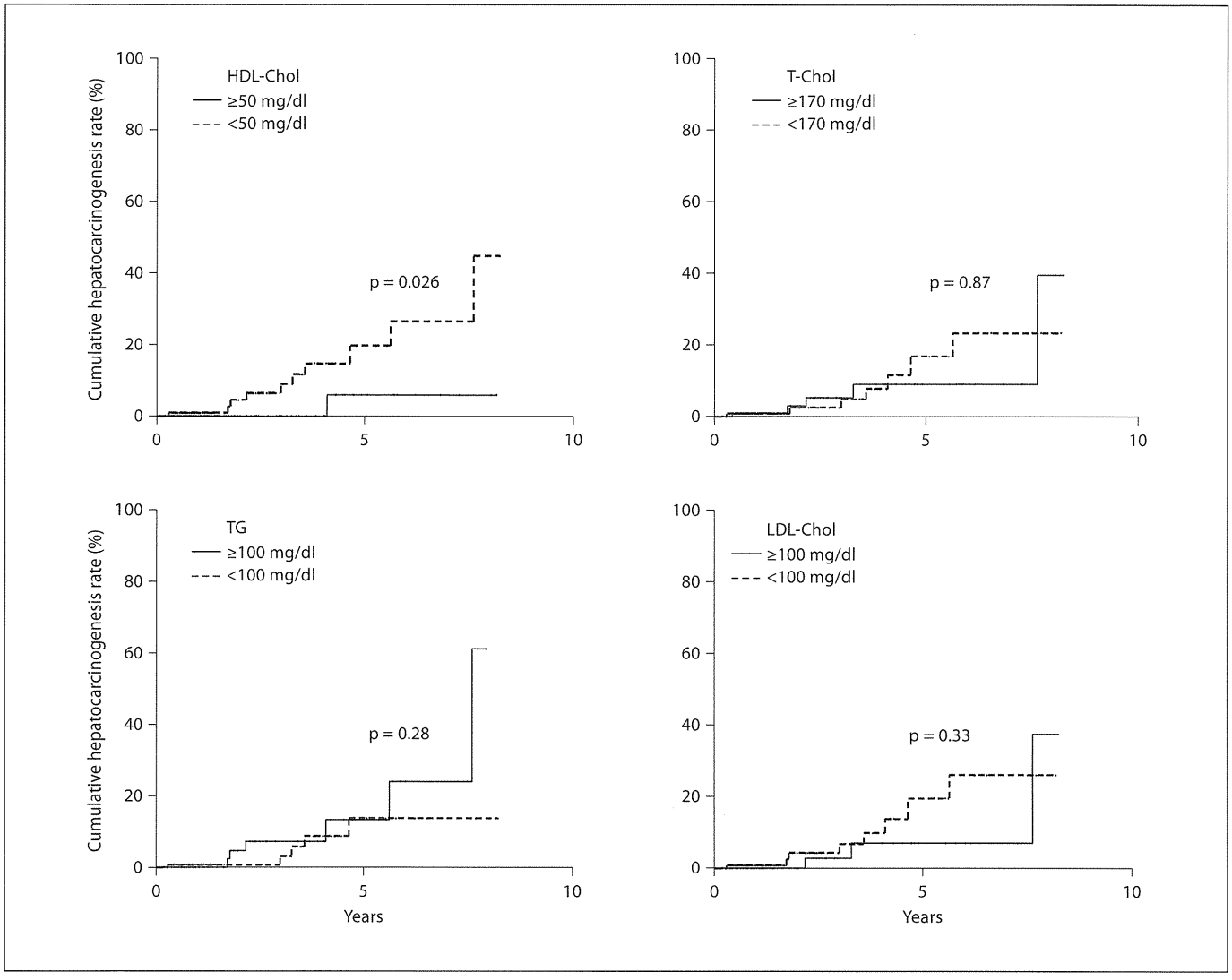

Fig. 1. Cumulative rate of HCC according to serum levels of HDL-Chol, low-density lipoprotein cholesterol (LDL-Chol), total cholesterol (T-Chol) and triglyceride (TG). The rate of HCC was significantly higher for low serum levels of HDL-Chol than high serum levels of HDL-Chol (p = 0.026, log-rank test).

