Statistical methods

The obtained clinical data were analyzed on an intention-to-treat basis. Standard statistical measures and procedures were used in the analysis. The chi-square, Fisher's exact test, and Mann-Whitney's *U*-tests were used to analyze the differences of background features and biochemical data between the two groups. HCC recurrence rate was calculated from the day of HCC treatment in both groups, using the Kaplan-Meier technique. The differences in recurrence curves were tested using the log-rank test. Cox proportional hazard analysis was performed to evaluate independent predictors of tumor recurrence after treatment. A *P*-value of less than 0.05 with two-tailed analysis was considered significant. Data analysis was performed using the computer program SPSS version 11 (SPSS Inc. Chicago, IL).[18]

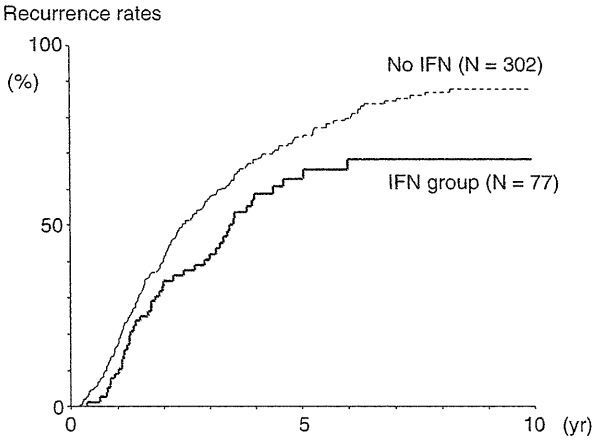

Figure 1 Cumulative recurrence rates of hepatocellular carcinoma in patients with and without interferon therapy.

RESULTS

Effects and toxicity of interferon

SVR WERE FOUND in 4 (5.2%) of 77 patients in IFN-treated group and none in untreated group. BR were found in 7 (9.1%), NR in 36 (46.8%), and undetermined judgment due to continuous administration currently in 30 (39.0%).

Almost all of the patients given IFN therapy showed varied degrees of fever, chills, myalgias, headache, and general malaise after the first injection of IFN. Most of patients revealed a various degree of leukocytopenia and thrombocytopenia. A total of 8 patients (10.4%) withdrew from IFN therapy before development of tumor recurrence. Three patients with depression or psychosis ceased the IFN therapy. The other 5 patients also stopped IFN administration because of varied degree of adverse effects: thrombocytopenia, insomnia, slight degree of hepatic encephalopathy, minor episode of cerebrovascular accident, and generalized fatigue with significant weight loss.

Recurrence rates of hepatocellular carcinoma

During the median observation period of 4.6 years, HCC recurred in 264 patients (69.7%); 45 patients belonged to the IFN group, and the other 219 patients to the untreated group. The cumulative recurrence rate in all patients was 16.2% at the end of the first year following the surgical treatment of HCC, 39.6% at the second year, 54.5% at the third year, 73.0% at the fifth year, 82.8% at the seventh year, and 85.5% at the 10th year. Crude recurrence rates in the IFN group and untreated group were 9.1% and 18.8% at the end of the first year, 33.3% and 42.1% at the second year, 41.1% and 58.1% at the third year, 63.0% and 76.6% at the 5th year, 68.5% and 86.2% at the seventh year, and 68.5% and 93.2% at the 10th year, respectively (Fig. 1). The recurrence rate in the IFN group was significantly lower than that of the untreated group (log-rank test: $P = 0.013$).

In univariate analysis, factors associated with tumor recurrence were explored in all of the 379 patients *en masse*. HCC recurrence was associated with high indocyanine green retention rate at 15 minutes (ICG R15) ($P = 0.004$), low albumin concentration ($P = 0.005$), no IFN therapy ($P = 0.010$), prolonged prothrombin time ($P = 0.041$), and RFA as treatment for HCC ($P = 0.046$).

Multivariate analysis disclosed that recurrence of HCC was independently associated with IFN therapy (hazard ratio 0.66, $P = 0.020$), a high ICG R15 of 20% or more (hazard ratio 1.43, $P = 0.008$), and RFA therapy (hazard ratio 1.32, $P = 0.041$). IFN treatment proved to prevent tumor recurrence after ablation of HCC in those patients with an early stage of HCC (Table 2).

Recurrence rates according to interferon effect

Tumor recurrence rates were evaluated according to judgment of IFN effect in the treated group: SVR ($n = 4$), BR ($n = 7$), NR ($n = 36$), continued IFN administration (N = 30), and untreated group.

Table 2 Independent factors affecting the recurrence of hepatocellular carcinoma after curative treatment

Factors	Category	Hazard ratio (95% CI)	*P*
Interferon therapy	1: No	1	
	2: Yes	0.66 (0.46–0.94)	0.020
ICG R15	1: <20%	1	
	2: ≥20%	1.43 (1.10–1.85)	0.008
Cancer treatment	1: Surgical resection	1	
	2: PRFA	1.32 (1.01–1.72)	0.041

ICG R15, indocyanine green retention rate at 15 minutes; PRFA, percutaneous radiofrequency ablation therapy.

Recurrence rates in the subgroup of SVR, BR, NR, continued administration, and untreated patients were 0%, 0%, 6.7%, 12.5%, and 18.8% at the end of the first year, 25.0%, 28.6%, 37.0%, 25.3%, and 42.1% at the second year, 25.0%, 42.9%, 52.0%, 32.6%, and 58.1% at the third year, 25.0%, 85.7%, 71.1%, 46.7%, and 76.6% at the fifth year, and 25.0%, 100%, 79.3%, 54.3%, and 86.2% at the seventh year, respectively (Fig. 2a). The recurrence rates in a combined group of SVR and continued IFN administration were significantly lower than those in a combined cohort of the other groups (log-rank test, $P = 0.0005$) (Fig. 2b). The recurrence rates of the former and the latter groups were 30.6% and 56.7% at the end of the third year, 43.3% and 75.0% at the fifth year, and 43.3% and 84.7% at the seventh year, respectively.

Recurrence rates according to length of interferon administration

Since HCV RNA eradication (SVR) was found in only four patients, significance of prolonged administration of IFN was assessed in those patients with positive HCV RNA during therapy ($n = 73$).

Recurrence rates in the subgroup with a long IFN therapy of 2 years or more ($n = 39$), a short IFN therapy of less than 2 years ($n = 34$), and in the untreated patients ($n = 302$) were 8.7%, 7.1%, and 18.8% at the end of the first year, 23.9%, 40.2%, and 42.1% at the second year, 39.3%, 46.2%, and 58.1% at the third year, 57.4%, 66.2%, 76.6% at the fifth year, and 66.0%, 77.5%, and 86.2% at the seventh year, and 66.0%, 77.5%, and 93.2%, respectively (Fig. 3). The recurrence rates in the long IFN-therapy group was significantly lower than those with a short therapy group and untreated group (log-rank test, $P = 0.012$).

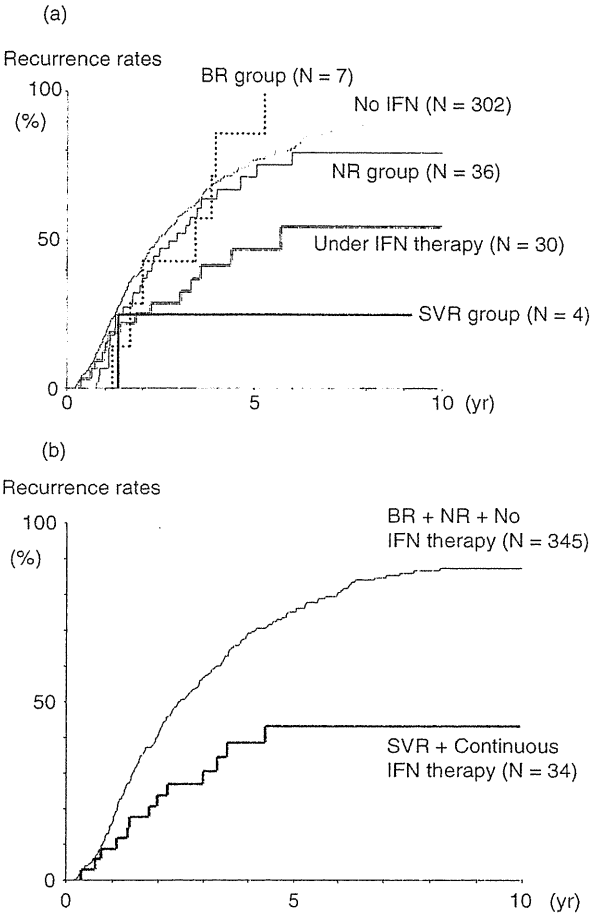

Figure 2 (a) Cumulative recurrence rates of hepatocellular carcinoma according to the effect of interferon. (b) Cumulative recurrence rates of hepatocellular carcinoma in a combined group of sustained virological response and continuous interferon administration and those in a combined group of biochemical response, no response, and no interferon therapy.

To elucidate the impact of a long-term administration of IFN in the prevention of HCC recurrence, multivariate hazard analysis was introduced in the IFN-treated patients without SVR effect ($n = 73$) and the untreated patients ($n = 302$). Multivariate analysis showed that a long-term IFN therapy significantly lowered the recurrence rate in patients with HCV-related HCC: hazard ratios of short-term therapy less than two years and long-term therapy for two years or longer of 2 years or more were 0.80 and 0.60, respectively ($P = 0.044$). The other covariates for recurrence rate included high ICGR15, high AFP value, and initial treatment modality (Table 3).

