

Influence of Amino-Acid Polymorphism in the Core Protein on Progression of Liver Disease in Patients Infected With Hepatitis C Virus Genotype 1b

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The substitution of amino acid (aa) 70 of arginine for glutamine and/or that of aa91 of leucine for methionine in the core protein in patients infected with hepatitis C virus (HCV) genotype 1b is associated with a poor response to pegylated interferon and ribavirin. Factors influencing these substitutions were sought in 1,097 patients infected with HCV-1b who had not received antiviral treatment. HCV variants with Arg70 and Leu91 (wild-type) decreased, while those with Gln70 and/or Met91 (mutant types) increased with age ($P < 0.001$). Of the 1,097 patients, 464 (42.3%) were infected with the Gln70 variant and the remaining 633 patients with the Arg70 variant. The proportion of patients with the Gln70 variant increased with the severity of liver disease ($P < 0.001$), elevated γ -glutamyl transpeptidase (γ -GTP) levels ($P < 0.001$) and a decrease in platelet count ($P = 0.008$). In univariate analysis patients with hepatocellular carcinoma, elevated aspartate aminotransferase (AST ≥ 58 IU/L) and γ -GTP (≥ 61 IU/L), and decreased albumin levels (< 3.9 g/dl) were more frequent in the patients with the Gln70 variant than the Arg70 variant ($P = 0.003$, 0.005 , < 0.001 , and 0.031 , respectively). In multivariate analysis HCC (odds ratio 1.829 [95% confidence interval 1.147–2.917]) and γ -GTP ≥ 61 IU/L (1.647 [1.268–2.139]) increased the risk for the Gln70 variant. In conclusion, the substitution of amino aa70 of Arg for Gln in patients infected with HCV-1b increases with age, and it is associated with severe liver disease accompanied by elevated AST and γ -GTP levels, as well as the development of hepatocellular carcinoma. **J. Med. Virol.** 82:41–48, 2010. © 2009 Wiley-Liss, Inc.

KEY WORDS: cirrhosis; core protein; hepatitis C; hepatocellular carcinoma; interferon; ribavirin

INTRODUCTION

Worldwide, an estimated 170 million people are infected with hepatitis C virus (HCV) persistently [Cohen, 1999]. Decompensated cirrhosis and hepatocellular carcinoma (HCC) can develop in about 30% of patients infected with HCV [Alberti et al., 1999; Seeff, 2002]. HCV has six major genotypes and dozens of subgenotypes, and they have distinct geographic distributions and are associated with the progression of liver disease [Simmonds, 1995]. Host and virological factors can influence the severity of liver disease and the response to antiviral treatment. HCV infection in the childhood and women runs a milder course than that in adulthood and men, and the intake of alcohol accelerates the progression of liver disease [Poynard et al., 1997; Kenny-Walsh, 1999; Vogt et al., 1999; Wiese et al., 2000]. Genotypes 1 and 4 aggravate liver disease and decrease the response to antiviral treatment, in comparison with genotypes 2, 3, and 6 [Tsubota et al., 1994; Hui et al., 2003; Hadziyannis et al., 2004; Legrand-Abraham et al., 2005; Yuen and Lai, 2006]. High levels of HCV RNA in the serum can induce severe liver disease and decrease treatment response [Tsubota et al., 1994].

In Japan, genotype 1b in a high viral load (> 100 KIU/ml) accounts for $> 70\%$ of HCV infection, and decreases the treatment response in patients with chronic hepatitis C [Kumada et al., 2006]. Even with pegylated interferon (PEG-IFN) combined with ribavirin, the sustained virological response for longer than 24 weeks after the withdrawal of treatment is achieved merely in

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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 radio-immunoassay (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.

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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). Arg70/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

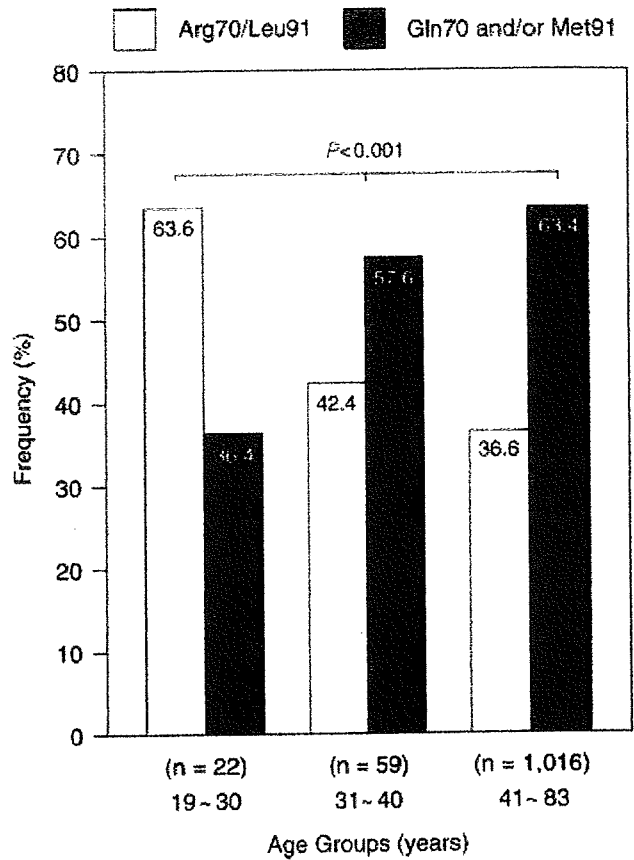


Fig. 1. The age-specific prevalence of Gln70 in treatment-naive patients infected with HCV-1b.

