50% of the patients with HCV-1b in high levels [Manns et al., 2001; Fried et al., 2002]. It is necessary to predict the response to PEG-IFN/ribavirin before the start of antiviral therapy, to avoid severe side-effects in the patients who will barely gain sustained virological response.

The core protein of HCV is coded for by the C gene, and consists of 191 amino acids (aa) [Rosenberg, 2001]. Although the core protein is conserved better than the other structural and non-structural proteins of HCV, polymorphisms of core protein are known, and they influence the response to antiviral treatment. In patients infected with HCV-1b, for example, the substitution of arginine at position 70 (Arg70) for glutamine (Gln70) and that of leucine at position 91 (Leu91) for methionine (Met70) decrease sustained virological response in the patients with chronic hepatitis C who are treated with PEG-IFN/ribavirin and increase the development of HCC [Akuta et al., 2007a,b,d, 2008].

In the Department of Hepatology at the Toranomon Hospital in Metropolitan Tokyo, the amino-acid sequence of the core-protein was determined in 1,079 patients infected with HCV-1b who had not received antiviral treatment. The substitution of Arg70 for Gln70 and that of Leu91 or Met 91 were correlated with the age at presentation, liver function tests and the severity of liver disease. Based on the results obtained, Gln70 would contribute to the progression of chronic hepatitis C.

MATERIALS AND METHODS

Patients

During 1966–2008, 1,097 patients infected with HCV-1b visited the Department of Hepatology at the Toranomon Hospital in Metropolitan Tokyo. They were: (1) negative for hepatitis B surface antigen by radioimmunoassay (Dainabot, Tokyo, Japan) or antibody to human immunodeficiency virus type-1; (2) positive for anti-HCV by a third-generation enzyme immunoassay (Chiron Corp., Emeryville, CA) and HCV RNA by the polymerase chain reaction (PCR) (Cobas Amplicor HCV Monitor ver.2.0, Roche Diagnostics, Tokyo, Japan); (3) infected with HCV genotype 1b but not with other genotypes; (4) without previous antiviral treatment; (5) without other forms of hepatitis, including hemochromatosis, Wilson's disease, primary biliary cirrhosis, alcoholic liver disease and autoimmune liver disease; and (6) had serum samples stored at −80°C. Of the 1,097 patients, 778 (70.9%) had chronic hepatitis, 221 (20.1%) cirrhosis, and 98 (8.9%) HCC. Amino acids in the core protein at positions 70 and 91 were determined, and were correlated with liver disease and biochemical and virological markers. Informed consent was obtained from each patient in this study, and the protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki as reflected by approval by Ethic Committee of the institution.

Histopathological Diagnoses of Liver Disease

Liver biopsy was performed under laparoscopy by a modified Vim Silverman needle (Tohoku University style, Kakinuma Factory, Tokyo). The sample was fixed in 10% formalin, and was stained with hematoxylin and eosin, Masson's trichrome, silver impregnation, and periodic acid-Schiff. It contained at least six portal areas. The pathological diagnosis was made by one of the authors (H.K.) who was blinded to the clinical data. Chronic hepatitis was diagnosed based on the scoring system of Desmet et al. [1994]. Cirrhosis was diagnosed by imaging on ultrasonography (US), computed tomography (CT), or magnetic resonance imaging (MRI). HCC was diagnosed by US and/or CT. Angiography was performed when HCC was strongly suspected by US, CT, MRI, or liver biopsy. An increasing trend of tumor markers was taken into consideration for the diagnosis of HCC.

Determination of Amino-Acid Substitutions in the Core Protein

Amino acid (aa) at position 70 of Arg or Gln and aa91 of Leu or Met were determined by PCR with primers specific for each of them [Okamoto et al., 2007]. It is highly reproducible, and has a sensitivity of 94.4% in the determination of aa70 or aa91 in samples with HCV RNA titers >10 KIU/ml. The concordance of the results of this method with those of direct sequencing reached 97.1%. Amino acids at positions 70 and 91 were confirmed by direct sequencing of most samples [Akuta et al., 2005].

Statistical Analysis

Changes of Arg70/Leu91 (wild-type) and Gln70 and/or Met91 (mutant types) with age were analyzed by the Cochran–Armitage trend test (SAS version 9.1.3; SAS Institute, Inc., Cary, NC). Frequencies were compared between groups by the Kruskal–Wallis test and Fisher's exact test. Univariate and multivariate logistic regression analyses were used for the evaluation of factors independently associated with the substitution of aa70. They included the following ten variables: age, sex, liver disease, platelet count, hemoglobin, albumin, aspartate aminotransferase (AST), alanine aminotransferase (ALT), γ-glutamyl transpeptidase (γ-GTP), and substitution of aa at position 91 in the core protein. Each variable was transformed into categorical data consisting of two simple ordinal numbers for univariate and multivariate analyses. Variables that achieved statistical significance on univariate analysis were tested by the multivariate Cox proportional hazard model to identify independent factors. Statistical comparisons were performed using SPSS ver.11.0 (SPSS, Inc., Chicago, IL). A P-value <0.05 by the two-tailed test was considered significant.

RESULTS

Clinical and Virological Characteristics of the 1,097 Patients Who Were Infected With HCV-1b

Table I lists the baseline characteristics of the 1,097 patients who were infected with HCV-1b and had not received antiviral treatment. They had the median age of 60 years and included 590 (53.8%) men. The median transaminase levels were elevated, and alpha-fetoprotein was within the normal limit (<10 μg/L). The majority of the patients (70.9%) had chronic hepatitis, while HCC had developed in 8.9% of the patients. Amino acids at positions 70 and 91 in the core protein were both the wild-type (Arg70 and Leu91) in 37.6% of them, and both mutant types (Gln70 and Met91) in 16.4%. The Gln70 variant was detected in 464 of the 1,097 (42.3%) patients.

The Prevalence of Amino-Acid Substitutions Stratified by Age and Sex

The 1,097 patients infected with HCV-1b were classified into three age groups, and the prevalence of Arg70/Leu91 (wild-type) and that of Gln70 and/or Met91 (mutant types) were compared (Fig. 1). Ag70/Leu91 decreased with age by trend analysis, from 63.6% in the patients aged ≤30 years to 36.6% in those ≥41 years ($P < 0.001$ by the Cochran–Armitage trend test). Table II lists the prevalence of the Gln70 variant in men and women stratified by the age. There were no sex differences in the prevalence of the Gln70 variant.

The Prevalence of the Gln70 Variant in Patients With Different Liver Diseases

Figure 2 compares the prevalence of the Gln70 variant among patients infected with HCV-1b who presented with different liver diseases at the baseline. The prevalence of the Gln70 variant increased with the progression of liver disease from chronic hepatitis (32.6%) to cirrhosis (43.0%) and HCC (53.1%) ($P < 0.001$ by the Kruskal–Wallis test). In patients with cirrhosis, the 126 patients with the Arg70 variant were aged with the mean of 62 years (range: 32–78 years) in comparison with the 95 patients with the Gln70 variant who were aged 59 years (25–80). In patients with HCC, the 47 patients with the Arg70 variant were aged with the mean of 66 years (range: 37–81 years) in comparison with the 51 patients with the Gln70 variant who were aged 66 years (46–78).

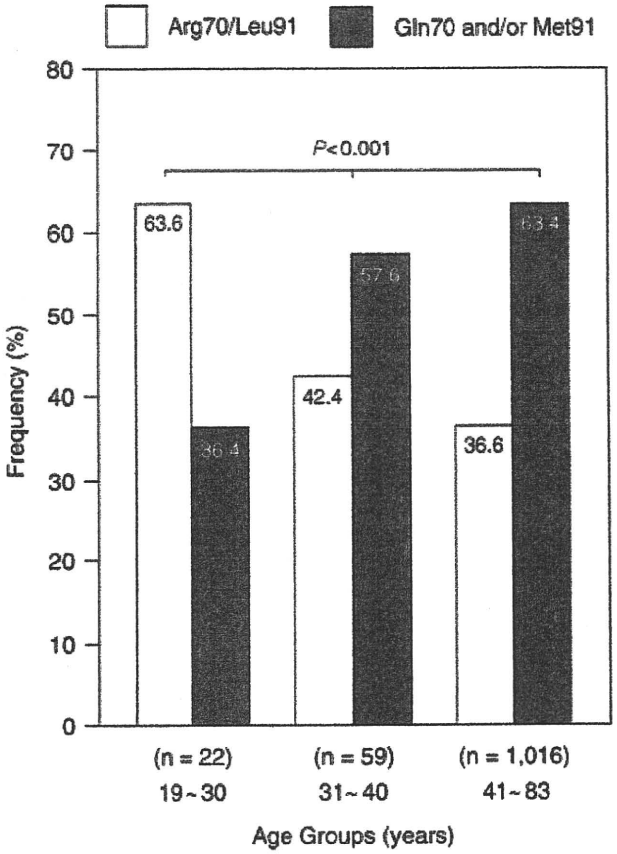

Fig. 1. The age-specific prevalence of Gln70 in treatment-naive patients infected with HCV-1b.

