The aim of this study was to evaluate the clinical profile in HCV-positive patients with elevated serum KL-6 level.

Patients and Methods

Patients

Selected subjects consisted of 502 HCV-positive patients who fulfilled the following criteria: (1) positive HCV-RNA for more than 6 months; (2) no HBsAg, and HBc antibody in the serum (determined by radioimmunoassay); (3) outpatients of our hospital from March 20 to 27, 2001; (4) no underlying systemic disease such as systemic lupus erythematosus and rheumatic arthritis, and (5) patients provided informed consent to KL-6 level studies. The diagnosis of IPF was based on history, physical examination, chest roentgenogram, pulmonary function tests, chest high-resolution computed tomography (HRCT), according to the criteria of the American Thoracic Society [20]. Diagnosis of HCC was based on the presence of typical hypervascular characteristics on angiography, in addition to the findings on CT and ultrasonography. Microscopic examination of fine-needle biopsy material was performed in patients whose angiograms did not demonstrate a typical image of HCC. Histopathological confirmation using surgically resected specimens was made in 12 patients.

The serum samples of these 502 patients were collected and stored at –80°C until the enzyme-linked immunosorbent assay (ELISA) for KL-6 was done. The study was approved by the institutional review board of our hospital. The physicians in charge explained the purpose and method of this clinical trial to each patient.

The HCV genotype was classified by a PCR using a mixture of primers for the five subtypes known to exist in Japan, as reported by Chayama et al. [21]. Using these blood samples, HCV-RNA levels were analyzed at the same time by a branched DNA probe assay (b DNA probe assay, version 2.0, Chiron, Tokyo, Japan), and the results were expressed as mega-equivalents/ml (MEq/ml) [22].

Determination of KL-6 Concentration

The serum concentration of KL-6 antigen was measured by a sandwich-type ELISA using immobilized KL-6 antibody (Eitest® KL-6, Eisai, Tokyo, Japan) as described previously [23, 24]. Moreover, the cutoff value of KL-6 was defined as 500 U/ml.

Statistical Analysis

We used Fisher's exact test, univariate analysis and multivariate analysis (multiple logistic regression analysis) to establish which factors contributed to the elevated KL-6 level. Results for each variable were transformed into categorical data consisting of two simple ordinal numbers for univariate and multivariate analyses. Variables that achieved statistical significance ($p < 0.1$) in univariate analysis were subjected to multiple logistic regression to identify significant independent predictors. $p < 0.05$ was considered statistically significant. All data analyses were conducted using the Statistical Package for Social Sciences (SPSS for Windows, version 9.0). A p value <0.05 was considered to be statistically significant.

Table 1. Clinical characteristics of the 502 study patients

Characteristics	
Patients	502
Sex, male/female	306/196
Age[a], years	61 (12–92)
Posttransfusion, %	38.3
HCV genotype, % (1b/2a/2b/others)	66/23.3/9/1.7
HCV-RNA[a], MEq/ml	4.6 (<0.2–88)
AST[a], IU/l	38 (13–442)
ALT[a], IU/l	43 (7–560)
Total protein[a], g/dl	7.6 (5.8–9.5)
Platelets[a], $\times 10^4$/mm^3	14.3 (3.3–36.9)

[a] Medians and ranges.
ALT = Alanine aminotransferase; AST = aspartate aminotransferase.

Results

Clinical Background

Clinical profile characteristics of the 502 study participants are shown in table 1. Serum KL-6 levels ranged from 71 to 2,295 (median; 223) U/ml, and the distribution of serum KL-6 levels is depicted in figure 1. In 32 of the 502 (6.4%) patients, the KL-6 level exceeded 500 U/ml. Three of these 32 (9.4%) patients had a diagnosis of IPF.

Predictive Factors for Elevated Serum KL-6 Level

Next, we assessed the factors predicting KL-6 levels >500 U/ml. A logistic model was used for the analysis of nonlinear data referring to various factors potentially related to elevated KL-6 level surpassing 500 U/ml. The following factors were evaluated: sex, age, post-transfusion, HCV genotype, viral load, alanine and aspartate aminotransferases, platelet counts, HCC and IPF. Univariate analysis (table 2) disclosed two factors which were significantly associated with elevated serum KL-6 level: (1) a history of HCC ($p < 0.0001$), and (2) age >60 years ($p = 0.0052$). Because the variables were mutually correlated, multivariate analysis was performed using the four significant variables in the model. Multivariate analysis (table 3) revealed the same significant factors: (1) a history of HCC ($p = 0.0007$), and (2) age >60 years ($p = 0.0085$).

Clinical Profiles in Patients with Elevated KL-6

The differences in clinical profiles were compared between patients with normal KL-6 and patients with elevated KL-6 (table 4). Regarding IPF, all 3 patients with IPF had elevated serum KL-6 levels. The median age of

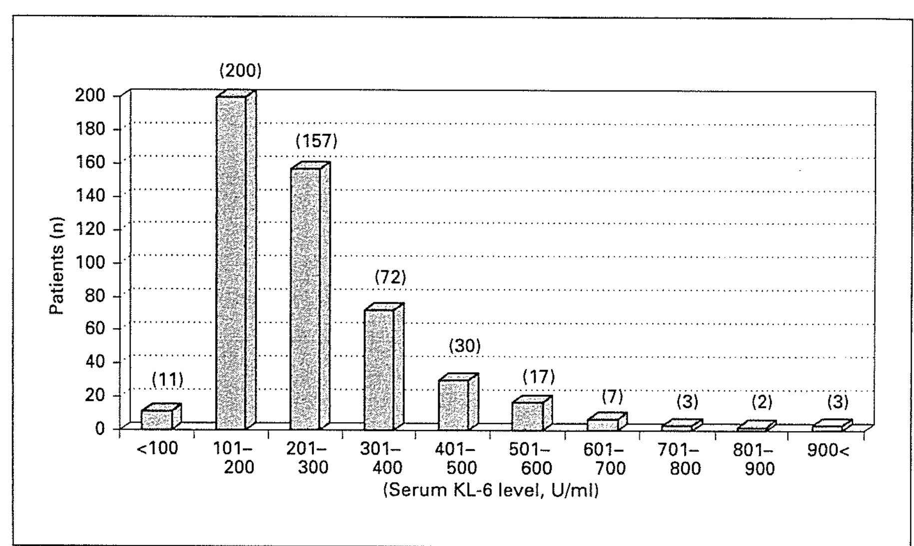

Fig. 1. Distribution of serum KL-6 levels in the patients with HCV.

Table 2. Factors associated with elevated KL-6 by univariate analysis

Factor	Category	Odds ratio	95% CI	p value
HCC	(–)/(+)	1/7.97	4.18–19.97	<0.0001
Age, years	<60/≥60	1/17.61	2.34–132.33	0.0053
Platelets, $\times 10^4$/mm^3	≥15/<15	1/2.94	0.60–14.49	0.182
AST, IU/l	≥38/<38	1/2.13	0.71–6.39	0.180
HCV genotype	2a, 2b/1b	1/2.00	0.22–18.12	0.538
ALT, IU/l	<50/≥50	1/1.58	0.49–5.00	0.450
Sex	female/male	1/1.45	0.60–5.23	0.104
HCV-RNA, MEq/ml	≥5/<5	1/1.41	0.31–6.43	0.655

CI = Confidence interval.

Table 3. Factors associated with elevated KL-6 by multivariate analysis

Factor	Category	Odds ratio	95% CI	p value
HCC	(–)/(+)	1/5.08	1.98–13.04	0.0007
Age, years	<60/≥60	1/15.37	2.01–117.66	0.0085

CI = Confidence interval.

Table 4. Difference in clinical profiles between patients with normal KL-6 and patients with elevated KL-6

Characteristics	Normal KL-6 group (<500 U/ml)	Elevated KL-6 group (≥500 U/ml)	p value
IPF (+/–)	0/470	3/29[a]	<0.0001
Median age, years (range)	60 (12–92)	70 (56–77)	0.0053
HCC (+/–)	37/431	12/20[a]	<0.0001

[a] Two patients had HCC and IPF.

the patients with elevated KL-6 exceeded that of the normal KL-6 patients by 10 years. The HCC rate was 37.5% (12/32) in the patients with elevated KL-6 and 8.3% (39/470) in the patients with normal KL-6. The median (range) age was 70 (56–77) years in the patients with elevated KL-6 and 60 (12–92) years in the patients with normal KL-6. The HCC rate of the patients with elevated KL-6 was significantly higher than that of the patients with normal KL-6. Twelve of 49 HCC patients demonstrated elevated KL-6.

