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.***

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′-CTAGCCATGGCGTTAGTATG-3′ and 5′-GTTCCCTGTTGCATAGTT-3′ as the first (outer) primer pair and 5′-GCCATAGTGGTCTGCGGAAC-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′-CTTGGGATATGATGATGAACTGG-3′ and 5′-CTGTCTCATTCTCCCCCCAGCTATA-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′-CCGAGGTCATCATAGACATC-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′-CCCGTCCATGTGTAGGACAT-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 genoytypes. In pattern 1 (spike type), serum ALT increased to ≥500 IU/L from normal levels. This subgroup included six patients with genotype 2 and two patients with

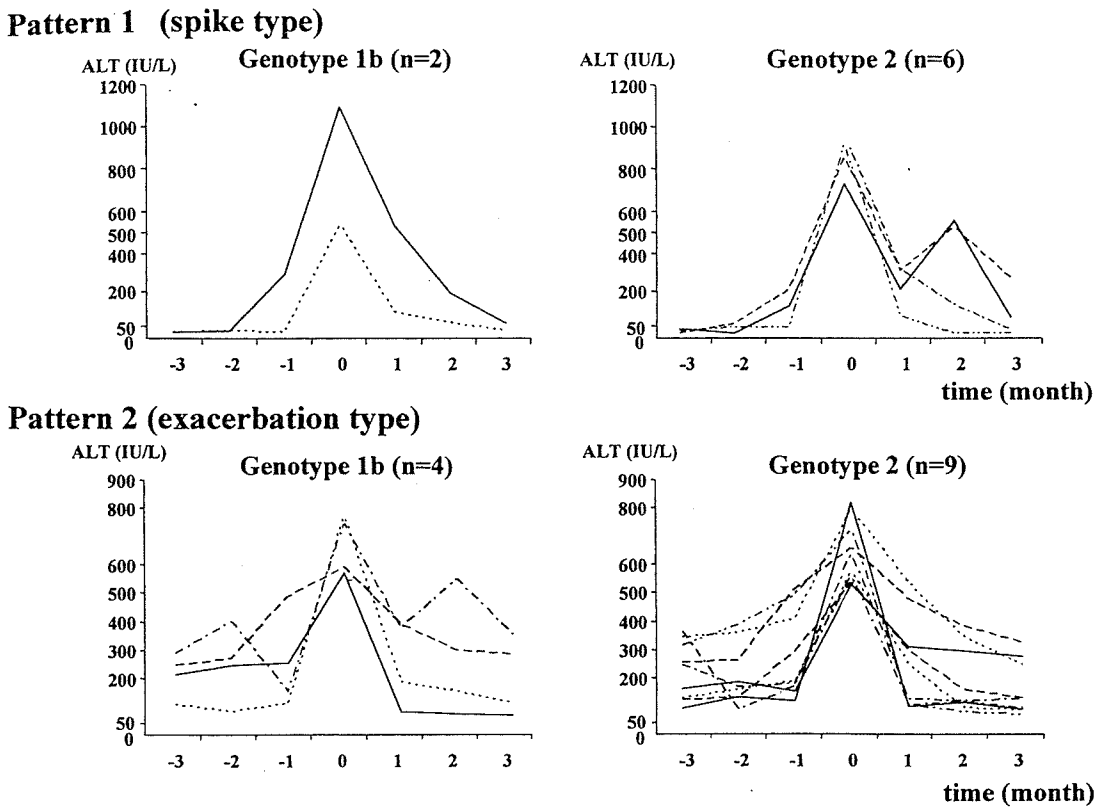

Fig. 1. Alanine aminotransferase (ALT) fluctuation patterns. Serum ALT flare-up to ≥500 IU/L showed two patterns. In pattern 1 (spike type), serum ALT increased to ≥500 IU/L from normal levels. This group included six patients with genotype 2 and two patients with genotype 1b. In pattern 2 (exacerbation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