Table 2. Factors associated with hepatocarcinogenesis in patients infected with HCV-1b who did not achieve SVR with IFN+RIB combination therapy, identified by multivariate analysis

Factor	Category	HR	p
Core aa 70	1: Arg70 2: Gln70/His70	1 4.64 (1.30–16.5)	0.018
HDL-Chol	1: ≥50 mg/dl 2: <50 mg/dl	1 9.35 (1.09–83.3)	0.041

Cox proportional hazard model. Values in parentheses represent 95% confidence intervals.

These included age (≥55 years; p = 0.093), body mass index (≥25; p = 0.013), HDL-Chol (<50 mg/dl; p = 0.026) and substitution of aa 70 in the HCV core region (Gln70/His70; p = 0.086). On the other hand, gender, stage of fibrosis and genetic variation near the IL28B gene showed no such correlation. These 4 factors were entered into multivariate analysis, which identified 2 parameters as significant and independent determinants of HCC, namely substitution of aa 70 in the HCV core region (Gln70/His70; HR 4.64, p = 0.018) and serum level of HDL-Chol (<50 mg/dl; HR 9.35, p = 0.041; table 2).

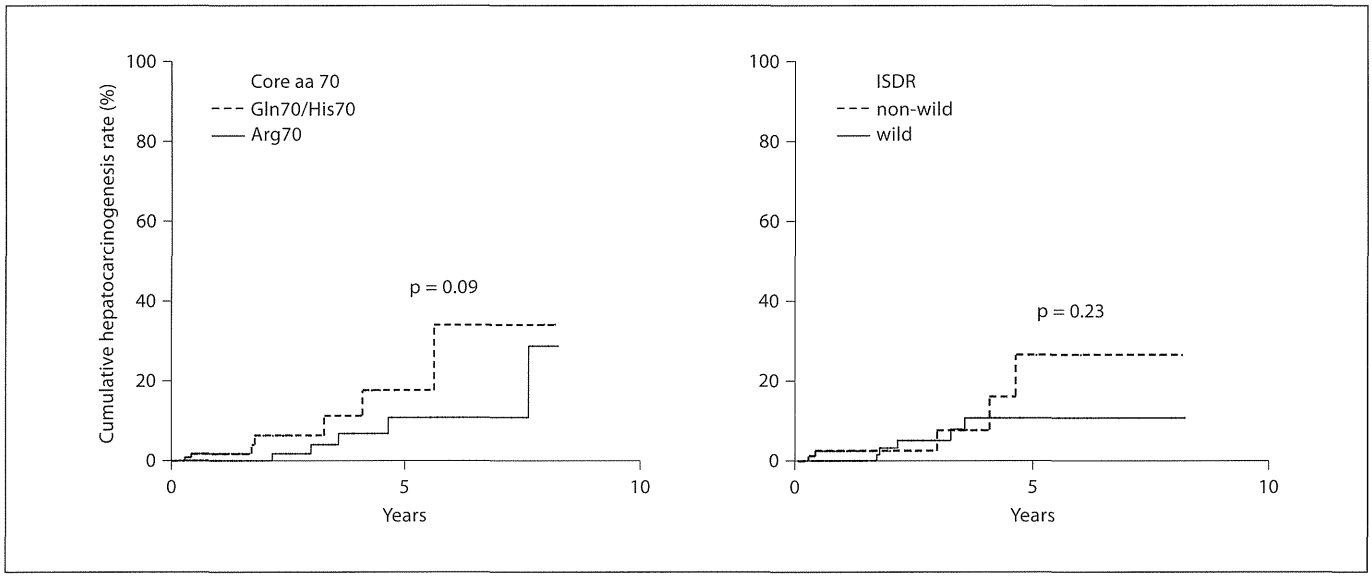

Fig. 2. Cumulative rate of HCC according to amino acid substitutions in the core region of HCV-1b and ISDR of NS5A. The rate of HCC for the Gln70/His70 substitution tended to be higher than that for Arg70 (p = 0.086, log-rank test). There was no significant relationship between ISDR substitution and HCC (p = 0.232, log-rank test).