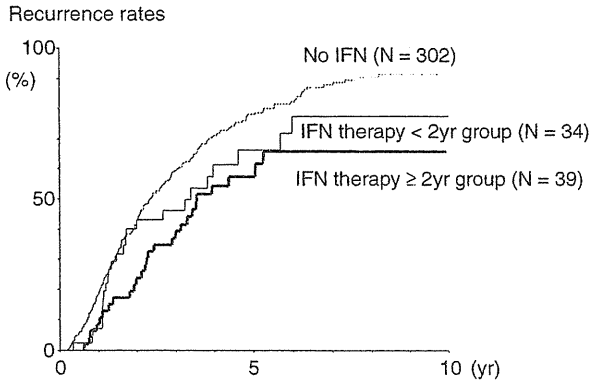

Figure 3 Cumulative recurrence rates of hepatocellular carcinoma in patients without sustained virological response. Recurrence rates were assessed according to the length of interferon administration.

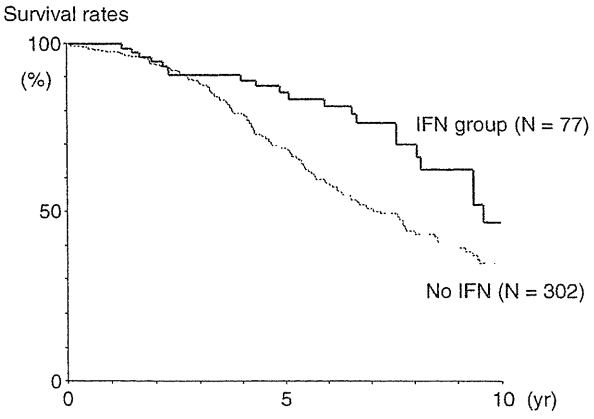

Figure 4 Overall survival rates of patients with or without interferon therapy after potentially curative therapy for hepatocellular carcinoma.

Overall survival rates

A total of 159 patients died during the observation period: 23 (29.9%) in the IFN-treated group and 136 (45.0%) in the untreated group. Crude survival rates of patients after potentially curative therapy for HCC in the IFN-treated and untreated patients were 90.7% and 88.5% at the end of the third year, 85.6% and 68.8% at the fifth year, 76.5% and 50.9% at the seventh year, and 47.0% and 34.7% at the tenth year, respectively (Fig. 4). The survival rates of IFN-treated group were significantly higher than that of those of untreated group (log-rank test, $P = 0.0044$).

Table 3 Independent factors affecting the recurrence of hepatocellular carcinoma after curative treatment, according the length of interferon administration†

Factors	Category	Hazard ratio (95% CI)	*P*
Interferon therapy	1: None	1	0.044
	2: <2 years	0.80 (0.51–1.24)	
	3: ≥2 years	0.60 (0.40–0.91)	
ICG R15	1: <20%	1	
	2: ≥20%	1.37 (1.06–1.77)	0.018
Alpha-fetoprotein	1: <40 mg/L	1	
	2: ≥40 mg/L	1.31 (1.00–1.71)	0.051
Cancer treatment	1: Surgical resection	1	
	2: PRFA	1.28 (0.98–1.65)	0.066

†Four patients with sustained virological response were excluded in the analysis. ICG R15, indocyanine green retention rate at 15 minutes; PRFA, percutaneous radiofrequency ablation therapy.

Multivariate analysis showed overall survival rates were significantly affected by interferon therapy ($P = 0.014$), albumin concentration ($P = 0.015$), platelet count ($P = 0.014$), and ICG R15 ($P = 0.0068$) (Table 4). Hazard ratio for death in those patients with IFN therapy was 0.55 (95% confidence interval 0.34–0.88).

DISCUSSION

ALTHOUGH THIS STUDY was not a prospective, randomized one, there was no significant difference in the background features and laboratory tests except for age, between the treated and untreated groups. This study was based on a long-term observation for a median of 4.6 years, and the number of patient was sufficiently large for sensitivity and reliabil-

Table 4 Independent factors affecting the survival rates of patients with hepatocellular carcinoma after curative treatment

Factors	Category	Hazard ratio (95% C.I.)	*P*
Interferon therapy	1: None	1	
	2: Yes	0.55 (0.39–0.88)	0.014
ICG R15	1: <20%	1	
	2: ≥20%	1.65 (1.15–2.37)	0.0068
Albumin	1: <3.5 g/dl	1	
	2: ≥3.5 g/dl	0.64 (0.44–0.92)	0.015
Platelet count	1: <100,000/mm3	1	
	2: ≥100,000/mm3	0.64 (0.45–0.91)	0.014

ICG R15, indocyanine green retention rate at 15 minutes.

ity for the data regarding recurrence and survival. We also analyzed only those patients with "an early stage" of HCC to minimize the influence of tumor recurrence due to small and undetectable metastatic tumors often found in patients with large or multiple tumors. In the establishment of the diagnosis of early stage of HCC, more than 93% of the patients underwent intensive imaging investigation with CT-HA and CT-AP, together with dynamic CT and dynamic MRI study. Therefore, the diagnosis of a few numbers with small-sized tumor was sufficiently reliable in the study.

This cohort study indicated IFN suppressed the recurrence rate after potentially curative treatment of HCC caused by HCV. Indeed SVR effect after IFN therapy did decrease recurrence rate, majority of patients were not tolerable for a large amount of IFN administration with or without ribavirin because of an old age or advanced liver disease with significant cytopenia. This study demonstrated interferon significantly decreased tumor recurrence rate, irrespective of "anti-viral interferon effect". This study also revealed relatively "rapid" anti-carcinogenic effect compared with the results of a study performed by Mazzaferro *et al.*[11] Most cases of late-phase recurrence are thought to be due to metachronous multicentric, or *de novo*, carcinogenesis. This is quite understandable, because the remaining liver, often cirrhotic, is still at high risk of carcinogenesis.

Our study also emphasizes that long-term, low-dose, intermittent administration of IFN was useful in prevention of tumor recurrence in patients without SVR, with a hazard ratio of 0.60 compared to those with no IFN administration.

The reason why IFN administration suppresses the recurrence rate in HCV-related liver disease remains uncertain. One reason may be anti-tumor activity in the early stage of HCC and another antiviral or anti-necroinflammatory effect for hepatitis. Our data did not disclose the relationship between ALT normalization and prevention of cancer recurrence, since the number of BR group was small (N = 7), and since many patients were currently continuing IFN therapy with normal ALT. Human lymphoblastoid IFN alpha has a powerful anti-proliferative effect on human hepatoma cell line PLC/PRF/5, both *in vitro* and *in vivo*, after implantation in nude mice.[19] Lai *et al.*[20] showed IFN induced objective tumor regression in a significant number of patients with inoperable hepatocellular carcinoma in a randomized controlled trial. Considering the short period to recurrence in our study, IFN may have a direct anti-tumor effect on clinically undetectable HCC. Wang *et al.*[21] showed anti-angiogenesis activity of IFN, and Wu *et al.*[22] demonstrated suppression of vascular endothelial growth factor and inhibition of tumor signaling pathways. Moreno *et al.*[23] reported that IFN induced remission of liver fibrosis irrespective of anti-viral effect. Control of necro-inflammatory process may therefore induce a suppression of the growth process of HCC. Tarao *et al.*[24] reported that high aminotransferase activity resulted in an increased HCC recurrence rate. A randomized controlled trial of IFN for patients with cirrhosis showed that IFN therapy decreased the HCC appearance rate in association with disappearance of HCV-RNA[3]. We also demonstrated IFN suppressed the carcinogenesis rate in patients with chronic hepatitis type C[5]. Taking into account that hepatocellular carcinogenesis in HCV-related chronic liver disease is accelerated by a prolonged period of necro-inflammation of hepatocytes, IFN is hypothesized to diminish the HCC appearance rate through suppression of excessive replication and turnover of hepatocytes. Since the entire process of hepatocellular carcinogenesis from initial transformation of a hepatocyte to detectable growth is considered to take at least several years, the influence of IFN on the carcinogenesis rate or recurrence rate might not be evaluated in as short period of three years or less. Aside from the exact mechanism of the prevention of HCC recurrence, our study demonstrated an encouraging result in the medical management of HCC.

Since these results were not generated from a prospective randomized study, we tried to adjust background biases using multivariate analysis between the treated and untreated group, if any. We should realize the significance of the decrease in recurrence rate by IFN therapy with a hazard ratio by 0.66. Cost-effectiveness and individual and social expenses should be evaluated in detail between those patients with reduction of recurrence rate and those with high recurrence rate with additional tumor ablation therapy. Considering that a long-term prospective trial with and without IFN arm seemed very difficult to perform ethically and economically, we should further accumulate these comparative studies and consider the efficacy of weekly injections of pegylated IFN and adequate dose and length of IFN therapy. Identification of suitable cases for IFN therapy and exact mechanisms of suppression of tumor recurrence are of paramount importance for increasing number of patients with HCC.

In conclusion, long-term intermittent IFN therapy reduced HCC recurrence rate in patients with HCV-related HCC.

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Diabetes Enhances Hepatocarcinogenesis in Noncirrhotic, Interferon-treated Hepatitis C Patients

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Diabetes Enhances Hepatocarcinogenesis in Noncirrhotic, Interferon-treated Hepatitis C Patients

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ABSTRACT

BACKGROUND: This retrospective cohort study assessed the impact of diabetes mellitus on hepatocarcinogenesis and determined the predictors of hepatocarcinogenesis in noncirrhotic, interferon-treated patients with hepatitis C virus infection.

METHODS: A total of 2058 hepatitis C virus-positive, noncirrhotic patients treated with interferon were enrolled. The median follow-up period was 6.7 years. The primary end point was the onset of hepatocellular carcinoma. The cumulative rate of new hepatocellular carcinoma cases was computed by the Kaplan–Meier method and Cox proportional hazard analysis according to diabetic state and response to interferon therapy.

