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HEPATOLOGY

Significance of hepatitis B virus DNA clearance and early prediction of hepatocellular carcinogenesis in patients with cirrhosis undergoing interferon therapy: Long-term follow up of a pilot study

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Abstract

Background and Aim: Because the anti-carcinogenic effect and mechanism of interferon (IFN) in patients with hepatitis B virus (HBV)-related cirrhosis have not been elucidated, quantitative analysis of HBV-DNA concentration was carried out sequentially.

Method: Of 60 consecutive patients with cirrhosis who began IFN therapy between 1986 and 1990, 57 patients were completely observed for the appearance of hepatocellular carcinoma (HCC). All patients underwent intermittent administration of IFN for a median period of 18 months. HBV-DNA was quantified using transcription mediated amplification and hybridization protection assay. A HBV-DNA count <3.7 log-genome equivalent (LGE)/mL (equivalent to $10^{3.7}$ or 5000 copies/mL) was considered to be a negative value.

Results: Of 25 patients who had HBV-DNA loss after IFN therapy, nine lost HBV-DNA during the therapy and 16 lost HBV-DNA after cessation of the therapy. The other nine patients showed a transient loss of HBV-DNA, and the remaining 23 retained persistently positive HBV-DNA during and after therapy. Although HCC developed in two (8.0%) of the 25 patients with HBV-DNA loss, carcinogenesis was found in 11 (34.4%) of 32 patients without HBV-DNA loss (Fisher's exact test, $P = 0.026$). In the two exceptional patients, HCC was detected at 1.2 and 3.6 years after loss of HBV-DNA, respectively. When the HBV-DNA concentration decreased by 2 LGE/mL (decrease to 1/100) at 6 months after initiation of interferon, HBV-DNA became negative eventually in 15 (60.0%) of 25 patients.

Conclusion: A significant decrease or loss of serum HBV-DNA prevents development of HCC, and sequential analysis of HBV-DNA could be very useful in both the prediction and the early detection of small HCC.

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Key words: cancer prevention, carcinogenesis, DNA, hepatitis B virus, hepatocellular carcinoma, interferon, liver cirrhosis.

INTRODUCTION

Hepatocellular carcinoma (HCC) is a leading cause of death in many parts of sub-Saharan Africa and Asia.[1,2] It is also one of the most common neoplasms in Japan. Abundant epidemiological and molecular biological evidence shows that the hepatitis B virus (HBV) is an important factor in the development of HCC,[3–6] but the precise role of HBV-DNA viruses in the oncogenesis of HCC is still unknown. Although increasing evidence indicates that the HBV plays an important role in the development of HCC, particularly after the discovery of integrated forms of HBV,[7,8] current serological and virological markers are still insufficient for establishing this relationship. Because a really curative therapy is not available for HCC at present, the accurate prediction

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and early detection of HBV-related HCC is essential in the current situation. Needless to say, a cohort of patients with HBV-related cirrhosis has a significantly high risk for the development of HCC,[6,9] but the degree of risk of carcinogenesis in an individual patient cannot be predicted as yet. Hepatocellular carcinogenesis in patients with HBV infection may be associated with persistence of aminotransferase, concentration of HBV-DNA, or merely the severity of the liver disease.

Interferon (IFN) has been reported to be effective in patients with HBV-related chronic hepatitis, which, on early control studies,[10–12] decreases serum HBV-DNA concentration and improves biochemical data and subsequently suppresses disease progression to cirrhosis.[13,14] Although the various effects of IFN in HBV infection have been well investigated from the virological, biochemical, and medico-economical viewpoints,[15–17] the influence of IFN on the long-term outcome for liver cirrhosis and on hepatocellular carcinogenesis is still controversial.[18–23] In order to clarify the mechanism of the anticarcinogenic activity of IFN, if any, we analyzed HBV-DNA concentration serially in a cohort of 60 patients with cirrhosis.

The purposes of this study are: (i) to elucidate the relation of hepatocellular carcinogenesis to longitudinal clinical courses of consecutive cirrhotic patients with IFN therapy; and (ii) to investigate a prediction of cancer preventative activity by early HBV-DNA elimination.

METHODS

Patients

Of 189 patients who were diagnosed as having HBV-related cirrhosis using peritoneoscopy and/or liver biopsy from 1983 to 1990 in our hospital, a total of 60 patients underwent IFN therapy from 1986 to 1990. Because three 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 three patients was simply relocating house.

Table 1 shows the demography and laboratory data of the consecutive 57 patients who began IFN therapy from 1986 to 1990. There were 45 men and 12 women, with an age range from 19 to 60 years and a median of 41 years. Median values of bilirubin and albumin were 0.9 mg/dL and 4.1 g/dL, respectively. All the patients had a high HBV-DNA concentration of 3.7 log-genome equivalent (LGE)/mL or more at the time of IFN therapy.

Table 1 Demography and laboratory data of 57 patients with hepatitis B virus-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)
Antibodies to hepatitis C virus positive	0
Hepatitis B e antigen positive	41 (71.9%)
Hepatitis B virus DNA (LGE/mL)	7.2 (3.9–> 8.7)
Observation period (year)	13.6 (6.5–16.1)

LGE/mL, log-genome equivalent, expressed as 10^n copy/mL.

Interferon treatment

IFN-α was administered in 35 patients (61.4%) and IFN-β in the remaining 22 patients (38.6%). The daily quantity of IFN was three million units in 22 (38.6%) and six million units in 35 (61.4%), twice a week administration was carried out in 54 (94.7%) and three times a week administration in three (5.3%). All patients received intermittent IFN 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 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 of patients and diagnosis of HCC

Follow up of the patients was made on a monthly basis after diagnosis of liver cirrhosis using monitoring virological, hematological, and biochemical data, including α-fetoprotein. All results for 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 alanine aminotransferase (ALT) fluctuation were also classified into four groups according to normalization of the ALT value.

Imaging diagnosis was made two or more times per year for each patient using computed tomography (CT), ultrasonography (US) or magnetic resonance imaging (MRI). HCC was diagnosed using typical hypervascular characteristics on angiography in addition to certain features of CT, US and MRI. Pathological confirmation of surgically resected specimens was carried out in six (46.2%) of 13 patients with HCC development.

Assays of HBV markers

Serum hepatitis B surface antigen was measured using radioimmunoassay (Dainabot, Tokyo, Japan) and reversed passive hemagglutination (Institute of Immunology, Tokyo, Japan) using commercial assay kits. hepatitis B e antigen (HBeAg) and antibody to HBeAg were determined using ELISA (Institute of Immunology) with commercial kits. Anti-hepatitis C virus antibody (third-generation anti-HCV) was assessed using ELISA kits (Dainabot).

HBV-DNA was assayed using frozen sera stored at −80°C, and quantified using transcription-mediated amplification and hybridization protection assay (Chugai Diagnostics Science, Tokyo, Japan), as described by Kamisango *et al.*[24] A HBV-DNA value of <3.7 LGE/mL (equivalent to $10^{3.7}$ copies/mL or 5000 copies/mL) was considered to be a low value. For all serial sera from the diagnosis of cirrhosis to the end of the observation period in each patient, the DNA quantification was simultaneously carried out using identical measurement kits.

Statistical analysis

Standard statistical measures and procedures were used. The Mann–Whitney *U*-test and χ^2 tests were employed for the examination of background characteristics between the groups with and without HBV-DNA elimination. Fisher's exact test was also used to analyze the relation of HBV markers to carcinogenesis. Rates of cumulative HBV-DNA disappearance, carcinogenesis and survival were calculated using Kaplan–Meier analysis,[25] and the differences between the analyzed groups were assessed using a log-rank test. A *P*-value of <0.05 using a two-tailed test was considered to be significant. Data analysis was carried out using the computer program SPSS version 11.[26]

RESULTS

HBV-DNA in clinical courses

HBV-DNA was positive in all patients at the initiation of IFN therapy (3.9–8.7 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 nine 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 nine 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).

The cumulative rate of HBV-DNA disappearance was calculated using Kaplan–Meier analysis (Fig. 1).

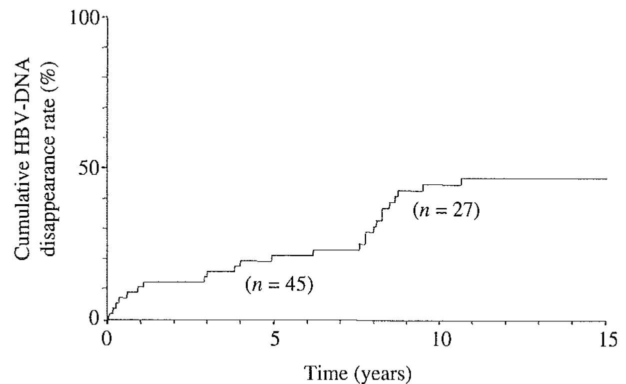

Figure 1 Cumulative hepatitis B virus (HBV)-DNA disappearance rate in the 57 cirrhotic patients with interferon therapy.

DNA became negative in 10.5% at the end of the first year after initiation of IFN therapy, in 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

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

The relationship between carcinogenesis and serial concentration of HBV-DNA was analyzed (Fig. 2). None of the nine patients in the type A group developed HCC. Two (12.5%) of 16 patients in the type B group developed HCC: HCC were detected 1.2 years after the disappearance of HBV-DNA in one patient, and 3.6 years after the disappearance of HBV-DNA in the other patient. Three (33.3%) of nine patients in the type C group showed carcinogenesis, and eight (34.8%) of 23 patients in the type D group developed HCC during the observation. Hepatocellular carcinogenesis was significantly associated with persistent positive HBV-DNA after initiation of IFN (2/25 *vs* 11/32; $P = 0.019$ using the $\chi 2$ test, $P = 0.026$ using Fisher's exact test).