TABLE I. Clinical and Virological Characteristics of the 1,097 Patients Who Were Infected With Hepatitis C Virus of Genotype 1b

Age (years)	60 (19–83)
Men	590 (53.8%)
Follow-up period (years)	8 (3–28)
Hemoglobin (g/dl)	14.0 (4.5–26.8)
Platelets ($\times 10^3/mm^3$)	15.4 (2.0–34.1)
Aspartate aminotransferase (IU/L)	58 (8–617)
Alanine aminotransferase (IU/L)	69 (6–776)
Alpha-fetoprotein (μg/L)	6 (2–65,700)
Liver disease	
Chronic hepatitis	778 (70.9%)
Cirrhosis	221 (20.1%)
Hepatocellular carcinoma	98 (8.9%)
Amino acids in the core protein	
Arg70/Leu91 (double wild-type)	412 (37.6%)
Gln70/Leu91 (mutant type)	284 (25.9%)
Arg70/Met91 (mutant type)	221 (20.1%)
Gln70/Met91 (double mutant type)	180 (16.4%)

Values are the median with range in parentheses or the number with percentage in parentheses.

TABLE II. Frequency of Gln70 in the Core Protein in Patients Infected With HCV-1b Stratified by Age and Sex

Age (years)	Men	Women	Differences
19–30	23.5% (4/17)	20% (1/5)	1.0
31–40	34.1% (14/41)	38.9% (7/18)	0.773
41–50	37.2% (45/121)	40% (14/35)	0.763
51–60	39.1% (72/184)	40.1% (63/157)	0.912
61–70	36.0% (62/172)	30.1% (74/246)	0.205
70–83	45.5% (25/55)	43.5% (20/46)	0.842
Total	37.6% (222/590)	35.3% (179/507)	0.451

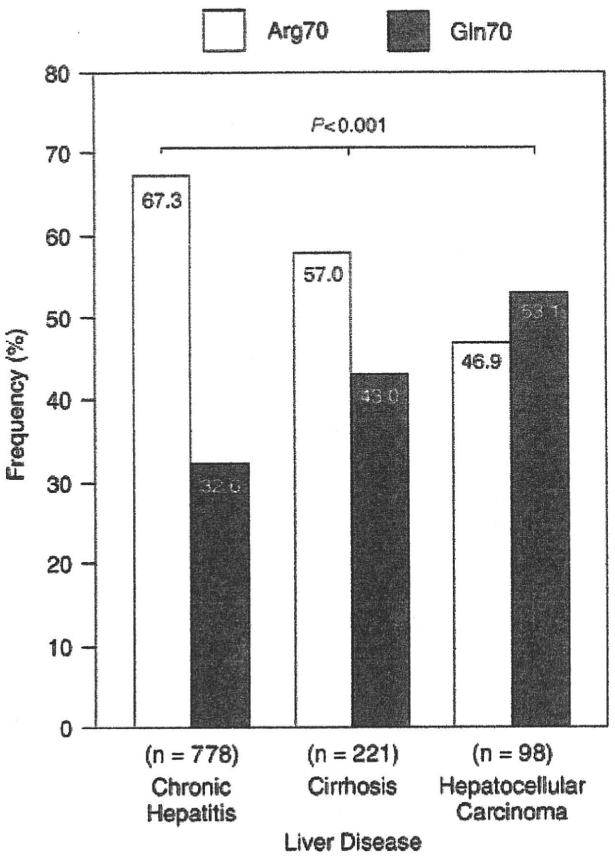

Fig. 2. The prevalence of the Gln70 variant among patients with chronic hepatitis, cirrhosis and hepatocellular carcinoma.

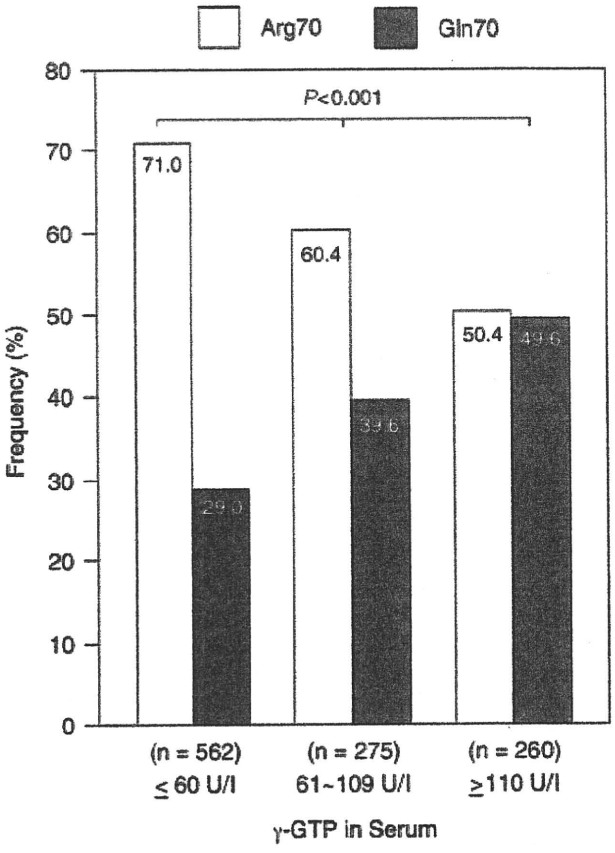

Fig. 3. The prevalence of the Gln70 variant among patients with different γ-GTP levels.

The Influence of γ-GTP Levels on the Prevalence of the Gln70 Variant

The prevalence of Gln70 was compared among patients with different γ-GTP levels at the baseline (Fig. 3). The prevalence of the Gln70 variant increased in parallel with the γ-GTP levels from 29.0% to 49.6% ($P < 0.001$ by the Kruskal–Wallis test).

The Influence of Platelet Count on the Prevalence of the Gln70 Variant

The prevalence of the Gln70 variant was compared among three groups of patients with various platelet counts at the baseline (Fig. 4). The prevalence of the Gln70 variant increased as the platelet count decreased ($P = 0.008$ by the Kruskal–Wallis test).

Factors Associated With the Gln70 Variant in Patients Infected with HCV-1b

Since the Gln70 variant, in comparison with the Arg70 variant, aggravated liver disease in patients infected with HCV-1b (Figs. 2–4), ten factors were evaluated for the association with the Gln70 variant by the univariate analysis (Table III); the cut-off value was set at the median of studied patients. Among them, HCC, elevated levels of AST (≥58 IU/L) and γ-GTP (>61 U/L), as well as decreased albumin concentration (<3.9 g/dl), were associated with the Gln70 variant ($P = 0.003$, 0.005, <0.001, and 0.031, respectively). A similar analysis was performed for the substitution of Leu91 for Met91 (Table IV). Except for the association with the substitution of Arg70 for Gln70, the Met91 variant had no influence on any variable examined.

Two factors associated independently with the Gln70 variant were identified by the multivariate analysis (Table V). The risk for the Gln70 variant was increased by HCC (odds ratio 1.829 [95% confidence interval 1.147–2.917], $P = 0.011$) and γ-GTP ≥61 IU/L (1.647 [1.268–2.139], $P < 0.001$).

DISCUSSION

The response to PEG-IFN and ribavirin is influenced by genotypes and viral load, and is poorest in patients with HCV-1b in high HCV RNA levels [Manns et al., 2001; Fried et al., 2002; Hadziyannis et al., 2004]. The prediction of sustained virological response would circumvent side-effects and costs in non-responders. Amino-acid substitutions in the core protein are useful

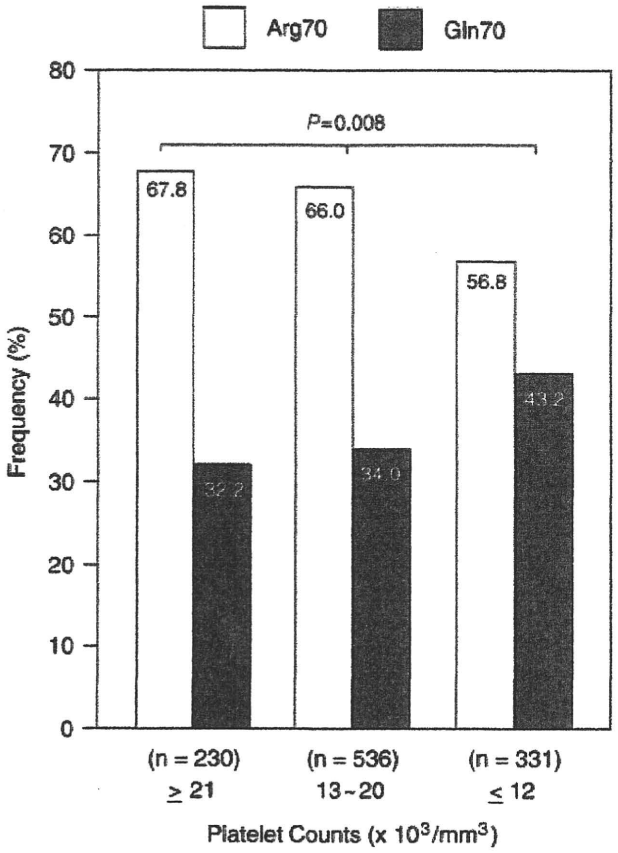

Fig. 4. The prevalence of the Gln70 among three groups of patients with different platelet counts.

for predicting the non-response in patients infected with HCV-1b. The substitution of Arg70 for Gln70 in the prototype sequence of HCV-1b [Kato et al., 1990] and/or that of Leu91 for Met91 can predict the non-response to IFN-based treatment [Akuta et al., 2005, 2006, 2007c,d]. It has been beyond the scope of previous studies, however, whether or not these amino-acid substitutions influence the progression of hepatitis C in the patients who have not received antiviral treatment. The availability of pre-treatment sera from many patients with chronic hepatitis C permitted the evaluation of the influence of aa substitutions in the core protein on the progression of liver disease without therapeutic intervention.