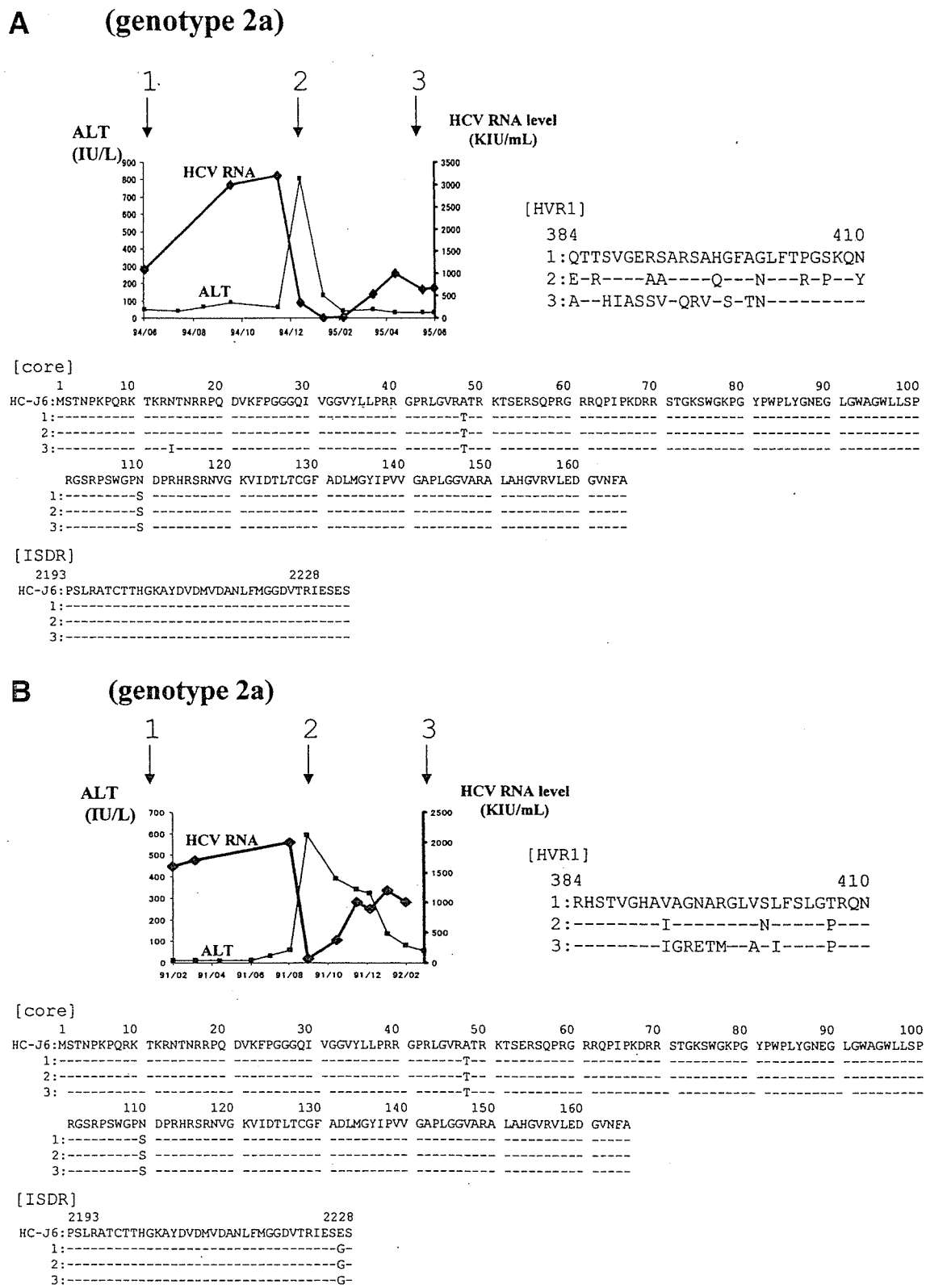

Fig. 2. Serial changes in ALT (thin line), hepatitis C virus (HCV) level (thick line), and amino acid sequences (by standard single letter codes) of core region (core), hypervariable region 1 (HVR1), and interferon sensitivity determining region (ISDR) in four patients (**A–D**). Serial changes in ALT (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).

