

All patients were followed up at our hospital at least twice a year. During each follow-up examination, platelet count, alanine aminotransferase (ALT), aspartate aminotransferase (AST), gamma glutamyl transpeptidase (γ -GTP), total bilirubin, cholinesterase, alkaline phosphatase (ALP), lactate dehydrogenase (LDH), albumin, total cholesterol, AFP, AFP-L3%, and DCP were measured. Platelet count and ALT, AST, γ -GTP, total bilirubin, cholinesterase, ALP, LDH, albumin, total cholesterol, AFP, AFP-L3%, and DCP values were expressed as average integration values [13, 14]. Briefly, using ALT as an example, the area of a trapezoid is calculated by multiplying the sum of two ALT values by one-half of the interval between the measurements. This value is then divided by the observation period to obtain the average integration value, and this technique provides a better representation of values over time when there are extremes of high and low values [14, 16]. In patients who developed HCC during the observation period, AFP, AFP-L3%, and DCP values obtained at least 1 year before the diagnosis of HCC were assessed. Serum AFP concentration was determined with a commercially available kit. AFP-L3% was measured by lectin-affinity electrophoresis and antibody-affinity blotting with the AFP Differentiation Kit L (Wako Pure Chemical Industries, Osaka, Japan) [10]. DCP was measured with a DCP reagent (Picolumi PIVKA-II; Eisai, Tokyo, Japan) [11]. Cutoff levels for AFP, AFP-L3%, and DCP were set at 20 ng/mL, 10%, and 40 mAU/mL, respectively, according to previous reports [10–12]. HCV genotype and quantification of HCV RNA (Amplicor 2; Roche Diagnostics, Tokyo, Japan) were determined in 513 cases. All patients underwent imaging modalities (US, computed tomography [CT], or magnetic resonance imaging [MRI]), every 3 months in patients with cirrhosis and every 6 months in patients with chronic hepatitis.

The diagnoses of HCC were confirmed by histologic examination of resected hepatic tumors or US-guided needle biopsy specimens. When biopsy of the tumor was contraindicated, the HCC diagnosis was made using clinical criteria and imaging findings obtained from B-mode US, CT angiography, or MRI [19, 20]. HCC was histologically diagnosed in 46 patients, and in the remaining 74 patients, the diagnosis was made based on clinical criteria [19, 20]. All tumors were 3 cm or less in maximum diameter, and there were 3 nodules or less on diagnosis.

One hundred eighty-nine patients received IFN therapy. Patients were classified into three groups according to the type of response to IFN therapy: sustained virologic response (SVR), defined as the absence of serum HCV RNA at 6 months after IFN therapy; the non-SVR group, defined as the presence of serum HCV RNA at 6 months after IFN therapy; and the no IFN therapy group.

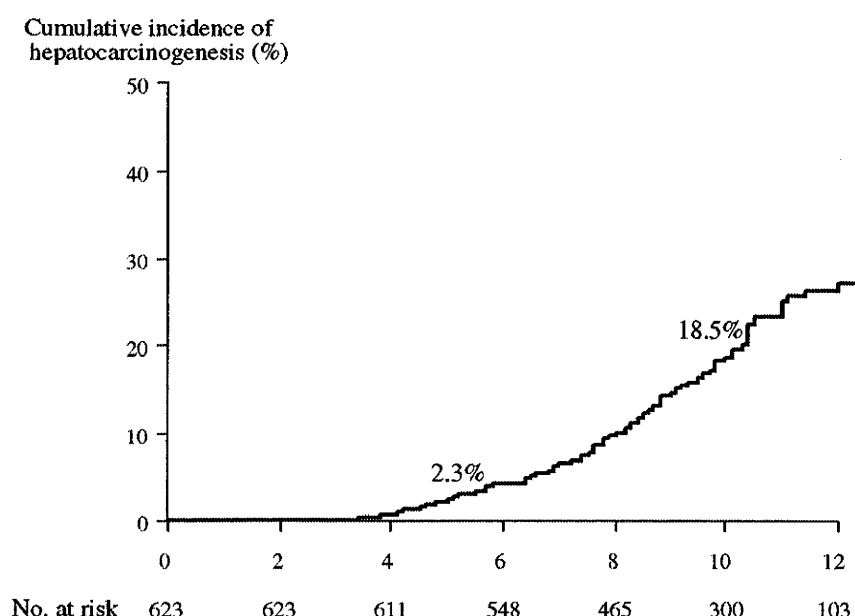
Patients were classified into three groups for each of the tumor markers according to the average integration values of AFP, AFP-L3%, and DCP: A1, <10 ng/mL ($n = 452$); A2, ≥ 10 , <20 ng/mL ($n = 80$); and A3, ≥ 20 ng/mL ($n = 91$); L1, <5% ($n = 588$); L2, ≥ 5 , <10% ($n = 18$); and L3, $\geq 10\%$ ($n = 17$); and D1, <20 mAU/mL ($n = 379$); D2, ≥ 20 , <40 mAU/mL ($n = 170$); and D3, ≥ 40 mAU/mL ($n = 51$), respectively.

The present study ended on 31 December 2008 or the date of identification of HCC occurrence. The median follow-up period was 9.0 years (range 3.0–13.0 years). The total number of blood examinations was 25,721, and the median number of blood examinations was 23 (range 6–105) per subject.

Statistical analysis

Statistical analysis was performed with the Statistical Program for Social Science (SPSS ver.17.0 for Windows; SPSS Japan, Tokyo, Japan). Continuous variables are shown as medians (ranges). The Mann–Whitney U -test was used for continuous variables, and Fisher's exact test was used for categorical variables. Actuarial analysis of the cumulative incidence of hepatocarcinogenesis was performed by the Kaplan–Meier method, and differences were tested by the log-rank test. The Bonferroni correction was performed for multiple comparisons. The Cox proportional hazards model and forward selection method were used to estimate the relative risk of HCC development associated with age (≤ 65 or > 65 years), sex (female or male), body mass index (BMI ≤ 25.0 or > 25.0 kg/m²), HCV genotype (type 1 or type 2), viral concentration (≤ 100 or > 100 KIU/mL), platelet count ($< 12.0 \times 10^4/\text{mm}^3$ or $\geq 12.0 \times 10^4/\text{mm}^3$), ALT (≤ 35 or > 35 IU/mL), AST (≤ 40 or > 40 IU/mL), total bilirubin (≤ 1.2 or > 1.2 mg/dL), γ -GTP (≤ 56 or > 56 IU/mL), ALP (≤ 338 or > 338 IU/mL), cholinesterase (< 431 or ≥ 431 IU/mL), LDH (≤ 250 or > 250 IU/mL), albumin (< 3.5 or ≥ 3.5 g/dL), total cholesterol (< 130 or ≥ 130 mg/dL), cirrhosis (presence or absence), and IFN treatment (no therapy, non-SVR, or SVR) for univariate and multivariate analyses. We used the lower or upper limit of the reference values at our institute as cutoff values for platelet count, ALT, AST, total bilirubin, γ -GTP, ALP, cholinesterase, LDH, albumin, and total cholesterol levels. Statistical significance was set at $P < 0.05$.

The study protocol was approved by the Ethics Committee at Ogaki Municipal Hospital in January 2009 and the study was performed in compliance with the Helsinki Declaration. Informed consent was obtained from each patient for analyzing patient records and images.

Fig. 2 Overall cumulative incidence rate of HCC**Table 1** Patient characteristics

Age (years)	61 (26–84)
Sex (F/M)	265/358
BMI (kg/m ²)	22.5 (12.0–34.9)
HCV genotype (type 1/type 2)	356/157
Viral concentration (KIU/mL)	270 (0.5–6300)
AFP (ng/mL)	4.8 (0.8–341.5)
AFP-L3 (%)	0.1 (0.0–32.5)
DCP (mAU/mL)	18.1 (8.5–99.6)
Platelets ($\times 10^4/\text{mm}^3$)	14.8 (3.0–33.9)
ALT (IU/L)	46.4 (10.1–340.4)
AST (IU/L)	48.5 (13.3–168.9)
γ -GTP (IU/L)	37.6 (9.9–2207)
Total bilirubin (mg/dL)	0.6 (0.2–2.7)
ALP (IU/L)	276.4 (86.8–845.5)
Cholinesterase (IU/L)	242.9 (38.8–545.30)
LDH (IU/L)	196.4 (118.4–650.1)
Albumin (g/dL)	4.0 (2.4–4.9)
Total cholesterol (mg/dL)	155.8 (77.9–264.1)
Fibrosis (F0/F1/F2/F3/F4) ^a	32/73/56/24/2
Cirrhosis (present/absent)	160/463
IFN therapy (none/non-SVR/SVR)	434/146/43

Continuous variables are quoted as medians (ranges)

BMI body mass index, HCV hepatitis C virus, AFP alpha-fetoprotein, AFP-L3 *Lens culinaris* agglutinin-reactive fraction of AFP, DCP des- γ -carboxy prothrombin, ALT alanine aminotransferase, AST aspartate aminotransferase, GTP gamma glutamyl transpeptidase, ALP alkaline phosphatase, LDH lactate dehydrogenase, IFN interferon, SVR sustained virologic response

^a Staging of chronic hepatitis according to Desmet et al. [15]

Results

HCC developed in 120 (19.3%) of the 623 patients. The 5- and 10-year cumulative incidences of HCC were 2.3 and 18.5%, respectively (Fig. 2). Demographic and medical data for the 623 patients are summarized in Table 1.