Rate of HCC according to Substitution of aa 70 in the HCV Core Region and Serum Level of HDL-Chol

The patients were divided into two groups according to the serum level of HDL-Chol using a cutoff value of 50 mg/dl [low HDL-Chol group (<50 mg/dl), n = 127, high HDL-Chol group (≥50 mg/dl), n = 115]. During the follow-up period, 10 patients (8.0%) in the low HDL-Chol group and 1 (1.0%) in the high HDL-Chol group developed HCC. The median interval between the completion of IFN+RIB therapy and detection of HCC was 3.1 years (range 0.0–7.6 years) and 4.1 years for the low and high HDL-Chol groups, respectively. The respective cumulative rates of HCC in the low and high HDL-Chol groups were 9.0 and 0% at the end of 3 years, 19.7 and 5.9% at the end of 5 years, and 26.4 and 5.9% at the end of 7 years. The rates were significantly different between the two groups (p = 0.026, log-rank test; fig. 1).

During the follow-up period, 7 patients (5.7%) who developed HCC had a Gln70/His70 substitution and 5 (3.5%) had an Arg70 substitution. The median interval between the completion of IFN+RIB therapy and detection of HCC in patients with Gln70/His70 and Arg70 was 1.8 years (range 0.0–5.6 years) and 3.6 years (range 0.0–7.6 years), respectively. The respective cumulative rates of HCC in these patients were 6.3 and 4.0% at the end of 3 years, 17.6 and 10.8% at the end of 5 years, and 34.1 and 10.8% at the end of 7 years. The rates tended to be different between the two groups (p = 0.086, log-rank test; fig. 2)

Discussion

Previous studies on Japanese patients infected with HCV-1b reported that IFN+RIB therapy increases the proportion of patients who achieve SVR [3, 28] and that the incidence of HCC among patients who achieve SVR is lower than that among patients who do not [7]. In the present study, we examined the incidence and risk factors of HCC in HCV-1b patients who did not achieve SVR after IFN+RIB therapy. Multivariate analysis identified amino acid substitution in the core region of HCV (Gln70/His70) and serum levels of HDL-Chol (<50 mg/dl) as determinants of HCC in such patients. We also examined the risk factors for HCC in HCV-1b patients treated with IFN+RIB therapy. Multivariate analysis identified age (>55 years), body mass index (>25), ISDR substitutions (wild type), amino acid substitution in the core region of HCV (Gln70/His70) and serum levels of HDL-Chol (<50 mg/dl) as determinants of HCC in such patients (data not shown). This result suggested that the effect of IFN+RIB therapy was independent of amino acid substitution in

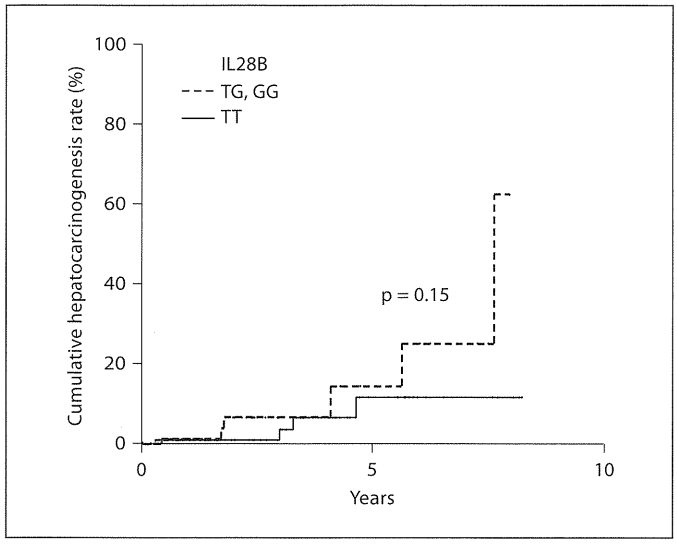

Fig. 3. Cumulative rate of HCC according to genetic variations near the IL28B gene. There was no significant relationship between genetic variations near the IL28B gene and HCC (p = 0.153, log-rank test).

the core region and serum levels of HDL-Chol. In this regard, previous reports identified severe fibrosis, male sex, old age, steatosis, HCV genotype [6, 8–12], ISDR substitutions [13], substitution of aa 70 in the HCV core region [14] and IL28B polymorphisms [15] as risk factors for HCC. In the present study, some of the above factors were not identified as significant predictors. The differences between the findings of the present study and the above reports are not clear at present, but they could reflect differences in the population samples, since we focused on Japanese patients with HCV-1b infection who were treated with IFN+RIB therapy and failed to respond to it. Further studies of larger population samples of other ethnicities are necessary.