RESULTS: The cumulative rates of hepatocellular carcinoma in diabetic patients (3.2% at 4 years, 8.5% at 8 years, and 24.4% at 12 years) were significantly higher than those of nondiabetic patients (1.3% at 4 years, 2.2% at 8 years, and 5.6% at 12 years, $P < .001$). In patients with a sustained virologic response, diabetes had no significant effect on the rate of hepatocarcinogenesis. In contrast, the rate in patients with a nonsustained virologic response was significantly higher in diabetic than in nondiabetic patients. Multivariate analysis identified lack of sustained virologic response (hazard ratio [HR] 7.28; 95% confidence interval [CI], 3.28-16.15; $P < .001$) and diabetes as independent risk factors for hepatocarcinogenesis (HR 2.00; 95% CI, 1.05-3.84; $P = .036$).

CONCLUSIONS: Our results highlight the enhancing effect of diabetes mellitus on hepatocarcinogenesis in noncirrhotic, interferon-treated patients with hepatitis C virus. The sustained virologic response induced by interferon therapy eliminates the influence of diabetes and markedly reduces the rate of hepatocarcinogenesis in such patients.

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KEYWORDS: Diabetes; Hepatocellular carcinoma; Interferon; Sustained virologic response

Hepatitis C virus is a common cause of chronic liver disease worldwide and a major risk of hepatocellular carcinoma.[1-10] The estimated incidence of hepatocellular carcinoma in patients with hepatitis C virus-related cirrhosis is 5% to 10% per year, and hepatocellular carcinoma is one of the major causes of death, especially in Asian countries.[10] In recent years, diabetes mellitus has attracted attention as a risk factor of hepatocarcinogenesis. Evidence suggests that in addition to various factors that affect liver fibrosis and hepatocarcinogenesis, diabetes and obesity are independent risk factors for the progression of liver fibrosis and development of hepatocellular carcinoma in chronic hepatitis C.[10-15] The majority of such clinical studies included patients with liver cirrhosis. However, for pathophysiologic reasons, liver cirrhosis increases the probability of impaired glucose tolerance. Therefore, in studies of cirrhotic patients,

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it is difficult to pinpoint the true effects of diabetes on hepatocarcinogenesis. On the other hand, we recently reported that a sustained virologic response to interferon therapy reduces the incidence of type 2 diabetes onset in chronic hepatitis C.[16] Thus, there is a gap in our knowledge on the exact effect of diabetes on hepatocarcinogenesis in interferon-treated patients.

The present retrospective study was designed to determine the effects of diabetes on hepatocarcinogenesis in noncirrhotic, interferon-treated patients with chronic hepatitis C virus infection, including the effects of viral clearance on diabetes-related hepatocarcinogenesis.

CLINICAL SIGNIFICANCE

- The hepatocarcinogenesis rate from first interferon therapy for noncirrhotic patients with chronic hepatitis C was 2 times greater in diabetic cases than in nondiabetic cases.
- Diabetes was an independent predictive factor of hepatocellular carcinoma in interferon-treated, noncirrhotic patients with chronic hepatitis C virus.
- In patients without a sustained virologic response from interferon therapy, the hepatocarcinogenesis rate of diabetic cases was approximately 15 times greater than that of nondiabetic, noncirrhotic patients with chronic hepatitis C and a sustained virologic response.

PATIENTS AND METHODS

Study Population

In this retrospective cohort study, we obtained the medical records of all patients in our database who had received interferon therapy for chronic hepatitis C between 1987 and 2007 at the Department of Hepatology, Toranomon Hospital, Tokyo, Japan. Of these patients, 2058 satisfied the following criteria: 1) no evidence of diabetes after termination of interferon; 2) laparoscopy or liver biopsy performed before initiation of interferon therapy confirmed the lack of liver cirrhosis; 3) measurement of serologic type and hepatitis C virus viral load before initiation of interferon therapy; 4) platelet count of $\geq 10 \times 10^4$/mL; 5) negativity for hepatitis B surface antigen, antinuclear antibodies, or antimitochondrial antibodies in serum, as determined by radioimmunoassay or spot hybridization; 6) no underlying metabolic disease, such as hemochromatosis, alpha-1-antitrypsin deficiency, or Wilson disease; 7) no underlying systemic disease, such as systemic lupus erythematosus or rheumatic arthritis; 8) no evidence of hepatocellular carcinoma on ultrasonography or computed tomography before the initiation of interferon therapy; and 9) follow-up period of ≥ 24 weeks.

All patients who did not show a sustained virologic response and persistently high alanine aminotransferase level (normal range: 6-50 IU/L) received liver protection therapy, consisting mainly of glycyrrhizin and ursodeoxycholic acid (300-600 mg/d), during this research.

In all patients, the observation starting point was the time of initiation of the first interferon treatment. All of the studies were performed retrospectively by collecting and analyzing data from the patient records. The study was approved by the institutional review board of the Toranomon Hospital.

Background and Laboratory Data

Table 1 (available online) summarizes the clinical profile and laboratory data of 2058 interferon-treated patients with chronic hepatitis C. The male to female ratio was 1.78:1. Of 2058 patients, 164 (8.0%) were alcoholic (total alcohol intake > 500 kg until the initiation of interferon therapy). Before the initiation of interferon therapy, 104 patients (5.1%) were known diabetics. Furthermore, 71.2% patients had a high viral titer (low viral load; Amplicor <100 KIU/mL [Cobas Amplicor HCV Monitor Test, version 2.0, Roche Molecular Systems, Inc, Belleville, NJ] or probe <1 MEq/mL [branched DNA probe assay; version 2,0; Chiron, Daiichi Kagaku, Tokyo], high viral load; Amplicor ≥ 100 KIU/mL or probe ≥ 1 MEq/mL).

Type of Interferon and Assessment of Response to Interferon Therapy

Among 2058 patients treated with interferon, 1207 (58.6%) received interferon-α, 329 (16.0%) received interferon-β, and the remaining 522 (25.4%) received a combination therapy of interferon and ribavirin. The response to interferon therapy was assessed on the basis of sustained virologic response (sustained virologic response was regarded as elimination of hepatitis C virus-RNA at 6 months after the termination of interferon treatment). After interferon therapy, 52.5% of the patients showed sustained virologic response.

Markers of Hepatitis B and C Viruses

Anti-hepatitis C virus was detected using a second-generation enzyme-linked immunosorbent assay (ELISA II; Abbott Laboratories, North Chicago, IL). Hepatitis C virus-RNA was determined by the Amplicor method (Cobas Amplicor HCV Monitor Test, version 2.0; Roche, Tokyo, Japan) or the branched DNA probe assay (branched DNA probe assay; version 2.0; Chiron). Hepatitis B surface antigen was tested via radioimmunoassay (Abbott Laboratories, Detroit, MI). The used serum samples were stored at −80°C at the first consultation. Diagnosis of hepatitis C virus infection was based on detection of serum hepatitis C virus antibody and hepatitis C virus RNA.

Histopathologic Examination of the Liver

Liver biopsy specimens were obtained percutaneously or at peritoneoscopy using a modified Vim-Silverman needle with an internal diameter of 2 mm (Tohoku University, Kakinuma Factory, Tokyo, Japan), fixed in 10% formalin, and stained with hematoxylin–eosin, Masson's trichrome, silver impregnation, and pe-

Table 1 Characteristics of 2058 Noncirrhotic, Interferon-Treated Patients with Chronic Hepatitis C Virus Infection at the Initiation of Interferon and Efficacy

Parameter	(n = 2058)
Gender (M:F)	1317:741
Age (y)†	50 (15-72)
Histopathologic grade (F1-2:F3)	1916:142
Total ethanol intake (≥500 kg) (yes/no)	164:1894
Follow-up period (d)†	2443 (170-7562)
Albumin (g/dL)†	4.2 (2.3-5.3)
Total bilirubin (mg/dL)†	0.7 (0.1-11.7)
AST (IU/L)†	68 (21-488)
ALT (IU/L)†	77 (5-1212)
γ-GTP (IU/L)†	43 (5-805)
Platelet count ($\times 10^4/\mu L$)†	18.3 (10.0-48.1)
AFP ($\mu g/L$)†	4 (1.0-780)
Fasting/casual plasma glucose (mg/dL)†	96 (66-376)/100 (49-415)
Diabetes (yes/no)	104:1954
Total cholesterol (mg/dL)†	172 (102-348)
Triglyceride (mg/dL)†	89 (32-325)
LDL cholesterol (mg/dL)†	105 (39-209)
HDL cholesterol (mg/dL)†	46 (8-107)
IFN (monotherapy/combination therapy)	1536:522
HCV serologic group (1:2)	1310:748
Viral load (low:high)	592:1466
Efficacy of IFN therapy acquired viral elimination* (yes:no)	1081:977

AST = aspartate aminotransferase; ALT = alanine aminotransferase; γ-GTP = gamma-glutamyl transpeptidase; AFP = alpha-fetoprotein; LDL = low-density lipoprotein; HDL = high-density lipoprotein; IFN = interferon; HCV = hepatitis C virus.
*Viral elimination means sustained virologic response.
†Expressed as median (minimum, maximum).

riodic acid-Schiff after diastase digestion. All specimens for examination contained at least 6 portal areas. Chronic hepatitis was diagnosed on the basis of histopathologic assessment according to the scoring system of Desmet et al.[17]

Definition of Diabetes Mellitus

Diabetes was diagnosed by the use of the 2003 criteria of the American Diabetes Association.[18] These criteria include 1) casual plasma glucose ≥ 200 mg/dL; 2) fasting plasma glucose ≥ 126 mg/dL; and 3) 2-hour post-glucose (oral glucose tolerance test) ≥ 200 mg/dL.