Factors associated with the incidence of hepatic carcinogenesis on univariate analysis

Factors associated with the incidence of HCC are listed in Table 2. Age ≥ 65 years, high AFP level, high AFP-L3% level, high DCP level, low platelet count, high ALT level, high AST level, high LDH level, high ALP level, low cholinesterase level, low albumin level, presence of cirrhosis, and response to IFN therapy were significantly associated with the development of HCC on univariate analysis.

The 5-, 7-, and 10-year cumulative incidences of HCC were 1.1, 2.1, and 7.5% in group A1; 2.6, 9.6, and 42.1% in group A2; and 6.6, 18.3, and 50.0% in group A3, respectively, and the cumulative incidence of HCC differed significantly between groups A1 and A2 and groups A1 and A3 (Fig. 3). The 5-, 7-, and 10-year cumulative incidences of HCC were 1.4, 4.6, and 15.6% in group L1; 19.6, 39.7, and 73.6% in group L2; and 12.5, 25.0, and 56.7% in group L3, respectively, and the cumulative incidence of HCC differed significantly between groups L1 and L2 and groups L1 and L3 (Fig. 4). The 5-, 7-, and 10-year cumulative incidences of HCC were 0.5, 4.6, and

Table 2 Factors associated with hepatocarcinogenesis (univariate analysis)

	Crude hazard ratio (95% CI)	P
Age (years)		
≤65	1	
>65	2.318 (1.580–3.400)	<0.001
AFP (ng/mL)		
A1; <10	1	
A2; ≥10, <20	6.061 (3.768–9.750)	<0.001
A3; ≥20	8.985 (5.874–13.744)	<0.001
AFP-L3 (%)		
L1; <5	1	
L2; ≥5, <10	8.032 (4.388–14.700)	<0.001
L3; ≥10	3.781 (1.838–7.778)	<0.001
DCP (mAU/mL)		
D1; <20	1	
D2; ≥20, <40	1.209 (0.788–1.855)	0.385
D3; ≥40	4.535 (2.840–7.241)	<0.001
Platelets ($\times 10^4/\text{mm}^3$)		
≥12.0	1	
<12.0	5.887 (3.982–8.702)	<0.001
ALT (IU/L)		
≤35	1	
>35	2.632 (1.574–4.400)	<0.001
AST (IU/L)		
≤40	1	
>40	8.120 (4.115–16.024)	<0.001
LDH (IU/L)		
≤250	1	
>250	1.970 (1.249–3.106)	<0.001
ALP (IU/L)		
≤338	1	
>338	2.509 (1.724–3.650)	<0.001
Cholinesterase (IU/L)		
>431	1	
≤431	3.288 (2.209–4.893)	<0.001
Albumin (g/dL)		
≥3.5	1	
<3.5	3.948 (2.635–5.917)	<0.001
Cirrhosis		
Absent	1	
Present	3.474 (2.413–5.002)	<0.001
IFN therapy		
No therapy	1	
Non-SVR	0.312 (0.180–0.539)	<0.001
SVR	0.215 (0.075–0.620)	0.004

Continuous variables are quoted as medians (ranges)

CI confidence interval, AFP alpha-fetoprotein, AFP-L3 *Lens culinaris* agglutinin-reactive fraction of AFP, DCP des-γ-carboxy prothrombin, ALT alanine aminotransferase, AST aspartate aminotransferase, LDH lactate dehydrogenase, ALP alkaline phosphatase, IFN interferon, SVR sustained virologic response

14.8% in group D1; 1.8, 4.3, and 16.3% in group D2; and 10.0, 25.0, and 48.2% in group D3, respectively, and the cumulative incidence of HCC differed significantly

between groups D1 and D3 and groups D2 and D3 (Fig. 5).

Factors associated with the incidence of hepatic carcinogenesis on multivariate analysis

Factors associated with the incidence of HCC as analyzed by the Cox proportional hazards model and the forward selection method are listed in Table 3. Age >65 years, low platelet count, high AST level, high AFP level, and high AFP-L3% level were significantly associated with the incidence of HCC. Factors associated with the incidence of HCC were analyzed in patients with chronic hepatitis and cirrhosis (Table 4). High age, low platelet count, high AST level, and high AFP level were significantly associated with the incidence of HCC in chronic hepatitis, and male sex, high age, low platelet count, high AFP level, and high AFP-L3% level were significantly associated with the incidence of HCC in cirrhosis. Factors associated with the incidence of HCC were analyzed in patients with and without IFN treatment (Table 5). Male sex, low platelet count, low cholinesterase level, and high AFP level were significantly associated with the incidence of HCC in patients with IFN therapy and male sex, high age, low platelet count, high AFP level, and high AFP-L3% level were significantly associated with the incidence of HCC in patients without IFN therapy.

Discussion

Advances in US, CT, and MRI have allowed for the more frequent and earlier detection of small HCC tumors less than 2 cm in diameter during the routine follow-up of patients with chronic liver disease [21–23]. However, the performance and resolution of the imaging device, the skills of individual operators, and the diagnostic acumen of the interpreting radiologist all affect the early detection of HCC. AFP, AFP-L3%, and DCP levels have been used as prognostic markers rather than diagnostic markers for HCC [9]. However, the detection rate of small HCC tumors with these markers is low; AFP-L3% and DCP have low sensitivity, and AFP has low specificity. Sassa et al. [12] reported detection rates of 22.6 and 48.4% for AFP-L3% and DCP, respectively, in patients with small HCC tumors. It is currently thought that serum markers are useful for follow-up after HCC therapy in patients with high tumor marker levels before treatment [24].

We have previously reported that the average integration value of ALT correlates with the cumulative incidence of hepatocarcinogenesis, even within the normal range [13, 14]. In the present study, the average integration value of AFP was not selected as a factor associated with the

Fig. 3 Incidence of HCC according to the average integration value of AFP. The cumulative incidence of HCC differed significantly between groups A1 (<10 ng/mL) and A2 (≥ 10 , <20 ng/mL) and groups A1 and A3 (≥ 20 ng/L)

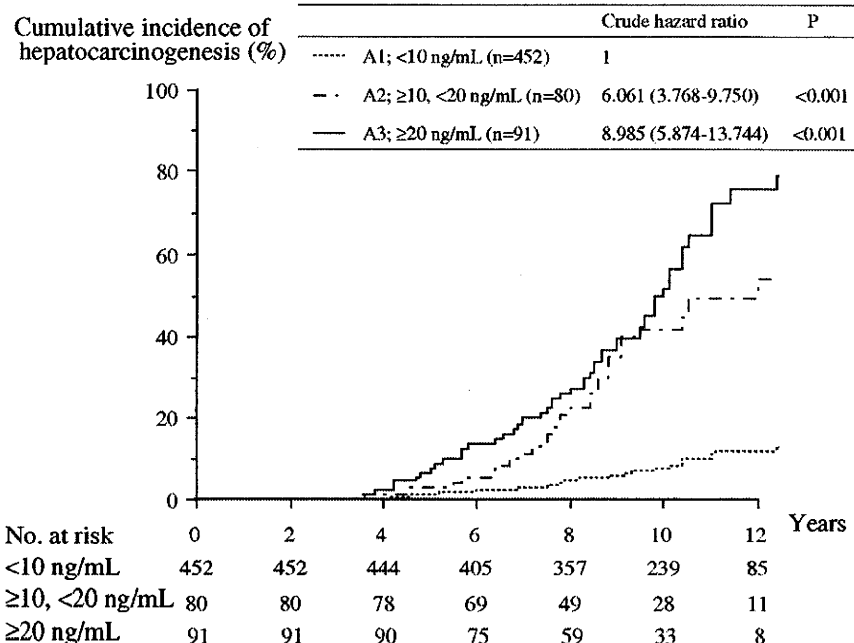
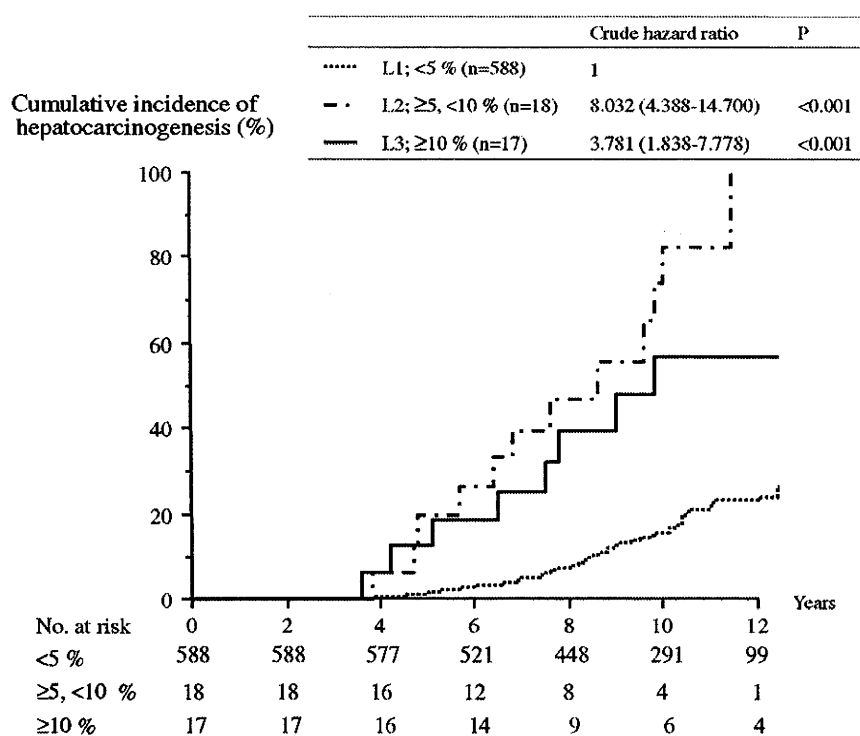


Fig. 4 Incidence of HCC according to the average integration value of AFP-L3%. The cumulative incidence of HCC differed significantly between groups L1 (<5%) and L2 (≥ 5 , <10%) and groups L1 and L3 (≥ 10 %)



incidence of HCC on multivariate analysis. AFP production is thought to be increased in response to injury, possibly due to increased hepatocyte turnover, in patients with HCV who do not have HCC [25]. In contrast, increased ALT levels are correlated with hepatocellular necrosis but not with hepatocyte proliferation. This difference may at

least partially explain the absence of correlation between ALT and AFP levels.