In this present study, substitution of aa 70 in the HCV core region was associated with the development of HCC after IFN+RIB therapy. Experiments in transgenic mice have provided evidence for the oncogenic role of the HCV core region [29]. Furthermore, patients infected by HCV-1b with amino acid substitutions in the core region are at high risk of HCC [14, 30–32], even after eradication of HCV RNA [33]. In the presence of amino acid substitutions in the core region, IFN-induced phosphorylation of STAT1 and STAT2 is lower, and the expression level of SOCS3, an IFN signal attenuator, was higher than in the wild type. Furthermore, the expression levels of IL-6, which upregulates SOCS3, and those of endoplasmic reticulum stress proteins were significantly higher in cells transfected with core mutant compared with the wild type [34]. These mechanisms may explain the resistance to IFN of HCV-1b with amino acid substitutions in the core region. Other studies also described the important role of a PA28γ-dependent pathway in the development of HCV-associated HCC. Moriishi et al. [35, 36] reported that knockout of the PA28γ gene induces accumulation of HCV core protein in the nuclei of hepatocytes of HCV core gene transgenic mice and disrupts the development of both hepatic steatosis and HCC. Furthermore, HCV core protein is also reported to enhance the binding of liver X receptor α/retinoid X receptor α to liver X receptor response element in the presence of PA28γ [36]. Thus, it seems that PA28γ plays a crucial role in the development of HCV-associated steatosis and HCC. Further studies should be performed to investigate the oncogenic potential of amino acid substitution in the core region of HCV detected at the start of antiviral therapy with regard to HCC after combination therapy.

The relationship 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 HCC in HCV-related liver disease independent of age, sex, body mass index, stage of fibrosis and response to antiviral therapy [9, 11]. Other reports indicated that obesity and diabetes mellitus are risk factors for HCC [37–39]. It is also reported that HCV core protein is involved in mitochondrial electron transfer system dysfunction and activation of peroxisome proliferator-activated receptor-α (PPARα). In the presence of mitochondrial dysfunction, PPARα exacerbates steatosis, and persistent activation of PPARα contributes to hepatocarcinogenesis by inducing overproduction of reactive oxygen species and cell growth signal activation [12]. In this present study, multivariate analysis identified amino acid substitution in the core region of HCV and low levels of HDL-Chol as determinants of HCC. These results are not inconsistent with previous studies. Interestingly, in our patients, the impact of amino acid substitution in the core region of HCV and low levels of HDL-Chol was more significant than that of gender, age and stage of fibrosis. One of the reasons for this finding could be the nature of the population study, i.e. Japanese patients treated with IFN+RIB.

Genetic variations near the IL28B gene are pretreatment predictors of a poor virological response to PEG-IFN/RIB combination therapy and triple therapy with telaprevir/PEG-IFN/RIB [22–25, 40]. It has recently been reported that the IL-28B rs12979860 C/T polymorphism

T allele is more prevalent in patients with HCV-related cirrhosis than other etiologies and mild chronic hepatitis C, and also in patients with HCC than in those without HCC [15]. However, the link between IL-28B and HCC remains unclear. In the present study, genetic variations near the IL28B gene did not significantly affect HCC (fig. 3). This discrepant result might be related to differences in the etiology, including hepatitis B virus, alcohol intake and HCV-related liver disease. The population of this study consisted of Japanese patients infected with HCV-1b who were treated with IFN+RIB. Further studies should be conducted to investigate the relationship between genetic variations near the IL28B gene and HCC.

Our study has certain limitations. Firstly, the study did not provide a comprehensive analysis of the viral factors and their role in the development of HCC. Experimental evidence suggests that the pathogenic role of HCV-1b strains in HCC is based on the secondary structure of the amino-terminal portion of the HCV NS3 protein [41]. In the present study, we did not investigate the roles of viral factors except for the HCV core region and NS5A region. Another limitation of the study is the lack of analysis of the clinical impact of lifestyle-related diseases (such as diabetes, insulin resistance, nonalcoholic steatohepatitis) on HCC, except for body mass index and cholesterol levels [38, 39, 42, 43]. Further studies are needed to investigate the clinical impact of viral factors and lifestyle-related diseases on HCC.