Follow-up and Diagnosis Procedure of Hepatocellular Carcinoma

The starting time of follow-up was the point of the initiation of the first interferon treatment. After that, patients were followed up monthly to tri-monthly in our hospital. Physical examination and biochemical tests were conducted at each visit together with regular checkups. Ultrasonography or computed tomography were performed every 3 to 6 months. The diagnosis of hepatocellular carcinoma was performed by biochemical examination (include alpha-fetoprotein and des-gamma carboxyprothrombin) and triple-phase dynamic computed tomography study. The number of cases lost to follow-up was 147 patients (7.1%) in this group.

Statistical Analysis

The cumulative rate of hepatocarcinogenesis (new cases of hepatocellular carcinoma) was calculated from the point of initiation of the first interferon treatment to the diagnosis of hepatocellular carcinoma using the Kaplan–Meier method. Differences in the development of hepatocellular carcinoma between different groups were tested using the log-rank test. Independent factors associated with the rate of hepatocellular carcinoma were analyzed by the Cox proportional hazard model. The following 19 variables were analyzed for potential covariates for incidence of hepatocellular carcinoma at the time of first interferon treatment initiation at Toranomon Hospital: gender, age, histologic stage of the liver, amount of total ethanol intake, existence of diabetes, viral serologic group, viral load, existence of sustained viral clearance by interferon therapy, serum concentration of albumin, total bilirubin, aspartate aminotransferase, alanine aminotransferase, gamma-glutamyl transpeptidase, alpha-fetoprotein, total cholesterol, triglyceride, low-density lipoprotein cholesterol, high-density lipoprotein cholesterol, and platelet count. A P value of less than .05 in a 2-tailed test was considered significant. Data analysis was performed using the Statistical Package for the Social Sciences version 11.0 for Windows (SPSS, Inc, Chicago IL).

RESULTS

Incidence of Hepatocellular Carcinoma in Noncirrhotic, Interferon-Treated Patients with Chronic Hepatitis C

In this cohort, hepatocellular carcinoma developed in 73 patients (3.5%) during a median observation period of 6.7 years. The cumulative rate of newly diagnosed hepatocellular carcinoma was 1.2% at 4 years, 2.6% at 8 years, and 6.8% at 12 years (**Figure 1**). The hepatocarcinogenesis rate according to interferon therapy was 2.1% at 4 years, 4.4% at 8 years, and 11.6% at 12 years in patients who did not acquire a sustained virologic response, and 0.7% at 4 years, 1.0% at 8 years, and 1.6% at 12 years in patients who acquired a sustained virologic response (**Figure 2**). The cumulative incidence rate of hepatocellular carcinoma was significantly lower in patients who acquired a sustained virologic response than in those who did not ($P < .001$).

Effect of Diabetes Mellitus on Hepatocarcinogenesis in Noncirrhotic, Interferon-Treated Patients with Hepatitis C

During the follow-up period, 58 of the 1954 nondiabetic patients (3.0%) developed hepatocellular carcinoma, and 15 of the 104

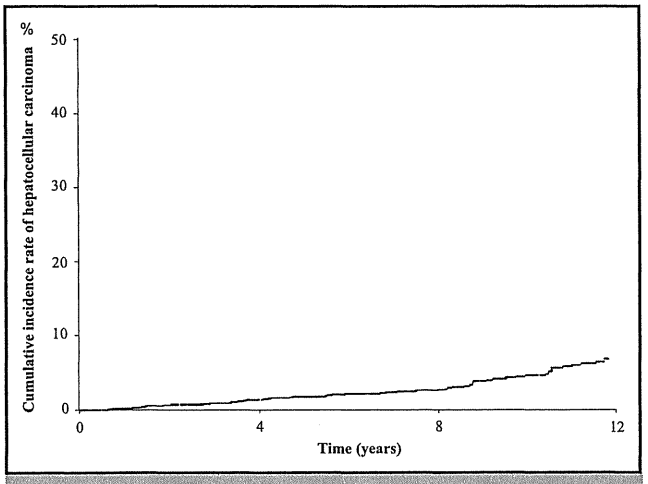

Figure 1 Cumulative rate of development of hepatocellular carcinoma from first interferon therapy in noncirrhotic patients with chronic hepatitis C infection.

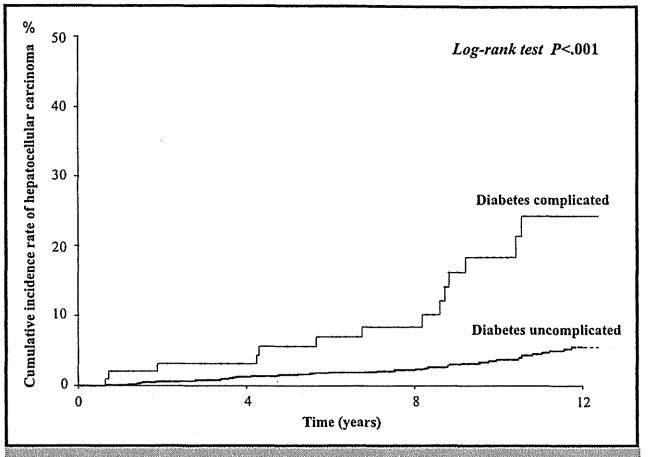

Figure 3 Cumulative rate of development of hepatocellular carcinoma from first interferon therapy in noncirrhotic patients with chronic hepatitis C infection according to the presence or absence of diabetes.

diabetic patients (14.4%) developed hepatocellular carcinoma. The cumulative rate of hepatocellular carcinoma in nondiabetic patients was 1.3% at 4 years, 2.2% at 8 years, and 5.6% at 12 years. For diabetic patients, these rates were 3.2%, 8.5%, and 24.4%, respectively (**Figure 3**). The cumulative rate of hepatocellular carcinoma was significantly higher in patients with diabetes than those without ($P<.001$).

Effect of Sustained Virologic Response on Rate of Hepatocarcinogenesis in Noncirrhotic, Interferon-Treated Patients with Hepatitis C According to Presence of Diabetes

In the nonsustained virologic response group (n = 977), 47 (5.2%) of the nondiabetic patients (n = 906) developed hepatocellular carcinoma during the observation period, whereas 14 (19.7%) of diabetic patients (n = 71) developed hepatocellular carcinoma. In the sustained virologic response group (n = 1081), 11 (1.0%) of the nondiabetic patients (n = 1048) developed hepatocellular carcinoma during the observation period, whereas 1 (3.0%) of the diabetic patients (n = 33) developed hepatocellular carcinoma.

Analysis of data according to the efficacy of interferon therapy in diabetic and nondiabetic patients showed that in patients with nonsustained virologic response, the cumulative rate of hepatocellular carcinoma in nondiabetic patients was 1.9% at 4 years, 3.6% at 8 years, and 9.6% at 12 years, whereas in diabetic patients, these rates were 4.7%, 12.1%, and 31.0%, respectively (**Figure 4**). The cumulative rate of hepatocellular carcinoma was significantly higher in diabetic patients with a nonsustained virologic response than in nondiabetic patients ($P<.001$). The same analysis in

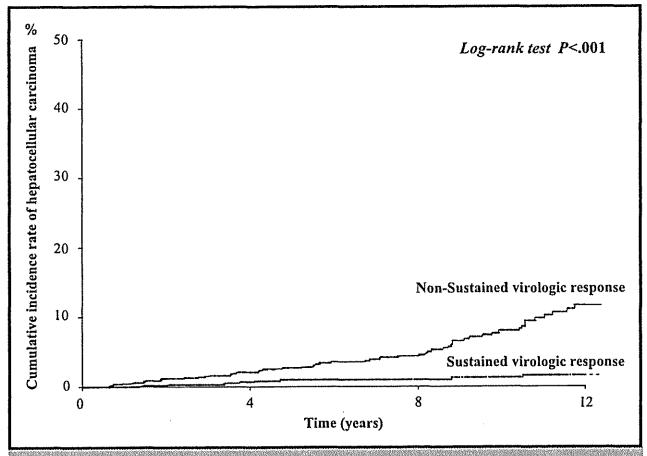

Figure 2 Cumulative rate of development of hepatocellular carcinoma from first interferon therapy in noncirrhotic patients with chronic hepatitis C infection according to effect of interferon therapy.

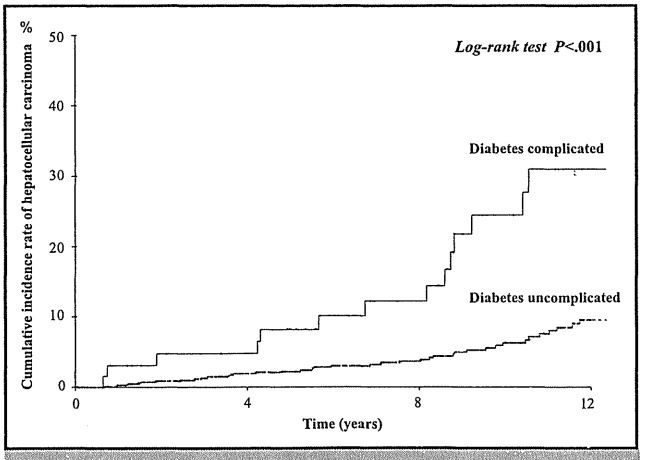

Figure 4 Cumulative rate of development of hepatocellular carcinoma from first interferon therapy in noncirrhotic patients with chronic hepatitis C infection who showed nonsustained virologic response to interferon therapy according to the presence or absence of diabetes.

patients with a sustained virologic response showed a cumulative rate of hepatocellular carcinoma of 0.7%, 1.0%, and 1.7% in nondiabetic patients, and 0.0%, 0.0%, and 0.0% in diabetic patients, respectively (Figure 5). There was no significant difference between diabetic and nondiabetic groups in patients with a sustained virologic response ($P = .249$).