The multivariate analysis in our series was carried out to minimize the influence of confounding factors, and 5 factors were selected by the forward selection method. Age >65 years, low platelet count, high AST value, high AFP

Fig. 5 Incidence of HCC according to the average integration value of DCP. The cumulative incidence of HCC differed significantly between groups D1 (<20 mAU/mL) and D3 (≥40 mAU/mL) and groups D2 (≥20, <40 mAU/mL) and D3

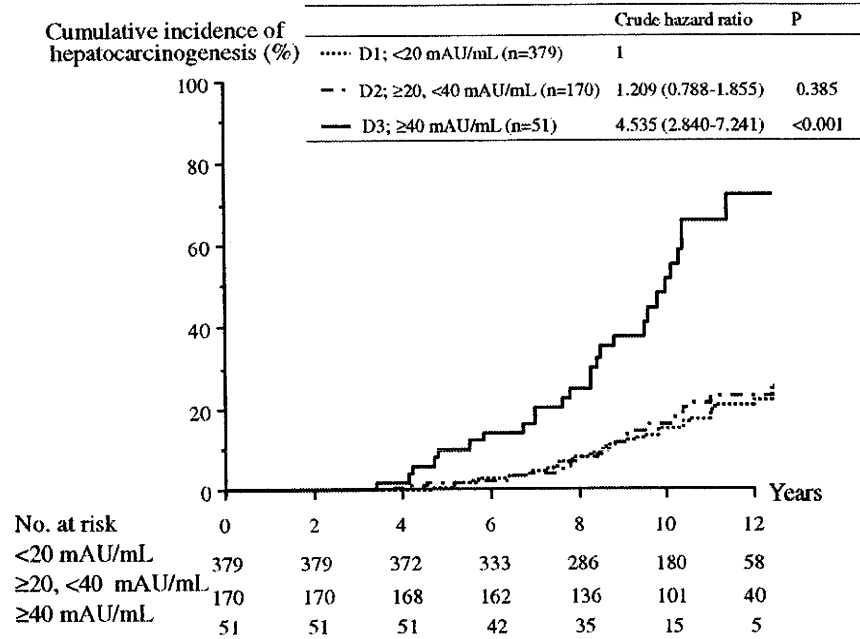


Table 3 Factors associated with hepatocarcinogenesis (multivariate analysis)

	Adjusted hazard ratio (95% CI)	P
Age (years)		
≤65	1	
>65	2.303 (1.551–3.418)	<0.001
Platelets (×10 ⁴ /mm ³)		
≥12.0	1	
<12.0	3.086 (1.997–4.768)	<0.001
AST (IU/L)		
≤40	1	
>40	3.001 (1.373–6.562)	0.006
AFP (ng/mL)		
A1; <10	1	
A2; ≥10, <20	2.814 (1.686–4.697)	<0.001
A3; ≥20	3.405 (2.087–5.557)	<0.001
AFP-L3 (%)		
L1; <5	1	
L2; ≥5, <10	2.494 (1.291–4.816)	0.007
L3; ≥10	3.555 (1.609–7.858)	0.002

AST aspartate aminotransferase, AFP alpha-fetoprotein, AFP-L3 *Lens culinaris* agglutinin-reactive fraction of AFP

level, and high AFP-L3% level were significantly associated with hepatic carcinogenesis in our multivariate analysis, but serum ALT level was not a risk factor for developing HCC. Ikeda et al. [26] reported that the cumulative incidence of HCC increased significantly in cirrhotic patients with an AFP level ≥10 ng/mL compared to those with an AFP level

<10 ng/mL, and the adjusted risk ratio was 15.788 in HCV patients. They speculated that AFP is a marker of disease activity or severity and cellular regeneration, and it acts as a better predictor of HCC with viral etiology of cirrhosis. As an index of hepatic regeneration, the AFP level better represents the risk of hepatic carcinogenesis than an index of liver injury (e.g., ALT level). In addition to AFP, AFP-L3% was identified as a factor predicting the development of HCC, and this is a specific marker for the existence of HCC. Therefore, elevations in AFP-L3% may reflect an occult cancer that is undetectable with current imaging modalities. More intensive surveillance is needed for patients such as those who fulfill the criteria of groups L2 and L3 in our series, although these groups were very small in size. However, similar to other laboratory values, as high AFP-L3% values may be associated with severe liver damage, it is necessary to interpret these values carefully. DCP is well known to be also a specific marker of HCC. DCP is more closely related to tumor size than AFP and AFP-L3% [27]. Therefore, it is thought that these were the reasons that DCP was not selected as a predictive marker for HCC in our multivariate analysis.

Among the other risk factors we identified for the development of HCC, a low platelet count stands out. The platelet count is a useful marker for the diagnosis of cirrhosis [28], and cirrhosis is an established risk factor for HCC in HCV carriers [26, 28–30]. Taken together with our other findings, the low platelet count suggests that HCC develops in patients with progressive or advanced liver disease. We additionally used ultrasound (US) to distinguish cirrhotic patients from non-cirrhotic patients [16–18]. The presence of cirrhosis on US was strongly associated with an increased

Table 4 Factors associated with hepatocarcinogenesis on multivariate analysis in patients with chronic hepatitis and cirrhosis

	Chronic hepatitis (n = 463)	Cirrhosis (n = 160)
Age (years): ≤ 65 vs. > 65	<0.001	0.008
Gender: female vs. male		<0.001
Platelets ($\times 10^4/\text{mm}^3$): ≥ 12.0 vs. < 12	0.001	0.007
AST (IU/L): ≤ 40 vs. > 40	0.043	
AFP (ng/mL): < 10 vs. ≥ 10 , < 20 vs. ≥ 20	<0.001	0.003
AFP-L3 (%): < 5 vs. ≥ 5 , < 10 vs. ≥ 10		0.017

AST aspartate aminotransferase, AFP alpha-fetoprotein, AFP-L3 *Lens culinaris* agglutinin-reactive fraction of AFP

Table 5 Factors associated with hepatocarcinogenesis on multivariate analysis in patients with and without IFN treatment

	With IFN (n = 189)	Without IFN (n = 434)
Age (years): ≤ 65 vs. > 65		0.001
Gender: female vs. male	0.005	<0.001
Platelets ($\times 10^4/\text{mm}^3$): ≥ 12.0 vs. < 12.0	0.047	<0.001
Cholinesterase (IU/L): ≥ 431 vs. < 431	0.007	
AFP (ng/mL): < 10 vs. ≥ 10 , < 20 vs. ≥ 20	<0.001	<0.001
AFP-L3 (%): < 5 vs. ≥ 5 , < 10 vs. ≥ 10		<0.001

IFN interferon, AFP alpha-fetoprotein, AFP-L3 *Lens culinaris* agglutinin-reactive fraction of AFP

incidence of HCC on univariate analysis, but US-determined cirrhosis was not identified as a risk factor on multivariate analysis. Histologic assessment of fibrosis and cirrhosis was obtained in only 187 patients (30.0%), and patients with F4 fibrosis had a higher incidence of HCC in our univariate analysis. However, the population of patients with material available for histologic review was only one-third the size of the entire study population, and this small number may have negatively affected our ability to detect the predictive nature of fibrosis at all levels of severity. In contrast to serum ALT, serum AST levels were significantly associated with the incidence of HCC. AST levels are often abnormal in patients with cirrhosis when ALT values are in the normal range, and the AST/ALT ratio is frequently greater than 1 in cirrhotic patients [31]. Elevated AST activity is a surrogate marker for cirrhosis. Aging is associated with a number of events at the molecular, cellular, and physiological levels that influence carcinogenesis and subsequent cancer growth [32]. It has been hypothesized that an age-associated decrease in DNA repair [33] contributes to the development of HCC.

Recent reports have shown that AFP levels fall following the administration of IFN with or without ribavirin [34, 35]. IFN has been shown to have antiviral, anti-inflammatory, and anticancer activities [36]. One study demonstrated an

anticancer effect of IFN when this agent was given following intrahepatic recurrence after HCC resection [37], and in our study, previous treatment with IFN was a factor associated with a reduced incidence of HCC on univariate analysis. The median ages of our patients with and without IFN treatment were 53 years (range 28–71) and 65 years (range 26–84), respectively; the age in those receiving IFN was significantly lower than the age in the group without IFN ($P < 0.0001$). It is thought that age and IFN therapy are confounding factors because IFN therapy has better results in younger patients. Although IFN was not identified as a predictive factor on multivariate analysis, the possibility cannot be denied that IFN may play an important role in modulating AFP levels prior to the onset of HCC.