We previously indicated that substitution of aa 70 in the HCV-1b core region might predict elevation of serum α-fetoprotein levels in non-HCC patients and that eradication of HCV-1b with Gln70/His70 seemed to induce normalization of α-fetoprotein [44]. To investigate α-fetoprotein during and after PEG-IFN+RIB therapy, according to the substitution pattern of aa 70, is important for evaluating the risk of hepatocarcinogenesis, especially in nonresponders. Further understanding of the complex interaction between α-fetoprotein levels and substitution of aa 70 in the HCV-1b core region should facilitate the development of more effective therapeutic regimens.

In conclusion, the present study identified amino acid substitution in the core region of HCV-1b and low levels of HDL-Chol as significant and independent predictors of HCC in nonresponders to the combination of IFN+RIB. The study emphasizes the importance of viral and lipid metabolic factors in hepatocarcinogenesis after combination therapy.

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Original Article

Serum albumin level is a notable profiling factor for non-B, non-C hepatitis virus-related hepatocellular carcinoma: A data-mining analysis

Shingo Yamada,[1] Atsushi Kawaguchi,[4] Takumi Kawaguchi,[1,2] Nobuyoshi Fukushima,[1,6] Ryoko Kuromatsu,[1] Shuji Sumie,[1] Akio Takata,[1] Masahito Nakano,[1] Manabu Satani,[1] Tatsuyuki Tonan,[3] Kiminori Fujimoto,[3,7] Hiroji Shima,[8] Tatsuyuki Kakuma,[4] Takuji Torimura,[1,5] Michael R. Charlton[9] and Michio Sata[1,2]

[1]*Division of Gastroenterology, Department of Medicine, and Departments of* [2]*Digestive Disease Information and Research and* [3]*Radiology, Kurume University School of Medicine,* [4]*Biostatistics Center,* [5]*Liver Cancer Research Division, Research Center for Innovative Cancer Therapy, Kurume University,* [6]*Department of Gastroenterology, National Hospital Organization, Kyushu Medical Center,* [7]*Center for Diagnostic Imaging, Kurume University Hospital,* [8]*St Mary's Hospital, Kurume, Japan; and* [9]*Division of Gastroenterology and Hepatology, Mayo Clinic, Rochester, Minnesota, USA*

Aim: Various factors are underlying for the onset of non-B, non-C hepatitis virus-related hepatocellular carcinoma (NBNC-HCC). We aimed to investigate the independent risk factors and profiles associated with NBNC-HCC using a data-mining technique.

Methods: We conducted a case-control study and enrolled 223 NBNC-HCC patients and 669 controls from a health checkup database (n = 176 886). Multivariate analysis, random forest analysis and a decision-tree algorithm were employed to examine the independent risk factors, factors distinguishing between the case and control groups, and to identify profiles for the incidence of NBNC-HCC, respectively.

Results: In multivariate analysis, besides γ-glutamyltransferase (GGT) levels and the Brinkman index, albumin level was an independent negative risk factor for the incidence of NBNC-HCC (odds ratio = 0.67; 95% confidence interval = 0.60–0.70; $P < 0.0001$). In random forest analysis, serum albumin level was the highest-ranked variable for distinguishing between the case and control groups (98 variable importance). A decision-tree algorithm was created for albumin and GGT levels, the aspartate aminotransferase-to-platelet ratio index (APRI) and the Brinkman index. The serum albumin level was selected as the initial split variable, and 82.5% of the subjects with albumin levels of less than 4.01 g/dL were found to have NBNC-HCC.

Conclusion: Data-mining analysis revealed that serum albumin level is an independent risk factor and the most distinguishable factor associated with the incidence of NBNC-HCC. Furthermore, we created an NBNC-HCC profile consisting of albumin and GGT levels, the APRI and the Brinkman index. This profile could be used in the screening strategy for NBNC-HCC.