Factors Associated with Rate of Hepatocarcinogenesis

Multivariate Cox proportional hazard analysis revealed the following independent factors for hepatocellular carcinoma development after the initiation of the first interferon therapy in patients who showed a nonsustained virologic response (hazard ratio 7.28; 95% confidence interval [CI], 3.28-16.15; $P < .001$): male (hazard ratio 4.90; 95% CI, 2.47-9.71; $P < .001$), aged ≥ 60 years (hazard ratio 3.28; 95% CI, 1.88-5.74; $P < .001$); aspartate aminotransferase ≥ 50 IU/L (hazard ratio 3.91; 95% CI, 1.81-8.43; $P = .001$); alpha-fetoprotein ≥ 20 mg/L (hazard ratio 2.89; 95% CI, 1.43-5.84; $P = .003$); diabetes (hazard ratio 2.00; 95% CI, 1.05-3.84; $P = .036$); and platelet count $< 17 \times 10^4$/mL (hazard ratio 1.96; 95% CI, 1.11-3.48; $P = .021$) (Table 2, available online).

Rate and Prognosis of Diabetic Patients with Marked Fatty Deposition at First Interferon Initiation

Fourteen of 104 diabetic patients (13.5%) had fatty deposition in hepatic cells of ≥ 30% before the initiation of interferon therapy. Of these 14 patients, 2 were diagnosed with hepatocellular carcinoma during the observation period. One patient underwent liver resection to treat hepatocellular carcinoma, and background liver tissue was liver cirrhosis. One patient did not receive a liver resection; however, this patient's platelet count was approximately $20 \times 10^4/\mu L$ at the time of diagnosis of hepatocellular carcinoma. Thus, severe fibrosis was not suspected in view of this platelet count level.

Rate of Liver Cirrhosis at Hepatocellular Carcinoma Diagnosis

In 23 of 73 patients with hepatocellular carcinoma (31.5%), hepatic resection was performed for treatment. Five of 23 resected patients (21.7%) had liver cirrhosis in background hepatic tissue. The remaining 50 of 73 patients (68.5%) did not receive hepatic resection, and these patients received other nonresection therapy. Because the platelet count level was less than $10 \times 10^4/\mu L$ in 17 of 50 patients without resection (34.0%), liver cirrhosis was suspected. In these patients with histologic or clinical diagnosis of liver cirrhosis at the time of onset of hepatocellular carcinoma, none had a sustained virologic response by interferon therapy.

DISCUSSION

The present study described the incidence of hepatocellular carcinoma after the initiation of interferon therapy in patients with chronic hepatitis C infection. The results indicate that the annual incidence of hepatocellular carcinoma over a prolonged follow-up from first interferon therapy among noncirrhotic patients with hepatitis C virus is 0.3% to 0.5%. The present study was limited by its retrospective design. Moreover, the number of diabetic and nondiabetic patients was markedly different, which might be a potential source of bias. Another limitation of the study was that patients received different types of antiviral therapies for different duration. Thus, we did not evaluate the effect of different interferon regimens but assessed the impact of having or not having a sustained virologic response. This heterogeneity makes it somewhat difficult to interpret the results. On the other hand, the strengths of the present study are the long-term follow-up in a large number of patients treated at the same institution. The present study highlights several new findings with regard to the development of hepatocellular carcinoma after interferon therapy in noncirrhotic patients with hepatitis C virus. First, in patients with a sustained virologic response, diabetes had no significant effect on the rate of hepatocarcinogenesis. Second, in patients with a nonsustained virologic response, the rate of hepatocarcinogenesis was significantly higher in diabetics; diabetes was associated with 2-fold increase in the incidence of hepatocellular carcinoma.

In the present study, no significant difference was noted in the rate of hepatocarcinogenesis in patients with a sustained virologic response with and without diabetes. However, at least 2 studies have described a relationship between diabetes and hepatocellular carcinoma in patients without viral hepatitis.[18,19] In our study, 7.3% of the patients with a nonsustained virologic response were diabetics, compared with approximately 3.0% in the group with a sustained virologic response. These rates were lower than those in the general Japanese population (~15% for men, 9% for women), especially in those with a sustained virologic response. With regard to interferon treatment, previous studies reported that insu-

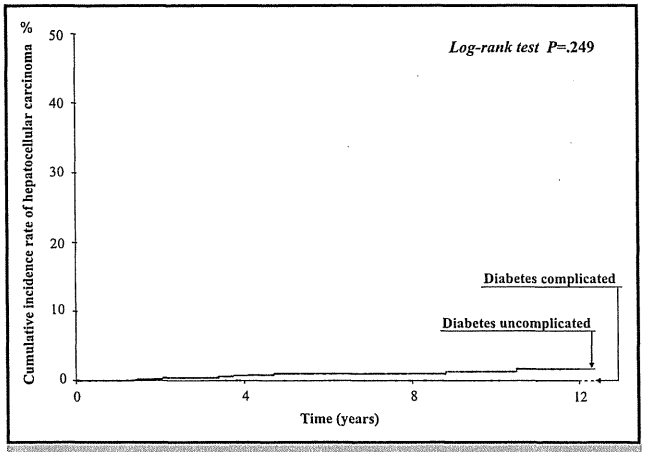

Figure 5 Cumulative rate of development of hepatocellular carcinoma from first interferon therapy in noncirrhotic patients with chronic hepatitis C infection who showed sustained virologic response to interferon therapy according to the presence or absence of diabetes.

lin resistance and diabetes lower the sustained virologic response rate in patients treated with peginterferon plus ribavirin.[20,21] Therefore, interferon therapy itself may explain the different rates of diabetes in the 2 groups.

Diabetes is an independent predictor of several types of cancers, including hepatocellular carcinoma in patients with or without viral infection.[19,22,23] However, the rate of hepatocarcinogenesis in our patients with a sustained virologic response was not significantly influenced by the presence or absence of diabetes. Our retrospective study included a low rate of diabetes compared with that of the general Japanese population. This lower rate of diabetes in patients with a sustained virologic response may explain the lack of effect of diabetes on the rate of hepatocarcinogenesis.

Several studies reported the relevance of hepatitis C virus core gene to insulin resistance in patients with chronic hepatitis C.[24-26] interferon therapy is considered to worsen blood glucose control, but if the cause of insulin resistance is based on the involvement of hepatitis C virus core gene, one could consider probable improvement of insulin resistance after a sustained virologic response. Further studies are necessary to examine in these points.

CONCLUSIONS

Our retrospective cohort study is the first to examine the effects of diabetes mellitus and sustained virologic response on hepatocarcinogenesis in noncirrhotic, interferon-treated patients with hepatitis C infection. Our results indicate that a sustained virologic response induced by interferon therapy eliminates the influence of diabetes mellitus and markedly reduces the rate of hepatocarcinogenesis in noncirrhotic, interferon-treated, hepatitis C virus-positive patients.

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Clinical and Virological Effects of Long-Term (Over 5 Years) Lamivudine Therapy

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Ideally, long-term lamivudine therapy should not induce tyrosine–methionine–aspartate–aspartate (YMDD) mutants (reverse transcription [rt]; rt M204I/V) in patients with chronic hepatitis B. There is little or no information on the clinical features of patients who do not develop such mutants. We analyzed 368 patients who received lamivudine therapy for more than 6 months between 1995 and 2003. Among them, 98 patients were negative for YMDD mutants during 5-year lamivudine therapy. Multivariate analysis identified hepatitis B e antigen (HBeAg) negativity, lack of cirrhosis, and high gamma glutamyltranspeptidase (GGTP) level as independent factors associated with lack of emergence of YMDD mutants during 5-year treatment. In these 98 patients, 21 patients developed YMDD mutants in the 5-year posttreatment follow-up. Old age was identified as the only factor associated with the emergence of YMDD mutants during that period. For all patients, 53 showed no elevation of alanine aminotransferase (ALT) or viral load after emergence of YMDD mutants during 5 years. Short latency to emergence of YMDD mutants, mixed (tyrosine–isoleucine–aspartate–aspartate (YIDD) [rtM204I]+tyrosine–valine–aspartate–aspartate (YVDD) [rtM204V]) type, and low ALT level were identified as independent factors associated with elevation ALT or viral load. HBeAg negativity, lack of cirrhosis, and high GGTP level were associated with lack of emergence of YMDD mutants during 5-year period. Young age protected against emergence of YMDD mutants over the 5-year period. Moreover, after the emergence of YMDD mutants, short latency to the emergence of YMDD mutant, mixed type mutants, and low baseline ALT level were associated with elevation of ALT or viral load. *J. Med. Virol. 82:684–691, 2010.*

KEY WORDS: YMDD mutant; HBV; lamivudine; GGTP; ALT; long-term

INTRODUCTION

Approximately 400 million people worldwide have chronic hepatitis B (CHB) infection, and 25–40% of these will develop hepatocellular carcinoma (HCC) and/or cirrhosis [Lee, 1997]. Prevention of disease progression is the primary target of treatment. To date, the nucleoside analogs, lamivudine, adefovir, dipivoxil, and entecavir, have been approved for the treatment of CHB [Zoulim and Perrillo, 2008]. Lamivudine markedly reduces viral load and hepatic necroinflammatory activity [Lai et al., 1998; Dienstag et al., 1999], and improves liver fibrosis [Dienstag et al., 2003a], and function. Unfortunately, failure of antiviral therapy is associated with the appearance of new viral variants, allowing hepatitis B virus (HBV) to become resistant. Lamivudine has the highest rate of drug resistance emergence. The number of patients with tyrosine–methionine–aspartate–aspartate (YMDD) mutation is higher with prolonged use of lamivudine. The cumulative rate of YMDD mutant reaches 60–70% after 4–5 years of treatment [Nafa et al., 2003; Suzuki et al., 2003]. On the other hand, 20–30% of patients continue long-term lamivudine therapy without YMDD mutations. There is little information at this stage about the

Abbreviations used: AFP, α-fetoprotein; ALT, alanine aminotransferase; AST, aspartate aminotransferase; CHB, chronic hepatitis B; DNA, deoxyribonucleic acid; GGTP, gamma glutamyltranspeptidase; HBeAg, hepatitis B e antigen; LC, liver cirrhosis; HBsAg, hepatitis B virus surface antigen; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; PCR, polymerase chain reaction; YIDD, tyrosine–isoleucine–aspartate–aspartate; YMDD, tyrosine–methionine–aspartate–aspartate; YVDD, tyrosine–valine–aspartate–aspartate.