Figure 2 depicts a flow chart to assess the clinical background in patients with elevated KL-6 level. Twenty-five

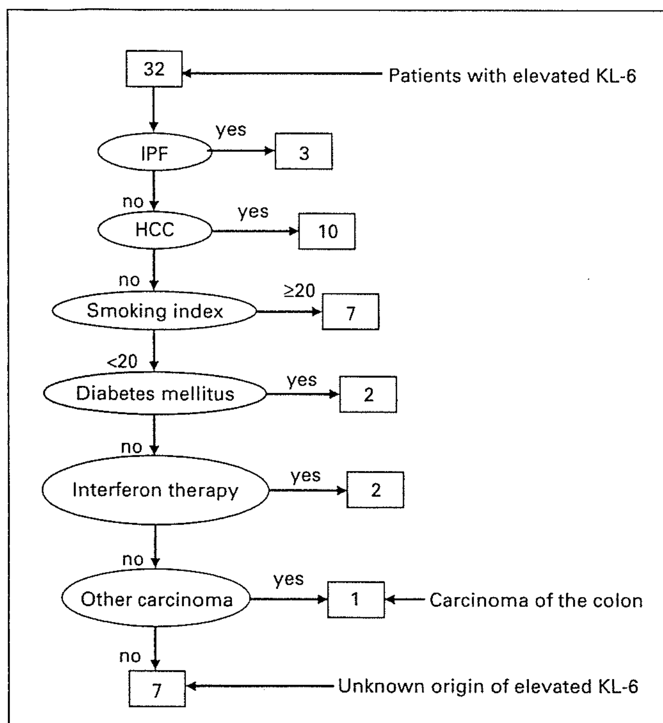

Fig. 2. Flow chart to assess the clinical background in patients with elevated KL-6 level. The smoking index was defined as the number of cigarettes smoked per day × years of smoking. Numbers in boxes indicate the numbers of patients.

of the 32 (78.1%) patients were diagnosed as having basic disease of IPF, HCC and diabetes mellitus, for example. However, the remaining 7 patients with elevated KL-6 did not have any underlying disease.

Discussion

Chest roentgenograms, CT, pulmonary function tests, gallium-67 lung scan, and bronchoalveolar lavage are used clinically for the diagnosis of IPF. However, the effect of exercise-induced changes and the repeatability of these examinations remain problems to be solved. KL-6 can be examined by blood sampling without discomfort to patients. Moreover, the serum level of KL-6 is elevated in the majority of patients with various interstitial lung diseases, including IPF [25]. In our opinion, serum KL-6 is generally a noninvasive, sensitive, diagnostic marker of IPF. The serum level of KL-6 can, therefore, provide useful information for an early diagnosis of IPF.

Ueda et al. [11] reported a higher prevalence of HCV antibody in patients with IPF compared with the general population. A total of 459 consecutive autopsy cases of chronic liver disease were examined at the Toranomon Hospital in Tokyo, Japan, from 1988 to 1998. The prevalence of IPF was 5% (13/261) in patients with HCV, 0% (0/58) in patients with hepatitis B virus (HBV) and 0.7% (1/140) in patients without HCV and/or HBV. The prevalence of IPF in HCV cases was significantly higher than those of other groups. Moreover, several studies have recently suggested that interferon therapy for chronic hepatitis C might induce interstitial pneumonitis [26, 27]. Recently, combination therapy of interferon and ribavirin has often been selected as the first choice for chronic hepatitis C in Japan. However, in about 10,000 patients treated with interferon and ribavirin, 16 patients had IFN-related IPF during therapy. Hence, preventive measure and early diagnosis of IPF are essential for the daily management of chronic hepatitis C.

Serum levels of KL-6 are generally increased in patients with fibrosing lung infection. However, serum levels of KL-6 are sometimes elevated in patients with certain malignancies such as adenocarcinoma of the lung, breast, pancreas and HCC [28]. In the present study, the serum KL-6 level in patients with HCC was higher than that of patients without HCC by univariate analysis. Therefore, we assessed the question of whether the serum KL-6 level is useful in the diagnosis of IPF in HCV-positive patients with chronic liver disease. The present study included only 3 patients with IPF of a total of 502 study patients. On the other hand, about 20% of the patients with HCC had elevated KL-6 levels. Our results indicate that serum KL-6 is a useful marker for HCC in patients with chronic liver disease and HCV. This result is in agreement with previous data reported Moriyama et al. [28].

Moreover, they have reported that serum levels of KL-6 are generally increased in patients with lung cancer compared to those without the disease. Consequently, an increase in the serum KL-6 level in patients with HCV should be associated with potential development of HCC, progression to liver disease or other mechanisms.

In conclusion, (1) patients aged ≥60 years having HCC show significantly elevated serum KL-6 levels, and (2) in patients with increasing KL-6 level and chronic hepatitis or liver cirrhosis, it is necessary to differentiate IPF, HCC, and other mechanisms.

Acknowledgments

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Hepatitis B Virus-Related Hepatocellular Carcinogenesis and Its Prevention

Kenji Ikeda Yasuji Arase Masahiro Kobayashi Takashi Someya
Tetsuya Hosaka Satoshi Saitoh Hitomi Sezaki Norio Akuta
Fumitaka Suzuki Yoshiyuki Suzuki Hiromitsu Kumada

Department of Gastroenterology, Toranomon Hospital, Tokyo, and Okinaka Memorial Institute for Medical Research, Tokyo, Japan

Key Words
Carcinogenesis · Hepatocellular carcinoma · Liver cirrhosis · Hepatitis B virus · DNA · Interferon · Cancer prevention

Abstract
To elucidate the influence of serum hepatitis B virus (HBV) load on hepatocellular carcinogenesis in cirrhotic patients, HBV-DNA was sequentially measured. In a nested, case-control study using 96 patients without antiviral therapy, high HBV-DNA ($\geq 10^{3.7}$ copies/ml) in the last 3 years was significantly associated with carcinogenesis (a patient group without hepatocellular carcinoma (HCC) development; 0/48 vs. a patient group with eventual HCC development; 22/48, $p < 0.0001$). No patient with a continuously low HBV-DNA for the last 3 years developed HCC. Persistence of high HBV-DNA concentration suggested an increased risk of carcinogenesis. In a retrospective cohort study using 57 patients with interferon therapy, HCC developed in 2 (8.0%) of the 25 patients with HBV-DNA loss, while carcinogenesis was found in 11 (34.4%) of 32 patients without HBV-DNA loss (Fisher's exact test, $p = 0.026$). A significant decrease or loss of serum HBV-DNA stops HCC development, and its sequential analysis could be very useful both in the prediction and early detection of small HCC.

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Introduction

Hepatocellular carcinoma (HCC) is a principal cause of death in many parts of sub-Saharan Africa and in Asia [1, 2]. It is also one of the most common neoplasms in Japan [3]. Abundant epidemiological and molecular biological evidence shows that hepatitis B virus (HBV) is an important factor in the development of HCC [4–6], but the precise role of HBV DNA viruses in oncogenesis is still unknown. Although increasing evidence indicates that the HBV plays an important role in the development of HCC after discovery of integrated forms of HBV [7–9], current serological and virological markers are still insufficient in establishing this relationship. Since a really curative therapy is not available for HCC at present, an accurate prediction and early detection of HBV-related HCC is essential in the current situation.

Hepatocellular carcinogenesis rates were estimated in patients with HBV-related chronic hepatitis (n = 297) and cirrhosis (n = 246), who have not received interferon (IFN), lamivudine, or steroid therapy. They were diagnosed by peritoneoscopy and/or biopsy as having chronic liver disease in the Toranomon Hospital, Tokyo, Japan, from 1974 to 1999. Cumulative carcinogenesis rates in F1 fibrosis, F2–3, and F4 were 0.5, 6.3, and 19.7% at the end of the 5th year, 2.7, 14.9, and 30.3% at the end of the 10th year, 4.1, 19.5, and 35.8% at the15th year, and 15.0, 29.6, 41.9% at the 20th year, respectively (fig. 1).

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Kenji Ikeda, MD
Department of Gastroenterology, Toranomon Hospital
Toranomon 2-2-2, Minato-ku
Tokyo, 105-8470 (Japan)
Tel. +81 44 877 5111, Fax +81 44 860 1623, E-Mail ikedakenji@tora.email.ne.jp

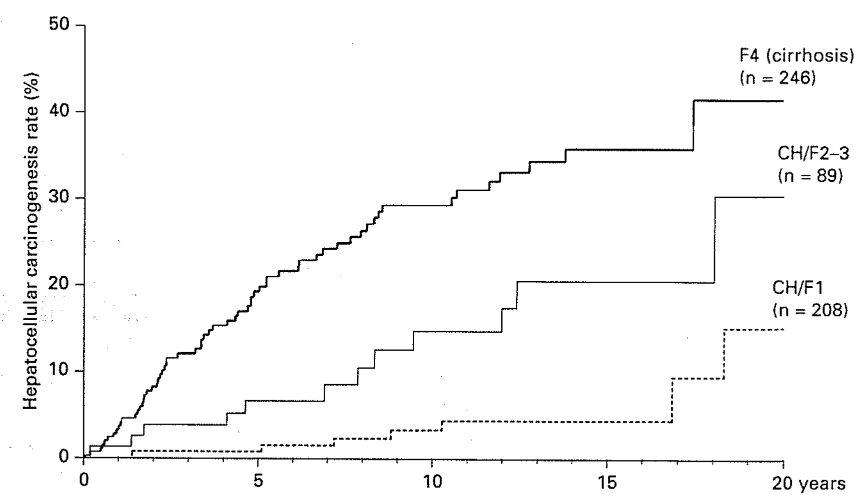

Fig. 1. Cumulative hepatocellular carcinogenesis rates in patients with chronic hepatitis or cirrhosis.