Cumulative carcinogenesis rates were analyzed according to the ultimate course of the serial assay of HBV-DNA (Fig. 3). Fifth-year hepatocellular carcinogenesis rates were 0% in patients with HBV-DNA loss, and 9.4% in patients without HBV-DNA elimination; 10-year 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 in those without DNA elimination ($P = 0.011$, using a log-rank test).

Hepatocellular carcinogenesis and HBeAg and aminotransferase

The relationship between carcinogenesis and HBeAg positivity during the clinical course was assessed.

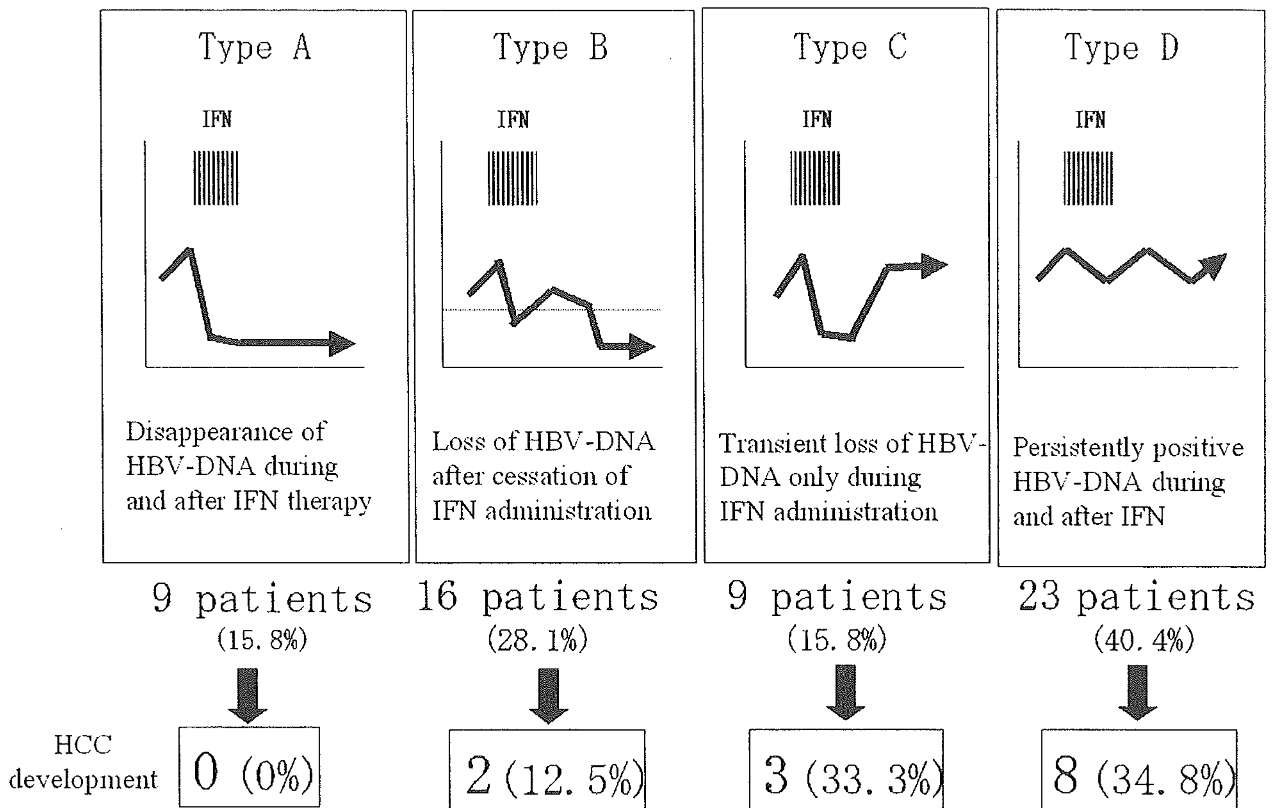

Figure 2 Relation between types of serial hepatitis B virus (HBV)-DNA concentration and carcinogenesis. HCC, hepatocellular carcinoma; IFN, interferon.

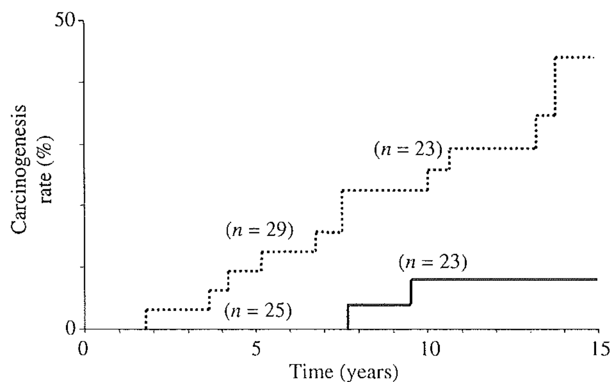

Figure 3 Cumulative hepatocellular carcinogenesis rates in patients (—; $n = 25$) with and (---; $n = 32$) without eventual hepatitis B virus (HBV)-DNA clearance.

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 four (25.0%) of the 16 patients without HBeAg from the beginning, four (14.3%) of the 28 patients with HBeAg clearance, and five (38.5%) of 13 patients with persistent HBeAg positivity. HBeAg clearance did not significantly decrease the incidence of carcinogenesis risk ($P = 0.12$ using the χ^2 test with Yates' correction).

The relationship between carcinogenesis and a longitudinal course of ALT after IFN therapy was also analyzed. Four (18.2%) of 22 patients with normalization of ALT after IFN therapy developed HCC; nine (25.8%) of 35 patients with persistently abnormal ALT levels developed HCC. The serial values of ALT were not significantly associated with carcinogenesis risk ($P = 0.075$ using the χ^2 test with Yates' correction).

The cumulative HBeAg disappearance rate, HBV-DNA disappearance rate, and ALT normalization rate were calculated in those patients with positive HBeAg at the beginning of IFN treatment (Fig. 4). The HBeAg disappearance rate and DNA disappearance rates were 55.4% and 14.6% at the end of the fifth year, and 55.4% and 40.1% at the tenth year, respectively. The ALT normalization rate at the fifth year was 25.4% and the tenth year rate was 41.2%. Although the incidence of virological and biochemical improvement gradually increased after therapy, the rates evidently differed between virological and biochemical responses.

Influence of the length of interferon therapy on HBV-DNA loss

The influence of the length of the therapy on virological response was assessed.

Although 25 (43.8%) of 57 patients cleared HBV-DNA on overall analysis, 21 (46.6%) of 45 patients who received IFN for more than 6 months and 20 (50%) of 40 patients who received IFN for more than 12 months lost HBV-DNA. Similarly, the HBV-DNA disappearance rate slightly increased correlating with the length of IFN administration: 55.5% in patients who were treated for more than 18 months, 56.0% with more than 24 months' treatment, 64.7% in more than 36 months' treatment, 58.3% in more than 48 months' treatment, and 71.4% in more than 60 months' treatment (Fig. 5). The longer the IFN therapy was carried out, the higher the rate of HBV-DNA disappearance.

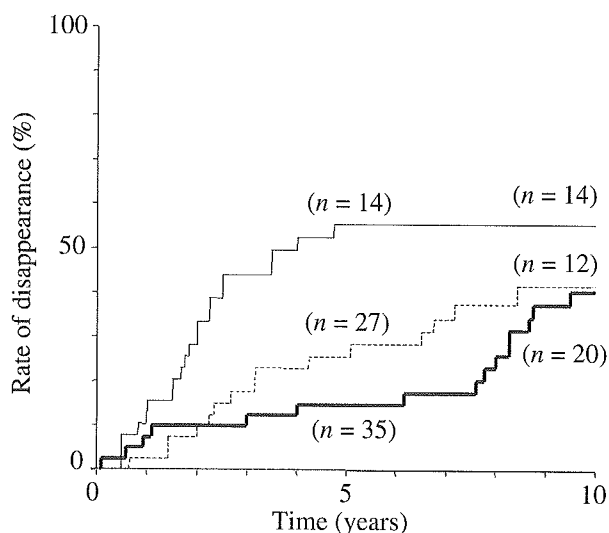

Figure 4 Cumulative (—) hepatitis B e antigen (HBeAg) disappearance rate, (—) hepatitis B virus (HBV)-DNA disappearance rate, and (---) alanine transaminase normalization rate in 41 patients with positive HBeAg at the initiation of interferon therapy.

Prediction of future HBV-DNA elimination

We assessed the relation between an early HBV-DNA response and a future HBV-DNA loss. When the HBV-DNA concentration decreased by ≥2 LGE/mL (decrease to 1/100) during the first 6 months, 15 (60.0%) of 25 patients eventually lost HBV-DNA. In contrast, when the HBV-DNA decrease was <2 LGE/mL during the period, HBV-DNA loss was found in 10 (31.3%) of 32 patients ($P = 0.036$, χ^2 test). Similarly, future HBV-DNA loss was estimated from a decrease in concentration of HBV-DNA at the end of 12 months: HBV-DNA eventually became negative in 15 (62.5%) of 24 patients with a larger DNA decrease of ≥2 LGE/mL at the end of 12 months, eventual DNA loss was found in only 10 (30.3%) of 33 patients with a smaller DNA decrease by <2 LGE/mL. The 12-month decrease of HBV-DNA was significantly associated with future DNA loss ($P = 0.030$, χ^2 test).