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clinical differences between patients with and without YMDD mutants on long-term lamivudine therapy.

After the emergence of YMDD mutant, breakthrough hepatitis occurs at a high frequency. This is important because breakthrough hepatitis can occasionally cause liver decompensation [Liaw et al., 2000]. However, alanine aminotransferase (ALT) and viral load are not elevated at least in some patients with YMDD mutant. The difference between these groups remains poorly defined. The aims of the present investigation were the following: (1) characterize the clinical and virological features of patients who do not show emergence of YMDD mutants during 5 years of lamivudine therapy. (2) Identify the factor(s) associated with the emergence of YMDD mutants in patients on >5 years of lamivudine therapy. (3) Determine the factors associated with elevation of ALT (>50 IU/L) and viral load (>5.0 log copies/ml) after the emergence of YMDD mutant.

PATIENTS AND METHODS

Patients

The study subjects were 368 patients (66 females and 302 males, median age 43 years [range 19–76]) who commenced treatment with lamivudine at the Department of Hepatology, Toranomon Hospital, Tokyo, between September 1995 and June 2003 and adhered to treatment for more than 6 months (Table I). All patients were followed from commencement of therapy at our hospital. Some of these patients have been reported previously [Chayama et al., 1998; Suzuki et al., 2003]. All patients were negative for hepatitis C serologic markers, but all had detectable hepatitis B virus surface antigen (HBsAg) for at least 6 months prior to commencement of lamivudine therapy. Lamivudine was administered orally at 100 mg/day. Chronic hepatitis or cirrhosis was confirmed by needle biopsy, peritoneoscopy, or clinically before treatment. The clinical criteria for chronic hepatitis included elevated ALT levels over 6 months and absence of clinical evidence of portal hypertension, such as esophageal varices, ascites, hepatic encephalopathy, and imaging features suggestive of cirrhosis on ultrasonography. Chronic hepatitis and cirrhosis were diagnosed in 309 and 57, respectively. Informed consent was obtained from each patient enrolled in the study; and the study protocol conformed to the ethical guidelines of Declaration of Helsinki and was approved by the human research committee of our institution.

Blood Tests, Serum Viral Markers, and Assessment of Response to Therapy

Routine biochemical tests were performed before and during therapy at least once every 2 months, using standard procedures. Serial blood samples were taken before and during therapy and stored at −80°C until used for HBV molecular analysis. Viral load was measured by polymerase chain reaction (PCR)-based method (Amplicor HBV monitor; Roche Diagnostics, Tokyo, Japan). Mutation of the HBV deoxyribonucleic acid (DNA) polymerase gene (rtM204I/V) was determined using PCR and restriction fragment length polymorphism, as described previously [Chayama et al., 1998] or PCR-ELMA method [Kobayashi et al., 2000]. The presence of YMDD mutation was determined at baseline and at yearly intervals. Resistance to lamivudine was determined annually before the development of mutations, and, if a mutation appeared, the time of appearance of resistance was confirmed by monthly measurement.

Statistical Analysis

Differences between groups were examined for statistical significance using the χ^2 test for categorical variables and Mann–Whitney U-test for continuous variables. The association of mutations with specific

TABLE I. Characteristics of Patients at Commencement of Lamivudine Therapy

Demography	
Total patients	368
Sex: female/male	302/66
Age (years)	43 (19–76)
Family history of HBV	245 (66.6%)
Cirrhosis	57 (15.5%)
Median duration of treatment, years (range)	5 (0.5–12.8)
Laboratory data	
Aspartate aminotransferase (IU/L)	80 (19–2,593)
Alanine aminotransferase (IU/L)	120.5 (12–2,274)
Bilirubin (mg/dl)	0.7 (0.2–16.5)
Gamma glutamyltranspeptidase (IU/L)	64 (13–475)
Albumin (g/dl)	3.9 (2.1–4.8)
Viral load (log copies/ml)	7.1 (<2.7 to >7.6)
HBeAg positive	187 (50.8%)
HBV genotypes: A/B/C/D/F/unknown	12/25/317/1/2/11

HBV, hepatitis B virus; HBeAg, hepatitis B envelope antigen.
The family history of six patients was not clear. Viral load was measured by PCR. All viral load values below the lower limit of detection (<2.7 log copies) were set to 2 while those over the upper limit of detection (>7.6) ware set to 8 for calculation purposes.
Data are median and range values except for the last two parameters.

predictive variables was assessed by Cox proportional hazard model. To determine the factors that affect YMDD mutation, multiple logistic regression analysis was carried out. Spearman correlation coefficient (two-tailed) was used to evaluate the correlation between gamma-glutamyltranspeptidase (GGTP) and other factors. Two-tailed P-value <0.05 was considered statistically significant. All data were analyzed using the statistical package SPSS (version 11.0, SPSS, Inc., Chicago, IL).

RESULTS

Clinical and Virological Features of Patients Free of YMDD Mutations

Lamivudine therapy was provided for a median duration of 5 years [range 0.5–12.8 years]. Forty patients discontinued lamivudine therapy due to pregnancy, expectation of a change to another therapy, or loss to follow-up. Among the remaining 328 patients, YMDD mutants were identified in 230 patients during the 5-year treatment. Table II summarizes the characteristics of patients without and with YMDD mutant during the 5-year treatment. There were more patients with genotype B, and fewer patients with genotype A in the former than in the latter group ($P < 0.001$). Furthermore, a high proportion of hepatitis B e antigen (HBeAg)-negative patients were noted in the former group than in the latter group ($P = 0.001$). In the latter group, the emergence of YMDD mutant was associated with elevated ALT and/or viral load in 177 patients while it was not in 53 patients. On the other hand, 98 patients showed no emergence of YMDD mutants during the 5-year treatment (Fig. 1).

Figure 2 shows the cumulative rate of patients who showed emergence of YMDD mutations duringlamivudine therapy [129, 74, and 48 patients developed tyrosine–isoleucine–aspartate–aspartate (YIDD), tyrosine–valine–aspartate–aspartate (YVDD), and mixed (YIDD + YVDD) mutants, respectively]. YMDD mutants were registered in 11 (92%) of 12 patients with genotype A, 13 (52%) of 25 patients with genotype B, 219 (69%) of 317 patients with genotype C, 0 (100%) of 1 patients with genotype D, 2 (100%) of 2 patients with genotype F, and 6 (55%) of 11 patients with unidentified genotype.

We then explored the factors associated without emergence of YMDD mutants. Patients free of YMDD mutants were considered to have ideal response to lamivudine therapy. The following significant independent factors for the lack of YMDD mutations during the 5-year treatment were identified in univariate analysis: HBV genotype B, lack of cirrhosis, HBeAg negativity, free family history of liver disease, high aspartate aminotransferase (AST) level (>75 IU/L), high ALT level (>180 IU/L), high GGTP level (>110 IU/L), high albumin level (3.7 g/dl), and low viral load (<5.9 log copies/ml). Multivariate analysis identified HBeAg negativity, high GGTP level (>110 IU/L), and lack of liver cirrhosis (LC) as significant determinants for the lack of YMDD mutations during the 5-year treatment (Table III).

GGTP is regarded as a marker of fatty liver and alcoholic liver disease [Patton et al., 2008]. Fatty liver disease correlates with liver fibrosis and carcinogenesis [Yuan et al., 2004; Yu et al., 2008]. However, the influence of treatment with nucleos(t)ide analog is not clear. Next, we investigated the correlation between GGTP and other factors (Table IV). GGTP correlated significantly with ALT ($r = 0.562$, $n = 355$, $P < 0.001$), AST ($r = 0.562$, $n = 355$, $P < 0.001$), α-fetoprotein (AFP) ($r = 0.430$, $n = 319$, $P < 0.001$), total bilirubin ($r = 0.264$, $n = 354$, $P < 0.001$), and platelet count ($r = -0.129$, $n = 330$, $P = 0.019$). GGTP did not correlate with liver fibrosis ($r = -0.28$, $n = 276$, $P = 0.641$), total cholesterol ($r = -0.77$, $n = 132$, $P = 0.379$), or blood glucose ($r = 0.118$, $n = 115$, $P = 0.355$) was. Based on the above results, GGTP correlated with ALT, AST, and other liver function-related parameters and does not seem to be related to other metabolic factors.