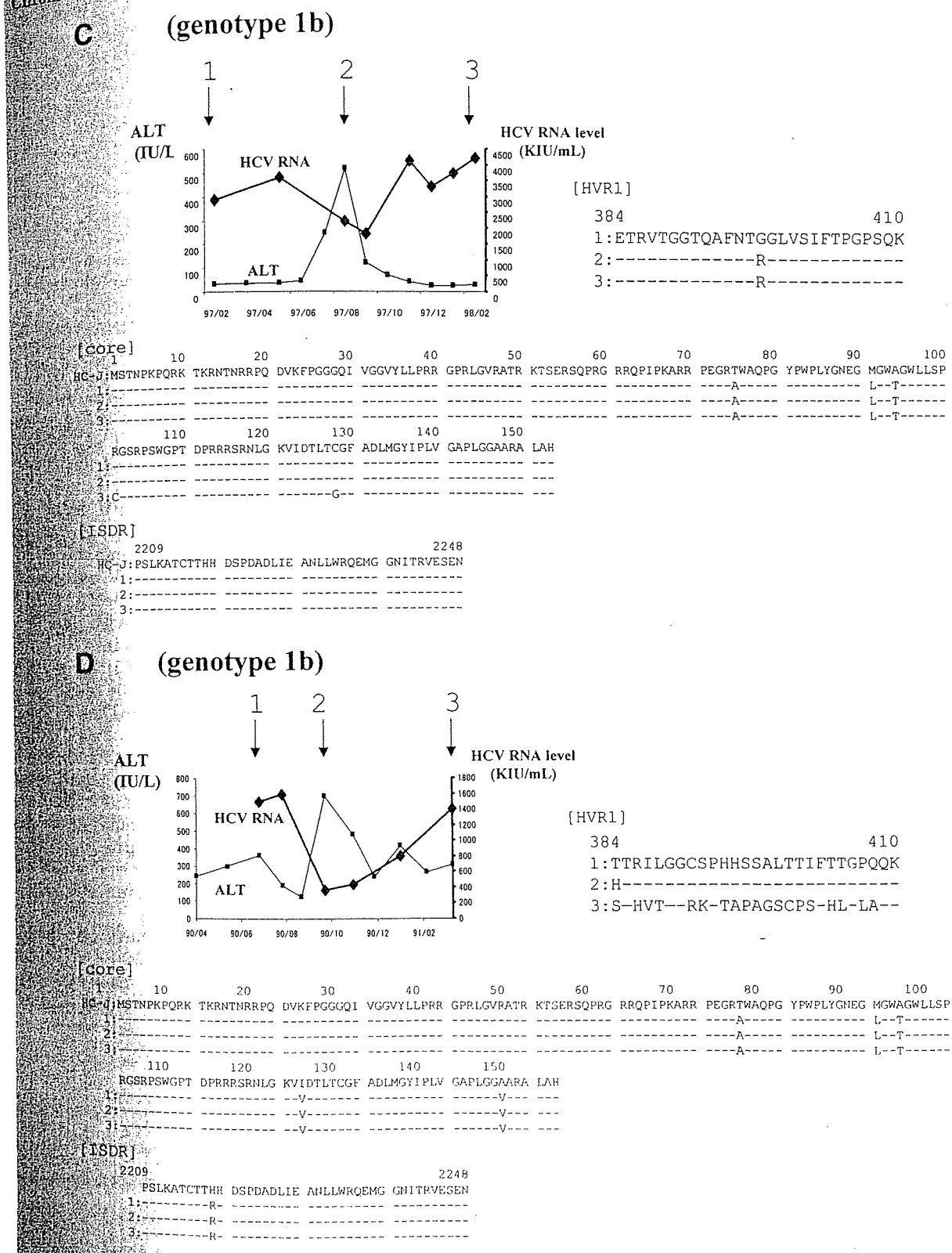

Fig. 2. (*Continued*)

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.***

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™, 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

at the start of treatment and reverse transcribed with random primer and MMLV reverse transcriptase (Takara Syuzo, Tokyo, Japan). Nucleic acids were amplified by PCR using the following primers.

Nucleotide sequences of the core region. Nucleic acids of the Core region were amplified by division into two parts of the N- and C-terminal regions. The first-round PCR in the N-terminal region was performed with 2aC1Fo (sense, 5′-TGCTAGCCGAGTAGCGTTGG-3′) and 2aC1Ro (antisense, 5′-TTCACTTCGGCAGCGGAGAC-3′), and the second-round PCR with 2aC1Fi (sense, 5′-CTTGTGGTACTGCCTGATAG-3′) and 2aC1Ri (antisense, 5′-CAGTGGAGCGCCGATCCTTA-3′). The first-round PCR in the C-terminal region was performed with 2aC2Fo (sense, 5′-CCAGATCGTTGGCGGAGTAT-3′) and 2aC2Ro (antisense, 5′-TCCAGCACCGAGATGTATTC-3′), and the second-round PCR with 2aC2Fi (sense, 5′-TATACTTGTTGCCGCGCAGG-3′) and 2aC2Ri (antisense, 5′-AGTCGTTGGTCACCATGTAG-3′).

Nucleotide sequences of the NS5A. The first-round PCR was performed with 5′ outer primer (sense, 5′-CCAGA(AG)TT(CT)TT(CT)TC(CT)TGGGTGGATGG-3′) and 3′ outer primer (antisense, 5′-GGTT(CG)(AG)TA(GA)(CT)C(CT)GGCCTCTTCCA-3′), and the second-round PCR with 5′ inner primer (sense, 5′-TGTAAAACGACGGCCAGTCAGCTCCCTTGCGATCCTGA-3′ with the sequence of the M13 forward primer underlined) and 3′ inner primer (antisense, 5′-CAGGAAACAGCTATGACC(AT)GG(GA)TTGTA(AG)TC(AT)GG(AC)CG(GT)GCCCA-3′ with the sequence of the M13 reverse primer underlined). All samples were initially denatured at 95°C for 15 min. Forty cycles of amplification were set as follows: Denaturation for 1 min at 94°C, annealing of primers for 1 min at 55°C, and extension for 1 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 reaction. Other conditions for the second PCR were the same as the first PCR, except that the second PCR primers were used instead of the first PCR primers. PCR-amplified complementary DNA (cDNA) was purified after agarose gel electrophoresis and used for direct sequencing using each of the second-round PCR primer or M13 primer as the sequencing primer with the Big Dye Deoxy Terminator Cycle Sequencing kit (Perkin-Elmer, Tokyo, Japan). To avoid false-positive results, the procedures recommended by Kwok and Higuchi [1989] to prevent contamination were strictly applied to these PCR assays. No false-positive results were observed in this study.