The early response of HBV-DNA and the length of IFN therapy were analyzed together for the prediction of eventual HBV-DNA loss. Of 25 patients with a HBV-DNA decrease of ≥2 LGE/mL during the initial 6 months, two (33.3%) of six patients with short IFN therapy of ≤6 months showed a HBV-DNA loss, but 13 (68.4%) of 19 patients with long-term IFN therapy of >6 months lost HBV-DNA. Of 32 patients with a HBV-DNA decrease of <2 LGE/mL in the first 6 months, one (20.0%) of five patients with short IFN therapy showed HBV-DNA loss, but nine (33.3%) of 27 patients with long-term IFN administration lost HBV-DNA. Therefore, according to the early HBV-DNA response and the duration of the therapy, the rate of sustained HBV-DNA decrease to <3.7 LGE/mL varied, with a range of 20.0–68.4%.

Prognosis after IFN therapy

A total of eight patients (14.0%) died in the period of observation: six from development of HCC and the

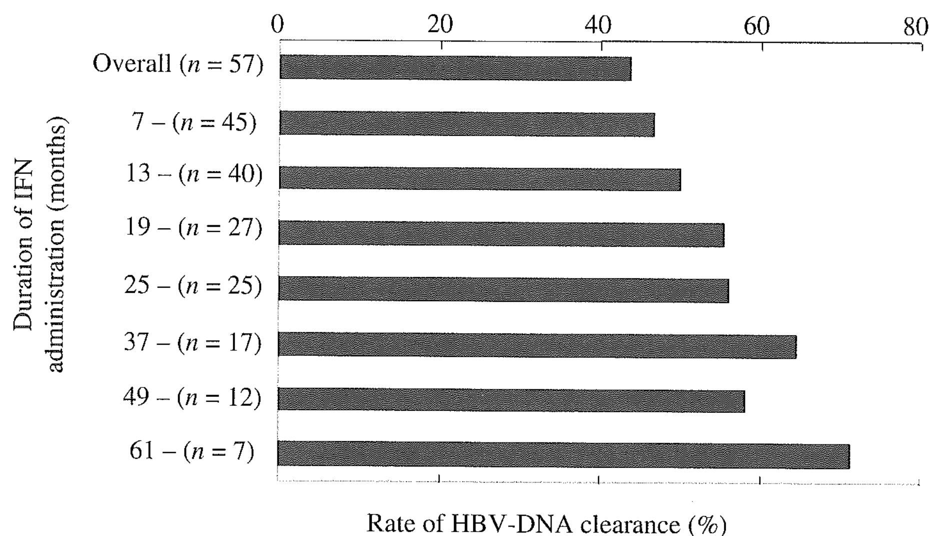

Figure 5 Influence of the length of interferon (IFN) therapy on hepatitis B virus (HBV)-DNA clearance.

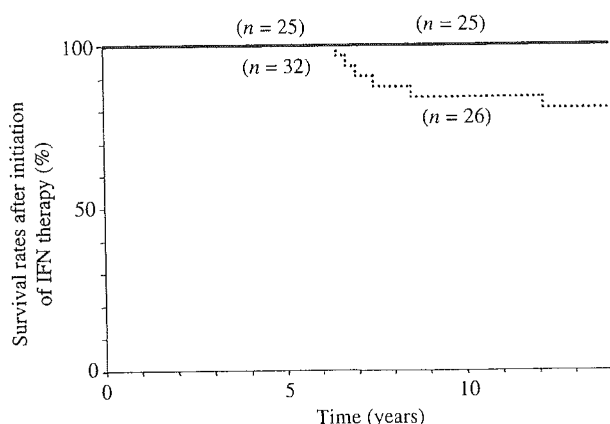

Figure 6 Cumulative survival rates after the initiation of interferon (IFN) therapy in patients (—; $n = 25$) with and (---; $n = 32$) without eventual hepatitis B virus DNA clearance.

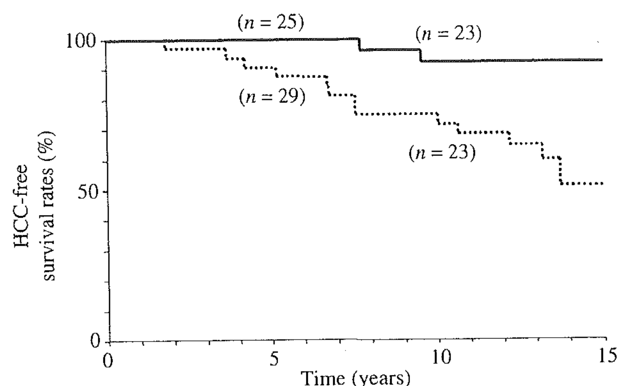

Figure 7 Hepatocellular carcinoma (HCC)-free survival rates in patients (—; $n = 25$) with and (---; $n = 32$) without eventual hepatitis B virus DNA clearance.

other two from liver failure due to aggravation of cirrhosis.

Of 13 patients with HCC development, two patients with HBV-DNA loss have not shown any tumor recurrence after surgical resection, and both patients are alive at the end of the observation. In contrast, nine (81.8%) of 11 patients with persistently high HBV-DNA developed HCC recurrence after therapy, and six (54.5%) of the patients died during the observation period. All six patients died from the development of HCC and none from aggravation of cirrhosis or extrahepatic disease.

Of 44 patients without HCC development until the end of the observation period, none of 23 patients with HBV-DNA loss died, but two (9.5%) of 21 patients with persistently positive HBV-DNA have died from liver failure.

Survival rates were compared between those patients with and without HBV-DNA loss (Fig. 6). Fifth-year survival rates in patients with and without HBV-DNA loss were 100% and 100%, seventh year rates were 100% and 90.5%, tenth year rates were 100% and 84.1%, and twelfth year rates were 100% and 80.6%, respectively. The cumulative survival rate in patients with HBV-DNA loss was significantly higher than that in patients without HBV-DNA clearance ($P = 0.0030$, log-rank test).

The HCC-free survival rates were also assessed in the two patient groups (Fig. 7). Fifth-year HCC-free survival rates in patients with and without HBV-DNA loss were 100% and 90.6%, seventh year rates were 100% and 81.3%, tenth year rates were 92% and 74.8%, and fifteenth year rates were 92% and 51.2%, respectively. The HCC-free survival rate in patients with HBV-DNA loss was significantly higher than that in patients without HBV-DNA clearance ($P = 0.0036$, log-rank test).

DISCUSSION

Until recently, several authors mentioned the anti-carcinogenic activity of IFN in patients with HBV-related cirrhosis. Oon[18] and Ikeda *et al.*[21] have shown that IFN significantly decreased carcinogenesis in patients undergoing IFN therapy with a relative risk of 0.03 and 0.39, respectively. Lin *et al.* 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.[23] Mazzella *et al.*,[19] Fattovich *et al.*[20] and the International Interferon-alpha Hepatocellular Carcinoma Study Group in Europe[22] demonstrated a low relative risk for carcinogenesis in patients with IFN therapy, but none could show a statistically significant difference. 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 carried out for the study of the clinical process and the mechanism of anti-tumor activity of IFN in HBV-positive cirrhosis patients.

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.[27] Although only two of 25 patients who developed HCC showed a disappearance of HBV-DNA during or after IFN therapy, 11 of 32 patients who showed carcinogenesis could not eliminate HBV-DNA using treatment with IFN ($P = 0.019$). A point in common found in the two exceptional patients with HCC development after elimination of HBV-DNA was that the HCC were detected immediately after a significant decrease in the HBV-DNA level after using IFN in the clinical courses: 1.2 years and 3.6 years after in each patient. We can reasonably consider that the discovered HCC in the patients already existed at an indiscernible size at the time of HBV-DNA elimination, and that the minimal HCC automatically grew gradually for the following few years after the decrease in HBV-DNA levels occurred. Even including these two patients with HCC development, the risk of hepatocellular carcinogenesis was significantly associ-

ated with the persistence of a high HBV-DNA concentration. Hepatocellular carcinogenesis was assessed using serial HBV-DNA assay with a cut-off value of 3.7 LGE/mL (or $10^{3.7}$ copy/mL) in this study. Although a detailed analysis of HBV-DNA concentration with a more sensitive measurement may demonstrate a better correlation with the carcinogenesis rate than the present study, setting the HBV-DNA concentration at this cut-off value was significantly valuable in the prediction for HCC appearance.

The mechanism of anticarcinogenic activity of IFN was regarded as an anti-necroinflammatory process through suppression of HBV-DNA concentration from these results. This study dealt with the relationship between carcinogenesis and HBV-DNA principally, but clinical courses of aminotransferase were also significantly related to the HCC development. Aminotransferase values were less valuable than HBV-DNA levels in the prediction of HCC development in the natural clinical course of HBV-cirrhosis,[27,28] and aminotransferase values were also less associated with the future rate of carcinogenesis in patients undergoing IFN therapy.

Although the mere use of IFN does not guarantee a decrease in the rate of carcinogenesis in patients with HBV-related cirrhosis, a serial course of HBV-DNA concentration was significantly correlated with future HCC development during and after treatment. The value of cancer prediction was much higher from 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 measuring HBV-DNA concentration were applied to the analysis, hepatocellular carcinogenesis could be more successfully predicted.

In conclusion, persistence of a high concentration of HBV-DNA was significantly associated with hepatocellular carcinogenesis in cirrhotic patients with IFN therapy, and its sequential analysis would be useful in the early detection of HCC. IFN therapy is recommended to be continued as long as possible until HBV-DNA loss occurs in HBV-cirrhosis patients, from the viewpoint of cancer prevention. Further studies with a greater number of patients are required to confirm the relationship, and future studies should be aimed at defining the role and basic mechanisms by which the carcinogenesis rate was suppressed by IFN in the cohort.