First, the prevalence of the Gln70 variant increased with the age of patients until they had reached 50 years (Fig. 1). It is not certain if HCV-1b with Arg70 underwent a point mutation for Gln70 (G-to-A at nucleotide 209), or these amino-acid residues were present in HCV-1b strains prevalent at the time of infection. Follow-up of patients for aa substitutions will resolve this issue. Another possibility for this difference would be a selection bias. If the patients with the Arg90 variant fare better than those with the Gln70 variant, they would not develop liver disease severe enough to visit hospital.

Secondly, the patients infected with HCV-1b with Gln70 increased in parallel with γ-GTP levels and the severity of liver disease from chronic hepatitis to cirrhosis and HCC, as well as with a decrease in platelet count (Figs. 2–4). Since the Met91 variant did not make such difference, the aggravation of liver disease would have been due to the Gln70 variant, but not to the Met91 variant. Increases in the γ-GTP level may have been related to the development of HCC; γ-GTP has been proposed as a sensitive marker of cirrhosis and HCC [Penn and Worthington, 1983]. Decreased platelet counts have been associated with HCC [Ikeda et al., 2001; Lu et al., 2006; Kumada et al., 2009]. Although the proportion of the Gln70 variant increases with the severity of liver disease (Fig. 2), the median age of patients with cirrhosis or HCC did not differ between the patients with the Arg70 variant and Gln70 variant who

TABLE III. Factors Associated With the Substitution of aa70 of Arginine for Glutamine in the Core Protein in 1,097 Patients Infected With HCV Genotype1b by Univariate Analysis

Factor	Category	Gln70	*P*-value
Sex	1: Male	38.6% (228/590)	0.663
	2: Female	37.3% (189/507)	
Age (years)	1: <60	40.6% (219/540)	0.093
	2: ≥60	35.5% (198/557)	
AST (IU/L)	1: <58	33.9% (184/543)	0.005
	2: ≥58	42.2% (234/554)	
ALT (IU/L)	1: <75	36.9% (213/578)	0.376
	2: ≥75	39.3% (204/519)	
Albumin (g/dl)	1: <3.9	42.5% (194/457)	0.031
	2: ≥3.9	35.8% (229/640)	
γ-GTP (IU/L)	1: <61	29.0% (163/562)	<0.001
	2: ≥ 61	44.4% (238/535)	
Hemoglobin (g/dl)	1: <14	35.1% (176/501)	0.083
	2: ≥14	40.4% (241/596)	
Platelet count (×10³/mm³)	1: <150	39.9% (207/519)	0.253
	2: ≥150	36.3% (210/578)	
Hepatocellular carcinoma	1: No	36.6% (366/999)	0.003
	2: Yes	53.1% (52/ 98)	
Substitutions of core aa91	1: Leucine	35.6% (227/638)	0.051
	2: Methionine	41.4% (190/459)	

TABLE IV. Factors Associated With the Substitution of aa91 of Leucine for Methionine in the Core Protein in 1,097 Patients Infected With HCV Genotype1b by Univariate Analysis

Factor	Category	Met91	*P*-value
Sex	1: Male	40.8% (241/590)	0.500
	2: Female	43.0% (218/507)	
Age (years)	1: <60	43.5% (235/540)	0.271
	2: ≥60	40.2% (220/517)	
AST (IU/L)	1: <58	43.6% (234/537)	0.196
	2: ≥58	39.7% (217/547)	
ALT (IU/L)	1: <75	42.4% (238/561)	0.618
	2: ≥75	40.8% (205/502)	
Albumin (g/dl)	1: <3.9	42.0% (177/421)	0.797
	2: ≥3.9	41.2% (249/604)	
γ-GTP (IU/L)	1: <61	40.4% (237/586)	0.327
	2: ≥61	43.4% (222/511)	
Hemoglobin (g/dl)	1: <14	40.8% (193/473)	0.658
	2: ≥14	42.3% (240/567)	
Platelet count ($\times 10^3/mm^3$)	1: <150	40.5% (202/499)	0.454
	2: ≥150	42.9% (240/559)	
Hepatocellular carcinoma	1: No	42.3% (423/999)	0.334
	2: Yes	36.7% (36/ 98)	
Substitutions of core aa71	1: Arginine	49.0% (269/680)	0.051
	2: Glutamine	45.6% (190/417)	

had cirrhosis (62 years vs. 59 years) of HCC (66 years vs. 66 years). This would indicate a possibility that the Gln70 variant would be a factor for the aggravation of liver disease that might be independent of age.

The distinct capacity of Gln70 and Met91 for decreasing the response to combined treatment in patients infected with HCV-1b was proposed in a recent study [Okanoue et al., 2008]. The Gln70 variant decreased sustained virological response, while the Met91 variant did not, although the Met91 variant reduced the rate of rapid virological response within 4 weeks after the start of therapy. The role of the Gln70 variant greater than that of the Met91 variant in the progression of liver disease has been confirmed in this study (Tables III and IV). In the multivariate analysis, the risk for Gln70 was increased by HCC (odds ratio 1.829 [95% confidence interval 1.147–2.917]) and γ-GTP ≥61 U/L (1.647 [1.268–2.139]). The Gln70 variant would aggravate liver disease toward the development of HCC in patients infected with HCV-1b who have not received antiviral treatment.

It would be a matter of conjecture how the Gln70 variant influences the severity of liver disease. Previous suggestions for a reduced response of patients with the Gln70 variant were confined to interaction of the core protein with IFN receptors and IFN-signaling pathways [Alexander, 2002; Blindenbacher et al., 2003; Bode et al., 2003]; these studies were restricted to patients receiving IFN-based treatments [Akuta et al., 2007a,b,d, 2008]. The ability of the Gln70 variant for accelerating the progression of liver disease, in the absence of exogenous IFN, has changed this issue into a wider perspective. There still remains a possibility, however, that the Gln70 variant would interact with the endogenous IFN induced by HCV infection, and aggravate liver disease.

Another possibility may be the cytotoxic T-cell (CTL) response, as has been demonstrated for the pathogenesis of chronic hepatitis B [Chisari and Ferrari, 1995]. Since both hepatitis B virus (HBV) and HCV do not have a cytopathic capacity, hepatitis B and C would be mediated by immune responses directed at viral proteins. Amino-acid sequences bearing a CTL epitope restricted by the MHC class-I are demonstrated in the HBV core protein [Bertoletti et al., 1993; Bertoletti and Gehring, 2006], and are implicated in liver disease in the patients with the HLA-2 phenotype [Penna et al., 1991; Bertoletti et al., 1994]. It is tempting to speculate that the substitution of Arg70 for Gln70 might generate a CTL epitope and stimulate cytotoxic lymphocytes toward inflammation of the liver [Kita et al., 1993; Jackson et al., 1999].

In conclusion, amino-acid substitutions in the core protein influence the progression of liver disease, and the Gln70 variant aggravates hepatic inflammation and increases the risk for HCC in the patients who have not received antiviral treatment. The ability of the Gln70

TABLE V. Factors Associated with the Substitution of aa70 of Arginine for Glutamine in the Core Protein in 1,097 Patients Infected with HCV Genotype1b by Multivariate Analysis

Factor	Category	Odds ratio (95%CI)	*P*-value
Hepatocellular carcinoma	1: No	1	0.011
	2: Yes	1.829 (1.147–2.917)	
γ-GTP (IU/L)	1: <61	1	<0.001
	2: ≥61	1.647 (1.268–2.139)	

variant to aggravate liver disease, in the absence of exogenous IFN, would lend further support on its capacity of predicting sustained virological response before the start of therapy. It is possible that mechanisms other than the resistance to IFN, such as cytotoxic T-cell responses, might be involved in an increased pathogenetic potential of HCV-1b with Gln70.

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Case report

Sustained virological response in a patient with chronic hepatitis C treated by monotherapy with the NS3-4A protease inhibitor telaprevir

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ABSTRACT

Here, we describe for the first time a case of sustained virological response (SVR) achieved in a patient with chronic hepatitis C (CH-C) by monotherapy with a NS3-4A protease inhibitor, telaprevir, without interferon therapy. A 59-year-old treatment-naïve Japanese man was enrolled in a phase II trial of telaprevir by repeat oral administration at a dose of 750 mg every 8 h for 24 weeks. At the start of treatment, he exhibited a low-level viremia with genotype 1b of the hepatitis C virus (HCV). After the first week of treatment with telaprevir, serum HCV RNA was undetectable, and negativity remained until the end of treatment. Moreover, he was evaluated as having a SVR after the post-treatment 24-week follow-up program. Two characteristics may explain the strong antiviral activity of telaprevir in the present case. First, although pre-treatment PCR-direct sequencing and cloning for the N-terminal in the NS3 region showed a protease inhibitor-resistant variant (T54A) in 1 of 32 independent clones, the T54A substitution has only a low-level resistance to protease inhibitors and his viral load was low. Second, when compared to a consequence sequence of 35 treatment-naïve patients with HCV genotype 1b, R130K and Q195K substitutions were unique to the present case. Although it is presently unknown whether the R130K and Q195K substitutions are related to SVR, this case suggests that long-term telaprevir monotherapy may be effective in CH-C patients with genotype 1 and a low viral load.

1. Introduction

The goals of antiviral treatment in patients with chronic hepatitis C (CH-C) are long-lasting eradication of the virus and a decrease in disease-related hepatic mortality. Standard treatment uses a combination of pegylated interferon and ribavirin (PEG-IFN-RBV), which provides a sustained virological response (SVR) rate of over 50%.[1,2] In Japan, approximately 70% of patients with CH-C are infected with genotype 1b, and those with a high titer of genotype 1b (≥100 KIU/mL [Amplicor; Roche Diagnostics K.K. Tokyo, Japan]) have lower rates of SVR (<50%), even on 48 weeks of PEG-IFN-RBV combination therapy.[3] Further, although treatment for CH-C is currently based on interferon (IFN), use of this agent is associated with serious adverse effects in some patients, such as mental disorders, apathy, and laboratory abnormalities.[1,2,4] Moreover, most CH-C patients in Japan over 70 years of age cannot receive IFN therapy due to either or both co-morbidities and the risk of adverse effects. For these reasons, a new treatment strategy is needed for patients with CH-C that displays high SVR rates and a favorable side-effect profile.