Statistical Analysis

Non-parametric tests were used to examine the background characteristics of patients and amino acids changes, including the chi-squared test or Fisher's exact probability test, and Mann–Whitney U-test. The cumulative HCV-RNA negative rates by qualitative analysis with PCR were calculated using the Kaplan–Meier technique, and differences between the curves were tested using the log-rank test. Univariate and multivariate logistic regression analyses were used to determine those factors associated with hepatocyte steatosis. All P-values of less than 0.05 by the two-tailed test were considered significant. The odds ratios and 95% CI were also calculated. 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 factors associated with hepatocyte steatosis included the following 16 variables: sex, age, history of blood transfusion, family history of liver disease, body mass index, body surface area, alanine aminotransferase (ALT), albumin, cholinesterase, hemoglobin, platelet count, serum iron, unsaturated iron binding capacity, ferritin, level of viremia, and pathological staging. Statistical comparisons were performed using the SPSS software (SPSS Inc., Chicago, IL).

RESULTS

Factors Associated With Hepatocyte Steatosis in Multivariate Analysis

Data of patients classified with low-grade steatosis (none [n = 44] to mild [n = 291]) and high-grade (moderate [n = 29] to severe [n = 0]) were examined to determine the factors associated with steatosis. Univariate analysis identified three parameters that significantly influenced hepatocyte steatosis. These included age ($P = 0.001$), serum ferritin level ($P = 0.002$), and body mass index ($P = 0.016$). Of these, multivariate analysis identified serum ferritin level ($P = 0.004$) and body mass index ($P = 0.049$) as two parameters that independently influenced hepatocyte steatosis (Table II).

Treatment Efficacy According to Severity of Steatosis and IFN Regimen

As a whole, 249 of 364 (68.4%) patients achieved sustained virological response, while the remaining 115

TABLE II. Factors Associated With Hepatocyte Steatosis in 364 Patients Infected With HCV Genotype 2a, Identified by Multivariate Analysis

Factor	(Category)	Odds ratio (95% CI)	P
Serum ferritin (μg/L)	1: <200	1	
	2: ≥200	3.48 (1.50–8.07)	0.004
Body mass index (kg/m^2)	1: <25.0	1	
	2: ≥25.0	2.37 (1.00–5.57)	0.049

Variables that achieved statistical significance ($P < 0.05$) on multivariate logistic regression are shown.

TABLE III. Sustained Virological Response Rates Estimated by Interferon Treatment Regimens in 364 Patients Infected With HCV Genotype 2a

		Continuous[a] + intermittent[b]	
Grade of steatosis	8 weeks continuous	24 weeks	>24 weeks
Moderate to severe	25.0% (1/4)	44.0% (11/25)	—
None to mild	61.8% (34/55)	72.1% (191/265)	80.0% (12/15)

[a]2- or 8-week continuous.
[b]Three times a week.

patients (31.6%) failed to respond to therapy. Among 335 patients with the low-grade hepatocyte steatosis (grade none to mild), 237 (70.7%) achieved sustained virological response, while of 29 patients with high-grade steatosis (grade moderate to severe), only 12 (41.4%) showed sustained virological response. The rate of sustained virological response in patients with low-grade steatosis was significantly higher than in those with high-grade steatosis ($P = 0.0017$). The clinicopathological characteristics (16 variables, evaluated as potential risk factors of steatosis) of non-sustained virological response patients (115 patients) at the start of treatment of low-grade (98 patients) and high-grade (17 patients) steatosis groups were not significantly different.

With regard to the efficacy of monotherapy according to IFN regimen in 335 patients with low-grade steatosis, sustained virological response rate was 61.8% in patients who received daily IFN therapy for only 8 weeks. In patients on continuous followed by intermittent therapy, 72.1% achieved sustained virological response after 24 weeks of treatment, and 80.0% showed sustained virological response after >24 weeks of treatment (Table III).

Early Viral Kinetics and Severity of Hepatocyte Steatosis

Table IV shows the features of patients who were evaluated for early viral kinetics. The cumulative HCV-RNA negative rates of the low- and high-grade hepatocyte steatosis were 10.0% and 11.1% at day 1; 10.0% and 13.0% at day 2; 20.0% and 40.7% at week 1; 30.0% and 75.9% at week 2; 60.0% and 94.4% at week 4; and 80.0% and 98.2% at week 8, respectively. Statistical analysis showed that the cumulative HCV-RNA negative rate was significantly higher in the low-grade group than the high-grade steatosis group ($P = 0.0055$; Log-rank test, Fig. 1).