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Clinical and Virological Characteristics of Untreated Patients With Chronic Hepatitis C Who Develop Serum Alanine Aminotransferase Flare-up

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Among patients with chronic hepatitis C virus (HCV) infection, serum alanine aminotransferase (ALT) rarely increases above 500 IU/L. We examined the clinical and virological features of untreated patients with serum ALT ≥ 500 IU/L. One thousand seven hundred and sixty adult patients with chronic HCV infection were followed-up. Among these patients, 22 developed ALT flare-up (M:F = 13:9, median age, 50.5 years). We evaluated liver function tests, genotype, and viral titer in these patients and 44 randomly selected age- and sex-matched control without ALT flare-up. In four patients with ALT flare-up, we examined changes in viral loads and sequential changes in amino acid sequences of the core region, hypervariable region 1 (HVR1), and interferon sensitivity determining region (ISDR) before and after ALT flare-up. Multivariate analysis identified genotype 2 as the only significant determinant of ALT flare-up. ALT flare-up occurred in three of four patients without increase in viral load. Several alterations in amino acids were noted in HVR1 before and within 6 months of ALT flare-up. One or two alterations in the core region and many alterations in HVR1 were noted after ALT flare-up in some patients. Genotype 2 is an important factor for ALT flare-up. However, we could not directly relate ALT flare-up to these alterations in amino acids of the core region, HVR1, and ISDR. ***J. Med. Virol. 75:240–248, 2005.***
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KEY WORDS: chronic hepatitis C; alanine aminotransferase; flare-up; genotype

INTRODUCTION

Hepatitis C virus (HCV) is a major public health problem, affecting an estimated 170 million people worldwide and more than 10% of the population in some countries [Cohen, 1999]. HCV frequently causes persistent infection in adults leading to chronic hepatitis, liver cirrhosis, and even hepatocellular carcinoma (HCC) [Dusheiko, 1998; Ikeda et al., 1998; Niederau et al., 1998; Kenny-Walsh, 1999]. In infected patients, the liver cell damage is caused by HCV, although the exact mechanism remains poorly characterized.

Alanine aminotransferase (ALT) is an enzyme produced mainly in the liver. In individuals with a normal liver function, the serum activity of this soluble enzyme is at low levels. With hepatic injury, ALT leaks from the liver, causing elevation of serum ALT activity [Sherman, 1991]. Patients with chronic hepatitis C have either normal or abnormal ALT levels, which rarely include flare-up. However, compared with hepatitis B viral (HBV) infection patients, serum ALT levels could be ≥500 IU/L (≥10 times the normal level) during the natural course of the disease in untreated patients with chronic hepatitis C [Liaw and Tsai, 1997], although we rarely experience untreated patients with hepatitis C with such high serum ALT level of ≥500 IU/L. The clinical and virological characteristics of untreated HCV patients with natural flare-up of serum ALT values are not well defined.

Several studies have indicated that amino acid substitutions in some portions of the viral protein are related to the host cell, the host immune response, and/or, viral load [Enomoto et al., 1996; Saito et al., 1996; Chayama et al., 1997; Murakami et al., 1999; Patel et al., 1999; Terazawa et al., 2000; Watanabe et al., 2001;

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Boulestin et al., 2002; Kobayashi et al., 2002]. Therefore, it is important to examine the changes in the core region, hypervariable region 1 (HVR1), and the interferon sensitivity determining region (ISDR). The core region encodes the viral capsid protein and produces the core protein. The latter is a multifunctional protein that interacts with numerous cellular signal proteins such as the tumor necrosis factor-receptor 1, apolipoprotein AII [Ray and Ray, 2001]. In addition, the core protein also affects important cellular signal pathways that regulate the activities of nuclear factor κ-B, AP-1, mitogen activated protein, and Raf-1 kinases, p53, signal transducer and activator of transcription family proteins [Otsuka et al., 2000]. The core protein is known to modulate apoptosis of hepatocytes [Patel et al., 1999]. To investigate the relationship between ALT flare-up and hepatocyte apoptosis, we examined the changes in the core region. The HVR1 is part of the E2 region, and is thought to form envelope proteins. Previous studies suggested that the HVR1 is an epitope area targeted by the host immune system [Hijikata et al., 1991; Weiner et al., 1992] and appears to be the only defined target for neutralizing antibodies [Saito et al., 1996; Boulestin et al., 2002]. Others reported that the ISDR correlates with viral titer [Enomoto et al., 1996; Chayama et al., 1997; Murakami et al., 1999; Terazawa et al., 2000; Watanabe et al., 2001; Kobayashi et al., 2002]. However, the sequential changes in the core region, HVR1 and ISDR during ALT flare-up are poorly defined in patients with hepatitis C infection.

The present retrospective study was designed to characterize the clinical and virological features of patients with HCV with ALT flare-up to ≥500 IU/L who had otherwise not received antiviral therapy.

PATIENTS AND METHODS

Patients

Between August 1969 and August 2002, 1,760 anti-HCV-positive adult patients were hospitalized at Toranomon Hospital, Tokyo, Japan, and underwent laparoscopy or liver biopsy and were diagnosed with chronic hepatitis C infection. Patients infected with both HCV and HBV, hepatitis A viral (HAV) and those with autoimmune diseases, previous interferon (IFN) treatment for hepatitis, history of heavy alcohol abuse, drug abuse, herbal remedies, liver cirrhosis, and HCC on ultrasonography, coexisting cardiac, renal, pulmonary endocrine conditions were excluded from this study. Past ALT values measured before August 1993 were converted into a present value by using an exchange rate. The present normal range of serum ALT is 6–50 IU/L. ALT flare-up was defined as an increase in serum ALT to ≥500 IU/L (≥10 times the normal level) from <300 IU/L 3 months before the study in patients with chronic hepatitis C infection. None of the flare-up patients and control subjects had superinfection with several genotypes.

We retrospectively identified 22 patients with ALT flare-up (M:F = 13:9, median age, 50.5 years), and were enrolled in the study. The median observational period from the first medical examination to ALT flare-up was 10 months (range, 6–97 months). On the other hand, among 136 patients who had not received IFN therapy for ≥100 months among the remaining 1,738 patients (excluding patients with ALT flare-up), 44 patients were selected at random as the control group. The ALT levels were measured once a month in these patients. Moreover, for a nested case-control study design, patients of the control group were sex- and age-matched to patients with ALT flare-up. The profile of each group at the first medical examination is summarized in Table I.

Histopathological Examination of Liver Biopsies

The baseline liver histology of chronic hepatitis was classified into four stages according to the extent of fibrosis and the criteria of Desmet et al. [1994]. Stage 0 (F0): no fibrosis; stage 1 (F1): periportal expansion; stage

TABLE I. Clinical and Virological Features of Patients With and Without Alanine Aminotransferase (ALT) Flare-up (Nested Case-Control Study)

	With ALT flare (n = 22)	Without ALT flare (n = 44)	*P* value
Age[a]	50.5 (21–62)	50.5 (21–62)	—
Gender (male/female)	13/9	26/18	—
Source of infection (blood transfusion/unknown)	9/13	12/30	0.266
HCV genotype (2/other than 2)	15/7	12/32	0.0035
HCV RNA level (kIU/ml)[a]	1,400 (30–5,000<)	825 (<5–3,800)	0.093
Liver histology (F1/F2/F3/F4)	15/6/1/0	22/20/2/0	0.254
Albumin (g/dl)[a]	4.3 (3.5–4.7)	4.4 (3.5–5.5)	0.443
Total bilirubin (mg/dl)[a]	0.7 (0.3–1.3)	0.7 (0.3–1.7)	0.94
AST level (IU/L)[a]	94.5 (18–273)	95 (13–336)	0.715
ALT level (IU/L)[a]	151 (12–498)	160 (12–432)	0.948
γ-GTP (IU/L)[a]	48.5 (11–262)	35.5 (15–149)	0.089
Platelet count (×1,000 μm/L)[a]	180 (8–282)	169 (97–306)	0.608
Duration of follow-up (month)	132 (63–247)	159 (100–350)	0.029

[a]Data are expressed as median (range) at the first medical examination.

2 (F2): portoportal septa; stage 3 (F3): portocentral linkage or bridging fibrosis; stage 4 (F4): liver cirrhosis.

HCV Genotype and Quantitation of HCV-RNA by PCR-Based Assay

The serum samples of 66 patients (ALT flare-up group = 22 patients, control group = 44 patients) were stored at −80°C until measurement of serum HCV RNA level. HCV genotype was determined by PCR using the method described previously by Chayama et al. [1993]. Serum HCV RNA levels were measured quantitatively by a PCR-based assay using the protocol provided by the manufacturer (Amplicor HCV Monitor assay version 2.0, Roche Diagnostics, Tokyo, Japan). The samples were tested after 10-fold dilution to maintain the linear range of the assay. The detection limit for serum HCV RNA in this assay was 5–5,000 kIU/ml [Pawlotsky et al., 2000]. Moreover, serum HCV RNA levels in samples of four patients were measured during 6 months before and after ALT flare-up.