(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).

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 ($\times 10^3/\text{mm}^3$)	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

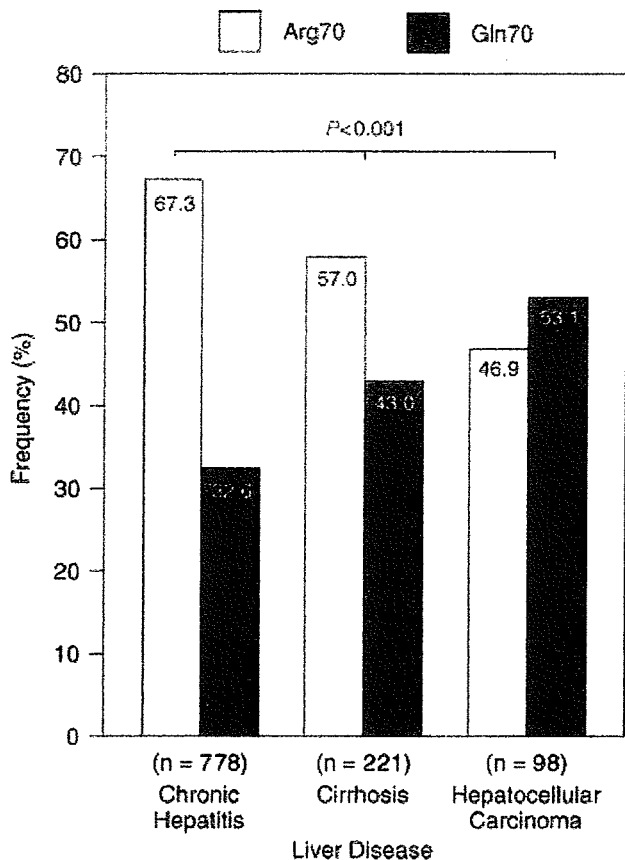


Fig. 2. The prevalence of the Gln70 variant among patients with chronic hepatitis, cirrhosis and hepatocellular carcinoma.

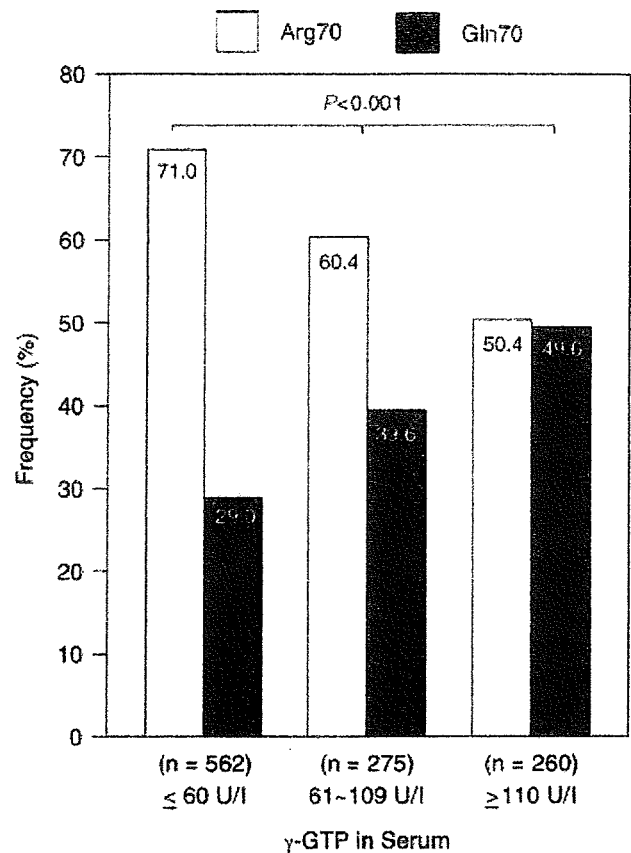


Fig. 3. The prevalence of the Gln70 variant among patients with different γ -GTP levels.