Among 163 patients who were positive for HBeAg at the commencement of lamivudine therapy, 35 (21%) did not show emergence of YMDD mutants during the 5-year treatment. Of these, 31 (89%) achieved HBeAg

TABLE II. Comparison of Patients With and Without YMDD Mutants During 5-Year Lamivudine Therapy

Category	Without YMDD mutation (n = 98)	With YMDD mutation (n = 230)	P-value
Age (years)[a]	43 (24–76)	44 (23–71)	0.783
Sex: male/female	77/21	194/36	0.206
Genotype: A/B/C/others	1/12/81/4	11/9/203/7	<0.001
Histology: chronic hepatitis/cirrhosis	88/10	185/43	0.052
Bilirubin (mg/dl)[a]	0.7 (0.2–12.2)	0.7 (0.2–16.5)	0.898
Alanine aminotransferase (IU/L)[a]	136 (16–2,077)	118.5 (14–2,274)	0.237
Gamma glutamyltranspeptidase (IU/L)[a]	72 (13–442)	58 (16–402)	0.197
Viral load (log copies/ml)[a]	7.1 (<2.7 to >7.6)	7.2 (<2.7 to >7.6)	0.136
HBeAg: positive/negative	35/63	128/102	0.001
Latency to emergence of YMDD mutation		2 (0–4.9)	

YMDD, tyrosine–methionine–aspartate–aspartate; HBV, hepatitis B virus; HBeAg, hepatitis B e antigen.
Viral load was measured by PCR. All viral load values below the lower limit of detection (<2.7 log copies) were set to 2 and those over the upper limit of detection (>7.6) ware set to 8 for calculation purposes.
[a]Data are median (range) values.

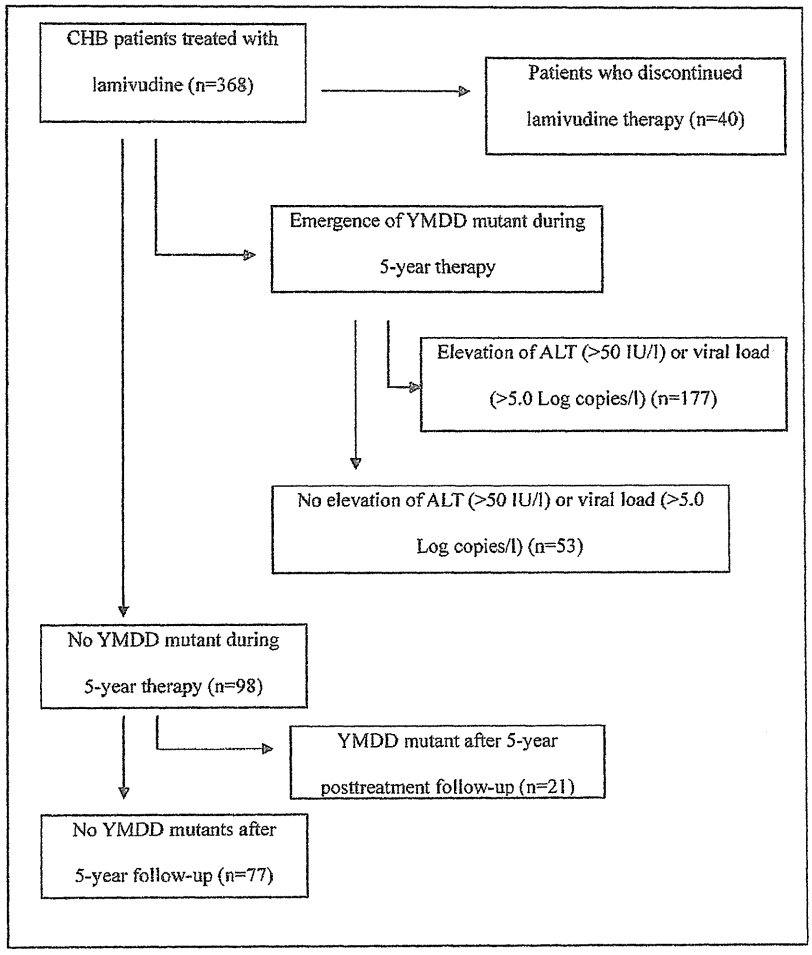

Fig. 1. Outcome of patients with lamivudine therapy. CHB, chronic hepatitis B; YMDD, tyrosine–methionine–aspartate–aspartate; ALT, alanine aminotransferase.

loss during 5-year treatment. On the other hand, in 128 patients who showed emergence of YMDD mutants, 42 (33%) achieved HBeAg loss. Analysis of various parameters showed that only the platelet count was different between the two HBeAg-positive groups; that is, in HBeAg-positive patients, those with high platelet counts were less likely to develop YMDD mutations ($P = 0.051$).

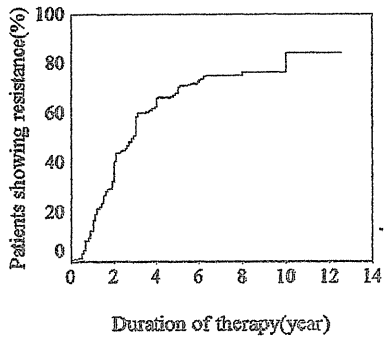

Fig. 2. Cumulative rate of patients who showed emergence of YMDD mutants during lamivudine therapy (Kaplan–Meier method). YMDD, tyrosine–methionine–aspartate–aspartate.

Emergence of YMDD Mutant After 5 Years of Lamivudine Therapy

As described above, 98 patients showed no emergence of YMDD mutants during the 5-year treatment. We investigated in this group the emergence of YMDD mutants after the 5-year treatment period. Twenty-one (21%) patients showed emergence of YMDD mutants following the completion of the 5-year treatment period (Table V). Univariate analysis showed only age (>50 years) influenced the emergence of the YMDD mutants after the 5-year treatment ($P = 0.012$). At time 5 years, 94 (96%) patients were negative for HBeAg. Therefore, the status of HBeAg at 5 years did not influence the emergence of YMDD mutant. After the emergence of YMDD mutant, 4 of the 21 patients had elevated ALT and viral load; they were further treated

TABLE III. Results of Multivariate Analysis of Factors Associated With Lack of Appearance of YMDD Mutants During 5-Year Lamivudine Therapy

Factors	Risk ratio (95% confidence interval)	*P*-value
Pretreatment HBeAg		
0: Positive	1	
1: Negative	2.492 (1.440–4.311)	0.001
Pretreatment GGTP (IU/L)		
0: <110	1	
1: ≥110	2.226 (1.296–4.900)	0.004
Pretreatment histology		
0: LC	1	
1: Not cirrhosis	2.254 (1.037–4.900)	0.04

YMDD, tyrosine–methionine–aspartate–aspartate; HBeAg, hepatitis B envelope antigen; GGTP, gamma glutamyltranspeptidase; LC, liver cirrhosis.

with a combination of adefovir and lamivudine. The remaining 17 patients showed no elevation of ALT or viral load, 3 of the 17 patients were treated with a combination of adefovir and lamivudine, treatment was switched in 6 of the 17 patients from lamivudine to entecavir, while 8 of the 17 patients continued lamivudine treatment. No emergence of YMDD mutant after the 5-year treatment period was noted in 77 patients, but 4 of 77 patients discontinued lamivudine therapy due to pregnancy, or loss to follow-up. Furthermore, treatment in 14 of the 77 patients was changed from lamivudine to entecavir while the remaining 59 patients continued lamivudine therapy.

Characteristics of Patients With Elevated ALT or Viral Load After Emergence of YMDD Mutant

As mentioned above, 230 (62.5%) of the 368 patients developed YMDD mutations during the 5-year treatment period, and 177 had elevated ALT or viral load level after the emergence of YMDD mutants, while 53 patients had neither ALT elevation (>50 IU/L) nor HBV DNA elevation (>5.0 log copies/ml) during the treatment period. We then explored the risk factors for the elevation of viral load and ALT level in these patients. In univariate analysis, the following seven factors correlated significantly with elevation of viral load or ALT level: HBeAg ($P < 0.001$), latency to emergence of YMDD mutant ($P < 0.001$), mixed type YMDD mutant (YIDD + YVDD) ($P < 0.001$), ALT level ($P = 0.003$), viral load ($P = 0.007$), and AFP level ($P = 0.021$). These variables were entered into multivariate analysis. In the last step of the analysis, the following three variables were identified as significant determinants of elevation of viral load or ALT level: latency to emergence of YMDD mutant ($P < 0.001$), mixed type YMDD mutant ($P < 0.001$), and ALT level ($P = 0.016$) (Table VI).

TABLE IV. Correlation Between GGTP and Laboratory Tests

Factors	r	n	*P*-value
ALT	0.562	355	<0.001
AST	0.562	355	<0.001
AFP	0.43	319	<0.001
Total bilirubin	0.264	354	<0.001
Platelet count	−0.219	330	0.019
Liver fibrosis	−0.28	276	0.641
Total cholesterol	−0.77	132	0.379
Blood glucose	0.118	115	0.355

ALT, alanine aminotransferase; AST, aspartate aminotransferase; AFP, α-fetoprotein.

Characteristics of Patients With YMDD Mutant During and After 5-Year Treatment

Throughout the present study, YMDD mutant developed in 251 patients. As described above, 230 of these 251 patients developed YMDD mutant during the 5-year period. The remaining 21 patients developed YMDD mutant after the 5-year period. Table VII summarizes the characteristics of patients with YMDD mutant during and after the 5-year treatment. HBV genotype and HBeAg negativity were found to correlate with the development of YMDD mutants after 5-year treatment.