Nucleotide and Amino Acid Sequence Analyses of Core, HVR1, and ISDR

Serum samples of four patients were collected 6 months before ALT flare-up, at ALT flare-up, and 6 months after ALT flare-up (for Patient B only, the serum samples were collected 3 months before ALT flare-up). HCV genotype of two of these four patients was 1b and that of the other two was 2a. The nucleotide sequences of the core region, HVR1, ISDR of HCV were determined by direct sequencing. The primers used to amplify the core region were 5′-CTAGCCATGGCGT-TAGTATG-3′ and 5′-GTTCCCTGTTGCATAGTT-3′ as the first (outer) primer pair and 5′-GCCATAGTGGTC-TGCGGAAC-3′ and 5′-GTTCCCTGTTGCATAGTT-3′ as the second (inner) primer pair. Thirty cycles of first and second amplifications were performed as follows: denaturation for 1 min at 94°C, annealing of primers for 2 min at 53°C, and extension for 3 min at 72°C. Final extension was performed at 72°C for 7 min. The primers used to amplify HVR1 of genotype 1b were 5′-CTTGGGATAT-GATGATGAACTGG-3′ and 5′-CTGTCTCATTCTCCC-CCCAGCTATA-3′. The primers used to amplify HVR1 of genotype 2a were 5′-TGTGATGTCCGCCACGCTCT-3′ and 5′-ATCCACGTGCAGCCGAACCA-3′ as the first (outer) primer pair and 5′-CCGAGGTCATCATAGA-CATC-3′ and 5′-GTCGAGTGCTGTTCAATAGG-3′ as the second (inner) primer pair. Forty cycles of amplification were performed as follows: denaturation for 1 min at 94°C, annealing of primers for 2 min at 52°C, and extension for 3 min at 72°C. Final extension was performed at 72°C for 7 min.

Hemi-nested PCR was performed to determine the sequence of ISDR for genotype 1b using the sense primer, 5′-GGGTCACAGCTCCCATGTGAGCC-3′ and two antisense primers, 5′-CCCGTCCATGTGTAGGA-CAT-3′ and 5′-GAGGGTTGTAATCCGGGCGTGC-3′. Thirty-five cycles of first and second amplifications were performed as follows: denaturation for 1 min at 94°C, annealing of primers for 2 min at 53°C, and extension for 3 min at 72°C. Final extension was performed at 72°C for 7 min. Determination of the sequence of ISDR of genotype 2a was conducted using the method described by Akuta et al. [2003].

Statistical Analysis

Differences between groups were examined for statistical significance using the Mann–Whitney test (U-test) and χ^2-test where appropriate. Independent predictive factors associated with untreated patients with chronic hepatitis C who develop ALT flare-up were determined using multivariate multiple logistic regression. The following nine potential predictors were assessed in this study: HCV genotype, HCV RNA level, liver histology, albumin, total bilirubin, aspartate aminotransferase (AST), ALT, gamma-glutamyl transpeptidase (γ-GTP), and platelet count. Variables that achieved statistical significance ($P < 0.05$) or marginal significance ($P < 0.20$) on univariate analysis were subjected to multiple logistic regression analysis to identify significant independent predictors. The odds ratio (OR) and 95% confidence interval (CI) were calculated to assess the relative risk confidence. All analyses described above were performed using the SPSS program (version 7.5, SPSS, Inc., Chicago, IL).

RESULTS

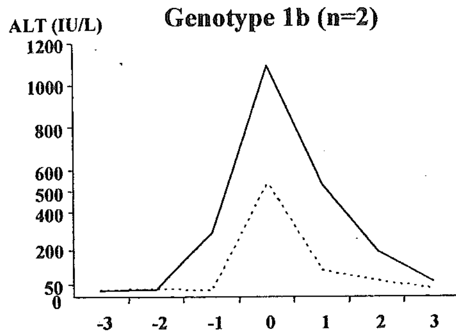
Clinical and Virological Features of Patients With ALT Flare-up

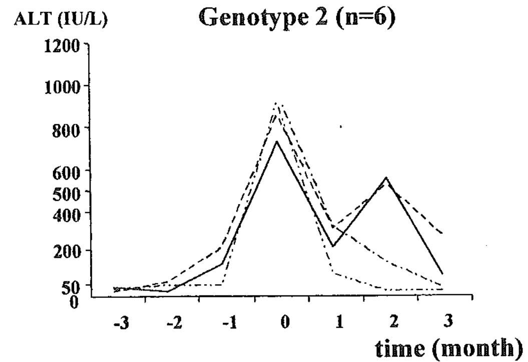
Table I lists the demographic and clinical characteristics of patients with ALT flare-up and the control group. Among the ALT flare-up group, 15 patients had HCV genotype 2 (13 patients with genotype 2a and 2 patients with genotype 2b), 6 patients had genotype 1b, and 1 patient had genotype 3b. On the other hand, among the control group, 12 patients had HCV genotype 2, 29 patients had genotype 1b, and 1 patient had genotype 3b. The proportions of patients of the ALT flare-up group with genotype 2 were significantly higher than those with other genotypes while the opposite was true for the control ($P = 0.0035$). HCV RNA levels and serum γ-GTP concentrations were higher in ALT flare-up group than control group ($P = 0.093$ and $P = 0.083$, respectively). On the other hand, there were no differences in the other factors between the two groups.

Pattern of ALT Changes

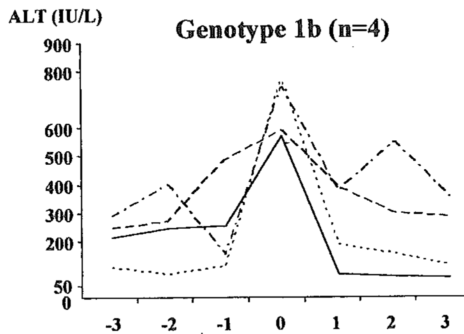
Among 22 patients of the ALT flare-up group, ALT levels were higher than normal in nine patients at 3 months prior to the present study. We classified patients of the flare-up group into two subgroups depending on the pattern of ALT flare-up. Figure 1 shows the fluctuation patterns of ALT concentrations in patients with ALT flare-up and different HCV genotypes. In pattern 1 (spike type), serum ALT increased to $\geq$500 IU/L from normal levels. This subgroup included six patients with genotype 2 and two patients with

Pattern 1 (spike type)





Pattern 2 (exacerbation type)



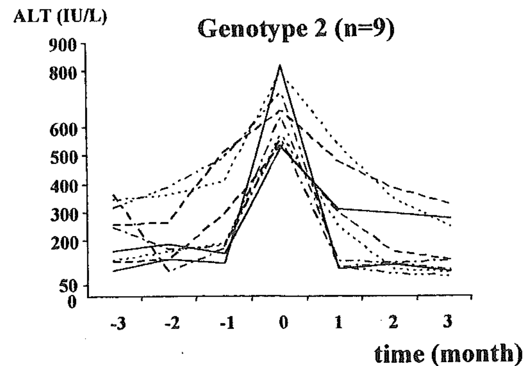


Fig. 1. Alanine aminotransferase (ALT) fluctuation patterns. Serum ALT flare-up to ≥500 IU/L showed two patterns. In pattern 1 (spike type), serum ALT increased to ≥500 IU/L from normal levels. This group included six patients with genotype 2 and two patients with genotype 1b. In pattern 2 (exacerbation type), ALT increased to ≥500 IU/L from a baseline of <500 to 50 IU/L. The group included nine patients with genotype 2 and four patients with genotype 1b.

genotype 1b. In pattern 2 (exacerbation type), ALT increased between <500 and 50 IU/L to ≥500 IU/L. This subgroup included nine patients with genotype 2 and four patients with genotype 1b. Using these definitions, 36.4% (8/22) showed pattern 1 and 63.6% (14/22) exhibited pattern 2. The genotype did not influence the ALT flare-up pattern.

Relationship Between Serum ALT Values and Serum HCV RNA Levels

Among 22 patients of ALT flare-up group, changes in HCV RNA levels were determined before and after (≥6 months) ALT flare-up in four patients. Figure 2 (A–D) shows the relationship between serum ALT concentrations and changes in viral load. Two patients (Patients B and C) showed ALT flare-up without an increase in viral load. On the other hand, the other patient (Patient A) developed ALT flare-up in association with increased viral load (an increase of 2,000 kIU/ml). However, the samples of Patient D taken 1 month before ALT flare-up were not available for analysis, therefore, changes in HCV RNA level could not be estimated in this patient. The viral load decreased after ALT flare-up in all patients. In particular, Patients A and B showed 2 log decreased in viral load.