Virological Features of Cases Resistant to IFN According to Severity of Steatosis

Figure 2A,B show the amino acid sequences of HCV core region/NS5A of 9 IFN-resistant patients. In eight patients, the level of viremia was more than 100 kIU/ml, while in the remaining patient (Case 5, who was free of liver steatosis), the level of viremia was 76 kIU/ml. Heterogeneity of two cases or more was found at the following amino acid positions: amino acid 4 (Cases 6

TABLE IV. Clinical and Virological Features of 64 Patients Who Were Evaluated for Early Viral Kinetics, According to the Grade of Hepatocyte Steatosis

	Grade of hepatocyte steatosis		
	None to mild	Moderate to severe	Differences
N	54	10	NS
Age (years)[a]	17–67 (50)	41–66 (52)	NS
Sex (M/F)	31/23	3/7	NS
Body mass index (kg/m^2)	16.8–32.6 (22.2)	19.2–31.1 (25.3)	$P = 0.049$
Level of viremia level (Meq/ml)[b]	<0.5–22.0 (2.2)	<0.5–18.0 (5.8)	NS
Histological staging (F1/F2/F3)[c]	45/6/3	7/1/2	NS
ALT level (IU/l)[a]	15–618 (64)	31–175 (64)	NS
Serum albumin (g/dl)[a]	3.1–4.5 (3.9)	3.3–4.0 (3.6)	$P = 0.024$
Cholinesterase (ΔpH)[a]	0.5–1.8 (1.1)	0.8–1.4 (1.1)	NS
Hemoglobin (g/dl)[a]	9.6–18.3 (14.0)	12.9–15.8 (14.0)	NS
Platelet count (×1,000 μ/L)[a]	89–304 (176)	113–229 (163)	NS
Serum iron (μg/dl)	31–264 (146)	64–283 (196)	NS
Unsaturated iron-binding capacity (μg/dl)	61–484 (198)	24–331 (112)	NS
Serum ferritin (μg/l)	<10–1,417 (102)	<10–698 (243)	NS
IFN dose per day (MU/day)	6–7.5 (6)	6–7.5 (6)	NS

ALT, alanine aminotransferase; IFN, interferon; MU, million units; NS, not significant.
[a]Expressed as range (median).
[b]Viral levels measured by branched DNA assay version 2.0.
[c]Stage of chronic hepatitis by Desmet et al. [1994].

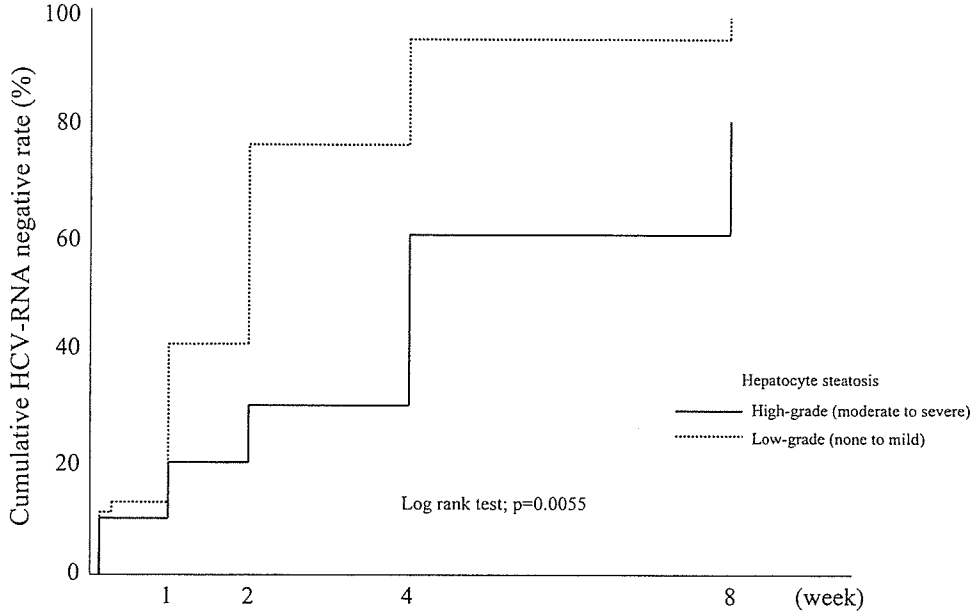

Fig. 1. In early viral kinetic study during IFN monotherapy, the cumulative HCV-RNA negative rate of the low-grade (none to mild) hepatocyte steatosis was significantly higher than that of the high-grade (moderate to severe) steatosis.

and 7; I/N), amino acid 10 (Cases 5 and 9; Q/K), amino acid 78 (Cases 2 and 4; R/K), amino acid 2240 (Cases 1, 2, and 4; V/A, T/A, and V/A, respectively), and amino acid 2243 (Cases 5, 6, and 7; E/R, M/R, and V/R, respectively). These results indicate that the amino acids sequences of four patients resistant to IFN monotherapy with the highest grade of liver steatosis (moderate grade) did not show specific amino acid substitutions, compared with the sequences of five patients also resistant to IFN monotherapy but had no hepatocyte steatosis (none grade).