Needless to say, patients with HBV-related cirrhosis have a significantly higher risk for HCC development [10, 11], but the degree of the carcinogenesis risk in an individual patient cannot be predicted as yet. How can we recognize a super-high-risk group or a rather low-risk group in HBV-related cirrhosis? Can we predict and specify a patient who is not likely to develop HCC in the future? Hepatocellular carcinogenesis in patients with HBV infection may well be associated with persistence of aminotransferase, concentration of HBV DNA, or merely the severity of the liver disease. One of the purposes of this article is, therefore, to elucidate the relationship of hepatocellular carcinogenesis with longitudinal clinical courses of biochemical data and HBV DNA concentration in consecutive patients with cirrhosis.

IFN has been reported to be effective in patients with HBV-related chronic hepatitis, which decreases serum HBV DNA concentration and improves biochemical data on early control studies [12–14], and subsequently suppresses disease progression to cirrhosis [15, 16]. Although various effects of IFN in hepatitis B virus infection have been well investigated from virological, biochemical, and medico-economical viewpoints [17–19], the influence on a long-term outcome of liver cirrhosis or on hepatocellular carcinogenesis is still controversial [20–25]. In order to clarify the mechanism of anti-carcinogenic activity of IFN, if any, we analyzed HBV DNA concentration serially in a cohort of 60 patients with cirrhosis. The other purposes of this study are to elucidate the relationship of hepatocellular carcinogenesis with longitudinal clinical courses in consecutive cirrhotic patients with interferon therapy and to investigate an early prediction of HBV DNA elimination and the cancer preventive activity.

Factors Affecting Hepatocellular Carcinogenesis in Cirrhosis (without Anti-Viral Therapy)

Patients and Methods

Analyzable Patients without Anti-Viral Therapy

Among 217 patients who were diagnosed as having HBV-related cirrhosis by peritoneoscopy and/or liver biopsy from 1976 to 1989 in our hospital, 160 patients had not undergone interferon or other antiviral therapy. Out of the consecutive 160 patients, sequential assay of serum HBV DNA using serial sera stored at −80 °C was available in 146 patients (91.3%). All 146 patients showed a positive hepatitis B surface antigen and negative anti-hepatitis C virus antibody in the assay of their initial sera. Among the 146 patients with HBV-related cirrhosis, 48 patients (32.9%) developed HCC during a median follow-up period of 7.2 years after the diagnosis of cirrhosis, and the other 98 patients (67.1%) have not developed HCC during 11.7 years.

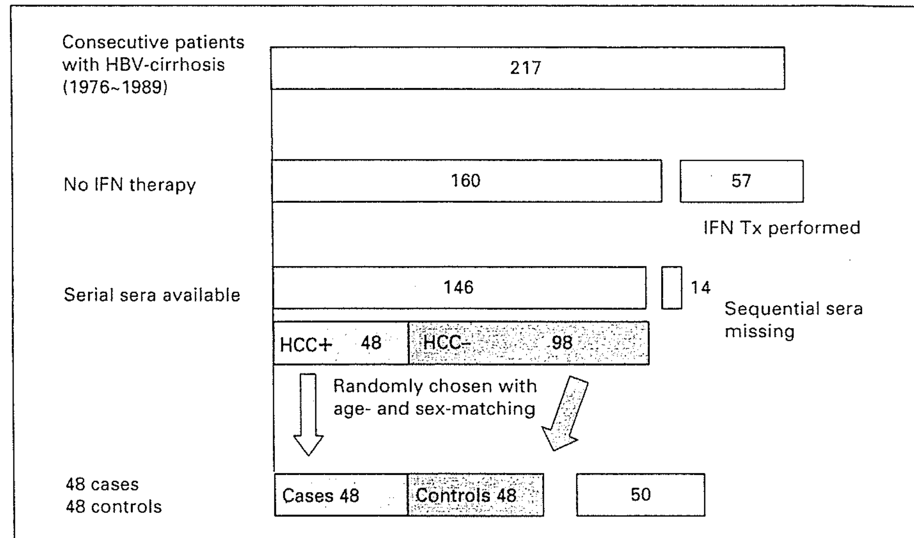

Fig. 2. Analyzed patients with HBV-related cirrhosis. Since almost all of consecutive untreated patients (146/160, 91.3%) were analyzable, a nested case-control study was established using a total of 96 patients.

Follow-Up of Patients and Diagnosis of HCC

Follow-up of the patients was made on a monthly or bi-monthly basis after diagnosis of liver cirrhosis by monitoring α-fetoprotein (AFP) and other biochemical data. Imaging diagnosis was carried out two or more times per year for each patient with computed tomography (CT), ultrasonography (US), or scintigraphy. HCC was diagnosed by typical hypervascular characteristics on angiography in addition to certain features of CT and US. A pathological confirmation of surgically resected specimens or autopsy was made in 38 (79.2%) of 48 patients with HCC development.

Nested Case-Control Study

In order to elucidate the relationship between hepatocellular carcinogenesis and longitudinal courses of clinical markers, a nested case-control study was introduced. Age- and gender-matched control patients were selected from the 98 HCC-free patients with a ratio of 1:1. The control patients were randomly selected using a computer-generated random number table, avoiding those with a short observation period of less than 3 years. Thus, a nested case-control study was made, consisting of 48 cases with cancer development (group A) and 48 demography-adjusted controls without sings of cancer (group B) (fig. 2).

Assays of HBV Markers

HBV DNA was assayed using frozen sera stored at −80°C, and quantified using transcription mediated amplification and hybridization protection assay described by Kamisango et al. [26] (TMA-HPA, Chugai Diagnostics Science, Tokyo, Japan). A lower value of HBV DNA of 3.7 LGE/ml (equivalent for $10^{3.7}$ copies/ml or 5,000 copies/ml) was considered as a low value. For annual sera from the diagnosis of cirrhosis to the end of observation period in each patient, the DNA quantification was simultaneously performed after fixation of the 48 cases and the 48 controls.

Statistical Analysis

Standard statistical measures and procedures were used. Mann-Whitney U test, χ^2 test, and Fisher's exact test were employed for examination of background characteristics of the patient groups with and without HCC development. $p < 0.05$ with the two-tailed test was considered significant. Data analysis was performed using the computer program SAS version 6.12 [27].

Results

Demography and Initial Laboratory Data of the Groups with or without HCC Development [28]

Table 1 shows the demography and initial laboratory data of the patients in groups A and B. The ratio of men was 39 of 48 (81.3%) in the both groups, and the median age was 49.5 and 49 in groups A and B, respectively. The proportion of decompensated cirrhosis, and a history of past alcohol consumption, were not significantly different

Table 1. Demography and initial laboratory data of 48 patients with HCC development and the 98 patients without HCC development during the observation period

	Group A HCC development (n = 48)	Group B no HCC (n = 48)	p
Demography			
Men:women	39:9	39:9	NS
Age, median (range)	49.5 (30–71)	49 (30–71)	NS
Decompensated cirrhosis	1 (2.1%)	7 (14.6%)	0.65
Past alcohol consumption of 500 kg or more	8 (16.6%)	9 (18.8%)	0.79
Initial laboratory data (median, range)			
Anti-HCV antibody positive	0	0	NS
HBe antigen positive	33/48 (68.8%)	17/48 (36.1%)	0.001
Bilirubin, mg/dl	1.0 (0.6–9.8)	1.0 (0.5–7.5)	0.46
Albumin, g/dl	3.95 (2.4–4.8)	4.0 (2.5–5.2)	0.23
Aspartic transaminase, IU	39.5 (15–820)	31.5 (13–376)	0.23
Alanine transaminase, IU	32 (8–740)	31 (9–313)	0.82
Platelet count, $\times 10^3/mm^3$	100 (28–225)	121 (49–255)	0.047
AFP, ng/ml	16 (3–785)	7 (3–1,520)	0.037

between the two groups. The prevalence of positive HBe antigen was, however, significantly higher in group A than that in group B. Although median platelet count was slightly lower, and alpha-fetoprotein concentration was higher in group A, there was no significant difference in bilirubin, albumin, aspartic transaminase, and ALT between the two groups.

Individual HBV DNA Concentration until the End of the Observation Period [28]

Quantitative HBV DNA assessment was sequentially performed until the diagnosis of HCC in each patient. In group A (HCC development), 9 patients showed intermittently high HBV DNA concentration and 39 patients showed a continuously high HBV DNA concentration from the diagnosis of cirrhosis to the development of HCC. All the patients experienced high HBV DNA during their clinical courses, and no patient showed low HBV DNA for a consecutive 3 years just before the detection of HCC.