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

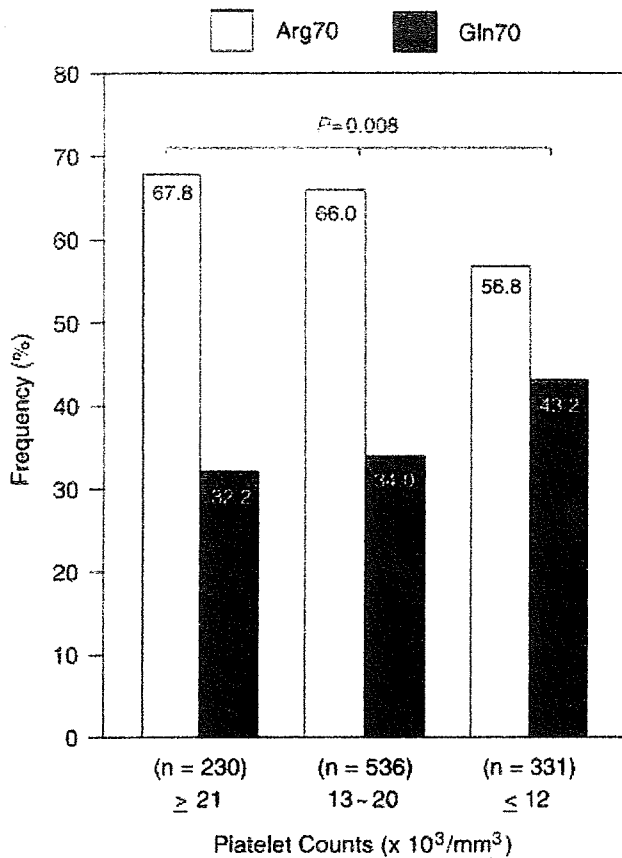


Fig. 4. The prevalence of the Gln70 among three groups of patients with different platelet counts.

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 (x10 ³ /mm ³)	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 (×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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Original Article

Development of HCC in patients receiving adefovir dipivoxil for lamivudine-resistant hepatitis B virus mutants

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Aim: To identify factors for the development of hepatocellular carcinoma (HCC) in the patients who receive adefovir add-on lamivudine for treatment of lamivudine-resistant hepatitis B virus (HBV) mutants.

Methods: A total of 247 patients who developed lamivudine-resistant HBV mutants, with an increase of HBV DNA ≥ 1 log copies/mL, received adefovir dipivoxil 10 mg add-on lamivudine 100 mg daily during a median of 115 weeks (range: 25–282 weeks). They were followed for the development of HCC by imaging modalities every 3–6 months.

Results: HCC developed in 18 of the 247 (7.3%) patients. Eight factors were in significant association with the development of HCC by the univariate analysis. They included age, cirrhosis, platelet counts, levels of bilirubin, aspartate aminotransferase (AST), alanine aminotransferase and α -fetoprotein, as well as YMDD mutants at the start of

adefovir dipivoxil. By the multivariate analysis, AST levels, YIDD mutants, cirrhosis and age were independent factors for the development of HCC. By the Kaplan-Meier analysis, AST levels ≥ 70 IU/L, YIDD mutants, cirrhosis and age ≥ 50 years increased the risk of HCC ($P = 0.018$, $P = 0.035$, $P = 0.002$ and $P = 0.014$, respectively). HCC developed more frequently in the patients with than without cirrhosis at the start of adefovir (10/59 [16.9%] vs. 8/188 [4.3%], $P = 0.002$).

Conclusion: HCC can develop in cirrhotic patients receiving adefovir add-on lamivudine. Hence, the patients with baseline AST ≥ 70 IU/L and YIDD mutants would need to be monitored closely for HCC.

Key words: adefovir dipivoxil, chronic hepatitis B, hepatitis B virus, hepatocellular carcinoma, lamivudine, rescue therapy

INTRODUCTION

WORLDWIDE, AN ESTIMATED 400 million people are infected with hepatitis B virus (HBV) persistently, and one million die of decompensated cirrhosis and/or hepatocellular carcinoma (HCC) annually.^{1,2} Interferon (IFN) was introduced for treatment of chronic hepatitis B, and it has been replaced for pegylated-IFN.³ Due to substantial side-effects and requirement for injection, however, IFN-based therapies are not favored.

In 1998, lamivudine was approved as the first nucleot(s)ide analogue for treatment of chronic hepatitis B,⁴ and then adefovir in 2002.⁵ Due to its lower costs and

safety records, lamivudine has gained a wide popularity for treatment of chronic hepatitis B. However, drug-resistant mutants arise in parallel with the duration of lamivudine, in 12.5% after 1 year, in 43.8% after 3 years, and 62.5–70.2% after 5 years.^{6,7} For preventing breakthrough hepatitis induced by lamivudine-resistant HBV mutants, additional adefovir dipivoxil 10 mg daily has been recommended;^{8,9} it is more effective than switching to adefovir monotherapy and has fewer chances of developing drug-resistant mutants.^{10,11}

Since 1995, 930 patients with chronic hepatitis have been treated with lamivudine in the Department of Hepatology at the Toranomon Hospital in Metropolitan Tokyo.¹² HBV mutants with mutations in the thymine-methionine-aspartic acid-aspartic acid (YMDD) motif elicited in the 247 (26.5%) patients, and they started to receive additional adefovir since December, 2002.^{13,14} However, HCC developed in 18 (7.3%) of them during the combination therapy for 25–282 weeks; HCC has

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not been reported in any of the patients who have received adefovir add-on lamivudine for 5 years.^{15–17} Hence, factors for the development of HCC in the patients receiving adefovir add-on lamivudine were sought for in a retrospective study.