DISCUSSION

In this study, the factors associated with hepatocyte steatosis were analyzed in HCV genotype 2a. Known factors associated with hepatocyte steatosis include chronic alcohol consumption, high body mass index (obesity), elevated serum concentrations of cholesterol or triglycerides, diabetes mellitus, hepatotoxic drugs, and HCV genotype 3 infection [Wanless and Lentz, 1990; Mihm et al., 1997; Sheth et al., 1997; Czaja et al., 1998; Westin et al., 2002; Castera et al., 2004; Rubbia-Brandt et al., 2004; Sharma et al., 2004]. Recent studies have focused on the mechanism of HCV-related liver damage associated with hepatocyte steatosis. Patton et al. [2004] discussed the possibility of non-inflammatory-mediated mechanism for fibrosis secondary to steatosis. They indicated that one possible mechanism might include lipid peroxidation by HCV, which could result in activation of hepatic stellate cells and subsequent collagen deposition, based on in vitro evidence [Paradis et al., 1997; Okuda et al., 2002; Patton et al., 2004]. Farinati et al. [1995] reported that HCV-related liver damage might be characterized by increased iron storage, which elicits a free-radical-mediated peroxidation, with consequent steatosis and activation of glutathione turnover. In a subsequent study, they also showed that the serum levels 8-hydroxydeoxyguanosine (8-OHdG), a reliable marker of oxidative stress in HCV patients, correlated with serum ferritin levels and the grade of hepatocyte steatosis [Farinati et al., 1999]. In the study of patients infected with HCV genotype 2a, multivariate analysis also identified high serum ferritin level as an independent factor associated with high-grade hepatocyte steatosis. Thus, the results of the present study confirm these early reports that hepatocyte steatosis associated with HCV-related liver damage, including HCV genotype 2 might be characterized by increased iron storage, which elicits a free-radical-mediated peroxidation. Interestingly, steatosis in this study of genotype 2a was not associated with fibrosis. Rubbia-Brandt et al. [2004] reported that liver fibrosis might be associated with steatosis only in genotype 3. Considered together, the results suggest that hepatocyte steatosis might influence progression of liver fibrosis in a viral genotype-specific manner.

Hepatocyte steatosis has been considered recently as an important pretreatment predictor of response to IFN therapy. It is reported to be an important predictive factor of sustained virological response in combination therapy of IFN-ribavirin in patients infected with HCV genotype 1 or 3 [Bjøro et al., 2002; Poynard et al., 2003; Patton et al., 2004; Zeuzem et al., 2004]. However, there is no information on whether hepatocyte steatosis in patients infected with genotype 2 affects the virological response to IFN therapy, a part from a previous report [Akuta et al., 2002]. Akuta et al. [2002] reported previously that hepatocyte steatosis was a negative predictor of sustained virological response to IFN monotherapy in patients infected with genotype 2a, based on