In conclusion, increased AFP or AFP-L3% levels were significantly associated with an increased incidence of HCC. Among HCV carriers, patients with ≥ 10 ng/mL AFP or patients with $\geq 5\%$ AFP-L3% are at very high risk for the development of HCC even if AFP is less than 20 ng/mL or AFP-L3% is less than 10%, which are the most commonly reported cutoff values. Intensive imaging modalities including US, CT, and MRI are recommended every 3–6 months for these patients.

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Conflict of interest There is no conflict of interest to disclose.

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mentioned in the recent editorial by McColl and Gillen (4). Howden and Kahrilas state that, "in summary, there is no clear clinical- or clinical trial-evidence of undue difficulty in reducing or discontinuing PPI treatment in GERD patients, apart from those with erosive esophagitis" (2). This is not correct as a placebo-controlled trial of discontinuation of PPIs in patients on long-term therapy was performed a few years ago (5). Most patients participating had gastroesophageal reflux disease (GERD) as the indication for the PPI and GERD patients had significantly more difficulties discontinuing PPI therapy as compared with patients with other indications (5). Exclusion criterion for participation was erosive esophagitis (5). In the articles mentioned above (2,3) there is no disagreement with the last paragraph of the editorial, that "PPI treatment remains an important, valuable and safe intervention for a multitude of patients with appropriate indications" (1). Finally, it is somewhat surprising that authors that are chosen to write the editorial of a study showing that PPI therapy can induce dyspeptic symptoms have strong and multiple conflicts of interest with the pharmaceutical companies producing PPIs (1).

CONFLICT OF INTEREST

Guarantor of the article: Einar Björnsson MD, PhD.

Financial support: None.

Potential competing interests: None.

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Withdrawing PPI Treatment

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To the Editor: Our editorial (1) sought to highlight both the strengths and limitations of the study by Niklasson *et al.* (2), of which Dr Björnsson was a co-author. We presume that Dr Björnsson would agree that the results of controlled studies should not be overinterpreted, which is precisely what we had observed with the previously reported study of Reimer *et al.* (3) and what we caution of here. We do not dispute that some (although by no means all) investigators have shown rebound acid hypersecretion following PPI withdrawal and that such an effect is biologically plausible. Rather, our concern regards the clinical relevance of this phenomenon. Furthermore, although the observations of Reimer *et al.* (3) and Niklasson *et al.* (2) might be explained on the basis of rebound acid hypersecretion, readers should understand that neither study actually measured this phenomenon.

Regarding our stated conflicts of interest, readers are free to make of them what they choose. We welcome and adhere to the Journal's policy of making a declaration of all relevant financial relationships mandatory ("strong" and otherwise); this helps to ensure transparency and objectivity. However, a thoughtful reading of our editorial would conclude that we advocate minimizing and withdrawing PPI treatment whenever appropriate, hardly a viewpoint steeped in bias. To re-state our main argument, this is generally easily accomplished in clinical practice.

CONFLICT OF INTEREST

Dr Howden has been a consultant for Takeda Pharmaceuticals North America, Takeda Global Research and Development, Santarus, XenoPort, Schering-Plough

Healthcare, Novartis Consumer Health, Novartis Oncology, Procter & Gamble, Eisai, Otsuka, and Boehringer Ingelheim. He has received speaking honoraria from Takeda Pharmaceuticals North America, Otsuka, and Novartis. He has received research support for an investigator-initiated project from AstraZeneca. Dr Kahrilas has been a consultant for AstraZeneca, Xenoport, ARYx Therapeutics, Eisai, EndoGastric Solutions, Novartis, Movetis, and Revaliesio. He has received research support for investigator-initiated studies from the National Institutes of Health and Reckitt Benckiser Group plc.

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Double-Contrast Ultrasound: A Novel Surveillance Tool for Hepatocellular Carcinoma

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This letter underwent AJG editorial review.

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To the Editor: Hepatocellular carcinoma (HCC) is the third most common cause of

Table 1. Results of surveillance by double contrast US: B-mode US vs. double contrast US

No.	Sex	Age	Virus	Location	Size (mm)	B-mode US	CEUS Kupffer phase	Double-contrast US	Pathological diagnosis
1	M	64	HCV	S6	6×6	Not detected	Defect	Positive	HCC
2	M	53	HCV	S8	7×7	Not detected	Defect	Positive	HCC
3	M	76	HCV	S6	8×8	Not detected	Defect	Positive	HCC
4	F	72	HCV	S7	8×7	Not detected	Defect	Positive	HCC
5	M	68	HBV	S5	8×8	Not detected	Defect	Positive	HCC
6	M	72	HCV	S2	9×8	Not detected	Defect	Positive	HCC
7	M	71	HCV	S3	10×9	Not detected	Defect	Positive	HCC
8	M	70	HBV	S8	10×10	Not detected	Defect	Positive	HCC
9	M	68	HCV	S2	10×7	Not detected	Defect	Positive	HCC
10	F	75	HCV	S6	11×11	Not detected	Defect	Positive	HCC
11	M	67	HCV	S6	11×10	Not detected	Defect	Positive	HCC
12	M	73	HBV	S7	12×11	Not detected	Defect	Positive	HCC
13	M	74	HCV	S5	12×11	Not detected	Defect	Positive	HCC
14	F	69	HCV	S2	12×10	Not detected	Defect	Positive	HCC
15	M	70	HCV	S6	12×11	Not detected	Defect	Positive	HCC
16	M	76	HCV	S8	13×12	Not detected	Defect	Positive	HCC

CEUS, contrast-enhanced US; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; US, ultrasound.

cancer death worldwide. Practice guidelines in the West (1) and East (2) recommend ultrasound (US) surveillance as a first-line test. However, despite the performance of periodic surveillance, some HCCs are still detected at advanced stages because of the coarse liver parenchyma. Furthermore, even HCCs detected at early stages, such as single nodular HCCs smaller than 3 cm, still show high annual recurrence rates (15–20%) after resection or ablation (3). These phenomena are attributed to the tumor biology of HCCs, which frequently metastasize via the portal vein even when they are less than 2 cm (4). Detection of much smaller HCC nodules that do not yet have microsatellites or vascular invasion is an urgent clinical need.

In 2007, Sonazoid, a second-generation US contrast agent, was approved for routine clinical use in Japan. The most important property of this agent is that it allows very stable Kupffer phase imaging for at least 60 min, which is tolerable for multiple scanning in addition to real-time imaging. From December 2007 to November 2009, Kupffer phase surveillance was performed for 292 consecutive patients with hepatitis B- or C-related cirrhosis, who are at very

high risk for HCC. At the outpatient clinic, 0.01 ml/kg of Sonazoid was injected, followed by entire liver scanning at the Kupffer phase. Among the 292 patients, 27 Kupffer defects that were not detected by B-mode US were detected by Kupffer phase surveillance. Of these defects, 16 hypervascular nodules (5.5%) were confirmed as HCC by re-injecting Sonazoid at the Kupffer phase (double-contrast US) (5). All 16 nodules were proven to be HCC histologically, with a size range of 6–13 mm (Table 1). After resection ($n=2$) or radiofrequency ablation ($n=14$), none of these nodules showed local recurrence or intrahepatic recurrence during a median follow-up period of 2.3 years. Only one HCC nodule located at the subphrenic region was missed during detection by double-contrast US. The sensitivity of detecting B-mode US-undetectable hypervascular HCC was 94% using double-contrast US.

In conclusion, Kupffer phase surveillance of the cirrhotic liver followed by re-injection of Sonazoid (double-contrast US) is a novel technique in the surveillance program for detecting small hypervascular HCCs that are in a completely curable state. Based on these findings, a prospective

randomized phase III multicenter controlled trial comparing B-mode and double-contrast US surveillance for virus-related cirrhotic patients is now ongoing (<http://www.clinicaltrials.com>; NCT 00822991).

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Incidence Reduction Following Colonoscopic Polypectomy

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To the Editor: In Dr Sandler's editorial (1) in which he reviewed the current controversy in screening colonoscopy, he stated that the National Polyp Study (NPS) finding that colonoscopic polypectomy reduces colorectal cancer (CRC) incidence has not been replicated (2). This is an inaccurate statement. An incidence and mortality reduction similar to that observed by NPS was replicated in two other studies of post polypectomy patients that showed a 67% incidence reduction and an 88% mortality reduction, respectively (3,4). The studies that he cited as having a similar design to the NPS in fact had different designs with respect to the initial colonoscopy that identified the adenoma patients. In the NPS, all patients referred to participating clinical centers for initial colonoscopy prospectively had a protocol colonoscopy that reached the cecum, all polyps detected were removed, and all colonoscopies were performed by experienced endoscopy investigators. Those patients identified as having adenomas at this initial examination were eligible for the NPS. The studies cited by Sandler (1) had adenomas identified from community-based practices and then, 1 year later, had a clearing colonoscopy performed by experienced endoscopy investigators. Interval cancers attributable to missed lesions are not uncommon in community-based practice (5). When the missed cancers of the first non-protocol colonoscopy were excluded, the post-polypectomy CRC rate dropped from 1.8 to 0.96 per 1000 person years of follow-up,

which is very similar to that of the NPS (0.6 per 1000). The CRC incidence reduction observed in the NPS compared with a simulated cohort of adenoma patients without their adenomas removed (90%) and compared with the general population Surveillance, Epidemiology and End Results rate (76%) was probably achieved as a result of the NPS design and methodology, which included rigorous baseline clearing with a 13% repeat for inadequate preparation.