METHODS

Patients

OVER A PERIOD of 13 years, from September 1995 to September 2007, 930 patients with chronic hepatitis B received long-term lamivudine treatment at the Department of Hepatology at the Toranomon Hospital in Metropolitan Tokyo. Drug-resistant YMDD mutants developed in 247 (26.5%) of them, accompanied by an increase in HBV DNA ≥ 1 log copies/mL, and they received adefovir 10 mg in addition to lamivudine 100 mg daily during the median of 115 weeks (range: 25–282 weeks). They have been followed for liver function and virological markers of HBV infection monthly, as well as blood counts and tumor makers including alpha-fetoprotein (AFP) and protein induced by vitamin K absence or antagonist-II (PIVKA-II). Cirrhosis was diagnosed by laparoscopy or liver biopsy, and in the patients who had not received them, by clinical data, imaging modalities and portal hypertension. HCC was diagnosed by hypervascularity on angiography and/or histological examination, characteristic features of computed tomography, magnetic resonance imaging and ultrasonography. An informed consent was obtained from each patient in this study, and the protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in a *priori* approval by the institution's human research committee.

Markers of HBV infection

Hepatitis B e antigen (HBeAg) was determined by enzyme-linked immunosorbent assay (ELISA) with commercial kits (HBeAg EIA, Institute of Immunology, Tokyo). HBV DNA was quantitated by the Amplicor monitor assay (Roche Diagnostics, Tokyo) with a dynamic range over 2.6–7.6 log copies/mL. Genotypes of HBV were determined serologically by the combination of epitopes expressed on the pre-S2 region product, which is specific for each of the seven major genotypes (A–G),^{18,19} with use of commercial kits (HBV Genotype EIA, Institute of Immunology).

Detection of YMDD mutants

YMDD mutants were determined by polymerase chain reaction (PCR)-based enzyme-linked mini-sequence

assay (PCR-ELIMA) with commercial kits (Genome Science Laboratories, Tokyo).

Statistical analyses

Categorical variables were compared between groups by the χ^2 test, and non-categorical variables by the Mann-Whitney *U*-test. A *P*-value < 0.05 was considered significant. Factors associated with HCC by univariate analysis were evaluated by the multivariate analysis by the stepwise Cox proportional hazard model. Development of HCC with time was analyzed by the Kaplan-Meier method, and differences were evaluated by the log-rank test. Data were analyzed by the SPSS software, version 11.0 (Chicago, IL).

RESULTS

Baseline characteristics of the patients who did and who did not develop hepatocellular carcinoma during adefovir add-on lamivudine treatment

TABLE 1 COMPARES characteristics at the start of adefovir between the 18 patients who developed HCC and the 229 who did not. Eight factors were associated with the development of HCC by the univariate analysis. They included age, cirrhosis, platelet counts, bilirubin, AST, alanine aminotransferase (ALT) and α -fetoprotein (AFP) levels, as well as YMDD mutants. HCC developed more frequently in the patients with than without cirrhosis at the start of adefovir (10/59 [16.9%] vs. 8/188 [4.3%], *P* = 0.002). There were 61 (26.6%) patients who had cirrhosis at the start of adefovir. Of them, one of the 18 (2.2%) with HCC and 18 of the 229 (2.2%) without HCC presented with decompensation; no patients developed decompensation after the start of adefovir.

Rates of HBV DNA disappearance from serum (< 2.6 log copies/mL) were: 55% (113/207) at 1 year, 71% (119/168) at 2 years, 77% (78/101) at 3 years and 85% (35/41) at 4 years. Rates of AST normalization (< 38 IU/L) were: 87% (179/207) at 1 year, 90% (151/168) at 2 years, 92% (93/101) at 3 years and 95% (39/41) at 4 years; and those of ALT normalization (< 50 IU/L) were: 88% (183/207) at 1 year, 91% (153/168) at 2 years, 93% (94/101) at 3 years and 98% (40/41) at 4 years. There were no differences in the rate of HBV DNA disappearance from serum between the patients with and without HCC: 57% (8/14) vs. 54% (105/193) at 1 year (*P* = 1.0); 86% (12/14) vs. 70% (107/154) at 2 years (*P* = 0.229); and 89% (8/9) vs.