Key words: lifestyle, metabolism, non-viral-related hepatoma, smoking

INTRODUCTION

LIVER CANCER IS the sixth most frequently diagnosed cancer worldwide and was the third most frequent cause of cancer-related death in 2008.[1] Although the incidence of liver cancer is increasing worldwide, the highest rate is found in East Asia. Hepatocellular carcinoma (HCC) accounts for 70–85% of the cases of primary liver cancer. The most significant risk factors for HCC are hepatitis C virus (HCV) and hepatitis B virus (HBV) infection. Although the incidence of HCV-related HCC has recently been decreasing,[2] the incidence of non-B, non-C hepatitis-related HCC (NBNC-HCC) in Japan has risen to 27.6% from 7.6% in the last 15 years.[2]

Correspondence: Dr Takumi Kawaguchi, Division of Gastroenterology, Department of Medicine, Kurume University School of Medicine. 67 Asahi-machi, Kurume 830-0011, Japan. Email: takumi@med.kurume-u.ac.jp

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Non-B, non-C hepatitis-related HCC can be caused by various non-viral chronic liver injuries, including those associated with alcoholic liver disease, non-alcoholic fatty liver disease, autoimmune liver diseases and hemochromatosis.[3] In addition, diabetes mellitus, use of exogenous insulin and smoking have been reported as risk factors for the development of HCC.[4–6] As NBNC-HCC is often diagnosed at an advanced stage, an efficient strategy for early detection is required.[7] Thus far, no case-control studies have been conducted to investigate the risk factors for NBNC-HCC; therefore, unidentified risk factors may exist. Moreover, the combined effect of these risk factors has not been examined. NBNC-HCC is thought to be caused by complicated interactions between multiple risk factors; hence, the identification of a risk profile for NBNC-HCC may aid the establishment of a novel strategy for the early detection of NBNC-HCC. The prevalence of NBNC-HCC may increase further due to the combined effects of more effective vaccine and treatment strategies for HBV and HCV coupled with the increasing prevalence of non-alcoholic fatty liver disease.

Two popular approaches to developing a risk profile for development of screening strategies are random forest analysis and decision-tree algorithms. Both approaches require identification of carefully matched cases and controls to avoid selection bias and to balance covariates.[8,9] Statistical matching techniques have been developed to facilitate this process. Genetic matching (GenMatch) is used to search the best pair on the basis of genetic Mahalanobis distance values. This process involves a multidimensional search to provide the near-optimal value of a fitness function in an optimization problem.[10,11] GenMatch is increasingly being employed in clinical practice and served to identify factors associated with gestational diabetes and multiple protein biomarkers for head and neck squamous cell cancer.[12,13] GenMatch has consistently been found to be more accurate than existing matching methods, such as propensity score.[14,15]

Random forest analysis is a data-mining technique that identifies the factors distinguishing between the case and control groups with an ordinal scale. A decision-tree algorithm is a data-mining technique that reveals a series of classification rules by identifying priorities, and therefore allows clinicians to choose an option that maximizes benefit for the patient. It has been used to identify the profiles associated with response to interferon therapy for chronic hepatitis C,[16,17] incidence of subclinical hepatic encephalopathy,[18] HCV carriers with persistently normal alanine aminotransferase (ALT) levels,[19] and the progression of NBNC-HCC.[7] Neither random forest analysis nor decision-tree algorithms have been applied to identify the clinical feature profile associated with NBNC-HCC incidence.

The aim of this study is to investigate independent risk factors associated with the incidence of NBNC-HCC by comparing cases to controls matched by GenMatch from a health checkup database. In addition, we also investigated a profile associated with NBNC-HCC incidence by using random forest analysis and a decision-tree algorithm.

METHODS

Ethics

THE STUDY PROTOCOL conformed to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in the prior approval given by each institutional review board. None of the subjects were institutionalized.

Subjects

We conducted a case–control study to examine NBNC-HCC risk factors. From 1995 to 2010, 1769 patients were diagnosed with HCC at Kurume University Hospital and all of the patients diagnosed with NBNC-HCC ($n = 223$) were enrolled in this study (no liver disease, $n = 109$; alcoholic liver disease, $n = 80$; schistosomiasis japonica, $n = 13$; autoimmune hepatitis, $n = 8$; non-alcoholic fatty liver disease, $n = 5$; hemochromatosis, $n = 2$; primary sclerosing cholangitis, $n = 2$; primary biliary cirrhosis, $n = 1$; sarcoidosis, $n = 1$; von Gierke disease, $n = 1$; Budd–Chiari syndrome, $n = 1$). NBNC-HCC patients were defined as those who were initially diagnosed with primary liver cancer with negative results for both serum hepatitis B surface antigen (HBsAg) and anti-HCV antibody. NBNC-HCC was diagnosed by a combination of tests for serum tumor makers such as α-fetoprotein and des-γ-carboxy prothrombin, and imaging procedures such as ultrasonography, computed tomography, magnetic resonance imaging and angiography. In addition, 48.9% (109/223) of the patients were pathologically diagnosed with HCC following ultrasonography-guided fine-needle tumor biopsy. No patients had malabsorption syndrome, protein-losing gastroenteropathy, and chronic kidney disease including nephrotic syndrome.