Serial HBV DNA concentration of each patient was also assessed in group B (no HCC development). HBV DNA was continuously low in 9 patients, and HBV DNA concentration showed a settling down and lowering for 3 years or more until the end of observation period in 13 patients. Nine patients showed a fluctuated HBV DNA concentration, and the remaining 17 patients had a continuously high HBV DNA during the observation period. Of the 48 patients, 9 patients never experienced a high HBV DNA load, and a total of 22 patients (45.8%) showed low HBV DNA values for a successive 3 years until the end of the observation (fig. 3).

Table 2. Demography and laboratory data of 57 patients with HBV-related cirrhosis undergoing interferon therapy

Demography	
Men:women	45:12
Age, median (range)	41 (19–60)
Decompensated cirrhosis	3 (5.3%)
Past alcohol consumption of 500 kg or more	3 (5.3%)
Laboratory data, median (range)	
Bilirubin, mg/dl	0.9 (0.4–2.6)
Albumin, g/dl	4.1 (3.0–4.9)
Aspartic transaminase, IU/l	65 (16–404)
Alanine transaminase, IU/l	74 (12–586)
Platelet count, $\times 10^3/mm^3$	125 (68–332)
Anti-HCV antibody positive	0
HBe antigen positive	41 (71.9%)
HBV-DNA, LGE/ml[1]	7.2 (3.9 to >8.7)
Observation period, years	13.6 (6.5–16.1)

[1] HBV-DNA (LGE/ml): log-genome equivalent, expressed as 10^n copies/ml.

The incidences of HBV DNA patterns were significantly different between the two groups (χ^2 test, $p < 0.001$). The rates of low or a settling down trend of HBV DNA concentration was significantly lower in group A

Serial course of HBV-DNA until the end of observation		HCC development	
		yes Case (n = 48)	no Control (n = 48)
3.7 LGE/ml	Consistently low (n = 9)	0	9
3.7 LGE/ml	Decreased DNA for latest 3 years or longer (n = 13)	0	13
3.7 LGE/ml	Intermittently high (n = 18)	9	9
3.7 LGE/ml	Persistently high (n = 56)	39	17
			χ^2: p < 0.001

Fig. 3. Patterns of longitudinal courses of HBV-DNA in groups A and B.

Serial course of ALT until the end of observation		HCC development	
		yes Case (n = 48)	no Control (n = 48)
50 IU	Consistently normal (n = 6)	2	4
50 IU	Normalized ALT for latest 3 years or longer (n = 19)	4	15
50 IU	Intermittently high ALT (n = 34)	21	13
50 IU	Persistently high (n = 37)	21	16
			χ^2: p = 0.022

Fig. 4. Patterns of longitudinal courses of alanine transaminase in groups A and B.

than in group B (0/48 in group A vs. 22/48 in group B, Fisher's exact test, p < 0.00001). Any patients with a continuously low HBV DNA concentration for 3 years or longer did not develop HCC during the clinical courses.

Patterns of Longitudinal Courses of Alanine Transaminase [28]

ALT values were also assessed sequentially throughout the entire clinical courses. In group A, ALT was continuously normal in 2 patients (4.2%), ALT was high initially but normalized for the last 3 years or longer in 4 (8.3%), it showed abnormal values intermittently in 21 (43.8%), and had a continuously high value during the observation period in the remaining 21 (43.8%). In group B, 4 patients (8.3%) showed consistently normal ALT, 15 (31.3%) showed a decrease in ALT values, 13 (27.1%) intermittent elevation, and the remaining 16 (33.3%) showed continuously high ALT values during the follow-up period (fig. 4).

The incidence of HBV DNA patterns was significantly different between the two groups (χ^2 test, p = 0.022). While persistently or intermittently elevated ALT value slightly favored higher carcinogenesis rate (42/48 in group A vs. 29/48 in group B), statistical significance was, however, not obtained between carcinogenesis and ALT values (χ^2 test, p = 0.077).

Discussion

Liver cirrhosis due to hepatitis C virus usually shows a rather steady and constant clinical course, which enables us to estimate the future carcinogenesis rate from only clinical information at the time of the diagnosis of cirrhosis. Disease activity and carcinogenic potency of HBV-related liver disease, on the contrary, often change in natural clinical courses, accompanying significant fluctuation of HBe antigen system or amount of HBV DNA. When we investigate the relationship between hepatocellular carcinogenesis and its affecting and contributing factors, explanatory parameters should include not only initial demographic data but also chronological clinical data after starting the observation [29]. A longitudinal analysis is, therefore, necessary for the study of carcinogenesis in chronic liver disease caused by HBV. We, therefore, established a nested case control study using longitudinal clinical data until the end of the observation period or just before carcinogenesis, including HBV DNA quantification and ALT.

In this study, the sequential trend of serum HBV DNA concentration was significantly associated with hepatocellular carcinogenesis, and the relationship of HBV DNA to the carcinogenesis was much stronger than that of ALT. Indeed, mere initial background features and laboratory data of the patients could predict a future risk of carcinogenesis, and the chronological analysis demonstrated more discrete differentiation of a high-risk group and provided more detailed information about HBV-related carcinogenesis. Although this study illustrates that a consistence of low HBV-DNA concentration for 3 years or longer saves cirrhotic patients from carcinogenesis, the combination of 'low HBV-DNA' and '3 years' might not avoid the carcinogenesis risk sufficiently, considering the fact that hepatocellular carcinoma does develop without hepatitis, without high ALT, or without high HBV-DNA. It is, however, true that HCC scarcely develops in a patient with HBV-related cirrhosis whose HBV-DNA concentration is consistently low for the recent 3 years or longer.

Although a high load of HBV-DNA seems to promote carcinogenesis or tumor growth, the reason why a high concentration of HBV-DNA affected hepatocellular carcinogenesis remains unknown. Taking into account that hepatitis patients with positive HBe antigen and fluctuated aminotransferase values often show a high serum HBV-DNA concentration, a large amount of HBV-DNA load may be associated with a high carcinogenesis rate through an active inflammatory state and indirect cancer promotion [30]. Relationship between hosts and hepatitis virus should also be considered in future studies on carcinogenesis.

Hepatocellular Carcinogenesis in Cirrhotic Patients with Interferon Therapy

Patients and Methods

Analyzed Patients

Among 189 patients who were diagnosed as having HBV-related cirrhosis by peritoneoscopy and/or liver biopsy from 1983 to 1990 in our hospital, a total of 60 patients underwent interferon therapy from 1986 to 1990. Since 3 patients were lost to follow-up, the remaining 57 patients (95.0%) were analyzed for virological outcome, carcinogenesis, and eventual prognosis: the reason for the dropout from the observation in the 3 patients was simply house moving.

Interferon Therapy

IFN-α was administered in 35 patients (61.4%) and IFN-β in the remaining 22 patients (38.6%). The daily amount of IFN was 3 million units in 22 (38.6%) and 6 million units in 35 (61.4%), and twice a week administration was performed in 54 (94.7%) and three times a week in 3 (5.3%). All patients received an intermittent interferon therapy for a median of 18 months (range 2–132 months), but the duration of the IFN therapy was arbitrary in this pilot study. Although the amount of daily dose of IFN and the duration of the therapy varied in this study, 52 (91.2%) of the 57 patients received IFN for 6 months or longer.

Follow-Up and Diagnosis of HCC

Follow-up of the patients was made on a monthly basis after diagnosis of liver cirrhosis by monitoring virological, hematological, and biochemical data including α-fetoprotein (AFP). All these laboratory tests including HBV-markers were obtained throughout the observation period in each patient. Patients were classified into four groups according to patterns of serial concentration of HBV DNA: type A, disappearance of HBV DNA during and after IFN therapy; type B, loss of HBV DNA after cessation of IFN administration; type C, transient loss of HBV DNA only during IFN administration; type D, persistently positive HBV DNA during and after the therapy. Clinical courses of ALT fluctuation were also classified into four groups according to normalization of ALT value.

Imaging diagnosis and establishment of diagnosis of HCC were carried out as shown above.

Assay of HBV DNA

HBV-DNA was assayed using frozen sera stored at −80 °C, and quantified using transcription mediated amplification and hybridization protection assay described by Kamisango et al. [26] as shown above.

Statistical Analysis

Standard statistical measures and procedures were used. Mann-Whitney's U and χ^2 tests were employed for examination of background characteristics between the groups with and without HBV DNA elimination. Fisher's exact test was also used to analyze the relationship of HBV markers with carcinogenesis. Cumulative HBV DNA disappearance rate, carcinogenesis rate, and survival rate were calculated by Kaplan-Meier technique [31], and the differences between the analyzed groups were assessed by log-rank test. $p < 0.05$ with the two-tailed test was considered to be significant. Data analysis was performed using the computer program SPSS version 11 [32].

Results

HBV DNA in Clinical Courses [33]

HBV DNA was positive in all the patients at the initiation of IFN therapy (3.9 to >8.7 LGE/ml). HBV DNA became negative (<3.7 LGE/ml) in 25 of 57 patients (43.9%) during the observation period with a median of 13.6 years. The remaining 32 patients did not show a sustained negative HBV DNA after the therapy, although 9 patients did show transient negative values for a limited period during the therapy.