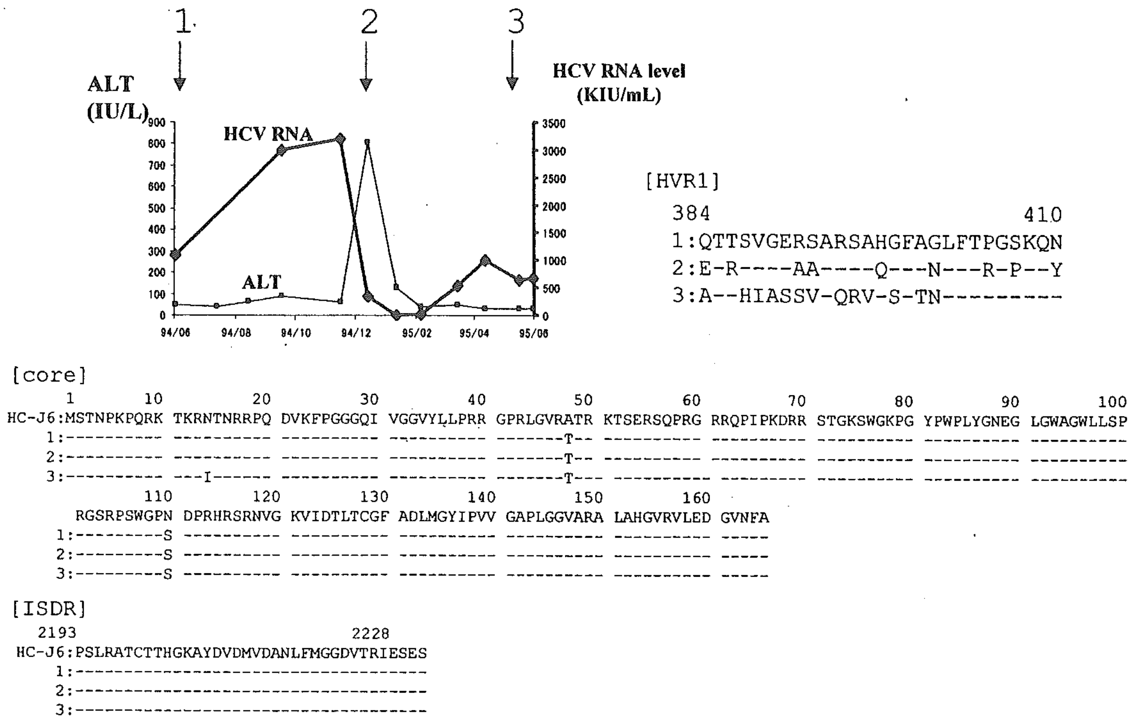
Amino Acid Sequence Substitutions in Core, HVR1, and ISDR

Figure 2 shows serial changes in ALT, HCV level, and amino acid sequences in four patients (A–D). Two patients were infected with genotype 2 of HCV (Patients A and B), while the others had HCV genotype 1 (Patients C and D). One mutation was noted in the core region in Patient A after ALT flare-up. In Patient C, two mutations were observed in the core region after ALT flare-up. However, no mutations of the core region were detected in Patients B and D at three points. In all patients, excluding patient C, various mutations in the HVR1 region were identified at 6 months after ALT flare-up. Amino acid alterations in HVR1 occurred sequentially between 6 months before and at ALT flare-up in these patients at a rate of 0.3–1.5 amino acids per month. However, amino acid alterations in HVR1 occurred sequentially between 6 months after and at ALT flare-up in three patients (Patients A, B, and D) at a rate of 1.3–3.1 amino acids per month. There was no mutation of ISDR region at all points in all patients.

Multivariate Analysis of ALT Flare-up

We explored the predictive factors for ALT flare-up. Among the nine factors examined in univariate analy-

A (genotype 2a)



B (genotype 2a)

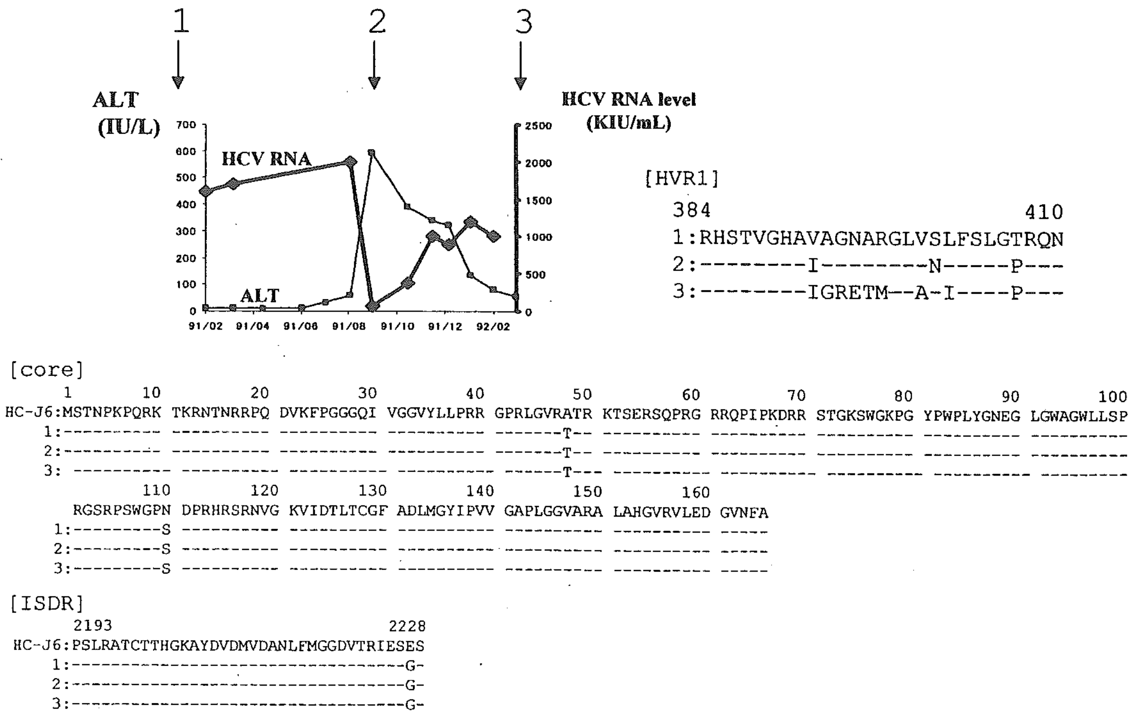
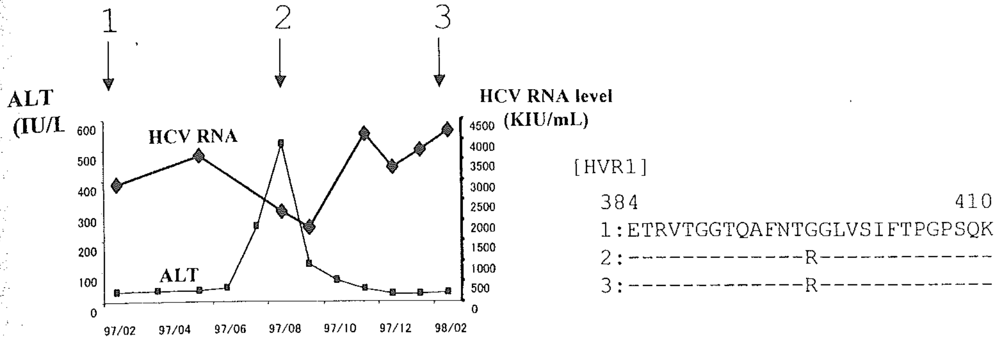


Fig. 2. Serial changes in ALT (thin line), hepatitis C virus (HCV) level (thick line), and amino acid sequences (by standard single letter codes) of core region (core), hypervariable region 1 (HVR1), and interferon sensitivity determining region (ISDR) in four patients (**A**–**D**). Serial changes in ALT (thin line) and HCV virus titer (thick line). All patients had high titers of HCV RNA load. Two patients were infected with genotype 2 of HCV (A and B), while the other patients were infected with genotype 1 (C and D).

C (genotype 1b)



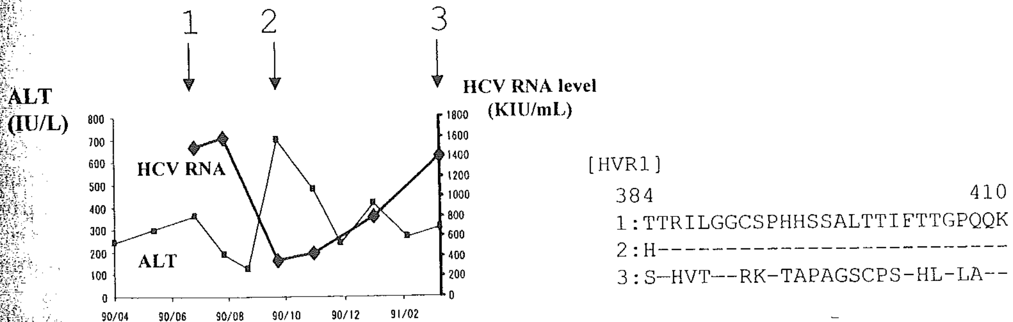
[core]

```
     1        10         20         30         40         50         60         70         80         90        100
HC-J:MSTNPKPQRK TKRNTNRRPQ DVKFPGGGQI VGGVYLLPRR GPRLGVRATR KTSERSQPRG RRQPIPKARR PEGRTWAQPG YPWPLYGNEG MGWAGWLLSP
   1:---------- ---------- ---------- ---------- ---------- ---------- ---------- ----A----- ---------- L--T------
   2:---------- ---------- ---------- ---------- ---------- ---------- ---------- ----A----- ---------- L--T------
   3:---------- ---------- ---------- ---------- ---------- ---------- ---------- ----A----- ---------- L--T------
            110        120        130        140        150
     RGSRPSWGPT DPRRRSRNLG KVIDTLTCGF ADLMGYIPLV GAPLGGAARA LAH
   1:---------- ---------- ---------- ---------- ---------- ---
   2:---------- ---------- ---------- ---------- ---------- ---
   3:C--------- ---------- -------G-- ---------- ---------- ---
```

[ISDR]

```
     2209                                 2248
HC-J:PSLKATCTTHH DSPDADLIE ANLLWRQEMG GNITRVESEN
   1:----------- --------- ---------- ----------
   2:----------- --------- ---------- ----------
   3:----------- --------- ---------- ----------
```

D (genotype 1b)