Control data from the period 1996–2007 were obtained from a health checkup database ($n = 176\,886$) at St Mary's Hospital, which is located in the same city as

Table 1 Genetic matching for case : control ratio

Case : control	*P*-value
1:1	0.3173
1:2	0.6834
1:3	0.7857
1:4	0.0460
1:5	0.1430

GenMatch was employed to investigate the proper ratio of the case : control number. *P*-value is the highest in "case : control = 1:3", indicating that the most matched control number is threefold the number of cases.

Kurume University Hospital, and selected using the following criteria: (i) no HCC; and (ii) negative results for both the serum HBsAg and anti-HCV antibody. In a case–control study, selection of controls is an important step. Because age and sex are well-known risk factors for hepatocarcinogenesis,[20,21] control subjects were matched to the cases by age and sex. The case : control ratio can also affect the results of a study.[22] To evaluate the case : control ratio, the smallest *P*-values obtained from all the matching balance tests, including Student's *t*-test and Kolmogorov–Smirnov test, were used. GenMatch was employed and demonstrated that the highest *P*-value was obtained for "case : control = 1:3", indicating that the most matched control number is threefold the number of cases in this study (Table 1). Thus, we randomly selected 669 subjects from 176 886 non-HCC subjects who underwent a medical checkup as the control group.

Clinical characteristics, lifestyle, complications and biochemical parameters

Data for clinical characteristics, lifestyle, complications and biochemical parameters were obtained at the time of HCC diagnosis and data from the health examination included information regarding the following variables: age; sex; height; weight; habitual intake of alcohol; cumulative cigarette consumption; history of fatty liver, hypertension and diabetes mellitus; use of antidiabetic agents (regardless of drug type); serum levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT), γ-glutamyltransferase (GGT) and albumin; hemoglobin level; platelet count; levels of blood glucose, hemoglobin A1c (HbA1c), total bilirubin, total cholesterol, triglyceride and HBsAg; and HCV antibody status. Biochemical parameters were measured using standard clinical methods (Department of Clinical Laboratory, Kurume University Hospital or St Mary's Hospital).

Assessment of body constitution, daily alcohol intake and cumulative cigarette consumption

Body mass index (BMI) was calculated as bodyweight in kilograms divided by the square of height in meters (kg/m^2). Daily alcohol intake was categorized as none, less than 60 g, 60–100 g and more than 100 g, as previously described.[23] The cumulative cigarette consumption was estimated by the Brinkman index (number of cigarettes consumed/day × years of smoking).

Assessment of fatty liver and hepatic fibrosis

Fatty liver was diagnosed by the presence of at least two out of three abnormal findings on abdominal ultrasonography: (i) diffusely increased hepatic echogenicity ("bright") that was greater than that for the kidney; (ii) vascular blurring; and (iii) deep attenuation of the ultrasound signal, as previously described.[24] Hepatic fibrosis was evaluated by the AST-to-platelet ratio index (APRI): serum AST level (U/L) / upper limit of normal AST level (33 U/L) × 100 / platelet count ($\times 10^4$/mL).[25] Liver cirrhosis is excluded by APRI of less than 1.5 and 61.9% (85/223) showed APRI of less than 1.5.

Diagnosis of hypertension and diabetes mellitus

Hypertension was diagnosed by a systolic blood pressure of more than 140 mmHg and/or diastolic blood pressure of more than 90 mmHg,[26] or by prescription of antihypertensive agents. Diabetes mellitus was diagnosed on the basis of fasting blood glucose levels of more than 126 mg/dL or HbA1c levels of more than 6.5% according to the Diagnostic Criteria for Diabetes Mellitus[27] or by use of antidiabetic agents.

Statistical analysis

Descriptive statistics were expressed as a number or mean ± standard deviation. GenMatch was used to determine the proper case : control ratio. Differences between the two groups were analyzed using the Mann–Whitney *U*-test. Variables or profiles associated with the incidence of NBNC-HCC were analyzed by data-mining techniques. The statistical methods are described in detail below.