Table 1 Characteristics of patients who did and did not develop hepatocellular carcinoma (HCC) at the start of adefovir†

	HCC developed (n = 18)	HCC did not develop (n = 229)	Differences P-value
Duration of lamivudine before the start of adefovir	128 (31-346)	144 (13-617)	0.321
Age (years)	52 (35-75)	45 (26-75)	0.008
Men	15 (83%)	183 (80%)	1.000
Cirrhosis	10 (56%)	51 (22%)	0.004
Platelets ($\times 10^3/\text{mm}^3$)	12.0 (4.6-19.7)	16.3 (3.1-31.9)	0.001
Albumin (g/dL)	3.6 (2.3-4.7)	3.9 (2.8-4.7)	0.073
Bilirubin (mg/dL)	0.8 (0.5-15.5)	0.7 (0.2-6.0)	0.046
Creatinine (mg/dL)	0.8 (0.5-1.0)	0.8 (0.4-1.6)	0.950
AST (IU/L)	119 (55-248)	66 (14-1413)	0.003
ALT (IU/L)	151 (61-576)	104 (13-1563)	0.035
AFP (ng/dL)	8 (2-130)	4 (1-282)	0.026
HBV genotypes			0.228
C	18 (100%)	189 (87%)	
Others	0	27 (13%)	
HBeAg	8 (44%)	132 (58%)	0.323
HBV DNA (log copies/mL)	7.1 (4.4->7.6)	7.1 (<2.6->7.6)	0.623
YMDD mutants			0.041
YIDD	13 (72%)	109 (45%)	
YVDD	5 (28%)	62 (25%)	
YI/VDD	0	56 (23%)	

†Values are the median with the range in parentheses or *n* with percent in parentheses.

AFP, alpha-fetoprotein; ALT, alaine aminotransferase; AST, aspartate aminotransferase; HBeAg, hepatitis B e antigen; HBV, hepatitis B virus.

92% (85/92) at 3 years ($P = 0.555$). Rates of normalized AST levels in the patients with and without HCC were: 50% (7/14) vs. 90% (173/193) at 1 year ($P < 0.001$); 79% (11/14) vs. 91% (140/154) at 2 year ($P = 0.166$); and 67% (6/9) vs. 95% (87/92) at 3 year ($P = 0.037$). Rates of ALT normalization in the patients with and without HCC were: 71% (10/14) vs. 90% (174/193) at 1 year ($P = 0.037$); 79% (11/14) vs. 90% (139/154) at 2 year ($P = 0.189$); and 56% (5/9) vs. 92% (85/92) at 3 year ($P = 0.015$). Thus, normalization of AST and ALT was less frequent in the patients with than without HCC.

Characteristics of the 18 patients who developed HCC are compared between the baseline and at the development of HCC (Table 2). At the start of adefovir, 10 (56%) of them had developed cirrhosis and 16 (89%) had AST levels ≥ 70 IU/L. HBV DNA was not detectable in 10 (56%) of them at the development of HCC. Of the eight patients with detectable HBV DNA levels (≥ 2.6 log copies/mL), five (63%) developed HCC within 1 year after the start of adefovir. AST was elevated (> 38 IU/L) in eight patients, including four (50%) without detectable HBV DNA levels.

Factors independently associated with the development of hepatocellular carcinoma

Eight factors associated with the development of HCC by the univariate analysis, including age, cirrhosis, platelet counts, bilirubin, AST, ALT and AFP levels, as well as YMDD mutants (Table 1), were evaluated by the multivariate analysis. AST ≥ 70 IU/L, YIDD mutants, age ≥ 50 years and cirrhosis at the baseline were independent risk factors for the development of HCC (Table 3). There were no differences in the distribution of YIDD, YVDD and the mixture thereof among the patients with distinct AST, ALT or HBV DNA levels or between those with and without cirrhosis at the start of adefovir. HBV mutants with mutations resistant to adefovir (rtA181T/S, rtN236T) occurred in two of the 247 (0.8%) patients; none of them developed HCC.

The median time between the elevation of HBV DNA > 5.0 log copies/mL and the administration of adefovir was 124 (range: 0-815) days for the 13 patients who developed HCC and 147 (0-3268) days for the 166 patients who did not ($P = 0.605$). The median time between the elevation of ALT > 43 IU/L and the start of

Table 2 Characteristics of the 18 patients at commencement of adefovir (ADV) and development of hepatocellular carcinoma (HCC)