There are three separate but related questions: first, does removal of adenomas reduce the incidence and mortality of CRC; second, what is the precise magnitude of this reduction; and third, what is the benefit of screening colonoscopy in the general population, of whom only a proportion have adenomas. The long-standing belief in the concept of the adenoma-carcinoma sequence and that its interruption reduces CRC incidence and mortality is supported by many studies, including the NPS (2–4,6). However, the precise magnitude of the colonoscopy effect in the general population has not been clearly established, and will not be established until completion 10 or 15 years hence of the European and American screening colonoscopy randomized controlled trials (RCTs). Data from the colonoscopy RCTs will also provide a comparison of the colonoscopy effect with the recently reported sigmoidoscopy effect (6). The NPS supports the importance of finding and removing adenomas with any screening method in addition to detecting early-stage cancers. The best method to do this needs to be established.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Appropriate Response to Influenza A (H1N1) Virus Vaccination in Patients With Inflammatory Bowel Disease on Maintenance Immunomodulator and/or Biological Therapy

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To the Editor: In April 2009 an outbreak of the novel influenza A (H1N1) virus infection occurred in Mexico and has assumed pandemic proportions soon. After initial controversial data, vaccines directed toward the influenza A (H1N1) virus have proven to be safe and efficient to prevent the complications of the infection.

Patients with inflammatory bowel diseases (IBD)—Crohn's disease (CD), ulcerative colitis) on immunosuppressive therapy are at increased risk for various infections, some of which can be prevented by immunization. Inactivated influenza vaccination

High ability to predict the treatment outcome of peginterferon and ribavirin combination therapy based on the reduction in HCV RNA levels at 4 weeks after starting therapy and amino acid substitutions in the hepatitis C virus in patients infected with HCV genotype 1b

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Abstract

Background The ability to predict the outcome of peginterferon (PEG-IFN) and ribavirin combination therapy based on the reduction in hepatitis C virus (HCV) RNA levels at 4 weeks after starting the therapy and amino acid substitutions in HCV was to be confirmed.

Methods We measured the reduction in HCV RNA levels at 4 weeks after starting the combination therapy, as well as examining amino acid substitutions at residue 70 in the HCV core and within the interferon sensitivity-determining region (ISDR) of HCV non-structural protein 5A (NS5A), for 101 patients infected with HCV genotype 1b. The ability of these factors to predict a sustained virologic response (SVR) was analyzed.

Results When a 3 log₁₀ reduction in HCV RNA levels at 4 weeks after starting therapy was set as the cut-off value, an SVR was achieved in 37 of the 46 patients (80.4%) with a ≥ 3 log₁₀ decrease and in 4 of the 55 patients (7.3%) with a < 3 log₁₀ decrease. All 4 patients who achieved an SVR despite a < 3 log₁₀ reduction in HCV RNA levels at 4 weeks had an arginine at residue 70 in the HCV core and a non-wild-type sequence for the ISDR of HCV NS5A.

Conclusion A ≥ 3 log₁₀ reduction in HCV RNA levels at 4 weeks after starting therapy indicates that a patient has a high likelihood of achieving an SVR as a final outcome. Additional information on the amino acid substitutions at

residue 70 in the HCV core and within NS5A-ISDR will further increase the ability to predict a clinical response.

Keywords Chronic hepatitis C · Four weeks · Peginterferon · Reduction in HCV RNA · Response-guided therapy · Ribavirin

Introduction

The current standard antiviral therapy for patients with chronic hepatitis C is combination therapy with peginterferon (PEG-IFN) and ribavirin [1]. This treatment regimen has markedly increased the rate of patients with a sustained virologic response (SVR), which indicates the eradication of hepatitis C virus (HCV). However, only approximately 50% of patients infected with HCV genotype 1 achieved an SVR.

Many investigators have studied the baseline virologic factors that predict the treatment outcome of PEG-IFN and ribavirin combination therapy in patients infected with HCV genotype 1 [2]. These factors include the amino acid substitutions in the core [3, 4], and envelope 1 (E1) [4], E2 [5–7], non-structural protein 4B (NS4B) [8], and NS5A regions of HCV [6, 7, 9–11]. Especially, strong associations between amino acid substitutions at residue 70 of the core region of HCV and the amino acid sequence of residues 2209–2248 of the NS5A region of HCV (i.e., the interferon sensitivity-determining region, ISDR) were reported in Japanese patients infected with HCV genotype 1b [12].

In addition to the baseline virologic characteristics, the response of HCV during combination therapy, i.e., the change in serum HCV RNA levels after starting therapy,

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has been shown to be an important predictor of the treatment outcome [13–16], and this has led to increased emphasis on “response-guided therapy” [16, 17]. An early virologic response (EVR), defined as either undetectable serum HCV RNA or HCV RNA levels decreased by >2.0 \log_{10} from the pretreatment level at 12 weeks after starting therapy, has reportedly been the most important predictor of an SVR in patients infected with HCV genotype 1 [18, 19]. Therefore, an EVR is a pivotal criterion for decision-making in treatment guidelines [1].

However, to obtain the information on EVR, patients should undergo 12 weeks treatment. There are many adverse effects associated with PEG-IFN and ribavirin antiviral therapy, and the treatment course is costly. For these reasons, it is important to predict, with high reliability, the likelihood that a patient will achieve an SVR to PEG-IFN and ribavirin combination therapy as early as possible in order to prevent unnecessary treatment. More recent reports have emphasized the importance of a rapid virologic response (RVR), in which serum HCV RNA levels are undetectable at 4 weeks after starting therapy, for predicting an SVR [20–23]. In the present study, we determined the predictive value of decreased serum HCV RNA levels at 4 weeks after starting therapy compared to that of an EVR at 12 weeks. In addition, we determined the predictive value of pretreatment information on the amino acid sequences of the HCV core and NS5A regions in combination with the decrease in HCV RNA levels at 4 weeks after starting therapy.

Patients and methods

Patients and treatment

Between January 2007 and June 2008, a total of 189 patients with chronic hepatitis C received antiviral combination therapy with PEG-IFN and ribavirin for HCV infection at Ogaki Municipal Hospital. Among these patients, 101 were infected with HCV genotype 1b and had pretreatment HCV RNA levels of >5.0 \log_{10} IU/mL, based on a quantitative real-time polymerase chain reaction (PCR)-based method for HCV (HCV COBAS AmpliPrep/COBAS TaqMan System; Roche Molecular Systems, Pleasanton, CA, USA; lower limit of quantification, 1.7 \log_{10} IU/mL; lower limit of detection, 1.0 \log_{10} IU/mL) [24, 25]. This study did not include any patients infected with HCV genotype 1a because this genotype is not found in the general Japanese population.

All patients were given PEG-IFN alpha-2b (Pegintron; Schering-Plough, Tokyo, Japan) weekly and ribavirin (Rebetol; Schering-Plough) daily. The PEG-IFN and ribavirin doses were adjusted based on the patient's body

weight. Patients weighing ≤ 45 kg were given 60 μ g of PEG-IFN alpha-2b once a week, those weighing >45 and ≤ 60 kg were given 80 μ g, those weighing >60 and ≤ 75 kg were given 100 μ g, those weighing >75 and ≤ 90 kg were given 120 μ g, and those weighing >90 kg were given 150 μ g. Patients weighing ≤ 60 kg were given 600 mg of ribavirin per day, those weighing >60 and ≤ 80 kg were given 800 mg per day, and those weighing >80 kg were given 1000 mg per day. Dose modifications of PEG-IFN or ribavirin were based on the manufacturer's recommendations. All patients were scheduled to undergo 48 weeks of treatment. Some patients had an extended treatment duration of up to 72 weeks. In some patients, treatment was discontinued before 48 weeks because they had a low likelihood of achieving an SVR.

An SVR was defined as undetectable serum HCV RNA at 24 weeks after ending the therapy. A patient was considered to have relapsed when serum HCV RNA levels were detectable between the end of treatment and 24 weeks after completing the treatment, even if serum HCV RNA levels had been undetectable during and at the end of therapy. A non-response was defined as detectable serum HCV RNA at 24 weeks after beginning therapy (i.e., null response or partial non-response according to the American guidelines [1]). Patients were considered to have an RVR if they had undetectable serum HCV RNA at 4 weeks after starting therapy. An EVR was defined as the disappearance of or decrease in serum HCV RNA levels by at least 2 \log_{10} at 12 weeks after starting therapy. Patients were considered to have a complete EVR if the serum HCV RNA levels were undetectable at 12 weeks after starting therapy and a partial EVR if the serum HCV RNA levels had decreased by at least 2 \log_{10} at 12 weeks after beginning therapy. A non-EVR was defined as a lack of decrease by more than 2 \log_{10} at 12 weeks compared to the pretreatment levels. Patients were considered to have a slow virologic response if the serum HCV RNA levels became undetectable between 12 and 24 weeks.

The study protocol was in compliance with the Helsinki Declaration and was approved by the hospital ethics committee. Prior to initiating the study, written informed consent was obtained from each patient to use their laboratory data and analyze stored serum samples.

Assessments of serum HCV RNA levels, amino acid substitution at residue 70 in the HCV core, and amino acid sequence of HCV NS5A-ISDR

After a patient had provided informed consent, serum samples were obtained at the patient's regular hospital visits, just prior to beginning treatment, every 4 weeks during the treatment period, and during the 24-week follow-up period after treatment. Serum samples were stored

at -80°C until future use. The HCV RNA levels were measured using a quantitative real-time PCR-based method for HCV (HCV COBAS AmpliPrep/COBAS TaqMan System; Roche Molecular Systems) [24, 25].