Multivariate stepwise analysis

A logistic regression model was used for multivariate stepwise analysis to identify any independent variables associated with the incidence of NBNC-HCC as previ-

ously described.[7] Data were expressed as odds ratio (OR) and 95% confidence interval (CI) values.

Random forest analysis

Random forest analysis was used to identify the factors distinguishing between the case and control with an ordinal scale, as previously described.[28] The procedure employed for building the random forest was as follows: First, n tree-models were created using bootstrap samples that were randomly chosen from the original dataset. Second, each classification or regression tree model was grown with no pruning. Instead of determining the best split among all potential predictors, we chose a random sample of these variables (one-third of the variables) to consider as potential splitting variables. Thus, the best split variable was chosen from among those variables. Third, new data were predicted by aggregating the predictions of the n trees. Finally, the error rate was estimated by predicting the data not in the bootstrap sample (out-of-bag) by using the tree grown with the bootstrap sample. The variable importance value reflecting the relative contribution of each variable to the model was estimated by randomly permuting its values and recalculating the predictive accuracy of the model, and was expressed as the mean difference of the Gini index.

Decision-tree algorithm

A decision-tree algorithm was constructed to reveal profiles associated with the incidence of NBNC-HCC. Predictive accuracy of the decision-tree model was validated by the area under the receiver–operator curve (AUROC) analysis using 10-fold cross-validation, as previously described.[7]

All P-values were two-tailed, and a level of less than 0.05 was considered to be statistically significant. Multivariate stepwise analysis was conducted using SAS ver. 9.2 (SAS Institute, Cary, NC, USA). GenMatch, random forest analysis and decision-tree analysis were conducted using the R packages (URL http://www.r-project.org/index.html).[29]

RESULTS

Characteristics of all subjects

THE CHARACTERISTICS OF the 223 patients and 669 control subjects are summarized in Table 2. There was no significant difference between the case and control groups in BMI, serum triglyceride levels and comorbidity with hypertension. The case group showed significantly higher comorbidity with fatty liver and serum levels of AST, ALT, GGT and total bilirubin, with reference to the corresponding values in the control group (Table 2). The case group also showed significantly higher alcohol intake, Brinkman index, comorbidity with diabetes mellitus, use of antidiabetic agents, fasting blood glucose levels, HbA1c value and APRI (Table 2). The case group showed significantly lower blood hemoglobin levels, platelet counts and serum levels of total cholesterol and albumin (Table 2).

Multivariate stepwise analysis for the incidence of NBNC-HCC

Multivariate stepwise analysis was performed to identify independent variables for the incidence of NBNC-HCC. The APRI and HbA1c values and platelet counts were not significant variables. However, GGT levels, the Brinkman index and use of antidiabetic agents were identified as independent positive risk factors for the incidence of NBNC-HCC (GGT, OR = 1.17, 95% CI = 1.08–1.21, $P < 0.0001$; Brinkman index, OR = 1.17; 95% CI = 1.05–1.30; $P = 0.0047$; use of antidiabetic agents, OR = 7.42, 95% CI = 2.42–22.76, $P = 0.0005$) (Table 3). On the other hand, total cholesterol, hemoglobin and albumin levels were identified as independent negative risk factors for the incidence of NBNC-HCC (total cholesterol, OR = 0.88, 95% CI = 0.79–0.98, $P = 0.0155$; hemoglobin, OR = 0.95, 95% CI = 0.93–0.97, $P < 0.0001$; albumin, OR = 0.67, 95% CI = 0.60–0.70, $P < 0.0001$) (Table 3).

Random forest analysis for distinguishing between the case and control groups

The results of random forest analysis are summarized in rank order in Figure 1. The analysis demonstrated that serum albumin level is the highest-ranked variable for distinguishing between case and control (Fig. 1). This is followed by APRI, GGT level, platelet count, hemoglobin level, AST level, HbA1c value, total cholesterol level and the Brinkman index (Fig. 1).

Decision-tree algorithm for the incidence of NBNC-HCC

With the dataset ($n = 892$), a decision-tree algorithm was created by using four variables to classify five groups of subjects (Fig. 2). The serum level of albumin was selected as the initial split variable with an optimal cut-off of 4.01 g/dL. When subjects showed albumin levels of 4.01 g/dL or more, 17.5% (39/223) of subjects were found to have NBNC-HCC. When the albumin