Patient no.	Age (years)	Sex	At the commencement of ADV						Period of ADV (years)			At the development of HCC		
			Liver disease	AST (IU/L)	ALT (IU/L)	HBeAg	HBV DNA (log copies/mL)	YMDD mutant	ADV (years)	AST (IU/L)	ALT (IU/L)	HBV DNA (log copies/mL)	AST (IU/L)	ALT (IU/L)
1	50	M	CH	248	576	-	6.9	I	4.5	26	27	<2.6		
2	35	M	LC	217	164	+	7.5	I	1.6	54	34	<2.6		
3	50	M	LC	192	272	+	>7.6	I	1.2	68	89	<2.6		
4	61	M	CH	192	332	-	6.9	I	2.8	22	23	<2.6		
5	65	M	CH	174	219	-	5.2	V	0.1	30	43	<2.6		
6	58	M	CH	160	216	-	6.5	V	2.2	41	32	<2.6		
7	53	M	LC	127	97	+	>7.6	I	0.5	55	41	3.2		
8	75	M	LC	119	209	+	>7.6	V	1.1	121	125	2.6		
9	58	F	CH	118	214	+	4.4	I	3.3	21	13	<2.6		
10	48	M	CH	116	99	+	>7.6	I	3.3	32	36	<2.6		
11	51	F	LC	111	130	-	5.3	I	0.9	88	95	<2.6		
12	47	M	CH	85	138	+	>7.6	I	1.3	28	29	3.1		
13	61	M	LC	81	65	-	5.6	I	0.2	32	27	2.9		
14	59	F	LC	80	132	-	>7.6	V	0.1	32	41	3.2		
15	40	M	LC	75	124	-	6.3	I	3.8	21	24	<2.6		
16	48	M	CH	71	61	-	6.6	I	0.6	48	26	3.7		
17	55	M	LC	55	76	+	7.3	I	0.2	50	64	5.4		
18	43	M	LC	27	21	-	5.4	V	1.6	30	23	3.7		

ALT, alanine aminotransferase; AST, aspartate aminotransferase; CH, chronic hepatitis; HBeAg, hepatitis B e antigen; HBV, hepatitis B virus; I, YIDD mutant; LC, cirrhosis; V, YVDD mutant.

Table 3 Independent risk factors influencing the development of hepatocellular carcinoma

Factors	Category	Hazard ratio (95% CI)†	P-value
AST (IU/l)	1: < 70	1	0.016
	2: ≥ 70	6.21 (1.40-27.5)	
YMDD mutants	1: YVDD or YV/IDD	1	0.012
	2: YIDD	3.97 (1.36-11.6)	
Age (years)	1: < 50	1	0.023
	2: ≥ 50	3.24 (1.17-8.95)	
Cirrhosis	1: Absent	1	0.030
	2: Present	1.42 (1.04-1.96)	

†Confidence interval.

adefovir was 59 (0-896) days for the patients who developed HCC and 54 (0-3240) days for those who did not ($P = 0.330$). Hence, exacerbation of hepatitis was not a risk factor for the development of HCC.

Age-specific risk factors for the development of HCC were evaluated by the multivariate analysis. In the patients < 50 years, platelet counts < $13 \times 10^3/\text{mm}^3$ was the only significant risk factor for HCC (hazard ratio 6.88 [95% confidence interval; 1.26-37.6]), while AST levels ≥ 70 IU/L was that in those ≥ 50 years (hazard ratio: 9.50 [95% confidence interval 1.20-74.9]).

Factors increasing the cumulative incidence of hepatocellular carcinoma

AST levels ≥ 70 IU/L at the start of adefovir increased the development of HCC during follow-ups ranging to 5 years (Fig. 1). HCC developed more frequently in the patients with YIDD mutants than in those with YVDD or the mixture of YVDD and YIDD mutants (Fig. 2). The cumulative incidence of HCC in the patients with YIDD mutants alone was: 4% at 1 year, 10% at 3 years and 43% at 5 years. In contrast, HCC never developed in the patients with the mixture of YIDD and YVDD mutants through 5 years of follow-up. HCC developed more frequently in the patients with cirrhosis and those aged ≥ 50 years (Figs 3,4, respectively).

DISCUSSION

HCC DEVELOPED IN 18 of the 247 (7.3%) patients who had received adefovir add-on lamivudine during a long-term ranging to 5 years. There were some differences in the characteristics at the start of adefovir dipivoxil between the patients who did and who did not

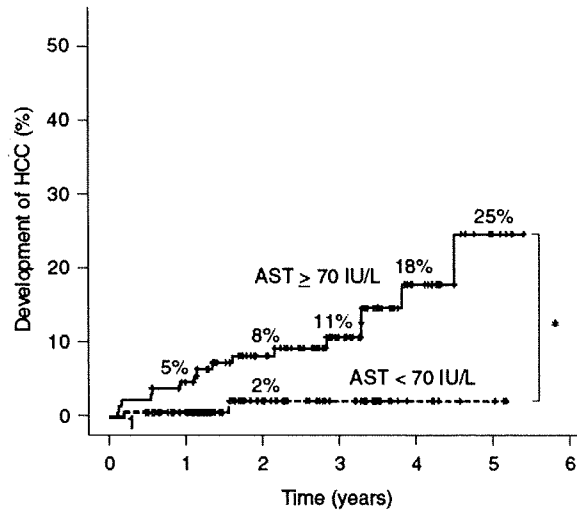


Figure 1 Kaplan-Meier life-table for the cumulative incidence of hepatocellular carcinoma (HCC) during adefovir add-on lamivudine in the patients with different baseline aspartate aminotransferase (AST) levels. * $P = 0.009$.