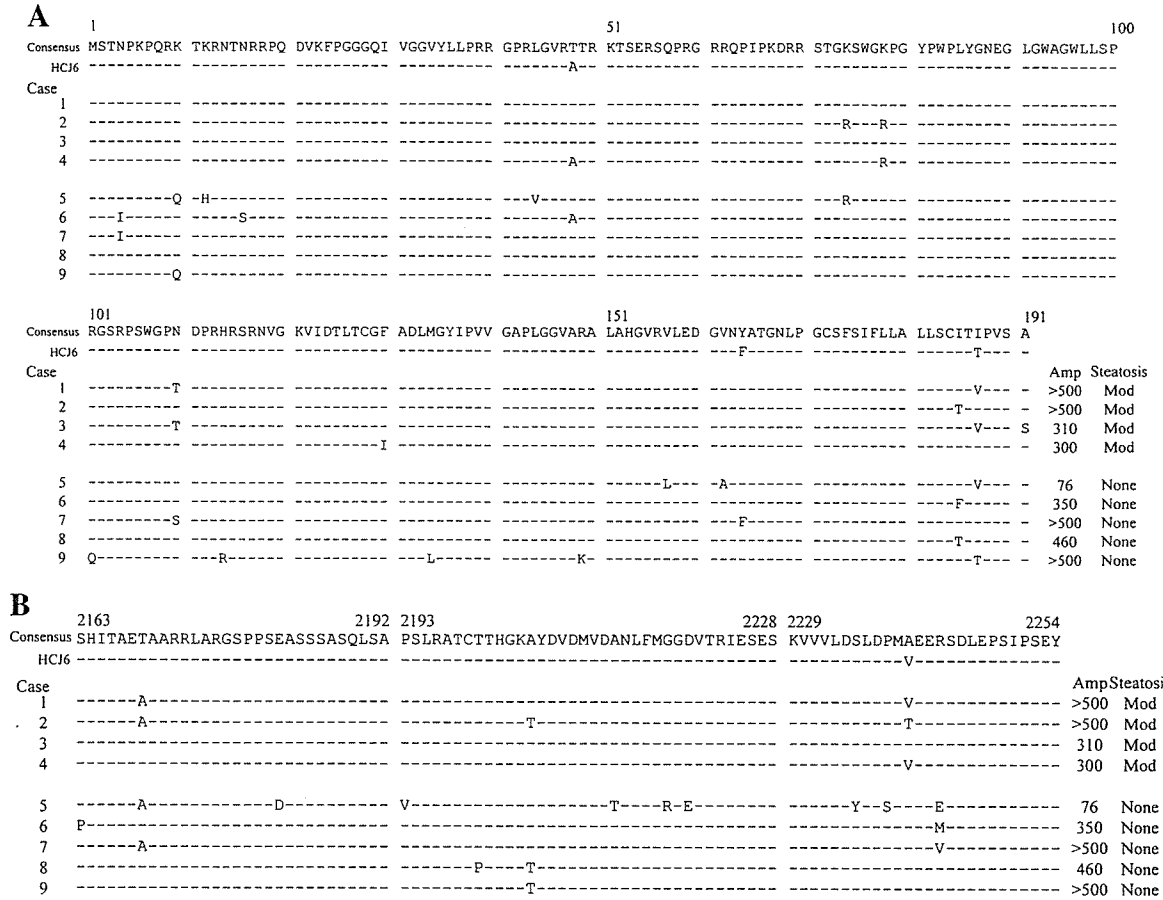

Fig. 2. Sequences of amino acids 1–191 in HCV core region (**A**) and amino acids 2163–2254 in HCV NS5A (**B**) at the start of IFN monotherapy of nine IFN-resistant patients infected with HCV genotype 2a with the highest grade of hepatocyte steatosis (moderate = Mod; Cases 1–4) or without steatosis (none; Cases 5–9). Dashes indicate amino acids identical to the consensus sequence of genotype 2a, and substituted amino acids are shown by standard single-letter codes. Eight IFN-resistant patients had high viremia (HCV more than 100 kIU/ml) while the viral level in the other IFN-resistant case (Case 5), free of steatosis, was 76 kIU/ml. Amp (kIU/ml) = quantitative analysis of HCV-RNA with PCR of Amplicor version 2.0.

multivariate analysis. A recent report [Patton et al., 2004] indicated that patients with genotype 1 who showed an early virologic response (defined as ≥ 2 $\log_{10}$ decline in HCV-RNA at week 12) were more likely to have low-grade steatosis than those who did not show an early response. The results of the present study of early viral kinetics and its relationship to the grade of steatosis in patients infected with genotype 2a were similar to the above study, and is the first report to indicate that the grade of steatosis influences early viral kinetics of HCV-RNA in patients infected with genotype 2a and treated with IFN. Further prospective studies should be performed based on a large number of patients who are matched for factors associated with hepatocyte steatosis (such as alcohol consumption, glucose/lipid metabolism) and pretreatment efficacy predictors (including IFN regimen, viral load, and serum albumin level [Akuta et al., 2002]).

Previous studies that patients infected with genotype 3 who could achieve sustained virological responses by IFN treatment often demonstrated a marked decrease in steatosis as confirmed by repeated posttreatment biopsies, and it was concluded that steatosis of genotype 3 might be considered as a consequence of viral infection [Kumar et al., 2002; Poynard et al., 2003; Castera et al., 2004; Patton et al., 2004]. However, it is not known whether steatosis of genotype 2a is also a consequence of viral infection or other non-viral factors (e.g., metabolic factors). Hence, it is important to investigate the relationship between treatment efficacy and improvement of steatosis grade, based on repeated biopsies in future studies.

IFN-resistance mechanisms specific for hepatocyte steatosis are so far unknown. Steatosis may be associated with reduced liver metabolism probably due to reduced activity of hepatic cytochrome induced by high liver fat content [Leclercq et al., 1998]. Previous studies showed that liver fibrosis correlates with steatosis and obesity [Hourigan et al., 1999; Clouston et al., 2001; Hwang et al., 2001]. Thus, lipid deposits within hepatocytes might cause functional disturbances by increasing the architectural distortion of the hepatic lobule caused by fibrosis and decrease in the contact area between drugs and hepatocytes [Taliani et al., 1995; Giannini

et al., 1999]. Another study reported a close correlation between the level of intrahepatic HCV-RNA and severity of steatosis [Rubbia-Brandt et al., 2000]. In the present study, no significant clinicopathological parameter (including serum HCV-RNA levels, fibrosis, and body mass index) was identified in non-sustained virological response patients with low-grade and high-grade steatosis. Thus, the results failed to establish a link between IFN-resistance and hepatocyte steatosis.