One recently introduced treatment strategy for CH-C is inhibition of the NS3-4A protease in the HCV polyprotein. Potential inhibitors include telaprevir (VX-950; MP-424; Mitsubishi Tanabe Pharma Co., Osaka, Japan), which has been selected as a clinical therapy candidate for the treatment of CH-C.[5] In some patients with genotype 1 and a high viral load, however, the efficacy of telaprevir monotherapy was limited, and combination therapy of telaprevir plus PEG-IFN-RBV is now standard.[6–10] On this background, we therefore report here for the first time a patient with CH-C who achieved a SVR following monotherapy with telaprevir.

2. Case report

A 59-year-old Japanese man was admitted to Toranomon Hospital, Tokyo in July 2007 following a positive result for HCV RNA at general check-up. Laboratory tests before treatment showed mild

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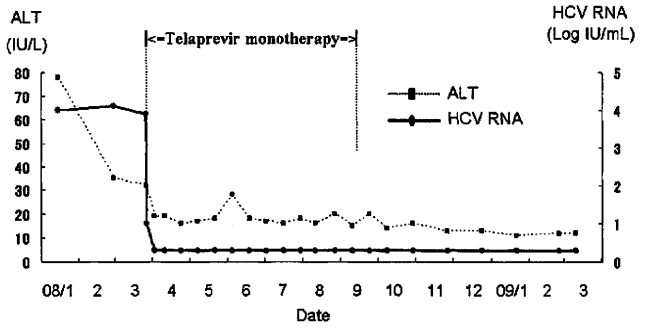

Fig. 1. Clinical course during and after 24 weeks of telaprevir monotherapy.

elevation of ALT (46 IU/L), and persistent HCV infection with genotype 1b and low-level viremia (<5 Log IU/mL [COBAS TaqMan HCV test, Roche Diagnostics K.K. Tokyo]) that continued to remain low until the start of treatment. He was diagnosed with CH-C by peritoneoscopy and liver biopsy (mild hepatitis [A1] and moderate fibrosis [F2]) at our hospital in February 2008. He had not received IFN therapy or any other antiviral drugs, and was enrolled in a phase II trial of telaprevir. Written informed consent was obtained, and the study was conducted in compliance with Good Clinical Practice and the Declaration of Helsinki. Treatment with telaprevir was started in March 2008, at which time serum HCV RNA was 3.9 Log IU/mL. Treatment was by repeat oral administration at a dose of 750 mg every 8 h for 24 weeks. Serum HCV RNA was undetectable after the first week and remained negative until the end of treatment (September 2008), and moreover remains undetectable as of March 2009. He was evaluated as having a SVR after the post-treatment 24-week follow-up program (Fig. 1).

The genome sequence for the N-terminal 609 nucleotides (203 amino acids) in the NS3 region of HCV isolates from the patient was analyzed before treatment with telaprevir. HCV RNA was extracted from 100 μL of serum and the nucleotide sequences were determined by direct sequencing and cloning. The primers used to amplify the NS3 region were NS3-F1 (5′-ACACCGCGGCGTGTGGGGACAT-3′; nucleotides 3295–3316) and NS3-AS2 (5′-GCTCTTGCCGCTGCCAGTGGGA-3′; nucleotides 4040–4019) as the first (outer) primer pair and NS3-F3 (5′-CAGGGGTGGCGGCTCCTT -3′; nucleotides 3390–3407) and NS3-AS2 as the second (inner) primer pair.[11] Thirty-five cycles of first and second amplifications were performed as follows: denaturation for 30 s at 95 °C, annealing of primers for 1 min at 63 °C, extension for 1 min at 72 °C, and final extension was performed at 72 °C for 7 min. PCR-amplified DNA was purified after agarose gel electrophoresis and amplification products of the second-round PCR were ligated with plasmid and transformed in *Escherichia coli* in a cloning kit (TA Cloning; Invitrogen, Carlsbad, CA). Dideoxynucleotide termination sequencing was performed with the BigDye Terminator v1.1 Cycle Sequencing kit (Applied Biosystems Japan, Tokyo). Sequences of 32 independent clones from the sample were determined and analyzed. The pre-treatment analyses by PCR-cloning showed a variant (T54A) resistant to protease inhibitors in 1 of the 32 clones.

We also made a consensus sequence of the NS3 region from the PCR-direct sequences of 35 treatment-naïve Japanese patients with HCV genotype 1b in our hospital (Fig. 2). Compared to the consensus sequence, there were a total of 5 identical substitution variants (V48I, P89S, S122G, R130K, Q195K) within the 32 independent clones from this patient, among which R130K and Q195K were unique to this patient.

3. Discussion

Previous studies showed that telaprevir monotherapy for HCV patients with genotype 1 and a high viral load demonstrated substantial antiviral activity, and the median maximum change was −4.77 Log IU/mL with administration at 750 mg every 8 h for 2 weeks.[6,7] In Reesink et al., HCV RNA decreased below the limit of

```
                1       10        20        30        40        50
CONSENSUS       APITAYSQQT RGLLGCIITS LTGRDKNQVE GEVQVVSTAT QSFLATCVNG
Case clone1     ---------- ---------- ---------- ---------- -------I--
Case clone2     ---------- ---------- ---------- ---------- -------I--
Case clone3     -----H---- ---------- ---------- ---------- -------I--
Case clone4     ---------- ---------- ---------- ---------- -------I--
Case clone5     ---------- ---------- ---------- ---------- -------I--
               51                                                  100
CONSENSUS       VCWTVYHGAG SKTLAGPKGP ITQMYTNVDQ DLVGWQAPPG ARSLTPCTCG
Case clone1     ---------- ---------- ---------- --------S- ----------
Case clone2     -----F---- ---------- ---------- --------S- ----------
Case clone3     ---A------ ---------- ---------- --------S- ----------
Case clone4     ---------- ---------- ---------- --------S- -----L----
Case clone5     -----F---- ---------- ---------- --------S- ----------
               101                  130                  150
CONSENSUS       SSDLYLVTRH ADVIPVRRRG DSRGSLLSPR PVSYLKGSSG GPLLCPSGHA
Case clone1     ---------- ---------- -G-------K ---------- ----------
Case clone2     ---------- ---------- -G-------K ---------- ----------
Case clone3     ---------- ---------- -G-------K ---------- ----------
Case clone4     ---------- ---------- -G-------K ---------- ----------
Case clone5     ---------- ---------- -G-------K ---------- ----------
               151                                      195  200
CONSENSUS       VGIFRAAVCT RGVAKAVDFI PVESMETTMR SPVFTDNSSP PAVPQTFQVA
Case clone1     ---------- ---------- ---------- ---------- ----K-----  15
Case clone2     ---------- ---------- ---------- ---------- ----K-----  14
Case clone3     ---------- ---------- ---------- ---------- ----K-----   1
Case clone4     ---------- ---------- ---------- ---------- ----K-----   1
Case clone5     ---------- ---------- ---------- ---------- ----K----V   1
```

Fig. 2. Evolution of the HCV NS3 gene sequence at the start of telaprevir monotherapy. Consensus sequence was made from the HCV RNA of 35 treatment-naïve Japanese patients with genotype 1b in our hospital. The number of clones within each sample of identical amino acid sequences is given on the right at the end of each sequence. Dashes indicate identical amino acid sequences.

detection (10 IU/mL) for 2 patients in the group receiving 750 mg every 8 h.[6] In some patients, however, HCV RNA levels increased between days 7 and 14, and mutations that confer resistance to telaprevir were detected. This trial of telaprevir monotherapy was therefore terminated after 2 weeks, and combination therapy of telaprevir plus PEG-IFN-RBV is now used in the USA and Europe.[8–10] Our case may therefore represent an unusual and possibly serendipitous response to long-term telaprevir monotherapy, and the efficacy of monotherapy remains unclear.

To our knowledge, this is the first report of a patient with CH-C achieving SVR by telaprevir monotherapy, without the use of IFN. Three treatment-naïve Japanese patients were enrolled in our hospital for this phase II trial of telaprevir monotherapy over 24 weeks. Before treatment, the 2 non-SVR patients had a high HCV RNA viral load (>5 Log IU/mL), while the viral load in the SVR patient remained low. Further, while HCV RNA decreased below the limit of detection (10 IU/mL) and negativity of HCV RNA remained until the end of treatment in 2 patients, HCV RNA in the other non-SVR patient reappeared after treatment cessation.

The development of drug resistance has been a challenge for treatment strategies in many viral infections. The high replication rate and the error-prone nature of viral RNA polymerases generate a viral quasi-species from which variants resistant to viral inhibitors can be selected. Recently, Kuntzen et al. reported that viral loads were high in the majority of treatment-naïve patients carrying mutations of protease and polymerase inhibitors.[12] Low viral load may therefore be an important factor for achieving SVR by telaprevir monotherapy.

It has recently been reported that CH-C patients never treated with an NS3-4A protease inhibitor may nevertheless possess variants resistant to protease inhibitors involving the HCV RNA NS3 region.[12–14] While there was a resistant variant (T54A) in this case, this mutation exhibits only low-level resistance,[7] and the number of mutant variants may have been few along with substantial suppression of HCV replication by telaprevir. This may also help to explain the effectiveness of telaprevir in this case.