Clinical courses of HBV DNA were classified into the four categories mentioned above. Nine patients (15.8%) lost HBV DNA during and after IFN therapy (type A), 16 patients (28.1%) lost HBV DNA after cessation of the therapy (type B). The other 9 patients (15.8%) showed a transient loss of HBV DNA (type C), and the remaining 23 (40.4%) retained persistently positive HBV DNA (type D).

Cumulative rate of HBV DNA disappearance was calculated using Kaplan-Meier technique. DNA became negative in 10.5% at the end of the first year after initiation of IFN, 12.3% at the third year, 21.0% at the fifth year, 43.7% at the tenth year, and 46.7% at the fifteenth year, respectively.

Hepatocellular Carcinogenesis and Serial Concentration of HBV DNA [33]

A total of 13 patients developed HCC during the observation period.

The relationship between carcinogenesis and serial concentration of HBV DNA was analyzed (fig. 5). No patients (0%) developed HCC among 9 patients in type A. Two (12.5%) of 16 patients developed HCC in type B: HCC were detected 1.2 year after disappearance of HBV DNA in one patient, and 3.6 years after disappearance of HBV DNA in the other patient. Three (33.3%) of 9 patients showed carcinogenesis in type C, and 8 (34.8%) of 23 patients developed HCC in type D during the observation. Hepatocellular carcinogenesis was significantly associated with persistent positive HBV DNA after initiation of IFN (2/25 vs. 11/32, $p = 0.019$ by χ^2 test, $p = 0.026$ by Fisher's exact test).

Cumulative carcinogenesis rates were analyzed according to the ultimate courses of serial assay of HBV DNA. Fifth-year hepatocellular carcinogenesis rate were 0% in patients with HBV DNA loss, and 9.4% in patients without HBV DNA elimination, 10-years rates were 8.0 and 22.5%, and 15-year rates were 8 and 44.0%, respectively. The carcinogenesis rate in patients with HBV DNA elimination was significantly lower than those without DNA elimination ($p = 0.011$, log-rank test).

Hepatocellular Carcinogenesis and HBe Antigen and Aminotransferase [33]

Relationship was assessed between carcinogenesis and HBeAg positivity during the clinical courses. HBeAg was positive in 41 patients (71.9%) and negative in 16 (28.1%) at the initiation of IFN therapy. Twenty-eight (68.3%) of the 41 patients showed continuous loss of HBeAg after IFN therapy. HCC developed in 4 (25.0%) of the 16 patients without HBeAg from the beginning, 4 (14.3%) of the 28 patients with HBeAg clearance, and 5 (38.5%) of 13 patients with persistent HBeAg positivity. HBeAg clearance did not significantly decrease the incidence of carcinogenesis risk ($p = 0.12$, χ^2 test with Yates' correction).

Relationship was also analyzed between carcinogenesis and a longitudinal course of ALT after IFN therapy. Four (18.2%) of 22 patients with normalization of ALT after IFN therapy developed HCC, 9 (25.8%) of 35 patients with persistent abnormal ALT developed HCC. Serial values of ALT was not significantly associated with carcinogenesis risk ($p = 0.075$, χ^2 test with Yates correction).

Cumulative HBe antigen disappearance rate, HBV-DNA disappearance rate, and ALT normalization rate

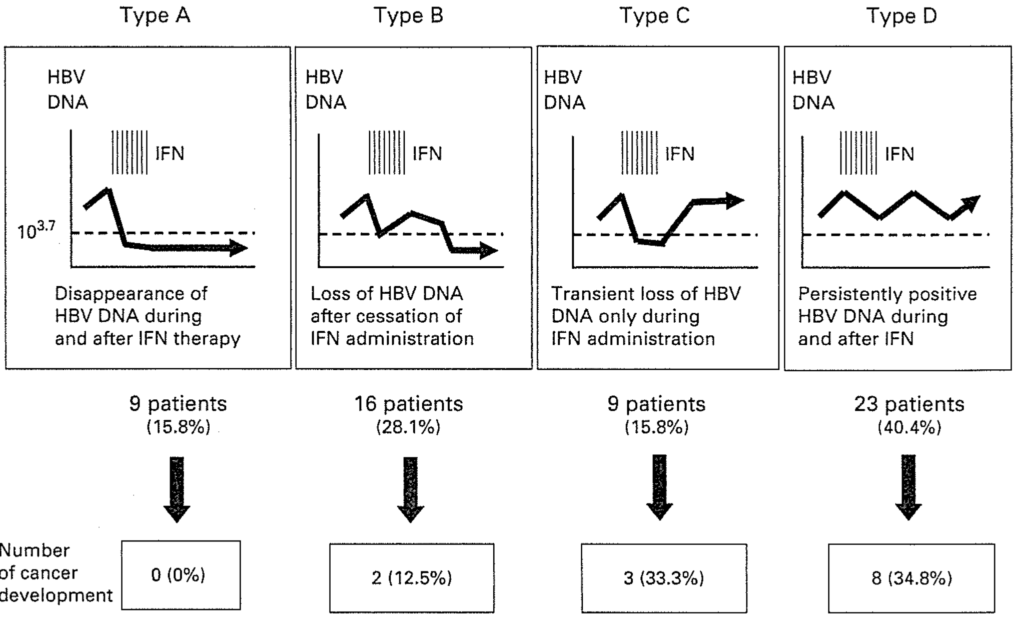

Fig. 5. Relationship between types of serial HBV-DNA concentration and carcinogenesis.

were calculated in those patients with positive HBe antigen at the beginning of IFN treatment. HBe antigen disappearance rate and DNA disappearance rate were 55.4 and 14.6% at the end of the 5th year, and 55.4 and 40.1% at the 10th year, respectively. ALT normalization rate at the 5th year was 25.4% and 10th-year rate was 41.2%. Although the incidence of virological and biochemical improvement gradually increased after the therapy, the rates evidently differed among them.

Discussion

Until recently, several authors mentioned the anti-carcinogenic activity of IFN in patients with HBV-related cirrhosis. Oon [20] and we [23] showed that IFN significantly decreased a carcinogenesis in patients with IFN therapy with a relative risk of 0.03 and 0.39, respectively. Lin et al. [25] also demonstrated an anti-tumor activity of IFN with a relative risk of 0.11 in a randomized controlled trial for patients with chronic hepatitis and cirrhosis. Mazzella et al. [21], Fattovich et al. [22], and the International Interferon-alpha Hepatocellular Carcinoma Study Group in Europe [24] demonstrated a low relative risk for carcinogenesis in patients with IFN therapy, but they could not show a statistical significance. Aside from the slightly inconsistent results after IFN therapy for cirrhosis, we tried to elucidate the relationship between virological response and HCC development, using a cohort of consecutive patients with cirrhosis who underwent IFN therapy more than 10 years ago. Considering that the disease activity and carcinogenic potency can change significantly in the course of HBV-related liver disease, a longitudinal analysis was performed for the study of clinical process and mechanism of anti-tumor activity of IFN in HBV-positive cirrhosis.

In this clinical study, sequential trends of HBV concentration were significantly associated with hepatocellular carcinogenesis, as was found in natural clinical courses of patients without IFN [28]. Although only 2 of 25 patients developed HCC who showed a disappearance of HBV-DNA during or after IFN therapy, 11 of 32 patients showed carcinogenesis who could not eliminate HBV DNA by the treatment with IFN ($p = 0.019$). Hepatocellular carcinogenesis was assessed using serial HBV DNA assay with a cut off value of 3.7 LGE/ml or $10^{3.7}$ copies/ml in this study. Although a detailed analysis of HBV-DNA concentration with more sensitive measurement may

demonstrate a better correlation with carcinogenesis rate than current one, this setting of HBV-DNA concentration as a cut-off value was significantly valuable in the prediction for HCC appearance.

Although the mere use of IFN does not guarantee the decrease of carcinogenesis in patients with HBV-related cirrhosis, a serial course of HBV DNA concentration was significantly correlated with the future HCC development during and after treatment. The value of cancer prediction was much higher in the assay of HBV DNA than that of HBe antigen. Indeed the cut-off values of HBV DNA concentration seemed to be discretionary, the advantage in clinical practice was marked and conspicuous. When more sensitive ways of HBV DNA concentration were applied to the analysis, hepatocellular carcinogenesis could be more successfully predicted.

Conclusions

Persistence of high concentration of HBV DNA was significantly associated with hepatocellular carcinogenesis in cirrhotic patients with and without IFN therapy and its sequential analysis would be useful in early detection of HCC. Further studies with a greater number of patients are required to confirm the relationship, and future studies should be aimed at defining the basic mechanism of hepatocellular carcinogenesis and the role of IFN by which the carcinogenesis rate was suppressed in the cohort.