DISCUSSION

The aim of the present study was to identify the factors associated with the lack of emergence of YMDD mutant during long-term lamivudine therapy. Our analysis showed that negativity for HBeAg, high GGTP level (≥110 IU/L), and lack of LC protected against the appearance of YMDD mutants during the 5-year lamivudine therapy. Since positivity for HBeAg is a well-known factor associated with emergence of YMDD mutant [Yuen et al., 2001; Suzuki et al., 2003], we focused on the correlation between GGTP and other factors (Table IV). The results showed that GGTP correlated with ALT, AST, and other liver function-related parameters. Previous studies identified high pretreatment ALT level as an independent factor associated with no appearance of YMDD mutant [Tsubota et al., 2004; Chang et al., 2005]. In this regard, GGTP is regarded as a marker of fatty liver and alcoholic liver disease [Patton et al., 2008]. Fatty liver disease correlates with liver fibrosis and carcinogenesis [Yuan et al., 2004; Yu et al., 2008]. However, the influence of treatment with nucleos(t)ide analog is not clear. Based on the above results, GGTP does not seem to be related to other metabolic factors (e.g., total cholesterol and blood glucose). However, further investigation of other

TABLE V. Comparison of Clinicopathological Features of Patients With and Without Emergence of YMDD Mutants After 5-Year Posttreatment Follow-Up

Category	Without YMDD mutation (n = 77)	With YMDD mutation (n = 21)	*P*-value
Age (years)[a]	42 (24–76)	50 (33–69)	0.032
Sex: male/female	63/14	14/7	0.134
Genotype: A/B/C/others	1/8/65/3	0/4/16/1	0.275
Histology: no cirrhosis/cirrhosis[b]	69/8	19/2	0.636
Bilirubin (mg/dl)[a]	0.7 (0.2–12.2)	0.7 (0.4–4.4)	0.644
ALT (IU/L)[a]	135 (16–1,975)	142 (25–2,077)	0.997
GGTP (IU/L)[a]	69 (13–442)	82 (24–264)	0.878
Viral load (log copies/ml)[a]	7 (<2.7 to >7.6)	7.4 (<2.7 to >7.6)	0.394
HBeAg: positive/negative	28/49	7/14	0.797
Status of HBeAg at 5 years: positive/negative	3/74	1/20	0.860
Latency to emergence of YMDD mutation		5.6 (5–10)	

YMDD, tyrosine–methionine–aspartate–aspartate; ALT, alanine aminotransferase; GGTP, gamma glutamyltranspeptidase; HBV, hepatitis B virus; CHB, chronic hepatitis B; HBeAg, hepatitis B envelope antigen.
[a]Data are median (range) values.
[b]Chronic hepatitis and cirrhosis were confirmed by needle biopsy, peritoneoscopy, or clinically before treatment. Diagnosis of chronic hepatitis was based on elevated ALT levels over 6 months and absence of clinical evidence of portal hypertension, such as esophageal varices, ascites, hepatic encephalopathy, and imaging features suggestive of cirrhosis on ultrasonography. Viral load was measured by PCR. All viral loads below the lower limit of detection (<2.7 log copies) were set to 2 and those over upper limit of detection (>7.6) were set to 8 for calculation purposes.

metabolic factors is needed, such as body mass index, HOMA-IR, and alcohol intake. The third factor, lack of liver fibrosis and cirrhosis based on histopathological examination, was associated with lack of YMDD mutants. Previous study reported that the presence of cirrhosis correlated with emergence of YMDD mutant [Ooga et al., 2004]. Moreover, among patients with LC, those who develop YMDD mutants are more likely to have high Child-Pugh scores than those without such mutants [Liaw et al., 2004]. On the other hand, viral load has been reported to promote the emergence of YMDD mutants [Yuen et al., 2001]. In the present study, although viral load was identified as a factor in univariate analysis, it was not identified as such in multivariate analysis.

We performed additional investigation on elevation of ALT or viral load after the emergence of YMDD mutants. In this analysis, 77% of these patients (n = 177) had elevated ALT or viral load, while 23% (n = 53) had not. We found several common characteristics among patients of the high ALT/viral load group. The latency to emergence of YMDD mutants and mixed type mutants (YIDD + YVDD) were significant factors in this group. Early emergence of YMDD mutant could reflect a rapid increase of HBV DNA. The mixed type was reported as a risk factor of HBV DNA breakthrough and breakthrough hepatitis [Akuta et al., 2003; Suzuki et al., 2006]. Previous studies reported that a low pretreatment ALT was an independent factor associated with appearance of YMDD mutants [Tsubota et al., 2004; Chang et al., 2005]. Based on the above findings, patients with low baseline ALT level and during treatment emergence of YMDD mutants seem to be at high risk of breakthrough hepatitis.

Younger patients had less opportunity to develop YMDD mutations after the 5-year treatment. We reported previously that age was not associated with emergence of YMDD mutant [Kawaoka et al., 2007]. However, the duration of treatment in our previous study was <5 years. Patients free of YMDD mutants during the 5-year treatment might have adequate immune response to suppress the development of YMDD mutants. The immune response is lower in elderly patients [Adler and Nagel, 1994; Marcus and Tur-Kaspa, 1997]. Considered together, younger patients seem to be more immune against the emergence

TABLE VI. Factors Associated With Elevation of ALT or Viral Load After Emergence of YMDD Mutant

Factors	Hazard ratio (95% confidence interval)	*P*-value
Latency to emergence of YMDD mutant		
0: ≥1 year	1	
1: <1 year	7.429 (4.769–11.572)	<0.001
YMDD mutant type		
0: YIDD or YVDD	1	
1: Mixed (YIDD + YVDD) type	2.939 (1.834–4.677)	<0.001
Pretreatment ALT level (IU/L)		
0: >160	1	
1: ≤159	1.583 (1.089–2.301)	0.016

YMDD, tyrosine–methionine–aspartate–aspartate; ALT, alanine aminotransferase; HBV, hepatitis B virus; YIDD, tyrosine–isoleucine–aspartate–aspartate; YVDD, tyrosine–valine–aspartate–aspartate.

TABLE VII. Comparison of Clinicopathological Features of Patients With YMDD Mutants During and After 5-Year Treatment Period

Category	With YMDD mutation during 5-year (n = 230)	With YMDD mutation after 5-year (n = 21)	*P*-value
Age (years)[a]	44 (23–71)	50 (33–69)	0.109
Sex: male/female	194/36	14/7	0.063
Genotype: A/B/C/others	11/9/203/7	0/4/16/1	0.0184
Histology: no cirrhosis/cirrhosis[b]	185/43	19/2	0.384
Bilirubin (mg/dl)[a]	0.7 (0.2–16.5)	0.7 (0.4–4.4)	0.570
ALT (IU/L)[a]	118.5 (14–2,274)	142 (25–2,077)	0.527
GGTP (IU/L)[a]	58 (16–402)	82 (24–264)	0.382
Viral load (log copies/ml)[a]	7.2 (<2.7 to >7.6)	7.4 (<2.7 to >7.6)	0.936
HBeAg: positive/negative	128/102	7/14	0.0496

YMDD, tyrosine–methionine–aspartate–aspartate; ALT, alanine aminotransferase; GGTP, gamma glutamyltranspeptidase; HBV, hepatitis B virus; CHB, chronic hepatitis B; HBeAg, hepatitis B envelope antigen.
[a]Data are median (range) values.
[b]Chronic hepatitis and cirrhosis were confirmed by needle biopsy, peritoneoscopy, or clinically before treatment. Diagnosis of chronic hepatitis was based on elevated ALT levels over 6 months and absence of clinical evidence of portal hypertension, such as esophageal varices, ascites, hepatic encephalopathy, and imaging features suggestive of cirrhosis on ultrasonography. Viral load was measured by PCR. All viral loads below the lower limit of detection (<2.7 log copies) were set to 2 and those over upper limit of detection (>7.6) were set to 8 for calculation purposes.

of YMDD mutant in long-term lamivudine treatment than elderly patients.

Several new nucleos(t)ide analogs, for example, adefovir and entecavir, are available at present [Gish et al., 2007; Marcellin et al., 2008]. These new drugs have greater inhibitory effects on HBV replication and their use is associated with a lower incidence of drug resistance. However, resistant to the new drugs has already been reported [Suzuki et al., 2007; Baldick et al., 2008]. Lamivudine was the first nucleoside analog and has been used over a long time worldwide. Based on the result of our study, younger patients (<50 years) who continued lamivudine monotherapy without emergence of YMDD mutant during 5-year period showed less opportunity to develop mutants after a 5-year follow-up and were able to continue lamivudine monotherapy. After the cessation of lamivudine therapy, flare up of ALT accompanied with elevation of HBV DNA was observed at a high frequency [Song et al., 2000; Dienstag et al., 2003a; Akuta et al., 2005]. Moreover, we reported previously HBsAg clearance from the serum in some patients who received long-term lamivudine therapy [Kobayashi et al., 2007]. Taken together, it seems that before any treatment, one can predict a less likelihood of development of YMDD mutants during long-term lamivudine therapy in young patients with genotype C who are HBeAg negative, have no cirrhosis, and no elevated GGTP level. Tables II and VII suggest that patients with genotype B are also less likely to develop YMDD mutant, but their numbers are too small to make a firm conclusion. Further studies of larger number of patients with genotype B, A, and others are needed to clarify this issue.

In conclusion, factors associated with lack of appearance of YMDD mutants during 5-year lamivudine therapy in patients with HBV infection are HBeAg negativity, lack of cirrhosis, and high GGTP level. Patients who do not show the emergence of YMDD mutants during 5-year lamivudine therapy, younger age protected against the emergence of such mutants during the following 5 years of follow-up. On the other hand, in those who show emergence of YMDD mutant, elevation of ALT or viral load correlate with a short latency to emergence of YMDD mutants, presence of mixed (YIDD + YVDD) type, and low baseline ALT level.

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