[core]

```
     1        10         20         30         40         50         60         70         80         90        100
HC-J:MSTNPKPQRK TKRNTNRRPQ DVKFPGGGQI VGGVYLLPRR GPRLGVRATR KTSERSQPRG RRQPIPKARR PEGRTWAQPG YPWPLYGNEG MGWAGWLLSP
   1:---------- ---------- ---------- ---------- ---------- ---------- ---------- ----A----- ---------- L--T------
   2:---------- ---------- ---------- ---------- ---------- ---------- ---------- ----A----- ---------- L--T------
   3:---------- ---------- ---------- ---------- ---------- ---------- ---------- ----A----- ---------- L--T------
            110        120        130        140        150
     RGSRPSWGPT DPRRRSRNLG KVIDTLTCGF ADLMGYIPLV GAPLGGAARA LAH
   1:---------- ---------- --V------- ---------- ------V--- ---
   2:---------- ---------- --V------- ---------- ------V--- ---
   3:---------- ---------- --V------- ---------- ------V--- ---
```

[ISDR]

```
     2209                                 2248
     PSLKATCTTHH DSPDADLIE ANLLWRQEMG GNITRVESEN
   1:---------R- --------- ---------- ----------
   2:---------R- --------- ---------- ----------
   3:---------R- --------- ---------- ----------
```

Fig. 2. (*Continued*)

..., only HCV genotype significantly influenced the ALT flare-up ($P = 0.0035$). The proportions of patients of the ALT flare-up group with genotype 2 were significantly higher than those with genotype 1b ($P = 0.0014$). In comparison, HCV RNA level and γ-GTP showed borderline significance with a higher chance of ALT flare-up ($P = 0.093$ and $P = 0.083$, respectively). As these three variables were mutually correlated, multivariate

analysis was performed. In the last step, the genotype was entered into the model and could not be removed ($P = 0.0033$).

DISCUSSION

Several studies reported ALT flare-up in hepatitis C patients during the natural course of the disease [Pontisso et al., 1999; Chen et al., 2001; Kuramoto et al., 2002; Fang et al., 2003; Hattori et al., 2003; Watanabe et al., 2003], but the ALT values of almost all reported patients were <500 IU/L. Only in one report the ALT value exceeded 500 IU/L [Rumi et al., 2002]. Moreover, ALT value of some patients with chronic hepatitis C infection flared-up to ≥500 IU/L from the normal range as measured at 3 months prior to the study. There are only a few reports of this phenomenon [Rumi et al., 2002]. In our study, 22 patients with chronic hepatitis C infection developed ALT flare-up and is thus the first large study on this phenomenon.

The pathogenetic mechanisms of ALT flare-up in HCV patients are difficult to explain. Previous studies have reported fluctuation of serum HCV RNA levels [Pontisso et al., 1999; Arase et al., 2000a; Kuramoto et al., 2002; Fang et al., 2003]. Arase et al. [2000a] reported that for patients with HCV RNA change of 1 log, they often show changes in ALT of >250 IU/L. Among our 22 patients, serum samples of only four patients were collected 6 months before ALT flare-up, at ALT flare-up, and 6 months after ALT flare-up. In one patient, the rise in serum ALT might have been due to increased viral levels. This conclusion is supported by the report of one patient in whom ALT increased with increased viral load as well as HBV [Liaw, 2003]. However, the serum level of ALT of the remaining three patients flared-up without a significant rise in viral load. Why did this happen? Hashimoto et al. [1999] examined the changes in HVR1 and ISDR in patients whose ALT changed approximately 100 IU/L. They reported that the rates of change of HVR1 were from 0.7 to 2 amino acids per month while there were no amino acid substitutions in ISDR. Our results regarding the rate of change of HVR1 and ISDR were similar to the above study. On the other hand, Kato et al. [1992] reported that amino acid alterations in HVR1 occurred sequentially during the chronic state of hepatitis at a rate of 0.5–1.7 amino acids per month. Our data showed the same rate of alterations between before and 6 months after ALT flare-up. Therefore, it is difficult to explain the mechanism of ALT flare-up by these alterations of amino acids of the core region, HVR1, and ISDR and it was unclear whether the virus factor was the cause of ALT flare-up.

The immune response against HCV is characterized by the generation of HCV-specific antibodies, cytotoxic T lymphocytes, and CD4 lymphocytes and by the production of IFN-γ [Freeman et al., 2001]. The importance of each of these components of the immune response, with regard to clearance of HCV infection is not clear because all have been demonstrated in individuals with chronic infection. Therefore, we assume that ALT flare-up is associated with activation of the aforementioned immunoresponses, resulting in reduction of HCV RNA level. On the other hand, it is thought that amino acids of the core region hardly mutate [Ina et al., 1994]. However, in two of four of our patients, part of the core protein was mutated and many amino acids of HVR1 were altered after ALT flare-up in three of four patients. This might also represent the outcome of extreme immune reaction by severe ALT flare-up. Unfortunately, we did not examine peripheral blood lymphocytes in this study. Further studies are required to clarify the relationship between the immune response and hepatitis.

We previously reported that HCV RNA level decreased by 2 log immediately after the increase in ALT [Hashimoto et al., 1999]. In the present study, we reported similar results after ALT flare-up. Another study from our laboratories showed that IFN therapy was effective in these patients [Arase et al., 2000b]. Thus, patients with ALT flare-up should be treated with IFN.

In Japan, about 70% of patients with HCV are genotype 1b, and about 25% are genotype 2a [Akuta et al., 2002]. The distribution of HCV genotype of our control group was as well as that of HCV patients in Japan. Why did HCV patients with genotype 2 have ALT flare-up more frequently than patients with genotype 1b? In general, the HCV gene is classified into six types by the classification of Simmonds [1995]. The HCV genotype has been reported to influence the response to IFN therapy and clinical course of infection [Tokita et al., 1994; Silini et al., 1995; Kobayashi et al., 1996; Akuta et al., 2002]. Previous studies showed a better response to IFN therapy in patients with genotype 2 than those with other genotypes [Tsubota et al., 1994; Akuta et al., 2002]. Moreover, differences between genotypes 1b and 2a were reported with regard to the immune reaction to HVR1 of *HCV* gene [Yoshioka et al., 1997]. Considered together, we speculate that the host immune response may be different among HCV genotypes.

In this study, we showed the clinical characteristics of 22 patients with ALT flare-up to ≥500 IU/L. The frequency of genotype 2 in these patients was about five times more than that of patients with genotype 1b.

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Hepatocyte Steatosis Is an Important Predictor of Response to Interferon (IFN) Monotherapy in Japanese Patients Infected With HCV Genotype 2a: Virological Features of IFN-Resistant Cases With Hepatocyte Steatosis

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The role of hepatocyte steatosis in interferon (IFN) resistance is still unclear, especially in patients infected with hepatitis C virus (HCV) genotype 2a. The present study was conducted in 364 consecutive non-cirrhotic naive patients infected with genotype 2a, who were evaluated for the severity of steatosis and response to IFN monotherapy after a 24-week median duration of therapy. The patients were examined for factors associated with steatosis and treatment efficacy according to the grade of steatosis. Early viral kinetics was also evaluated in 64 patients for predictors of response to therapy. Nine IFN-resistant patients were assessed for the relationship between amino acid sequence of HCV core region/NS5A and severity of steatosis. Multivariate analysis identified two independent factors associated with steatosis; serum ferritin $\geq$200 μg/l and body mass index $\geq$25.0 kg/m^2. The sustained virological response rate in patients with high-grade steatosis was significantly lower than in the low-grade group. Study of early viral kinetics showed a significantly lower cumulative HCV-RNA negative rate for the high-grade than low-grade steatosis group. Sequence analysis of HCV core region/NS5A in IFN-resistant patients with or without steatosis failed to identify steatosis-specific amino acid substitutions associated with resistance. This study of HCV genotype 2a suggested that steatosis is associated with excess iron storage, and that it is an important predictor of efficacy of IFN monotherapy. Further large-scale studies are warranted to examine the role of amino acid substitutions on IFN resistance specific for steatosis. ***J. Med. Virol. 75:550–558, 2005.***
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KEY WORDS: HCV; genotype 2a; interferon monotherapy; hepatocyte steatosis; body mass index; ferritin; early viral kinetics; core; NS5A

INTRODUCTION

The response to interferon (IFN) therapy varies according to the genotype of hepatitis C virus (HCV) [Simmonds, 1997; Haydon et al., 1998]. In Japan, about 70% of patients with chronic hepatitis C are infected with HCV genotype 1b, and about 25% are genotype 2a. Sustained virological response to IFN monotherapy is as low as 10–20% in genotype 1b infection, but is more than 60% in genotype 2 infection [Kanai et al., 1992; Hino et al., 1994; Mahaney et al., 1994; Tsubota et al., 1994; Akuta et al., 2002]. However, physicians also sometime encounter IFN resistant patients infected with genotype 2a [Akuta et al., 2002, 2003].

Hepatocyte steatosis (i.e., fatty degeneration of hepatocytes) was highlighted recently as an important pretreatment predictor of response to IFN therapy [Akuta et al., 2002; Poynard et al., 2003; Patton et al., 2004]. Akuta et al. [2002] evaluated previously 394 consecutive non-cirrhotic naive patients infected with genotype 2a, who received IFN monotherapy for 24 weeks, including initial aggressive induction therapy. That study was the first report to show that hepatocyte steatosis was a

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negative predictor of sustained virological response to IFN monotherapy in patients infected with genotype 2a, based on multivariate analysis. However, how hepatocyte steatosis alters the response to IFN therapy in patients infected by genotype 2a is still unclear. Hence, evaluation of the clinical and virological differences between IFN resistant cases with and without hepatocyte steatosis may be important for identifying the resistance mechanism specific for hepatocyte steatosis.