It is also not clear whether the virological characteristics of HCV play any pathogenic role in the derangement of lipid metabolism, and hence contribute to hepatocyte steatosis. Experiments conducted in vitro and in transgenic mice suggested that HCV core protein and NS5A region might be involved in the pathogenesis of lipid accumulation [Barba et al., 1997; Moriya et al., 1997; Shi et al., 2002]. On the other hand, Rubbia-Brandt et al. [2000] reported that steatosis might be a morphological expression of viral cytopathic effect in patients infected with HCV genotype 3, but that analysis of the HCV core protein failed to identify a sequence specially associated with the development of steatosis [Rubbia-Brandt et al., 2000]. No study has investigated the effects of HCV core protein and NS5A region on IFN efficacy in patients with hepatocyte steatosis. In this context, the relationship between amino acid substitutions in HCV core protein/NS5A region and the grade of hepatocyte steatosis was analyzed in the present study in IFN-resistant patients. However, the analysis showed no specific amino acid substitutions in these regions that could establish a role for hepatocyte steatosis in IFN-resistance. It should be noted, however, that the present study was based on a small group of patients and sequence analysis of the defined regions should be investigated in large-scale studies to confirm the present findings.

β IFN is rarely used and is not licensed outside Japan. It was reported previously that the type of IFN (α vs. β) is not a predictor of sustained virological response to IFN monotherapy in 394 patients infected with genotype 2a, based on multivariate analysis [Akuta et al., 2002], and accordingly when the present study was designed, it was thought that the type of IFN should not affect the outcome of patients infected with genotype 2a. Incidentally, based on data from Toranomon Hospital, the frequency of β IFN-related adverse events seems to be lower than those by α IFN, especially in elderly patients (unpublished data). Therefore, the use of β IFN rather than α IFN is recommended at least for elderly patients.

In conclusion, the present study of patients infected with HCV genotype 2a suggested that hepatocyte steatosis is possibly associated with excessive iron storage, and that it might be an important predictor of the efficacy of IFN monotherapy. Further studies should be performed to investigate whether hepatocyte steatosis associated with HCV genotype 2a might be also a predictor of other treatments, including IFN-ribavirin combination therapy and pegylated IFN. In this study, amino acid substitutions associated with IFN-resistance specific for hepatocyte steatosis could not be identified, and large-scale studies should be conducted to confirm the present findings. Further analysis of IFN-resistance mechanisms should be conducted in future studies taking into consideration pharmacokinetic, viral, and host-related factors.

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Virological and Biochemical Relapse after Discontinuation of Lamivudine Monotherapy for Chronic Hepatitis B in Japan: Comparison with Breakthrough Hepatitis during Long-Term Treatment

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Key Words

Chronic hepatitis B · Lamivudine monotherapy · Biochemical and virological relapse · Basic core promoter · YMDD motif mutant · Breakthrough hepatitis · Retreatment · HBV genotype

Abstract

Objective: Comparison of virological and biochemical relapse in patients with chronic hepatitis B, based on continuation or discontinuation of lamivudine monotherapy. ***Methods:*** In Japanese genotype C-dominant hepatitis B patients, 25 patients who stopped treatment at normal levels of alanine transferase (ALT) were retrospectively compared with 75 patients who continued treatment. Both groups were matched for age, sex, and observation period after start of treatment. We investigated the relapse rates, and evaluated predictive factors for relapse and efficacy of retreatment of the discontinuous group. ***Results:*** Virological and biochemical relapse occurred significantly earlier in the discontinuous than continuous group, and the peak levels and ratios of peak to pretreatment levels of serum bilirubin and ALT after relapse were not significantly different between the two groups. Multivariate analysis identified three independent factors at discontinuation of treatment associated with early biochemical relapse: HBeAg positivity, presence of liver cirrhosis, detection of basic core promoter mutant. Normalization of ALT levels with retreatment occurred in 62.5% of patients, but 2 HBeAg-positive patients retreated after the emergence of YMDD motif mutant developed severe relapse with hyperbilirubinemia. ***Conclusion:*** Our results in Japanese patients with genotype C-dominant hepatitis B suggest that discontinuation of lamivudine monotherapy, and retreatment after the emergence of YMDD mutant should be given attention.

Introduction

Lamivudine, an oral cytosine nucleoside analog clinically used for the treatment of chronic hepatitis B virus (HBV) infection, potently inhibits HBV replication by interfering with HBV reverse transcriptase activity [1–4],

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