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Association of Amino Acid Substitution Pattern in Core Protein of Hepatitis C Virus Genotype 1b High Viral Load and Non-Virological Response to Interferon-Ribavirin Combination Therapy

Norio Akuta[a] Fumitaka Suzuki[a] Hitomi Sezaki[a] Yoshiyuki Suzuki[a] Tetsuya Hosaka[a] Takashi Someya[a] Masahiro Kobayashi[a] Satoshi Saitoh[a] Sachiyo Watahiki[b] Junko Sato[b] Marie Matsuda[b] Mariko Kobayashi[b] Yasuji Arase[a] Kenji Ikeda[a] Hiromitsu Kumada[a]

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

Key Words

Hepatitis C virus · Genotype 1b · Albumin · Core region · Interferon sensitivity-determining region · Interferon · Ribavirin · Non-virological responder · Viral kinetics

Abstract

Objective: Patients with high titer (≥100 kIU/ml) of hepatitis C virus (HCV) genotype 1b do not achieve highly sustained virological response rates to combination therapy with interferon plus ribavirin. Non-virological responders (NVRs, namely ultimate resistant cases) who do not achieve HCV-RNA negativity during treatment are also encountered. We investigated the pretreatment virological features of NVRs. ***Methods:*** We evaluated 50 consecutive Japanese adults with high titer of HCV genotype 1b who received combination therapy for 48 weeks. We investigated the pretreatment substitution patterns in amino acids 1–191 of the core region and amino acids 2209–2248 of NS5A, and early viral kinetics. ***Results:*** Overall, a non-virological response was noted in 12 (24%) patients. Multivariate analysis identified serum albumin <3.9 g/dl, substitutions of amino acid 70 in the core region, and substitutions of amino acid 91 as independent and significant factors associated with a non-virological response. Especially, substitutions of arginine (R) by glutamine (Q) at amino acid 70, and/or leucine (L) by methionine (M) at amino acid 91 were significantly more common in NVRs. The falls in HCV-RNA levels during treatment in patients with specific substitutions in the core region were significantly less than in those without such substitutions. ***Conclusions:*** Our results suggest that serum albumin and amino acid substitution patterns in the core region in patients with high titers of HCV genotype 1b may have an effect on combination therapy in NVRs. Further large-scale studies are required to examine the role of amino acid substitutions specific to a non-virological response to combination therapy.

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Introduction

Hepatitis C virus (HCV) usually causes chronic infection which can result in liver cirrhosis and hepatocellular carcinoma (HCC) [1–4]. The aims of interferon (IFN) therapy for chronic hepatitis C include a reduction in the risk of development of HCC and liver-related death by

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Norio Akuta, MD
Department of Gastroenterology, Toranomon Hospital
2-2-2 Toranomon, Minato-ku
Tokyo 105-0001 (Japan)
Tel. +81 44 877 5111, Fax +81 44 860 1623, E-Mail akuta-gi@umin.ac.jp

viral clearance, and then by normalization of alanine aminotransferase (ALT) even if viral clearance cannot be achieved [5].

The most effective initial therapy for viral clearance is the combination of IFN and ribavirin (RBV) administered for 48 weeks [6, 7]. In Japan, about 70% of patients with chronic hepatitis C are infected with HCV genotype 1b, and a sustained virological response (SVR) to IFN monotherapy for 24 weeks is as low as 10–20% in patients with genotype 1b infection [8–11]. Moreover, patients with a high titer of genotype 1b (≥100 kIU/ml) do not achieve high SVR rates (<50%), even when the most effective combination treatment (IFN plus RBV) is administered for 48 weeks [6, 7]. Furthermore, in genotype 1b, we also often encounter non-virological responders (NVRs) who do not achieve HCV-RNA negativity as determined by polymerase chain reaction (PCR) during treatment, compared with only 1.0% (non-virological response rate) of patients infected with genotype 2a and treated with IFN monotherapy [12]. The underlying mechanism(s) of the different virological responses to treatment in patients with 1b strain infection is still unclear. Hence, in the present study, we investigated the pretreatment virological features of NVRs.

The present study included 50 consecutive Japanese adults with chronic hepatitis C of genotype 1b and a high viral load who received combination therapy for 48 weeks. The aims of the study were: (1) to investigate the rate of non-virological responses in this group; (2) to analyze the predictive factors associated with a non-virological response, including pretreatment virological features, and (3) to examine the pretreatment virological features associated with early viral kinetics. Previous studies have shown that the HCV core region might be associated with resistance to IFN therapy involving the Jak-STAT signaling cascade [13–16], and have also shown that the number of substitutions in amino acids 2209–2248 (IFN-sensitivity determining region, ISDR, of NS5A in HCV genotype 1b) [17, 18] might be associated with the efficacy of IFN therapy and viral load. Therefore, we analyzed the amino acid substitutions of the core region and NS5A in patients with genotype 1b and high viral load to identify the virus-related factors apart from the genotype and viral load.

Materials and Methods

Study Population

Fifty-seven HCV-infected adult Japanese patients were consecutively recruited into the study protocol of combination therapy with IFN (peginterferon (PEG)-IFNα-2b or IFNα-2b) plus RBV for 48 weeks between 2001 and 2004 at Toranomon Hospital, Tokyo, Japan. Among these, 50 patients were selected in the present study based on the following criteria. (1) They were negative for hepatitis B surface antigen (radioimmunoassay, Dainabot, Tokyo, Japan), positive for anti-HCV (third-generation enzyme immunoassay, Chiron Corp, Emerville, Calif., USA), and positive for HCV-RNA qualitative analysis with PCR (Amplicor, Roche Diagnostic Systems, Pleasanton, Calif., USA). (2) They were naive to RBV therapy. (3) They were infected with HCV genotype 1b alone. (4) Each had a high viral load (≥100 kIU/ml) by quantitative analysis of HCV-RNA with PCR (Amplicor HCV-RNA kit, version 2.0, Roche Diagnostics) within the preceding 2 months of enrolment. (5) Each had chronic hepatitis, without cirrhosis or HCC, as confirmed by biopsy examination within the preceding 12 months of enrolment. (6) They had abnormal serum ALT levels (the upper limit of normal for ALT, 45 IU/l) within the preceding 2 months of enrolment. (7) In each patient, the hemoglobin (Hb) concentration was ≥12.0 g/dl, platelet count $\geq 100 \times 10^3/mm^3$, and neutrophil count $\geq 1.5 \times 10^3/mm^3$ within the preceding 2 months of enrolment. (8) Their body weight was >40 kg. (9) All were free of co-infection with human immunodeficiency virus. (10) None had been treated with antiviral or immunosuppressive agents within the preceding 3 months of enrolment. (11) None was an alcoholic; lifetime cumulative alcohol intake was <500 kg (mild to moderate alcohol intake). (12) None had diabetes, other forms of hepatitis, such as hemochromatosis, Wilson disease, primary biliary cirrhosis, alcoholic liver disease, and autoimmune liver disease. (13) None of the females was pregnant or lactating. (14) All accepted treatment for 24 weeks or more as outlined in the study protocol, as well as repeated evaluation of HCV-RNA levels during treatment (at least once every month). (15) Each signed a consent form of the study protocol that had been approved by the Human Ethics Review Committee of Toranomon Hospital.

With regard to the treatment protocol, 34 (68.0%) patients received the PEG-IFNα-2b treatment protocol at dose of 1.5 μg/kg subcutaneously each week plus oral RBV at 600–800 mg/day for 48 weeks. The remaining 16 (32.0%) patients received 6 million units of IFNα-2b intramuscularly each day for 48 weeks (6 times per week for initial 2 weeks, followed by 3 times per week for 46 weeks), and oral RBV at a dose of 600–800 mg/day for 48 weeks. The RBV dose was adjusted according to body weight (600 mg for weight ≤60 kg, and 800 mg for weight >60 kg).

Table 1 summarizes the profiles and data of the 50 patients at the commencement of combination therapy with IFN plus RBV. They included 31 men and 19 women, aged 20–65 (median 53) years. The median total duration of treatment was 48 (range 28–48) weeks. In 14 of the 50 (28.0%) patients, the dose of RBV was reduced during treatment due to a fall in Hb concentration.

Patients who remained positive for HCV-RNA based on quantitative and/or qualitative analyses with PCR during and at the end of combination therapy were defined as NVRs (namely ultimate resistant cases), while the other patients who could achieve negative HCV-RNA by qualitative analysis with PCR during and/or at the end of treatment were defined as virological responders (VRs).

Laboratory Tests

Blood samples were obtained at least once every month before, during, and after treatment, and were analyzed for ALT and HCV-RNA levels. The serum samples were frozen at –80°C within 4 h of collection and were thawed at the time of measurement. HCV

genotype was determined by PCR using a mixed primer set derived from nucleotide sequences of NS5 region [19]. HCV-RNA levels were measured quantitatively by PCR (Amplicor HCV-RNA kit, version 2.0, Roche Diagnostics) at least once every month before, during, and after therapy. The lower limit of the assay was 0.5 kIU/ml. Samples collected during and after therapy that showed undetectable levels of HCV-RNA (<0.5 kIU/ml) were checked also by qualitative PCR (Amplicor, Roche Diagnostic Systems), which has a higher sensitivity than quantitative analysis, and the results are expressed as positive or negative. The lower limit of the assay was 100 copies/ml.