develop HCC. The patients who developed HCC were older, more frequently had signs of early cirrhosis with less platelet counts, as well as higher levels of AST, ALT and AFP, than those who did not develop HCC. By multivariate analysis, AST ≥ 70 IU/L, YIDD mutants in

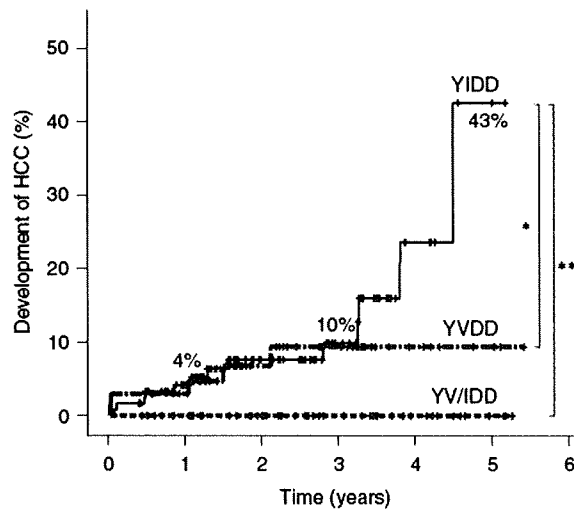


Figure 2 Kaplan-Meier life-table for the cumulative incidence of hepatocellular carcinoma (HCC) during adefovir add-on lamivudine in the patients with distinct YMDD mutants. * $P = 0.035$; ** $P = 0.003$.

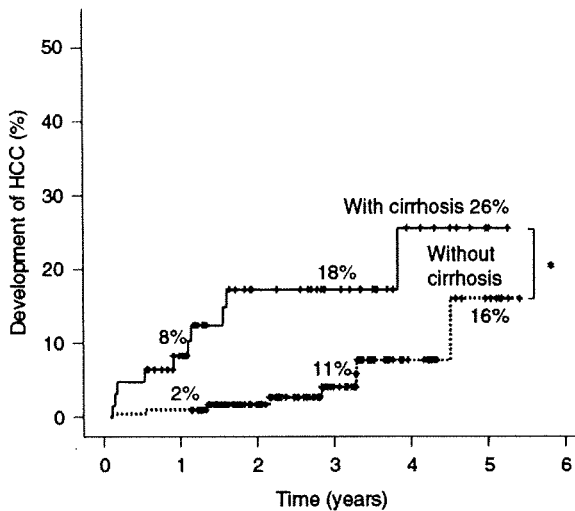


Figure 3 Kaplan-Meier life-table for the cumulative incidence of hepatocellular carcinoma (HCC) during adefovir add-on lamivudine in the patients with and without cirrhosis at the baseline. **P* = 0.002.

comparison with YVDD or the mixture of YVDD and YIDD mutants, age ≥ 50 years and cirrhosis were independent risk factors for the development of HCC. By the Kaplan-Meier life-table analysis, the cumulative incidence of HCC during 5 years in the patients receiving

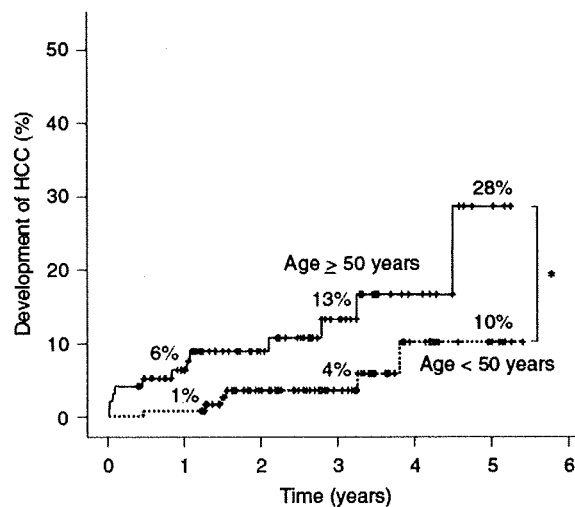


Figure 4 Kaplan-Meier life-table for the cumulative incidence of hepatocellular carcinoma (HCC) during adefovir add-on lamivudine in the patients aged ≥ 50 years and < 50 years at the baseline. **P* = 0.014.

adefovir add-on lamivudine was significantly higher in those with AST ≥ 70 IU/L, YIDD mutants, cirrhosis and aged ≥ 50 years at the start of adefovir.