Moreover, two amino acid substitutions (R130K and Q195K) were unique to this patient. We therefore checked the nucleotide sequence data in the DDBJ/EMBL/GenBank databases and found a previous report by Franco et al. on the R130K substitution (EF013801, EF013863, EF013867, EF013869).[15] Interestingly, although only a minor clone (4% of total) in that study, the viral load of the patient with the R130K substitution was also low (2364 IU/mL). To date, however, the Q195K substitution has not been reported. Their presence in this case may indicate that telaprevir has a stronger antiviral activity against HCV with these substitutions.

The NS3-4A protease targeted by protease inhibitors is required for viral polyprotein processing, an essential step in viral replication, but is also responsible for disrupting IFN responses to the infection.[16] Previous studies have shown that high concentrations of protease inhibitors may restore retinoic acid-inducible gene I (RIG-I) signaling in HCV replicon cells, [16–18] and Liang et al. also recently reported that protease inhibitors could restore interferon regulatory factor 3 (IRF-3) signaling in HCV-infected cells.[19] In our patient, telaprevir may have therefore rescued the NS3-4A-mediated blockade of IRF-3 signaling *in vivo*.

Further studies are required, such as sequencing analyses of the HCV NS3 region, and research into the rescue of IFN-β signaling through the RIG-I pathway. It is foreseeable in the future for CH-C patients to be treated by one or a combination of two or more oral drugs with high efficacy and genetic barriers to resistance and low side-effect profiles. Telaprevir may hold promise for being one of these drugs, even if only within a subset of patients, and further studies into telaprevir monotherapy or combination therapy with other oral drugs is therefore warranted. Although still an isolated response, based on our current molecular understanding of HCV infection and pharmacotherapy, this case suggests that long-term telaprevir monotherapy may be effective in other CH-C patients with genotype 1 and a low viral load.

Conflict of interest

The authors have no commercial or other associations that may pose a conflict of interest.

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Virus Clearance Reduces Bone Fracture in Postmenopausal Women With Osteoporosis and Chronic Liver Disease Caused by Hepatitis C Virus

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Osteoporosis is often present in postmenopausal women. The aim of this retrospective cohort study was to assess the cumulative incidence and predictive factors for bone fracture after cessation of interferon (IFN) in postmenopausal women with osteoporosis and chronic liver disease caused by hepatitis C virus (HCV). A total of 420 postmenopausal women treated with IFN monotherapy were enrolled. The mean observation period was 7.2 years. The primary goal was the development of bone fracture. Evaluation was carried out by using the Kaplan–Meier method and the Cox proportional hazards analysis. Thirty-one out of 420 patients sustained bone fracture. The cumulative development rate of bone fracture was 3.6% at 5th year, 9.2% at 10th year, and 17.4% at 15th year. Multivariate Cox proportional hazards analysis showed that bone fracture after cessation of IFN therapy occurred when histological staging of the liver was advanced (hazard ratio (HR): 2.54; 95% confidence interval (CI) = 1.21–5.31; $P = 0.013$), serum albumin level was < 3.5g/dl (HR: 2.25; 95% CI = 1.10–4.59; $P = 0.026$), and virus clearance was not achieved (HR: 3.65; 95% CI = 1.11–12.05; $P = 0.033$). The results indicate that virus clearance causes a reduction of two-thirds in the risk of bone fracture after cessation of IFN therapy in postmenopausal women with osteoporosis and chronic liver disease caused by HCV. ***J. Med. Virol. 82:390–395, 2010.***

KEY WORDS: chronic hepatitis C; osteoporosis; interferon; bone fracture

INTRODUCTION

Hepatitis C virus (HCV) is one of the more common causes of chronic liver disease in all countries. Chronic hepatitis C is a progressive form of liver disease that progresses relentlessly but silently to cirrhosis and/or hepatocellular carcinoma (HCC) over a period of 10–30 years [Kiyosawa and Furuta, 1991; Alter et al., 1992; Ikeda et al., 1993; Tsukuma et al., 1993]. Additionally, chronic infection due to HCV has been associated with a variety of extrahepatic complications such as essential mixed cryoglobulinemia, membranoproliferative glomerulonephritis, autoimmune thyroiditis, and sialadenitis [Johnson et al., 1993; Gumber and Chopra, 1995; Pawlotsky et al., 1995].

Bone disease is one of the major complications of chronic liver disease. The rate of bone fracture is increased in chronic liver disease, especially in postmenopausal women [Rouillard and Lane, 2001; Shiomo et al., 2002; Arase et al., 2008]. Although there is growing evidence to support the concept that HCV infection is a risk factor for bone fracture, there have been a few interventional studies confirming this issue. This

Abbreviations used: ALT, alanine aminotransferase; AST, aspartate aminotransferase; CI, confidence interval; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; HR, hazard ratio; IFN, interferon.

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requires confirmation by a long-term follow-up of patients with a high risk of developing bone fracture.

The present retrospective cohort study was, therefore, undertaken to determine the cumulative incidence and risk factors of bone fracture after cessation of interferon (IFN) monotherapy among postmenopausal women with osteoporosis and chronic liver disease caused by HCV.

MATERIALS AND METHODS

Patients

The number of patients who were diagnosed with chronic HCV infection and treated with IFN between April 1994 and March 2007 in the Department of Hepatology, Toranomon Hospital, Tokyo, Japan was 6,003. Out of these, 420 postmenopausal women with the following criteria were enrolled in this retrospective cohort study. The enrollment criteria were: age of 55–75 years; postmenopausal osteoporosis; features of chronic hepatitis or cirrhosis diagnosed by laparoscopy, liver biopsy, ultrasonography clinical features, and/or laboratory tests; positive for HCV-RNA; treatment with IFN monotherapy, negative for hepatitis B surface antigen, antinuclear antibodies, or antimitochondrial antibodies in serum, as determined by radioimmunoassay or spot hybridization; no evidence of HCC nodules as shown by ultrasonography and/or computed tomography; no underlying systemic disease, such as systemic lupus erythmatosus, rheumatic arthritis. The diagnosis of osteoporosis was based to X-ray evidence of vertical trabecular and/or loss bone mineral density of spine or femur (AP spine by dual-energy X-ray absorptiometry, DEXA) >2 SD of young adult mean. A total of 234 patients were diagnosed by standard X-ray examination. The remaining 186 patients were diagnosed by standard X-ray and DEXA. Patients with either of the following criteria were excluded from the study: (1) malignant tumor, (2) advanced and decompensated stage of cirrhosis with encephalopathy, icterus, or refractory ascites.

In the present study, predictive factors for bone fracture after cessation of IFN therapy were assessed. All of the studies were performed retrospectively by collecting and analyzing data from the patient records. This study had been approved by Institutional Review Board of the hospital.

Viral Markers of HCV

Diagnosis of HCV infection was based on detection of serum HCV antibodies and HCV RNA. Anti-HCV antibodies were detected using a second-generation enzyme-linked immunosorbent assay (ELISA II) (Abbott Laboratories, North Chicago, IL). HCV-RNA was determined by the Amplicor method (Cobas Amplicor HCV Monitor Test, v2.0, Roche Molecular Systems, Inc., Branchburg, NJ).

Evaluation of the Stage of Liver Disease

The stage of liver disease was determined partly on the basis of peritoneoscopy and/or liver biopsy. The 291 patients out of 420 were diagnosed by peritoneoscopy and/or liver biopsy. Liver biopsy specimens were obtained 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–eosin, Masson's trichrome, silver impregnation, and periodic acid-Schiff after diastase digestion. The size of specimens for examination was more than six portal areas [Desmet et al., 1994]. The remaining patients were diagnosed by clinical features, laboratory tests, and ultrasonographic findings.

Follow-Up

Patients were followed-up monthly to tri-monthly after the cessation of IFN therapy at the Toranomon hospital. Physical examination and biochemical tests were conducted at each examination together with a regular follow-up using abdominal ultrasonography and/or computed tomography imaging in each patient. When a patient had any symptoms relating to bone fracture, the physicians in charge explored further the possibility of bone fracture. Forty-seven patients were lost to follow-up. Because bone fracture and death were not identified in these 47 patients, they were regarded as withdrawals at the time of the last visit at the Toranomon hospital in statistical analysis [Harrington and Fleming, 1983].

Statistical Analysis

The cumulative development rate of bone fracture was calculated from the time of cessation of IFN therapy by using the Kaplan–Meier method. Differences in the development of bone fracture were tested using the log rank test. Independent factors associated with the development of bone fracture were analyzed by the Cox proportional hazard model. The following 12 variables were analyzed for potential covariates for development of bone fracture: age, body mass index, state of liver disease (chronic hepatitis or liver cirrhosis), HCV load, HCV-genotype, platelet count, albumin, total-cholesterol, alanine aminotransferase (ALT), kind of IFN, total dose of IFN, and efficacy of IFN therapy. A P-value of <0.05 in the two-tailed test was considered significant. Data analysis was performed using the computer program SPSS version 11.0.

RESULTS

Characteristics of the Patients

Table I shows the characteristics of the 420 women with postmenopausal osteoporosis and type C chronic liver disease. The number of patients with virus clearance was 111 (26.4%). The observation period (mean ± standard deviation) was 7.2 ± 3.5 years.