Histopathological Examination of Liver Biopsies

Liver biopsy specimens were obtained percutaneously or at peritoneoscopy using a modified Vim Silverman needle with an internal diameter of 2 mm (Tohoku University style, Kakinuma Factory, Tokyo), fixed in 10% formalin, and stained with hematoxylin and eosin, Masson's trichrome, silver impregnation, and periodic acid-Schiff after diastase digestion. All specimens for examinations contained 6 or more portal areas. Histopathological diagnosis was confirmed by an experienced liver pathologist (H.K.) who was blinded to the clinical data. Chronic hepatitis was diagnosed based on histological assessment according to the scoring system of Desmet et al. [20]. Hepatocyte steatosis was graded as either none (absent), mild (<1/3 of hepatocytes involved), moderate (>1/3 but <2/3 of hepatocytes involved), or severe (>2/3 of hepatocytes involved) [21].

Nucleotide Sequencing of the Core and NS5A Gene

We determined the sequences of amino acids 1–191 in the core and amino acids 2209–2248 (ISDR) in the NS5A by the direct sequencing method using pretreatment sera of 50 patients. These sequences were compared with the consensus sequence of genotype 1b, which was determined by comparing the sequences obtained in this study and prototype sequence (HCV J) [22]. HCV-RNA was extracted from serum samples at the start of treatment and reverse transcribed with random primer and MMLV reverse transcriptase (Takara Syuzo, Tokyo). Nucleic acids were amplified by PCR using the following primers. (a) Nucleotide sequences of the core region: the first-round PCR was performed with CC11 (sense, 5'-GCC ATA GTG GTC TGC GGA AC-3') and e14 (antisense, 5'-GGA GCA GTC CTT CGT GAC ATG-3') primers, and the second-round PCR with CC9 (sense, 5'-GCT AGC CGA GTA GTG TT-3') and e14 (antisense) primers. (b) Nucleotide sequences of ISDR in NS5A: the first-round PCR was performed with ISDR1 (sense, 5'-ATG CCC ATG CCA GGT TCC AG-3') and ISDR2 (antisense, 5'-AGC TCC GCC AAG GCA GAA GA-3') primers, and the second-round PCR with ISDR3 (sense, 5'-ACC GGA TGT GGC AGT GCT CA-3') and ISDR4 (antisense, 5'-GTA ATC CGG GCG TGC CCA TA-3') primers (hemi-nested PCR and nested PCR). All samples were initially denatured at 95°C for 15 min. The 35 cycles of amplification were set as follows: denaturation for 1 min at 94°C, annealing of primers for 2 min at 55°C, and extension for 3 min at 72°C with an additional 7 min for extension. Then 1 μl of the first PCR product was transferred to the second PCR. Other conditions for the second PCR were the same as the first PCR, except that the second PCR primers were used instead of the first PCR primers. The amplified PCR products were purified by the QIA quick PCR purification kit (Qiagen, Tokyo) 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).

Table 1. Patient profile and laboratory data at commencement of combination therapy with interferon plus ribavirin for 48 weeks in 50 patients infected with HCV genotype 1b

Demographic data	
Number	50
Sex, M/F	31/19
Age, years[a]	53 (20–65)
History of blood transfusion	14 (28.0%)
Family history of liver disease	16 (32.0%)
Body mass index, kg/m^2 [a]	23.2 (18.7–32.0)
Laboratory data[a]	
Serum alanine aminotransferase, IU/l	97 (35–276)
Serum albumin, g/dl	3.8 (3.1–4.2)
Hemoglobin, g/dl	14.4 (12.0–17.4)
Platelet count, $\times 10^4$/mm^3	17.4 (10.1–30.9)
ICG R15, %[b]	13 (7–41)
Serum iron, μg/dl	140 (52–308)
Serum ferritin, μg/l	150 (<10–644)
Creatinine clearance, ml/min	101 (46–142)
Viremia level, KIU/ml	710 (49–2,800)
Number of amino acid substitutions in ISDR (0/1–3/≥4)	27/20/3
Histological findings	
Stage (F1/F2/F3)[c]	31/15/4
Hepatocyte steatosis (none/mild/moderate/severe)	3/40/7/0
Treatment	
PEG-IFNα-2b/IFNα-2b	34/16
Ribavirin dose, mg/kg[a]	11.3 (9.7–14.2)

ALT levels were abnormal (the upper limit of normal for ALT; 45 IU/l) and viremia levels were high titer (≥100 kIU/ml), when all patients were recruited in this study. Normal reference ranges: 3.9–5.2 g/dl for albumin.

[a] Expressed as median (range).

[b] ICG R15: indocyanine green retention rate at 15 min.

[c] Stage of chronic hepatitis by Desmet et al. [20].

To avoid false-positive results, the procedures recommended by Kwok and Higuchi [23] to prevent contamination were strictly applied to these PCR assays. No false-positive results were observed in this study.

Viral Kinetic Study

Viral kinetic study was evaluated at three time points (4, 8 and 12 weeks during treatment). Falls in HCV-RNA levels from baseline were expressed using $\log_{10}$ of viral loads at each time point, in comparison with the pretreatment viral load. For data analysis, we used the $\log_{10}$ of the cutoff value (500 IU/ml) for HCV-RNA values below the limit of detection.

Statistical Analysis

Non-parametric tests were used to analyze the decline in HCV-RNA levels and amino acid substitutions in HCV core and NS5A between the each groups, including the Mann-Whitney U test, χ^2 test and Fisher's exact probability test. Univariate and multivariate logistic regression analyses were used to determine the factors that significantly contributed to a non-virological response. We also calculated the odds ratios and 95% confidence intervals (95% CI). All p values of <0.05 by the two-tailed test were considered significant. Variables that achieved statistical significance ($p < 0.05$) or marginal significance ($p < 0.10$) on univariate analysis were entered into multiple logistic regression analysis to identify significant independent factors. Potential predictive factors associated with NVR included the following variables: sex, age, history of blood transfusion, familial history of liver disease, body mass index, ALT, albumin, Hb, platelet count, indocyanine green retention rate at 15 min (ICG R15), serum iron, serum ferritin, creatinine clearance, viremia level, pathological staging, hepatocyte steatosis, type of IFN, RBV dose according to body weight, treatment term, dose reduction, and pretreatment amino acid substitution in the core and ISDR of NS5A. Statistical analyses were performed using the SPSS software (SPSS Inc., Chicago, Ill., USA).

Results

Virological Response Rates by Combination Therapy

The virological response could be evaluated in all 50 patients. In this study, 38 of 50 (76.0%) patients achieved a virological response while the remaining 12 (24.0%) patients were considered NVRs.

Predictive Factors Associated with a Non-Virological Response in Multivariate Analysis

We then analyzed the data of the whole population sample to determine those factors that could predict a non-virological response. Univariate analysis identified 5 parameters that tended to or significantly influenced the non-virological response. These included serum albumin ($p = 0.008$), presence of amino acid substitution in HCV core in the pretreatment sample (substitution of amino acid 70, $p = 0.003$, and amino acid 91, $p = 0.044$), RBV dose according to body weight ($p = 0.044$), and serum ferritin ($p = 0.095$).

Multivariate analysis identified three parameters that independently influenced the non-virological response, including serum albumin ($p = 0.004$), substitutions of amino acids 70 ($p = 0.013$) and 91 ($p = 0.016$; table 2).

Table 2. Factors associated with non-virological response to combination therapy with interferon plus ribavirin for 48 weeks in 50 patients infected with HCV genotype 1b, identified by multivariate analysis

Factor	Category	Odds ratio (95% confidence interval)	p
Albumin, g/dl	1: <3.9	1	
	2: ≥3.9	0.009 (0.000–0.227)	0.004
Substitution of aa 70	1: Absent	1	
	2: Present	22.2 (1.905–258.3)	0.013
Substitution of aa 91	1: Absent	1	
	2: Present	19.5 (1.737–219.3)	0.016

Only variables that achieved statistical significance ($p < 0.05$) on multivariate logistic regression are shown. aa = Amino acid.

Treatment Efficacy according to Substitution Patterns in Amino Acids of HCV Core

Figure 1 shows the sequences of amino acids 61–110 of the HCV core in 50 patients at the commencement of combination therapy. Substitutions at amino acid 70 of the HCV core were significantly more frequent in NVRs (n = 8, 66.7%) than VRs (n = 7, 18.4%; $p = 0.003$). Similarly, substitutions at amino acid 91 were significantly more frequent in NVRs (n = 9, 75.0%) than VRs (n = 14, 36.8%; $p = 0.044$). Furthermore, dual substitutions at amino acids 70 and 91 were significantly more frequent in NVRs (n = 5, 41.7%) than VRs (n = 5, 13.2%; $p = 0.046$). Thus, substitutions at amino acid(s) 70 and/or 91 were found in all 12 (100%) NVRs while only 16 (42.1%) of the VRs had such substitutions ($p < 0.001$). There were no significant differences in other substitution sites and treatment efficacy between NVR and VR groups (table 3).