The amino acid at residue 70 of the core region of HCV and the amino acid sequences of residues 2209–2248 of the NS5A region of HCV (ISDR) were analyzed by direct nucleotide sequencing of each region based on previous reports [3, 9]. The following PCR primer pairs were used for direct sequencing of the HCV core region:

5'-GCCATAGTGGTCTGCGGAAC-3' (outer, sense primer),
 5'-GGAGCAGTCCTTCGTGACATG-3' (outer, antisense primer),
 5'-GCTAGCCGAGTAGTGTT-3' (inner, sense primer), and
 5'-GGAGCAGTCCTTCGTGACATG-3' (inner, antisense primer).

The following PCR primers were used for direct sequencing of HCV NS5A-ISDR:

5'-TTCCACTACGTGACGGGCAT-3' (outer, sense primer),
 5'-CCCGTCCATGTGTAGGACAT-3' (outer, antisense primer),
 5'-GGGTCACAGCTCCCTGTGAGCC-3' (inner, sense primer), and
 5'-GAGGGTTGTAATCCGGGCGTGC-3' (inner, antisense primer).

When evaluating HCV-ISDR, HCV was defined as wild-type when there were 0 or 1 amino acid substitutions in residues 2209–2248 of the NS5A region compared with the HCV-J strain [26], and as non-wild-type when the number of substitutions was >1 .

Statistical analyses

Quantitative values are reported as means \pm SD. Between-group differences were analyzed by the χ^2 test. Univariate and multivariate analyses using a logistic regression model were performed to identify factors that predicted an SVR, including age, sex, body weight, serum alanine aminotransferase activity, serum aspartate aminotransferase activity, serum gamma-glutamyl transpeptidase levels, serum alkaline phosphatase values, serum albumin levels, total serum bilirubin values, white blood cell counts, hemoglobin, platelet counts, hepatitis activity grade (A0 and A1 vs. A2 and A3), liver fibrosis grade (F0 and F1 vs. F2 and F3), pretreatment HCV RNA levels, reduction in HCV RNA levels at 4 weeks after starting therapy ($\geq 3 \log_{10}$ vs. $< 3 \log_{10}$), amino acid substitution at residue 70 in the HCV core (arginine vs. glutamine), and the amino acid

sequence of HCV NS5A-ISDR (non-wild-type vs. wild-type). All *P* values were two-tailed, and *P* < 0.05 was considered statistically significant.

Results

The characteristics of the patients examined in this study are shown in Table 1. The patients consisted of 49 males (48.5%) and 52 females (51.5%) with a mean age of 58.7 ± 9.1 years. Among the 95 patients who underwent a pretreatment liver biopsy, the grade of liver fibrosis according to the METAVIR score [27] was F0 in 3 patients (3.1%), F1 in 57 patients (60.0%), F2 in 24 patients (25.3%), and F3 in 11 patients (11.6%). Although 28 patients (27.7%) had a reduction in the PEG-IFN dose and 50 patients (49.5%) had a reduction in the ribavirin dose during therapy, all patients except for those who discontinued the therapy had more than 80% adherence to both the PEG-IFN and ribavirin regimens. No patients discontinued the therapy because of adverse effects. As the final outcome, 41 patients

Table 1 Characteristics of all study patients

	All patients (<i>n</i> = 101)
Age (years)	58.7 ± 9.1
Sex (female/male)	52 (51.5)/49 (48.5)
History of interferon therapy (naive/retreatment)	68 (67.3)/33 (32.7)
Body weight (kg)	59.1 ± 10.4
Alanine aminotransferase (IU/L)	64.3 ± 64.0
Aspartate aminotransferase (IU/L)	54.6 ± 42.6
Gamma-glutamyl transpeptidase (IU)	54.3 ± 54.7
Alkaline phosphatase (IU/L)	263.2 ± 82.0
Albumin (g/dL)	4.11 ± 0.36
Total bilirubin (mg/dL)	0.67 ± 0.26
White blood cell count (/ μL)	5152 ± 1212
Hemoglobin (g/dL)	14.1 ± 1.3
Platelet count ($\times 10^3/\mu\text{L}$)	166 ± 50
Liver histology-activity (A0/A1/A2/A3) ^a	2 (2.1)/50 (52.6)/55 (36.9)/8 (8.4)
Liver histology-fibrosis (F0/F1/F2/F3) ^a	3 (3.1)/57 (60.0)/24 (25.3)/11 (11.6)
Pretreatment HCV RNA level (\log_{10} IU/mL)	6.21 ± 0.55
Reduction in the peginterferon dose	28 (27.7)
Reduction in the ribavirin dose	50 (49.5)
Final outcomes (SVR/relapse/NR)	41 (40.6)/31 (30.7)/29 (28.7)

HCV hepatitis C virus, SVR sustained virologic response, NR no response

Percentages are shown in parentheses

^a Liver biopsy was not performed in 6 patients

(40.6%) achieved an SVR, 31 patients (30.7%) relapsed, and the remaining 29 patients (28.7%) had a non-response.

Reduction of serum HCV RNA levels at 4 weeks after starting therapy and treatment outcome

Serum HCV RNA became undetectable in 9 patients (8.9%) at 4 weeks after starting therapy. In the remaining 92 patients, the decrease in serum HCV RNA levels at 4 weeks after starting therapy ranged from 0.15 log₁₀ to 5.14 log₁₀ (mean 2.61 log₁₀). The reduction in serum HCV RNA levels was ≥ 3 log₁₀ in 37 patients (36.6%), < 3 log₁₀ and ≥ 2 log₁₀ in 24 patients (23.8%), < 2 log₁₀ and ≥ 1 log₁₀ in 19 patients (18.8%), and < 1 log₁₀ in 12 patients (11.9%). Figure 1 shows the rates of SVRs according to the HCV RNA levels at 4 weeks after starting therapy. The rates were significantly higher in patients who achieved an RVR or had a decrease in serum HCV RNA levels of ≥ 3 log₁₀ at 4 weeks compared to those with a decrease in serum HCV RNA levels of < 3 log₁₀ ($p < 0.0001$). When a 3 log₁₀ decrease in serum HCV RNA levels was defined as the cut-off, 45.5% of patients were considered to have a ≥ 3 log₁₀ decrease in serum HCV RNA levels. The sensitivity, specificity, positive predictive value, and negative predictive value for an SVR were 90.2, 85.0, 80.4, and 92.7%, respectively.

Based on the univariate analysis, the factors that were associated with an SVR included serum albumin, platelet count, reduction in HCV RNA levels at 4 weeks after starting therapy, and amino acid substitutions at residue 70 in the HCV core (Table 2). In addition, serum gamma-glutamyl transpeptidase, liver fibrosis grade, and the amino

acid sequences of HCV NS5A-ISDR tended to affect an SVR. In the multivariate analysis, a reduction in HCV RNA levels at 4 weeks after starting the therapy and the HCV NS5A-ISDR amino acid sequences were independent factors that were significantly associated with an SVR. A reduction in HCV RNA levels at 4 weeks after starting therapy was the strongest factor that affected an SVR.

Association between reduction in serum HCV RNA levels at 4 weeks after starting therapy and an EVR at 12 weeks

Serum HCV RNA became undetectable in 42 patients at 12 weeks after starting therapy, and they achieved a complete EVR. In 47 patients, the serum HCV RNA levels did not become undetectable but decreased by ≥ 2 log₁₀ and these patients achieved a partial EVR. In the remaining 12 patients, the decrease in serum HCV RNA levels was < 2 log₁₀ and they had a non-EVR. The percentages of virologic responses at 12 weeks after starting therapy are shown in Fig. 2 in relation to the reduction in serum HCV RNA levels at 4 weeks. Serum HCV RNA remained undetectable at 12 weeks after starting therapy in all patients who had an RVR. More than 80% of patients whose serum HCV RNA levels decreased by ≥ 3 log₁₀ achieved a complete EVR, whereas more than 90% of patients with a decrease in serum HCV RNA levels at 4 weeks of < 3 log₁₀ and ≥ 2 log₁₀ or < 2 log₁₀ and ≥ 1 log₁₀ achieved a partial EVR. In contrast, more than 80% of the patients with a decrease in serum HCV RNA levels at 4 weeks of < 1 log₁₀ had a non-EVR. The sensitivity, specificity, positive predictive value, and negative predictive value of a complete EVR for an SVR were 85.4, 88.3, 83.3, and 89.8%, respectively. Serum HCV RNA became undetectable between 12 and 24 weeks in 28 of the 47 patients with a partial EVR and they had a slow virologic response. The treatment duration was extended from 48 to 72 weeks in 16 of these 28 patients (57.1%) who demonstrated a slow virologic response. Treatment was discontinued before 48 weeks in 5 of 12 patients (41.7%) with a non-EVR, because their serum HCV RNA levels remained detectable at 24 weeks after starting the therapy (non-response).