Bone Fracture

Thirty-one out of 420 patients sustained bone fracture. Seventeen patients had vertebral fracture alone

TABLE I. Characteristics of Subjects Enrolled

Characteristic	
N	420
Age (years)	64.1 ± 3.5
BMI	22.1 ± 3.6
HCV-genotype (1b/2a/2b/others)	237/110/46/27
HCV RNA level (KIU/ml)	1,193 ± 1,151
Staging (chronic hepatitis/liver cirrhosis)	310/110
AST (IU/L)	80 ± 56
ALT (IU/L)	101 ± 70
Albumin (g/dl)	4.0 ± 0.3
Total cholesterol (mg/dl)	165 ± 30
Platelet count ($\times 10^4$/mm^3)	13.6 ± 4.8
IFN-alpha[a]/IFN-beta[a]	300/120
Total dose of IFN (Megaunit)	582 ± 204
Efficacy of treatment (virus clearance/ non-virus clearance)	111/309
Follow-up period (year)	7.2 ± 4.4

ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; IFN, interferon.
Data are number of patients, median (range), or mean ± standard deviation.
[a]Outbreak of IFN monotherapy: recombinant IFN alpha 2a, 35 cases; recombinant IFN alpha 2b, 23 cases; natural IFN alpha, 242 cases; natural IFN beta, 120 cases.

and seven patients had hip fracture alone. One patient had both vertebral and hip fracture. Remaining six patients had bone fractures except in vertebral or hip. The cumulative appearance rate of bone fracture was 3.6% at 5th year, 9.2% at 10th year, and 17.4% at the 15th year in all the patients (Fig. 1).

Determinants of Bone Fracture

Table II shows the factors associated with bone fracture after cessation of IFN therapy in all the 420 women with postmenopausal osteoporosis and chronic liver disease caused by HCV. Univariate analysis identified the following four factors that influenced incidence of bone fracture: liver staging ($P = 0.002$), serum albumin level ($P = 0.016$), efficacy of IFN ($P = 0.039$), and kind of IFN ($P = 0.095$). These four parameters were entered into multivariate Cox proportional hazard analysis. Multivariate Cox proportional hazards analysis showed that bone fracture occurred when patient had liver cirrhosis (hazard ratio (HR): 2.54; 95% confidence interval (CI) = 1.21–5.31; $P = 0.013$), serum albumin level of <3.5 mg/dl (HR: 2.25; 95% CI = 1.10–4.59; $P = 0.026$), and non-virus clearance (HR: 3.65; 95% CI = 1.11–12.05; $P = 0.033$).

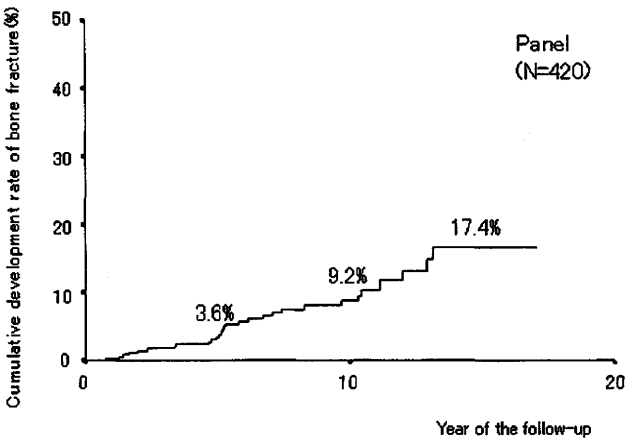

Fig. 1. Cumulative development rate of the bone fracture after the termination of IFN treatment in a total of women with osteoporosis and type C chronic liver disease.

Causes of Death After Bone Fracture

During the observation period after an episode of bone fracture, 10 of the 31 patients died. Four patients died of liver-related disease (HCC, decompensated liver cirrhosis, rupture of esophageal varices). On the other hand, six patients died of infection and deterioration of general condition. In a total of 10 patients died after the development of bone fracture, liver-related death corresponded to 40% (4/10) of all deaths.

DISCUSSION

Bone fracture after cessation of IFN monotherapy in postmenopausal women with osteoporosis and chronic liver disease caused by HCV is described in the present study. The study was limited because a retrospective cohort trial. Postmenopausal women aged 55–75 years and diagnosed as having osteoporosis were selected. The reason for the selection of women aged 55–75 years as follows; (1) Onset of bone fracture based on osteoporosis is rare in young females with <55 years and/or males, (2) patients over the age of 75 years have a tendency to avoid IFN therapy due to some IFN-related side effects. Other limitations are the followings: (1) serum levels of vitamin D were not measured, (2) bone density measurement was not measured.

However, several findings were obtained with regard to bone fracture after cessation of IFN in postmenopausal women with osteoporosis and chronic liver disease caused by HCV. First, the annual rate of bone fracture among female patients with osteoporosis and chronic liver disease caused by HCV was about 1%. Second, the development rate of bone fracture in patients with virus clearance was low with statistical significance compared to that without virus clearance. In a previous study, it was reported that virus clearance reduce the onset of malignant lymphoma and/or type 2 diabetes in HCV patients treated with IFN [Kawamura et al., 2007; Arase et al., 2009a]. The present study shows that virus clearance reduces the development of bone fracture in HCV patients. The reasons for the reduction of bone fracture in patients with virus clearance are unclear. Possible reasons are that improvement of nutrition and physical activity after virus clearance might reduce the development of bone fracture. Third, in addition to virus clearance, slight fibrosis of the liver and serum albumin level of ≥3.5 g/dl reduced the onset of bone fracture in HCV patients treated with IFN. These results suggest that maintaining a serum albumin level of ≥3.5 g/dl is important for

TABLE II. Predictive Factors for Appearance of Bone Fracture*

Variables	Univariate analysis		Cox-regression	
	HR (95%CI)	*P*	HR (95%CI)	*P*
Age (years) (≥65/<65)	1.90 (0.93–3.89)	0.078		
BMI (≥25/<25)	1.46 (0.65–3.21)	0.362		
HCV load (KIU/ml) (≥1,000/<1,000)	1.12 (0.96–1.32)	0.155		
Genotype (1/2)	0.74 (0.35–1.57)	0.431		
ALT (IU/L) (<50/≥50)	0.50 (0.14–1.77)	0.289		
Platelet count ($\times 10^4/mm^3$) (<15/≥15)	1.52 (0.64–3.61)	0.345		
Albumin (g/dl) (<3.5/≥3.5)	2.37 (1.17–4.80)	0.016	2.25 (1.10–4.59)	0.026
Cholesterol (mg/dl, ≥180/<180)	0.33 (0.04–2.72)	0.305		
Staging (liver cirrhosis/chronic hepatitis)	2.85 (1.38–5.90)	0.005	2.54 (1.21–5.31)	0.013
Kind of IFN (beta/alpha)	0.44 (0.17–1.15)	0.095	0.40 (0.15–1.05)	0.062
Total dose of IFN (MU) (≥500/<500)	0.91 (0.59–1.40)	0.672		
Efficacy (non-virus clearance/virus clearance)	3.50 (1.06–11.53)	0.039	3.65 (1.11–12.05)	0.033

ALT, alanine aminotransferase; BMI, body mass index; HR, hazards ratio; IFN, interferon.
*Data are number of patients or mean ± standard deviation.

protecting the bone fracture in HCV patients. Definitive treatment of maintaining a serum albumin level of ≥3.5 g/dl is unclear. However, the use of branched-chain amino acid granules has been shown to improve hypoalbuminemia in decompensated patients with cirrhosis and hypoalbuminemia despite adequate dietary intake [Yoshida et al., 1989; Muto et al., 2005].

Bone fracture in patients treated with IFN-beta was slightly lower compared with that in patients treated with IFN-alpha as shown in Figure 2 in spite of $P > 0.05$. Takayanagi et al. [2002] have reported that administration of IFN-beta into the site of inflammation resulted in marked inhibition of osteoclast formation and bone resorption. Although the role of IFN-beta in the bone