A marked difference in the development of HCC between the present study (7.3% [18/247]) and two studies reported from Europe and the US (0/70 and 0/65, respectively)^{16,17} would be accounted for, at least in part, by the age of patients who developed HCC in this study that was older than in those in previous reports (the median of 52 years vs. means of 36 and 47 years, respectively). This view would be supported by the age of patients with long-term adefovir add-on lamivudine that was higher in those with than without the development of HCC (52 vs. 45 years [median], *P* = 0.008). HBV infection in Asia is acquired by the perinatal infection, while that in Western countries is gained after the adolescence ~ 20 years after birth. Hence, the duration of HBV infection would have been > 20 years longer in Japanese than Western patients. In addition, genotypes of HBV may give an additional account on the difference in development of HCC between them. All the 18 patients who developed HCC in this study were infected with genotype C; it is associated with HCC more closely than the other genotypes.²⁰⁻²³ By contrast, by far the most patients from Western countries would have been infected with genotypes A and D.^{24,25}

HCC developed more frequently in patients with than without cirrhosis at the start of adefovir (10/61 [16.4%] vs. 8/186 [4.3%], *P* = 0.002). Hence, cirrhosis increased the risk of HCC in patients receiving adefovir add-on lamivudine. This view is supported by the development of HCC in 11 of the 94 (11.7%) patients with cirrhosis who received adefovir add-on lamivudine from Italy.¹⁰ Although HCC did not develop in any of the 39 Italian patients with chronic hepatitis, it did in eight of the 186 (4.3%) Japanese patients in the present study. There were, however, marked differences in the median baseline ALT levels between Italian and Japanese patients (58 vs. 108 IU/L); the grade of liver inflammation would have been higher in the Japanese patients. In actuality, all the eight patients with chronic hepatitis who developed HCC had high AST and ALT levels at the start of adefovir (Table 2).

In the natural history of persistent HBV infection, HCC develops more frequently in the patients with persistently high ALT levels than in those with normal levels. Hence, necroinflammation in the liver would contribute to carcinogenesis.^{26,27} Although adefovir add-on lamivudine may prevent virological breakthroughs, it would not be able to suppress the pre-

neoplastic state induced by exacerbation of hepatitis. It would be necessary therefore to identify the patients with chronic hepatitis at an increased risk for HCC during adefovir add-on lamivudine, such as those with cirrhosis or aged ≥ 50 years, and take special care of them toward early detection of HCC and immediate therapeutic intervention. They need to be monitored frequently for any increase in HBV DNA and aminotransferase levels that herald breakthrough hepatitis during lamivudine therapy.

In the present study, HCC developed more frequently in the patients with YIDD mutants than in those with YVDD or the mixture of YVDD and YIDD; there have been no studies correlating YMDD mutants and the development of HCC. No patients with the mixture of YVDD and YIDD mutants developed HCC, despite the predominance of YIDD mutants in the patients with HCC. This might have been due to the assay used for YMDD mutants by the commercial kit; it can miss YVDD mutants in samples in which YIDD mutants account for the great majority. By the assay method specific for either mutant, YIDD was detected either alone or accompanied by small amount of YVDD in the patients who have received adefovir add-on lamivudine treatment.²⁸ Sensitive and specific quantification of YIDD and YVDD mutants are necessary for further evaluating a role for YIDD mutants in hepatocarcinogenesis, as well as for identifying factors promoting the generation of both YIDD mutants and HCC.

Some points of clinical importance have emerged in the present study. First, patients who receive a long-term adefovir add-on lamivudine and have developed YMDD mutants need to be screened for HCC on the regular basis. This is required especially for the patients who have signs of cirrhosis and/or high AST levels, or aged ≥ 50 years. In these high-risk patients, adefovir has to be started promptly when HBV DNA levels increase, even before transaminase levels elevate in them. Secondly, it would be a matter of concern if adefovir is involved in the development of HCC. Should it be the case, tenofovir or newer potent antivirals, either as a monotherapy or add-on lamivudine, would deserve considerations. Thirdly, it needs to be evaluated if YIDD mutants have any significance in the development of HCC. Although nucleot(s)ide analogues may suppress hepatic inflammation and are expected to improve the prognosis of patients with chronic hepatitis B, they need to be monitored closely for HCC. The development of HCC has to be identified, as early as possible, for timely treatment toward longevity with minimal morbidity and improvement of the quality of life.

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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 hepatitis B virus (HBV)-positive patients for nucleoside analogue treatment for patients with chronic HBV receiving lamivudine therapy

Lamivudine therapy		< 3 years	≥ 3 years
HBV DNA			
Keep < 2.6 log copies/mL ≥ 2.6 log copies/mL	No BTH†	Switch to entecavir 0.5 mg/day	Continue lamivudine 100 mg/day
	With BTH	Switch to entecavir 0.5 mg/day 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, none 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 mini-sequence 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		Differences (P-value)
	< 3 years n = 125	≥ 3 years n = 234	
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 hepatitis B virus (HBV) DNA and alanine transaminase (ALT) level in patients during lamivudine treatment for < 3 years (125 patients)

HBV DNA† (Amplificor: 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.