Amino acid substitutions in the HCV core and NS5A region

The amino acid at residue 70 in the core region of HCV was arginine in 63 patients and glutamine in 38 patients. The analysis of the amino acid sequence of the ISDR in the HCV NS5A region showed that 60 patients had the wild-type sequence and 41 patients had a non-wild-type sequence. The rates of patients with an RVR, complete

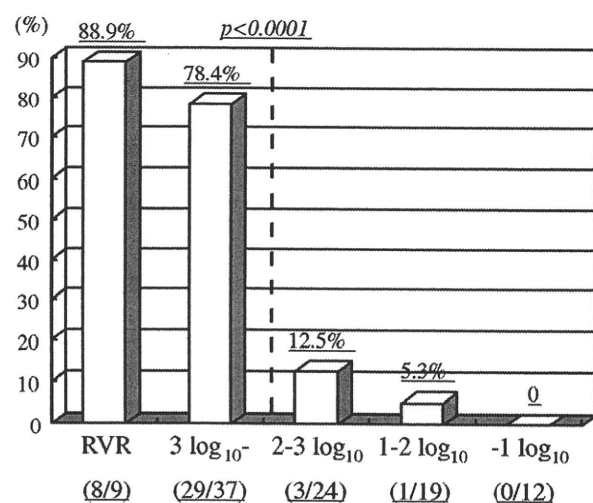


Fig. 1 The rates of sustained virologic responses based on the reduction in serum hepatitis C virus (HCV) RNA levels at 4 weeks after starting therapy. RVR Rapid virologic response

Table 2 Univariate and multivariate analyses of factors associated with a sustained virologic response to peginterferon and ribavirin combination therapy

	Univariate analysis	Multivariate analysis	Odds ratio (95% confidence interval)
Age (years)	0.2359	–	
Sex (female/male)	0.3932	–	
Body weight (kg)	0.4445	–	
Alanine aminotransferase (IU/L)	0.6398	–	
Aspartate aminotransferase (IU/L)	0.7663	–	
Gamma-glutamyl transpeptidase (IU)	0.0590	0.7415	
Alkaline phosphatase (IU/L)	0.1277	–	
Albumin (g/dL)	0.0017	0.1688	
Total bilirubin (mg/dL)	0.9611	–	
White blood cell count (/μL)	0.3019	–	
Hemoglobin (g/dL)	0.1967	–	
Platelet count ($\times 10^3/\mu\text{L}$)	0.0470	0.3076	
Liver histology-activity (A0-1/A2-3) ^a	0.3555	–	
Liver histology-fibrosis (F0-1/F2-3) ^a	0.0853	0.3414	
Pretreatment HCV RNA level ($\times 10^3$ IU/mL)	0.6442	–	
Reduction in the peginterferon dose	0.5279	–	
Reduction in the ribavirin dose	0.4444	–	
Reduction in HCV RNA level at 4 weeks after starting therapy ($\geq 3 \log_{10}$ vs. $< 3 \log_{10}$)	< 0.0001	< 0.0001	61.758 (14.259–423.13)
Amino acid at residue 70 in the HCV core (arginine vs. glutamine)	0.0008	0.1663	
Amino acid sequences of HCV NS5A-ISDR (non-wild-type vs. wild-type)	0.0741	0.0427	5.1375 (1.1959–31.367)

HCV hepatitis C virus, NS5A non-structural protein 5A, ISDR interferon sensitivity-determining region

^a Liver biopsy was not performed in 6 patients

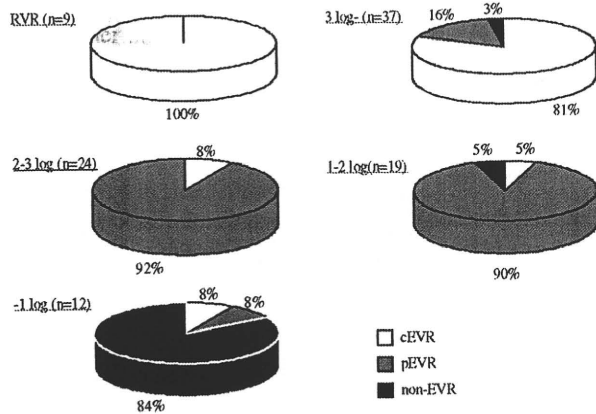


Fig. 2 The association between the virologic responses at 12 weeks after starting therapy and the reduction in serum HCV RNA levels at 4 weeks after starting therapy. RVR Rapid virologic response, cEVR complete early virologic response, pEVR partial early virologic response, non-EVR non-early virologic response

EVR, $\geq 3 \log_{10}$ decrease in HCV RNA level at 4 weeks after the starting therapy, and SVR, according to these amino acid substitutions, are shown in Table 3. An SVR was achieved in 34 of the 63 patients (54.0%) with arginine at residue 70 in the HCV core and in 7 of the 38 patients

(18.4%) with glutamine at this residue. The rate of achieving an SVR was significantly higher in patients with arginine at this residue ($P = 0.0009$). An SVR was achieved in 20 of the 60 patients (33.3%) with a wild-type HCV-ISDR and in 21 of the 41 patients (51.2%) with a non-wild-type HCV-ISDR. There were no differences in the rates of SVRs between these two patient groups. When we analyzed the association between a $\geq 3 \log_{10}$ decrease in HCV RNA level at 4 weeks after starting therapy and amino acid substitutions in the HCV core and NS5A region, 37 of the 63 patients (58.7%) with arginine at residue 70 in the HCV core achieved a $\geq 3 \log_{10}$ decrease. Nine of the 38 patients (23.7%) with glutamine at this residue achieved a $\geq 3 \log_{10}$ decrease, the rate being significantly different from that in the former group ($P = 0.0013$). A $\geq 3 \log_{10}$ decrease in HCV RNA level at 4 weeks was achieved in 26 of the 60 patients (43.3%) who had the wild-type sequence of the ISDR in the HCV NS5A region and a $\geq 3 \log_{10}$ decrease was achieved in 20 of the 41 patients (48.8%) who had a non-wild-type sequence. There were no differences in the rates of patients with a $\geq 3 \log_{10}$ decrease between these two patient groups.

The rates of an SVR based on both the amino acid substitutions at residue 70 of the HCV core and within the ISDR are shown in Table 4. In patients with a $\geq 3 \log_{10}$

Table 3 The rates of rapid virologic response, $\geq 3 \log_{10}$ decrease in HCV RNA level at 4 weeks after starting therapy, complete early virologic response, and sustained virologic response according to the amino acid substitutions at residue 70 in the HCV core region and HCV NS5A-ISDR

	Residue 70* in the HCV core		HCV NS5A-ISDR	
	Arginine	Glutamine	Non-wild-type	Wild-type
Rapid virologic response	6/63 (9.5)	3/38 (7.9)	5/41 (12.2)	4/60 (6.7)
$\geq 3 \log_{10}$ decrease in HCV RNA level at 4 weeks after starting therapy	37/63 (58.7)	9/38 (23.7)	20/41 (48.8)	26/60 (43.3)
Complete early virologic response	33/63 (52.7)	9/38 (23.7)	18/41 (43.9)	24/60 (40.0)
Sustained virologic response	34/63 (54.0)	7/38 (18.4)	21/41 (51.2)	20/60 (33.3)

*Arginine versus glutamine, $p = 0.0013$ at $\geq 3 \log_{10}$ decrease in HCV RNA level at 4 weeks after starting therapy; $p = 0.0086$ at complete early virologic response; $p = 0.0009$ at sustained virologic response

Percentages are shown in parentheses

HCV hepatitis C virus, NS5A non-structural protein 5A, ISDR interferon sensitivity-determining region

Table 4 Rates of sustained virologic response to peginterferon and ribavirin combination therapy based on amino acid substitutions at residue 70 in the HCV core and within HCV NS5A-ISDR in patients with a $\geq 3 \log_{10}$ or $< 3 \log_{10}$ reduction in HCV RNA levels at 4 weeks after starting therapy

Residue 70 in the HCV core	HCV NS5A-ISDR	Rate of SVR
(A) $\geq 3 \log_{10}$ reduction in serum HCV RNA level at 4 weeks after starting therapy ($n = 46$)		
Arginine	Non-wild-type	14/15 (93.3)
	Wild-type	16/22 (72.7)
Glutamine	Non-wild-type	3/5 (60.0)
	Wild-type	4/4 (100)
(B) $< 3 \log_{10}$ reduction in serum HCV RNA level at 4 weeks after starting therapy ($n = 55$)		
Arginine	Non-wild-type	4/11 (36.4)
	Wild-type	0/15
Glutamine	Non-wild-type	0/10
	Wild-type	0/19

Percentages are shown in parentheses

HCV hepatitis C virus, NS5A non-structural protein 5A, ISDR interferon sensitivity-determining region, SVR sustained virologic response

reduction in HCV RNA levels at 4 weeks after starting therapy, there was a high rate of an SVR regardless of the amino acid sequence at residue 70 in the HCV core and within the ISDR. In contrast, in patients with a $< 3 \log_{10}$ reduction in HCV RNA levels at 4 weeks after starting therapy, there was no SVR in those with glutamine at residue 70 of the HCV core or in those with arginine at residue 70 of the HCV core but a wild-type HCV-ISDR sequence. We detected an SVR only in patients with both arginine at residue 70 of the HCV core and the non-wild-type HCV-ISDR sequence.

We examined the characteristics of all 4 patients who had a $< 3 \log_{10}$ decrease in HCV RNA levels at 4 weeks after starting therapy and yet achieved an SVR (Table 5). All 4 patients were female and showed a partial EVR at 12 weeks after starting therapy. In all of these patients, the HCV RNA levels became undetectable by 24 weeks after starting therapy and 2 of the 4 patients had an extended

treatment duration of 72 weeks. In the amino acid sequence analyses of the HCV core and NS5A regions, all of these patients had arginine at residue 70 of the HCV core and all had a non-wild-type HCV-ISDR.