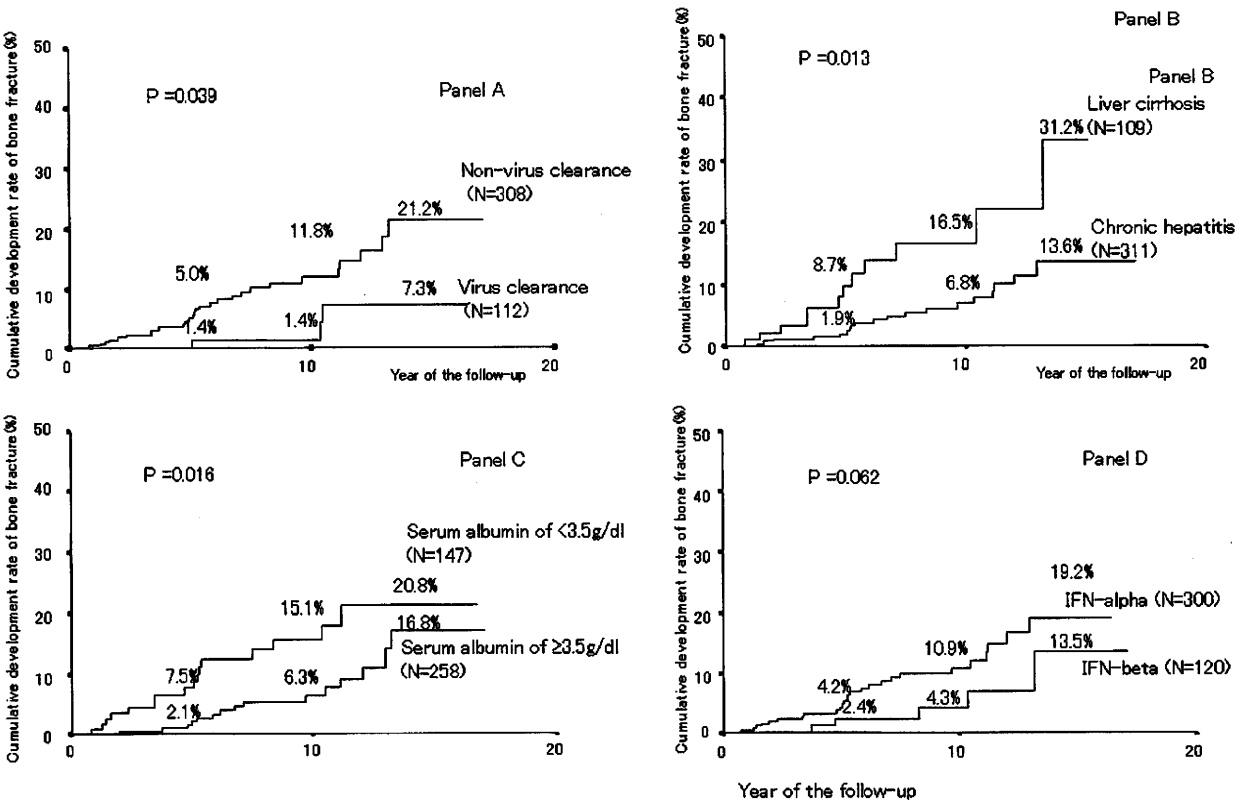

Fig. 2. **Panel A**: Cumulative development rate of the bone fracture based to difference of treatment efficacy. **Panel B**: Cumulative development rate of the bone fracture based to difference of histological staging of the liver. **Panel C**: Cumulative development rate of the bone fracture based to difference of serum albumin level. **Panel D**: Cumulative development rate of the bone fracture based to difference of kind of IFN.

metabolism remains speculative, the following possible mechanism have been considered [Abraham et al., 2009], (1) binding of IFN-beta to its biological receptor of nuclear factor-kappaB (RANK) ligand (RANKL) initiates a signal transduction cascade through the classic JAK/STAT pathway, causing an inhibition of osteoclast proliferation and differentiation; (2) another mechanism pertinent to the anti-resorptive effect of IFN-beta is the induction of nitric oxide which has been shown to inhibit osteoclast formation. On the other hand, IFN-alpha did not inhibit osteoclast formation and bone resorption. This result may indicate that the role of IFN-beta in bone metabolism way warrant systematic evaluation as a potential adjunct to therapeutic regimens of osteolytic diseases

IFN-beta should be given intravenously. Intravenous injection is not convenient for treatment compared to intramuscular or subcutaneous injection. However, IFN-beta-related side effects are mild and few compared to combination therapy with IFN-alpha. IFN-beta-induced mental disorders are milder than those induced by IFN-alpha [Katamura et al., 2008]. IFN-beta could also be given in elderly patients of 70 or older years because of mild side effects [Arase et al., 2009b]. Thus, about 10% of HCV patients are given IFN-beta in Japan.

Recent studied have reported that osteodystrophy occurs not only in patients with alcoholic cirrhosis, but also in those with cirrhosis caused by hepatitis C or B virus. Due to improvement of treatment, patients with cirrhosis live longer; an increasing proportion of such patients are found to have bone disease [Tsuneoka et al., 1996]. Thus, physicians undertaking the daily management of patients with hepatitis virus should check the bone condition of the patients in addition to the liver.

In conclusion, the present retrospective study shows that the annual incidence of bone fracture after cessation of IFN therapy among postmenopausal women with osteoporosis and chronic liver disease caused by HCV was about 1%. Virus clearance causes a two-thirds reduction in the risk of bone fracture after cessation of IFN in postmenopausal women with osteoporosis and HCV.

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Original Article

Correlation of YMDD mutation and breakthrough hepatitis with hepatitis B virus DNA and serum ALT during lamivudine treatment

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Aim: Continuous lamivudine treatment is associated with high frequency of drug resistance. We analyzed the incidence of tyrosine-methionine-aspartate-aspartate (YMDD) motif mutant and breakthrough hepatitis (BTH) in hepatitis B virus (HBV) DNA positive patients receiving lamivudine for > 1 year and correlated it with HBV DNA and alanine aminotransferase (ALT) levels to evaluate if these measurements can provide a practical option for monitoring patients in clinical practice and define early switch from lamivudine therapy.

Methods: Of the 929 patients receiving lamivudine for > 1 year, 359 patients who maintained an ALT level of ≤ 40 IU/L during the course of lamivudine treatment were stratified into two groups based on the duration of lamivudine treatment – one receiving lamivudine for < 3 years and the other for ≥ 3 years.

Results: The incidence of YMDD motif in patients receiving lamivudine for < 3 years was 27% in patients with ALT ≤ 20 IU/L, 58% with ALT ≤ 30 IU/L, and 63% with ALT ≤ 40 IU/L, ($P = 0.002$). The corresponding incidence of BTH was 2%, 7%, and 48% ($P < 0.001$). The incidence of YMDD motif and BTH in these patients was 7% and 2% with HBV DNA < 2.6 (log copies/mL) and ALT ≤ 20 IU/L, while with ALT at 21–30, the YMDD motif mutant was 16% and BTH was 0%.

Conclusion: Correlation of ALT and HBV DNA levels with YMDD motif mutant and BTH indicates that these measurements can be used in clinical practice for deciding early switch from lamivudine to other suitable antiviral therapies.

Key words: alanine transaminase, breakthrough hepatitis, hepatitis B virus, lamivudine, mutation, viral DNA

INTRODUCTION

LAMIVUDINE HAS GAINED increasing popularity since its approval in 1998 for the treatment of chronic hepatitis B virus (CHBV).[1–4] Lamivudine blocks HBV replication, reduces HBV DNA levels, normalizes alanine aminotransferase (ALT) levels, thereby resulting in histological improvement of the liver.[5] It is a reverse transcriptase inhibitor that acts by competing with the natural polymerase substrate deoxycytidine triphosphate (dCTP) and thus inhibits the elongation of HBV DNA minus strand. It incorporates into the nascent DNA strand and thereby acts as a chain terminator. Although lamivudine is very effective in inhibiting viral replication, the incidence of resistance is high, with an estimated 14–32% of patients developing resistance after 1 year of treatment, 38% after 2 years of treatment, and 53–76% after 3 years of treatment.

Resistance to lamivudine, which increases over years is due to development of mutations in the tyrosine-methionine-aspartate-aspartate (YMDD) motif in the DNA polymerase/reverse transcriptase, which is the main target of lamivudine.[4,6–9] This amino acid sequence in YMDD motif is predominantly involved in deoxynucleoside triphosphate (dNTP) binding in the catalytic site of the HBV DNA polymerase.

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Table 1 2007 Ministry of Health, Labour and Welfare of Japan guidelines for hepatits B virus (HBV)-positive patients for nucleoside analogue treatment for patients with chronic HBV receiving lamivudine therapy

Lamivudine therapy HBV DNA		< 3 years	≥ 3 years
Keep < 2.6 log copies/mL		Switch to entecavir 0.5 mg/day	Continue lamivudine 100 mg/day
≥ 2.6 log copies/mL	No BTH†	Switch to entecavir 0.5 mg/day	
	With BTH	Adefovir 10mg/day (duo therapy with lamivudine)	Adefovir 10 mg/day (duo therapy with lamivudine)

†After checking for absence of tyrosine-methionine-aspartate-aspartate (YMDD) motif mutation. BTH, breakthrough hepatitis.

Long-term lamivudine therapy is associated with amino acid substitutions mainly in the YMDD motif and also in the proximal FLLAQ (phenylalanine, leucine, alanine, glutamine) motif.[10] Common mutation may occur in the YMDD motif where the methionine residue is replaced either by valine (rtM204V) or isoleucine (rtM204I).[11] These amino acid substitutions form the basis of emergence of lamivudine-resistant strains of HBV and when these occur, the clinical condition may worsen, which is usually accompanied by increase in viral load and serum aminotransferase levels. YMDD mutants cause breakthrough hepatitis (BTH) and, therefore, require withdrawal or switch-over from lamivudine treatment. The American Association for the Study of Liver Diseases (AASLD) and the United States Algorithm for Management of Patients with Drug Resistance recommend either switching over to entecavir or adding adefovir in the event of lamivudine resistance.[12] The 2007 Japanese guidelines of the study group (Ministry of Health, Labour and Welfare of Japan)[13] on standardization of treatment for HBV positive patients for nucleoside analogue treatment for patients with CHBV receiving lamivudine therapy are explained below and also summarized in Table 1.

According to the 2007 guidelines for patients on lamivudine therapy, switching over criteria from lamivudine therapy has been changed from BTH to HBeAg status in patients maintaining HBV DNA copies ≥ 2.6 log copies/mL. Patients on lamivudine for < 3 years and maintaining HBV DNA copies ≥ 2.6 log copies/mL can be switched over to entecavir 0.5 mg/day if they are also HBeAg negative, whereas HBeAg-positive patients can be co-administered adefovir 10 mg/day in both the treatment duration groups (> 3 years or < 3 years).

Unfortunately, the cost of measuring HBV resistance to lamivudine by molecular methods is high and is not presently covered by Japanese reimbursement system in clinical practice. Development of HBV resistance to lamivudine is typically indicated by an increase in HBV DNA followed by an increase in serum ALT levels. Increase in HBV DNA represents active viral replication whereas serum ALT levels provide an indirect assessment of the degree of liver injury.[14]

Hence, in this study, we analyzed the correlation of the incidence of YMDD motif mutant and BTH with HBV DNA and serum ALT levels, either separately or together, in HBV DNA-positive patients who are treated with lamivudine for ≥ 1 year and who had maintained an ALT level of ≤ 40 IU/L until the development of BTH during the course of lamivudine treatment.