At amino acid 70, the substitution in which arginine (R) was replaced by glutamine (Q) was significantly more frequent in NVRs (n = 7, 58.3%) than VRs (n = 5, 13.2%; $p = 0.004$). At amino acid 91, the substitution in which leucine (L) was replaced by methionine (M) was significantly more frequent in NVRs (n = 9, 75.0%) than VRs (n = 14, 36.8%; $p = 0.044$). At amino acid 110, the substitution in which threonine (T) was replaced by asparagine (N) was significantly more frequent in NVRs (n = 3, 25.0%) than VRs (n = 2, 5.3%; $p = 0.082$). Substitutions Q–M instead of R–L at amino acids 70 and 91 were significantly more frequent in NVRs (n = 5, 41.7%) than VRs (n = 3, 7.8%; $p = 0.014$). Thus, 11 (91.7%) NVRs and 16 (42.1%) VRs ($p = 0.003$) had a substitution of Q at amino acid 70 and/or M at amino acid 91. There were no significant differences in other substitution patterns and treatment efficacy between NVRs and VRs (table 3).

```
                  70         80         90         100        110
Consensus RRQPIPKARR PEGRTWAQPG YPWPLYGNEG LGWAGWLLSP RGSRPSWGPT  Efficacy
HCJ       ---------- ---------- ---------- M--------- ----------
Case 1    ---------Q ---------- --L------- M--------- ----------  NVR
2         ---------- ----D----- ---------- M--------- ----------  NVR
3         ---------- ---------- ---------- M--------- ---------N  NVR
4         ---------- ---------- ---------- M--------- ----------  NVR
5         ---------- ---------- ---------- M--------- ----------  NVR
6         ---------Q ----A----- ---------- M--------- ---------N  NVR
7         ---------Q ----A----- ---------- ---------- ---------S  NVR
8         ---------Q ----A----- ---------- ---------- ----------  NVR
9         ---------Q ----P----- ---------- M--------- ----------  NVR
10        ---------Q ----A----- ---------- M--------- ---------N  NVR
11        ---------Q ----A----- ---------- M--------- ----------  NVR
12        ---------H ----A----- ---------- ---------- ----------  NVR
13        ---------- ---------- ---------- M--------- ----------  VR
14        ---------- ----A----- ---------- ---------- -----N----  VR
15        ---------- ---------- ---------- M--------- ----------  VR
16        ---------H ----D----- ---------- M--------- ----------  VR
17        ---------- ----S----- ---------- ---------- H---------  VR
18        ---------- ----A----- ---------- ---------- ----------  VR
19        ---------- ---------- ---------- ---------- ----------  VR
20        ---------- ---------- ---------- ---------- H---------  VR
21        ---------- ----A----- ---------- ---T------ ----------  VR
22        ---------- ----A----- ---------- ---------- ---------S  VR
23        ---------- ---------- ---------- ---------- ----------  VR
24        ---------- ---------- ---------- ---------- ----------  VR
25        ---------- ---------- ---------- ---------- ----------  VR
26        ---------- ----A----- ---------- ---------- ----------  VR
27        ---------- ----A----- ---------- ---------- ----------  VR
28        ---------- ----V----- ---------- M--------- -----N----  VR
29        ---------Q ----A----- ---------- M--------- ----------  VR
30        ---------Q ---------- ---------- ---------- ----------  VR
31        ---------Q ----A----- ---------- M--------- ----------  VR
32        ---------H ---------- ---------- M--------- ----------  VR
33        ---------- ---------- ---------- M--------- ----------  VR
34        ---------- ---------- ---------- M--------- ---------N  VR
35        ---------- ----A----- ---------- ---------- ----------  VR
36        ---------- ----A----- ---------- ---------- ----------  VR
37        ---------- ---------- ---------- ---------- ----------  VR
38        ---------- ----P----- ---------- ---------- ----------  VR
39        ---------- ---------- ---------- M--------- ----------  VR
40        ---------Q ----A----- ---------- ---------- -----N----  VR
41        ---------- ---------- ---------- M--------- H----N----  VR
42        ---------- ---------- ---------- ---------- -----N---S  VR
43        ---------- ---------- ---------- M--------- ----------  VR
44        ---------Q ---------- ---------- M--------- ----------  VR
45        ---------- ---------- ---------- ---------- ----------  VR
46        ---------- ----A----- ---------- ---------- ---------N  VR
47        ---------- ---------- ---------- ---------- ----------  VR
48        ---------- ----A----- ---------- ---------- ----------  VR
49        ---------- ---------- ---------- M--------- ---------A  VR
50        ---------- ----A----- ---------- ---------- ----------  VR
```

Fig. 1. Sequences of amino acids 61–110 in the core region at the commencement of combination therapy in 50 patients infected with high HCV viral load genotype 1b. Dashes indicate amino acids identical to the consensus sequence of genotype 1b, and substituted amino acids are shown by standard single-letter codes. The amino acid patterns at positions that are probably associated with sensitivity to therapy are shown in boldface characters. NVR = Nonvirological responder; VR = virological responder.

Viral Kinetics according to Substitution Patterns in Amino Acids of HCV Core

Table 4 shows HCV-RNA levels at 4, 8, and 12 weeks relative to baseline as a function of pretreatment amino acid substitutions in the core region. The fall in HCV-RNA level at each time point was significantly lower in patients with specific substitution patterns (Q at amino acid 70, M at amino acid 91, N at amino acid 110, Q–M at amino acid 70 and 91, Q at amino acid 70 and/or M at amino acid 91) than in those without them.

Table 3. Amino acid substitutions in the core region in non-virological responders (NVR) and virological responders (VR) to combination therapy of interferon plus ribavirin for 48 weeks in 50 patients infected with HCV genotype 1b

	NVR (n = 12)	VR (n = 38)	p*
Presence of substitution site			
aa 70	8 (66.7%)	7 (18.4%)	0.003
aa 91	9 (75.0%)	14 (36.8%)	0.044
aa 70 and 91	5 (41.7%)	5 (13.2%)	0.046
aa 70 and/or 91	12 (100%)	16 (42.1%)	<0.001
Presence of substitution pattern			
Q at aa 70	7 (58.3%)	5 (13.2%)	0.004
M at aa 91	9 (75.0%)	14 (36.8%)	0.044
N at aa 110	3 (25.0%)	2 (5.3%)	0.082
Q-M at aa 70 and 91	5 (41.7%)	3 (7.8%)	0.014
Q at aa 70 and/or M at aa 91	11 (91.7%)	16 (42.1%)	0.003

Q = Glutamine; M = methionine; N = asparagine; aa = amino acid.
* NVR vs. VR (Fisher's exact probability test).

Table 4. Decline levels of HCV-RNA from baseline at 4, 8 and 12 weeks according to the amino acid substitutions in the core region during combination therapy of interferon plus ribavirin for 48 weeks in 50 patients infected with HCV genotype 1b

Presence of substitution pattern	Decline levels of HCV-RNA from baseline, $\log_{10}$ IU/ml[1]		
	4 weeks	8 weeks	12 weeks
Q at aa 70			
Absent[2]	2.49 (–0.024 to 3.41)]a	3.02 (0.25 to 3.41)]b	2.98 (0.30 to 3.45)]c
Present	0.58 (0.11 to 3.13)	1.18 (–0.095 to 3.16)	1.99 (0.34 to 3.19)
M at aa 91			
Absent	2.49 (0.12 to 3.41)]d	3.14 (1.69 to 3.41)]e	3.13 (0.49 to 3.45)]f
Present	0.85 (–0.024 to 3.16)	1.56 (–0.095 to 3.41)	2.40 (0.30 to 3.41)
N at aa 110			
Absent	2.36 (0.10 to 3.41)]g	3.02 (–0.095 to 3.41)]h	2.96 (0.48 to 3.45)]i
Present	0.28 (–0.024 to 0.86)	0.32 (0.25 to 1.43)	0.70 (0.30 to 2.46)
Q-M at aa 70 and 91			
Absent	2.49 (–0.024 to 3.41)]j	3.04 (0.25 to 3.41)]k	2.98 (0.30 to 3.45)]l
Present	0.58 (0.11 to 2.34)	0.50 (–0.095 to 2.34)	1.99 (0.34 to 3.19)
Q at aa 70 and/or M at aa 91			
Absent	2.49 (0.88 to 3.41)]m	3.11 (1.69 to 3.41)]n	3.18 (2.40 to 3.45)]o
Present	0.85 (–0.024 to 3.16)	2.01 (–0.095 to 3.41)	2.40 (0.30 to 3.41)

Q = Glutamine; M = methionine; N = asparagine; aa = amino acid.
[1] Decline levels of HCV-RNA from baseline are shown in $\log_{10}$ of viral loads at each time point in comparison to pretreatment viral loads. For HCV-RNA quantitative values below the limit of detection, we used the $\log_{10}$ of the cutoff value (500 IU/ml) for data analysis. Data are expressed as median (range).
[2] Absent vs. Present of substitution pattern (Mann-Whitney U test): [a] p = 0.025; [b] p = 0.019; [c] p = 0.011; [d] p = 0.049; [e] p = 0.001; [f] p = 0.007; [g] p = 0.010; [h] p = 0.002; [i] p = 0.004; [j] p = 0.018; [k] p = 0.001; [l] p = 0.019; [m] p = 0.028; [n] p = 0.006; [o] p = 0.001.