Discussion

Several previous studies have reported that patients infected with HCV who achieved an RVR, in which serum HCV RNA levels became undetectable at 4 weeks after starting PEG-IFN and ribavirin combination therapy, had a high likelihood of achieving an SVR [20–23]. More recent studies have suggested the possibility of shortening the treatment duration from 48 to 24 weeks in patients who had HCV genotype 1 but had a low pretreatment HCV RNA level and had achieved an RVR [28–33]. However, there is not a high prevalence of patients with an RVR

Table 5 Patients who achieved a sustained virologic response despite the absence of a ≥ 3 log₁₀ reduction in serum HCV RNA levels at 4 weeks after starting therapy

	Age (years)	Sex	Liver histology	Pretreatment HCV RNA level (log ₁₀ IU/mL)	Amino acid at residue 70 in the HCV core	NS5A-ISDR sequences	Reduction in HCV RNA level (log ₁₀ IU/mL) at 4 weeks	Response at 12 weeks	HCV RNA became undetectable (weeks)	Treatment duration (weeks)
1.	62	F	A3/F3	6.19	Arginine	Non-wild	2.19	Partial EVR	20	48
2.	31	F	A1/F1	6.13	Arginine	Non-wild	1.62	Partial EVR	16	48
3.	62	F	A1/F1	6.32	Arginine	Non-wild	2.41	Partial EVR	20	72
4.	59	F	A3/F3	6.21	Arginine	Non-wild	2.70	Partial EVR	24	72

HCV hepatitis C virus, NS5A non-structural protein 5A, ISDR interferon sensitivity-determining region, EVR early virologic response

among patients infected with HCV genotype 1 that is resistant to therapy. A considerable percentage of patients achieved an SVR even though they did not achieve an RVR. Therefore, an RVR has high specificity but low sensitivity as a predictive factor for an SVR. Several previous studies from Asia have evaluated the predictive value of an RVR and the degree of reduction in serum HCV RNA levels at 4 weeks after starting therapy [34, 35]. However, the number of patients in these studies was small and the analyses were not sufficient to form reliable conclusions.

In the present study, we evaluated the ability of the decrease in serum HCV RNA levels at 4 weeks after starting therapy to predict the likelihood of an SVR as a final outcome in Japanese patients infected with HCV genotype 1b. We used a highly sensitive real-time PCR-based quantification method to measure serum HCV RNA levels. The rate of patients with an RVR was 8.9%, which was lower than the rates reported in previous studies. This difference could have arisen because our study patients did not include HCV genotype 1b-infected patients with low pretreatment HCV RNA levels (<5.0 log₁₀). The Japan National Medical Insurance System does not allow patients with pretreatment HCV RNA levels of <5.0 log₁₀ to be treated with ribavirin in combination with PEG-IFN as an initial treatment. When our patients were stratified according to the reduction in serum HCV RNA levels at 4 weeks, there was a marked difference in the rate that patients achieved an SVR when the cut-off value for the decrease in serum HCV RNA levels was fixed at 3 log₁₀. An SVR was achieved in 78.4% of patients with a ≥ 3 log₁₀ decrease at 4 weeks, despite the fact that these patients did not achieve an RVR. The sensitivity, specificity, positive predictive value, and negative predictive value were comparable or even superior to those of a complete EVR that is evaluated at 12 weeks after starting therapy. In contrast to the low percentage of patients with an RVR, the percentage of patients with a ≥ 3 log₁₀ decrease at 4 weeks was much higher. Therefore, this cut-off value will be useful to identify patients with a high likelihood of achieving an SVR. A previous study by Deltenre et al. [36] suggested a

≥ 2 log₁₀ decrease at 4 weeks as the best predictor of an SVR in patients with HCV genotype 1 and with normal alanine aminotransferase, a finding that was not consistent with our results. The reason for this discrepancy is unclear and further studies, including consideration of ethnicity, will be needed to investigate this difference.

Only 4 of our 55 patients (7.3%) who had a <3 log₁₀ decrease in their serum HCV RNA levels at 4 weeks achieved an SVR as a final outcome. These patients had distinctive features in the HCV sequence. All 4 patients had arginine at residue 70 in the HCV core and all had a non-wild-type sequence for HCV-ISDR, both of which factors reportedly indicate a higher likelihood of achieving an SVR [3, 9]. Our results indicate that it is difficult to achieve an SVR in patients with a <3 log₁₀ reduction in HCV RNA levels at 4 weeks after starting therapy unless the patients have arginine at residue 70 in the HCV core and a non-wild-type ISDR sequence. According to the current American Association for the Study of the Liver Diseases (AASLD) guidelines, discontinuing therapy should be considered for patients whose serum HCV RNA remains detectable at 24 weeks after starting therapy, and discontinuing therapy can also be considered for patients with a non-EVR at 12 weeks after starting therapy. Based on these data, it may be possible to determine whether therapy should be discontinued at 4 weeks in patients in whom there is a potential for adverse effects.

There are several limitations of our study. Nearly half of the patients who demonstrated a slow virologic response did not have a treatment duration that was extended from 48 to 72 weeks. This is because the effectiveness of a 72-week combination therapy regimen for patients with HCV genotype 1 with a slow virologic response [37, 38] had not been established in Japan in the earlier part of the period when this study was conducted. In addition, the data were based on Japanese patients infected with HCV genotype 1b. Therefore, the results of our study should be confirmed in patients of other ethnicities and patients infected with HCV genotype 1a.

In conclusion, in addition to an RVR, a reduction in HCV RNA levels of $\geq 3 \log_{10}$ at 4 weeks after starting therapy indicates a high likelihood that patients will achieve an SVR as a final outcome of PEG-IFN and ribavirin combination therapy for HCV infection. In contrast, a reduction in HCV RNA levels of $< 3 \log_{10}$ at 4 weeks after starting therapy indicates that a patient has a very low likelihood of achieving an SVR. Patients with a reduction in HCV RNA levels of $< 3 \log_{10}$ at 4 weeks after starting therapy achieved an SVR only when they had both arginine at residue 70 in the HCV core and a non-wild-type HCV NS5A-ISDR sequence. The combination of the reduced HCV RNA levels at 4 weeks after starting therapy and substitutions at residue 70 in the HCV core and in NS5A-ISDR can further identify patients who are unlikely to achieve an SVR.

Conflict of interest None.

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Mutations in the core and NS5A region of hepatitis C virus genotype 1b and correlation with response to pegylated-interferon-alpha 2b and ribavirin combination therapy

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SUMMARY. Mutations in two regions of hepatitis C virus (HCV) have been implicated in influencing response to interferon (IFN) therapy. Substitutions in the NS5A region of HCV have been associated with response to IFN therapy, and this region has been known as the IFN sensitivity-determining region (ISDR). The mutations in the core region of HCV have also been reported to predict IFN response. The aim of this study was to investigate whether amino acid substitutions in the core region and ISDR among patients with HCV genotype 1b affect the response to IFN therapy. A total of 213 patients who completed IFN treatment were randomly selected. All patients received pegylated-IFN-alpha 2b once each week, plus oral ribavirin daily for 48 weeks. Of the 213 patients, 117 (54.9%) showed early virologic response (EVR), with HCV-negativity, at 12 weeks. Factors related to EVR on multivariate analysis were non-Gln70 and Leu91 in the core

region, and ISDR mutant-type. One hundred and two (47.9%) showed a sustained virologic response (SVR). SVR occurred more frequently in patients without Gln70 (55.4%) than in those with Gln70 (21.3%) ($P < 0.0001$). SVR was achieved in 43.6% of patients with wild-type ISDR and 62.5% of patients with mutant-type ($P = 0.0227$). Of the 34 patients who simultaneously had non-Gln70 and mutant-type ISDR, 26 (76.5%) achieved SVR. Factors related to SVR on multivariate analysis were non-Gln70 and ISDR mutant-type. In conclusion, amino acid substitutions in the core region and ISDR were useful for predicting the response to IFN in patients with HCV genotype 1b.

Keywords: core region, genotype 1b, hepatitis C virus, interferon sensitivity-determining region, interferon therapy, NS5A.

INTRODUCTION

Hepatitis C virus (HCV) is a member of the Flaviviridae family and causes chronic hepatitis that can develop into potentially fatal cirrhosis and hepatocellular carcinoma [1]. It has been estimated that 170 million people are infected with HCV worldwide. Therefore, HCV infection is a major global health problem. HCV consists of four structural proteins (core,

envelope 1, envelope 2 and p7) and six nonstructural proteins (NS2–NS5) [2]. HCV core protein was thought to inhibit the antiviral action of interferon (IFN) through down-regulation of transcription of IFN-induced antiviral genes [3,4]. The NS5A region includes the PKR-binding domain, which is associated with viral replication that is affected by IFN [5]. Thus, the core and NS5A regions of HCV appear to be important factors that may affect the response to IFN therapy, and mutations in the core and NS5A regions of HCV have been reported to affect response to IFN therapy [6–10]. The core region of HCV is well conserved, but substitutions of amino acid (aa) 70 and aa 91 are frequently found. Several studies reported a relation between these substitutions in the core region and IFN responsiveness [8,10]. The substitutions in the NS5A region of HCV have been closely associated with response to IFN therapy, and this region is known as the IFN sensitivity-determining region (ISDR) [6]. However, these

Abbreviations: Aa, amino acid; ALT, alanine aminotransferase; EVR, early virologic response; HCV, hepatitis C virus; IFN, interferon; ISDR, interferon sensitivity-determining region; SVR, sustained virologic response.

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