METHODS

Patients

THIS WAS A retrospective, nonrandomized study that enrolled 929 HBV DNA-positive-patients receiving 100 mg of lamivudine daily and followed up for a period of 1 year or longer between 1995 and 2006. Since long-term treatment with lamivudine was associated with a high frequency of YMDD motif mutant and BTH (BTH can be defined as abnormal variations in serum transaminase level due to YMDD motif mutant), we analyzed patients who had a possibility to switch to other nucleoside analogues. Patients ($n = 395$) with ALT ≤ 40 IU/L during follow-up (for 48 patients who developed BTH, data was used until 1 month before the patient developed BTH). Patients were not treated with either adefovir or entecavir during follow-up (for patients who used adefovir or entecavir because of BTH development, data was used until the point before the patient started adefovir or entecavir treatment). Patients were negative for anti-hepatitis C virus (HCV) (third-generation enzyme immunoassay; Chiron, Emerville, CA) and negative for HCV RNA with PCR (Amplicor; Roche Diagnostic Systems, Pleasanton, CA), did not have hepatocellular carcinoma, nore other forms of liver injury such as hemochromatosis, Wilson's disease,

primary biliary cirrhosis, alcoholic liver disease, and autoimmune liver disease.

Informed consent was obtained from each patient included in the study. The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in a priori approval by the institution's human research committee.

Patients were stratified into 2 groups based on the duration of lamivudine treatment – one receiving lamivudine for < 3 years ($n = 125$) and the other for ≥ 3 years ($n = 234$). In addition, we also analyzed patients based on their ALT level (IU/L) grouped into ≤20, 21–30, and 31–40, and HBV DNA (log copies/mL) divided into < 2.6, 2.6–5.0, and ≥ 5.1.

During treatment, patients were followed up each month for liver function and serum markers of HBV infection. The serum sample of the patients were collected and preserved at −80 °C. All the collected samples up to this time period were analyzed for HBV DNA in June 2001. From July 2001, the serum samples were collected and analyzed once a month at the clinical treatment facility.

YMDD motif mutants were determined at the baseline and monitored at 6 months and during the study as well as at the development of breakthrough hepatitis. YMDD motif mutants were analyzed in the serum preserved at −80 °C altogether.

Markers of HBV infection

The HBeAg was estimated by enzyme-linked immunosorbent assay (ELISA) (F-HBe; Sysmex, Kobe). HBV DNA was determined by PCR followed by hybridization (Amplicor HBV Monitor: Roche Molecular Systems, Branchburg, NJ), and the results were expressed in log copy per milliliter over a range of 2.6–7.6. The 6 major genotypes of HBV (A–F) were determined serologically by ELISA (HBV GENOTYPE EIA; Institute of Immunology) and the PCR-invader method with genotype-specific probes.[15] YMDD motif mutants were determined by PCR followed by restriction fragment length polymorphism (RFLP)[8] or enzyme-linked minisequence assay with commercial assay kits (PCR-ELMA; Genome Science).

Statistical analyses

Frequencies were compared between groups by the χ^2-test, Fisher's exact test, and HBV DNA values by Mann–Whitney *U*-test. Emergence of YMDD motif mutants and BTH were compared in the Kaplan–Meier life table by using the production limit method. A *P*-value < 0.05 was considered significant. Analyses of all data were performed with SAS 9.1.3.

RESULTS

DURING THE PERIOD of 12 years from 1995 to 2006, 929 HBV DNA-positive patients received 100 mg of lamivudine daily. From the total of 929 patients who received lamivudine for 1 year or more, 359 patients who maintained an ALT level of ≤ 40 IU/L were stratified based on the duration of lamivudine treatment and divided into 2 groups – one receiving lamivudine for < 3 years ($n = 125$) and the other for ≥ 3 years ($n = 234$). Demographic features and clinical background of the two study groups were uniformly matched with no significant differences in age, sex, serum transaminase levels, HBV DNA, hepatitis B e-antigen (HBeAg), and HBV genotype (Table 2). The median ALT values were 112 IU/L and 145 IU/L in both the groups, respectively, and the median HBV DNA level was identical at 6.1 log copies/mL in both the groups.

Incidence of YMDD motif mutant and BTH after lamivudine treatment for < 3 years

The incidence of YMDD motif mutant within 3 years of treatment with lamivudine by ALT (IU/L) level was 27% in 53 patients maintaining an ALT level of ≤ 20 (group A), 58% in 46 patients maintaining an ALT level of ≤ 30 (group B); and 63% in 26 patients maintaining an ALT level of ≤ 40 (group C), with statistical differences among the 3 groups ($P = 0.002$). The incidence of BTH was 2% in group A, 7% in group B, and 48% in group C ($P < 0.001$). The lowest incidence of YMDD motif mutant and BTH was noted in patients with ALT level of ≤ 20 (IU/L) (Fig. 1a,b). Follow-up for patients who developed BTH was discontinued upon the detection of YMDD motif mutant.

The incidence of YMDD motif mutant within 3 years of treatment with lamivudine based on the HBV DNA (log copies/mL) level was 28% in patients maintaining an HBV DNA level of < 2.6; 83% in patients maintaining an HBV DNA level of 2.6–5.0; and 100% in patients maintaining an HBV DNA level of ≥ 5.1, with significant differences among the 3 groups ($P < 0.001$). The incidence of BTH was 4%, 30%, and 40%, respectively, in patients with HBV DNA level of < 2.6, 2.6–5.0, and ≥ 5.1 log copies/mL ($P = 0.004$) (Fig. 2a,b). The lowest incidence of YMDD motif mutant and BTH was seen in patients maintaining an HBV DNA level of < 2.6 log

Table 2 Background of 359 patients using lamivudine treatment for ≥ 1 year at the start of lamivudine therapy

Factors	Duration of lamivudine therapy		
	< 3 years *n* = 125	≥ 3 years *n* = 234	Differences (*P*-value)
Age (years)	23–75 (43)†	18–76 (43)†	NS‡
Male	93 (73%)	182 (77.1%)	NS‡
HBV infection in mother	47 (37%)	82 (35%)	NS‡
Chronic hepatitis	109 (85%)	212 (90%)	NS‡
AST (IU/L)	15–866 (80)†	19–2593 (83)†	NS‡
ALT (IU/L)	11–2092 (112)†	14–2142 (145)†	NS‡
Total bilirubin (mg/dL)	0.2–3.8 (0.7)†	0.2–10.6 (0.7)†	NS‡
γ-GTP (IU/L)	16–440 (54)†	13–468 (65)†	NS‡
HBV DNA (log copy/mL)	<2.6–>7.6 (6.1)†	<2.6–>7.6 (6.1)†	NS‡
HBeAg	66(52%)	107 (45%)	NS‡
HBV genotype (A, B, C, ND)	4:15:98:8	5:21:207:1	NS‡

†Median value where indicated. ‡Not significant. ALT, alanine transaminase; AST, aspartate aminotransferase; HBeAg, hepatitis B e-antigen; HBV, hepatitis B virus; γ-GTP, gamma glutamyl transferase.

copies/mL. The BTH incidence was particularly high in patients with an HBV DNA level of ≥ 5.1, which was 40% within 1 year.

The incidence of YMDD motif mutant within 3 years of treatment with lamivudine in patients based on both the ALT (IU/L) and HBV DNA (log copies/mL) level during the course of lamivudine treatment was evaluated (Table 3).

In patients maintaining HBV DNA < 2.6 and ALT ≤ 20, the incidence of YMDD motif mutant and BTH was 7% and 2%, respectively. Whereas in patients with HBV DNA level of < 2.6 and ALT 21–30, the incidence of YMDD motif mutant was higher at 16% and BTH was 0%, and in patients with ALT 31–40, YMDD motif mutant and BTH was further higher at 42% and 17%, respectively.

In patients with HBV DNA level at 2.6–5.0 and ALT ≤ 20, the incidence of YMDD motif mutant was 33% in patients with 0% incidence of BTH. Nevertheless, in patients maintaining HBV DNA at 2.6–5.0 but with ALT 21–30, the incidence of YMDD motif mutant was 73% and BTH was 18%; whereas in patients with ALT 31–40, the incidence of YMDD motif mutant was 50% and BTH was 42%.

In patients maintaining HBV DNA ≥ 5.1 and ALT 31–40, both YMDD motif mutant and BTH was 100%.

Incidence of YMDD motif mutant and BTH after lamivudine treatment for ≥ 3 years

In patients treated with lamivudine for 3 years or more, the incidence of YMDD motif mutant by ALT (IU/L) level was 58% in 113 patients in group A, 60% in 84

Table 3 Incidences of tyrosine-methionine-aspartate-aspartate (YMDD) mutant and breakthrough hepatitis (BTH) by hepatits B virus (HBV) DNA and alanine transaminase (ALT) level in patients during lamivudine treatment for < 3 years (125 patients)

HBV DNA† (Amplicor: log copies/mL)	ALT level (IU/L)†					
	≤ 20		21–30		31–40	
	YMDD	BTH	YMDD	BTH	YMDD	BTH
< 2.6	3/41 (7%)	1/41 (2%)	5/32 (16%)	0/32 (0%)	5/12 (42%)	2/12 (17%)
2.6–5.0	4/12 (33%)	0/12 (0%)	8/11 (73%)	2/11 (18%)	6/12 (50%)	5/12 (42%)
≥ 5.1	0	0	3/3 (100%)	0/3 (0%)	2/2 (100%)	2/2 (100%)

†The HBV DNA and ALT levels are shown based on the treatment duration of lamivudine.