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

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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 ra-

tio 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.

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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].

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.

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 IFN α -2b or PEG-IFN α -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 coinfection 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.

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, $\times 10^4/\text{mm}^3$	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.

Of the total 292 patients, 226 (77%) received PEG-IFN α -2b at a median dose of 1.4 $\mu\text{g/kg}$ (range 1.3–1.9 $\mu\text{g/kg}$) subcutaneously each week for a median duration of 47 weeks (range 28–48 weeks). The remaining 66 patients (23%) received 6 million units of IFN α -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	1	0.018
	2: Gln70/His70	4.64 (1.30–16.5)	
HDL-Chol	1: ≥50 mg/dl	1	0.041
	2: <50 mg/dl	9.35 (1.09–83.3)	

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 re-

ticulum 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 PA28 γ -dependent pathway in the development of HCV-associated HCC. Moriishi et al. [35, 36] reported that knockout of the PA28 γ 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 PA28 γ [36]. Thus, it seems that PA28 γ 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 (PPAR α). In the presence of mitochondrial dysfunction, PPAR α exacerbates steatosis, and persistent activation of PPAR α 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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