Previous reports have shown that HCV core region and NS5A might be associated with the pathogenesis of derangement of lipid metabolism, contributing to hepatocyte steatosis in hepatitis C [Barba et al., 1997; Moriya et al., 1997; Shi et al., 2002], but it is still unknown whether these regions could affect the IFN efficacy in patients with hepatocyte steatosis infected by genotype 2a. Therefore, in order to examine the IFN-resistance mechanism specific for hepatocyte steatosis, the relationship between amino acid substitutions of HCV core region/NS5A and the severity of hepatocyte steatosis in IFN-resistant cases was studied.

The present study included 364 consecutive naive patients with chronic hepatitis C of genotype 2a strain, who were treated with IFN alone. The aims of the study were as follows: (1) To examine the factors associated with hepatocyte steatosis in HCV genotype 2a, including viral and host factors; (2) to investigate the sustained virological response rates and the early viral kinetics as early predictors of sustained virological response to IFN monotherapy in HCV genotype 2a [Akuta et al., 2002], according to the grade of hepatocyte steatosis; and (3) to study the virological differences between IFN-resistant cases in HCV genotype 2a with and without hepatocyte steatosis.

PATIENTS AND METHODS

Study Population

Three hundred ninety-four Japanese non-cirrhotic naive patients infected with HCV genotype 2a, among 2,264 consecutive HCV-infected patients who underwent IFN monotherapy were evaluated between 1987 and 2001 at Toranomon Hospital [Akuta et al., 2002]. In the present study, 364 of 394 patients of the previous study were selected based on the following criteria: (1) Patients infected with HCV genotype 2a only; (2) patients naive to IFN therapy; (3) patients evaluated for the grade of hepatocyte steatosis; (4) patients with chronic hepatitis, without cirrhosis or hepatocellular carcinoma (HCC), as confirmed by biopsy examination within 6 months of enrolment; (5) patients negative for hepatitis B surface antigen (radioimmunoassay, Dainabot, Tokyo, Japan), positive for anti-HCV (third-generation enzyme immunoassay, Chiron Corp, Emerville, CA), and positive for HCV-RNA qualitative analysis with PCR (nested polymerase chain reaction or Amplicor^{TM}, Roche Diagnostic Systems, CA); (6) patients free of coinfection with the human immunodeficiency virus; (7) patients who have not been treated with antiviral or immunosuppressive agents within 6 months of enrolment; (8) lifetime cumulative alcohol intake <500 kg (mild to moderate alcohol intake); (9) patients free of other forms of hepatitis, including hemochromatosis, Wilson disease, primary biliary cirrhosis, alcoholic liver disease, and autoimmune liver disease; and (10) patients without or with well-controlled diabetes.

In this protocol, 59 patients (16.2%) received IFN therapy every day for 8 weeks; while 290 patients (79.7%) received IFN for 24 weeks (every day for 2 or 8 weeks, followed by three times per week for 22 or 16 weeks); and the remaining 15 patients (4.1%) received IFN therapy for more than 24 weeks (every day for 2 or 8 weeks, followed by three times per week). Furthermore, 270 patients (74.2%) received IFN-α alone; 82 patients (22.5%) received IFN-β alone; while the remaining 12 patients (3.3%) received IFN-β followed by IFN-α. A median IFN dose of 6 million units (MU) per day (range, 6–10 MU) was administered. As a whole, a median total dose of IFN of 600 MU (range, 306–1,815 MU) was administered during a median period of 24 weeks (range, 8–78 weeks).

The study protocol was approved by the Human Ethics Review Committee of Toranomon Hospital, and an informed consent was obtained from each subject. Table I summarizes the profiles and data of the 364 patients at the start of IFN monotherapy. The study included 239 men and 125 women, aged 17–68, of a median age of 51 years. None of the patients was an intravenous drug user.

The primary measure of efficacy of treatment was sustained virological response, defined as negative by HCV-RNA qualitative analysis with PCR at 24 weeks after cessation of IFN therapy.

Early Viral Kinetic Studies Based on Severity of Hepatocyte Steatosis

Early viral kinetic studies according to the grade of hepatocyte steatosis were performed. Of the 364 patients, 64 were selected based on the following criteria: (1) Consecutive patients closely monitored by HCV-RNA qualitative analysis with PCR before therapy (day 0); at day 1 and, 2, and week 1, 2, 4, 8 of therapy; and 24 weeks after the completion of therapy between 1996 and 2001; (2) patients received initial induction therapy every day for 8 weeks; and (3) patients received IFN dose of 6 or 7.5 MU per day. They were divided into two groups of low-grade hepatocyte steatosis (none to mild) and high-grade (moderate to severe), and were compared for the cumulative HCV-RNA negative rates by qualitative analysis with PCR.

Virological Studies in IFN Resistant Cases Based on Grade of Hepatocyte Steatosis

Virological studies were conducted in cases resistant to IFN therapy according to the grade of hepatocyte steatosis. Nine of 364 patients were selected as resistant cases based on the following criteria: (1) Patients who could not achieve sustained virological response despite the ideal total IFN dose of more than 500 MU;

TABLE I. Patient Profile and Laboratory Data at Commencement of Interferon Monotherapy

Demography	
Number	364
Sex (M/F)	239/125
Age (years)[a]	51 (17–68)
History of blood transfusion	150 (41.2%)
Familial history of liver disease	65 (17.9%)
Body mass index (kg/m^2)[a]	23.1 (16.3–35.7)
Body surface area (m^2)[a]	1.70 (1.22–2.19)
Laboratory data[a]	
Alanine aminotransferase (IU/l)	83 (8–642)
Albumin (g/dl)	4.0 (2.7–5.1)
Cholinesterase (ΔpH)	1.1 (0.4–1.9)
Hemoglobin (g/dl)	14.6 (9.6–18.3)
Platelet count (×1,000 μ/L)	178 (43–331)
Serum iron (μg/dl)	153 (16–355)
Unsaturated iron-binding capacity (μg/dl)	192 (24–509)
Serum ferritin (μg/l)	128 (<10–2008)
Level of viremia (Meq/ml)	0.8 (<0.5–43.5)
Histological findings	
Stage (F1/F2/F3)[b]	259/81/24
Hepatocyte steatosis (none/mild/moderate/severe)	44/291/29/0

[a]Expressed as median (range).
[b]Stage of chronic hepatitis by Desmet et al. [1994].

(2) patients were cases without steatosis (none) or with the highest grade of steatosis (moderate); and (3) the body mass index was less than 25.0 kg/m^2 (i.e., non-obese patients). Previous studies showed that the body mass index might influence the grade of hepatocyte steatosis and the response to IFN therapy [Akuta et al., 2002; Bressler et al., 2003]. Therefore, to examine the IFN-resistance mechanism specific for hepatocyte steatosis, the relationship between amino acid substitutions of HCV core region/NS5A and the grade of hepatocyte steatosis was assessed only in resistant non-obese cases so as to minimize the influence of obesity.

Laboratory Investigations

Blood samples were frozen at −80°C within 4 hr of collection and were not thawed until used for testing. HCV genotype was determined by PCR using a mixed primer set derived from nucleotide sequences of NS5 region [Chayama et al., 1993]. In all cases, HCV-RNA viremia level was measured by branched DNA assay version 2.0 (Chiron Corp., Emeryville, CA) at commencement of therapy using frozen samples, and the results were expressed as 10^6 genomic equivalents per milliliter (Meq/ml). The lower limit of the assay was 0.5 Meq/ml. Samples were evaluated by HCV-RNA qualitative analysis with PCR (nested polymerase chain reaction or Amplicor™, Roche Diagnostic Systems, CA) during and after therapy, and the results were expressed as positive or negative. The lower limit of the assay was 100 copies/ml. With regard to the IFN resistant cases, samples obtained at the commencement of therapy were also evaluated by quantitative analysis of HCV-RNA with PCR (Amplicor HCV-RNA kit, version 2.0, Roche Diagnostics). The lower limit of the assay was 0.5 kIU/ml.

Histopathological Examination of the Liver

Liver biopsy specimens were obtained percutaneously or at peritoneoscopy using a modified Vim Silverman needle with an internal diameter of 2 mm (Tohoku University style, Kakinuma Factory, Tokyo, Japan), fixed in 10% formalin, and stained with hematoxylin and eosin, Masson's trichrome, silver impregnation, and periodic acid-Schiff after diastase digestion. All specimens for examination contained six or more portal areas. Histopathological diagnosis was made by an experienced liver pathologist (H.K.) who was blinded to the clinical data. Chronic hepatitis was diagnosed based on histopathological assessment according to the scoring system of Desmet et al. [1994]. Hepatocyte steatosis was classified into four grades based on the criteria of D'Alessandro et al. [1991]: none (absent), mild (involvement of less than 1/3 of hepatocytes), moderate (involvement of greater than 1/3 but less than 2/3 of hepatocytes), or severe (involvement of greater than 2/3 of hepatocytes).

Nucleotide Sequencing of the Core Region and NS5A Gene

As described in previous reports with some modifications [Chayama et al., 1997; Murakami et al., 1999], the sequences of amino acids 1–191 in the core region [Rubbia-Brandt et al., 2000] and amino acids 2163–2254 in the NS5A [Akuta et al., 2003] were determined by the direct sequencing method using sera of nine patients. The sequences of amino acids were compared with the consensus sequence of genotype 2a, which were determined by comparing the sequences obtained in this study and prototype sequence (HC-J6) [Okamoto et al., 1991]. HCV-RNA was extracted with a SepaGene RV-R kit (Sanko Junyaku, Tokyo, Japan) from serum samples