CLINICAL RESEARCH STUDY

THE AMERICAN JOURNAL of MEDICINE®

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.

 • *The American Journal of Medicine (2010) 123, 951-956*

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,

Funding: Okinaka Memorial Institute for Medical Research and Japanese Ministry of Health, Labour and Welfare.

Conflict of Interest: None of the authors have any conflicts of interest associated with the work presented in this manuscript.

Authorship: All authors had access to the data and played a role in writing this manuscript.

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0002-9343/$ -see front matter © 2010 Elsevier Inc. All rights reserved.
doi:10.1016/j.amjmed.2010.05.013

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 (μg/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

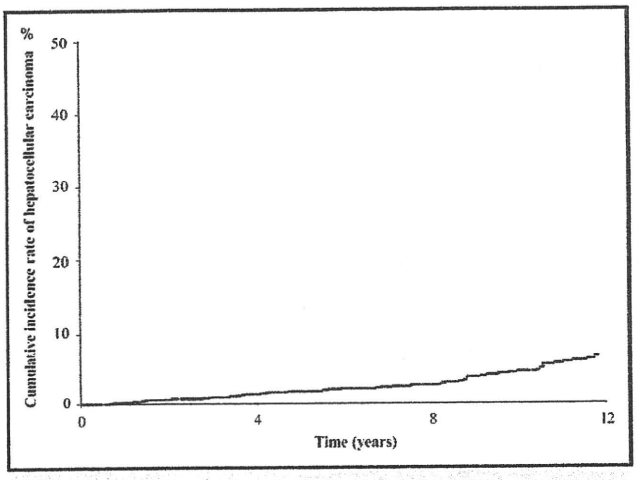

Figure 1 Cumulative rate of development of hepatocellular carcinoma from first interferon therapy in noncirrhotic patients with chronic hepatitis C infection.

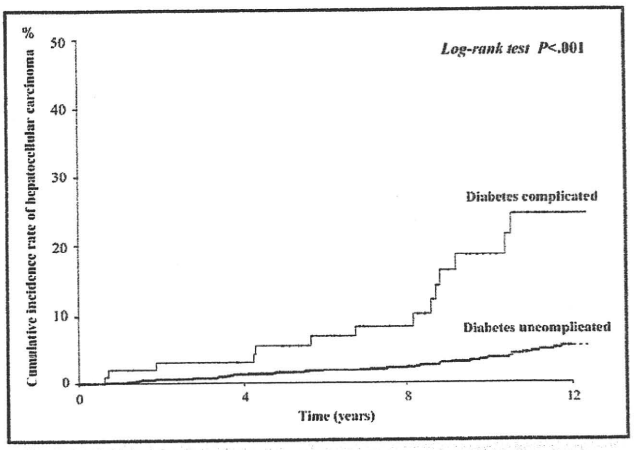

Figure 3 Cumulative rate of development of hepatocellular carcinoma from first interferon therapy in noncirrhotic patients with chronic hepatitis C infection according to the presence or absence of diabetes.

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

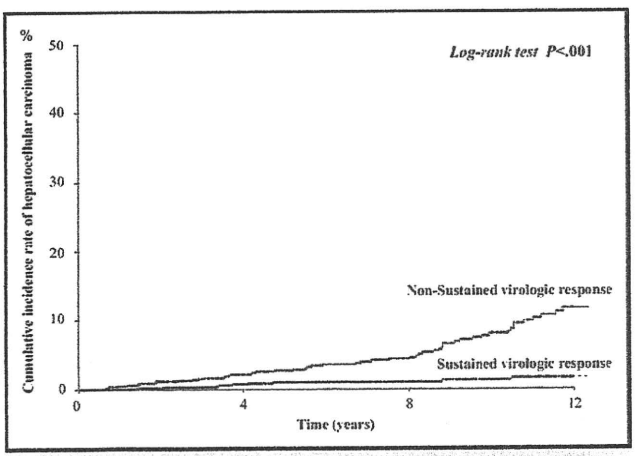

Figure 2 Cumulative rate of development of hepatocellular carcinoma from first interferon therapy in noncirrhotic patients with chronic hepatitis C infection according to effect of interferon therapy.

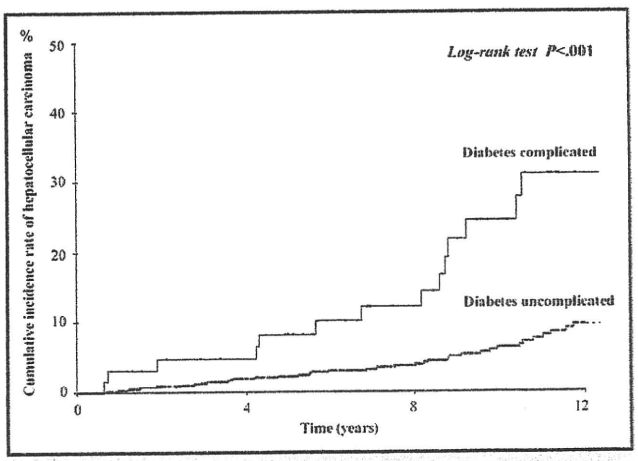

Figure 4 Cumulative rate of development of hepatocellular carcinoma from first interferon therapy in noncirrhotic patients with chronic hepatitis C infection who showed nonsustained virologic response to interferon therapy according to the presence or absence of diabetes.

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-

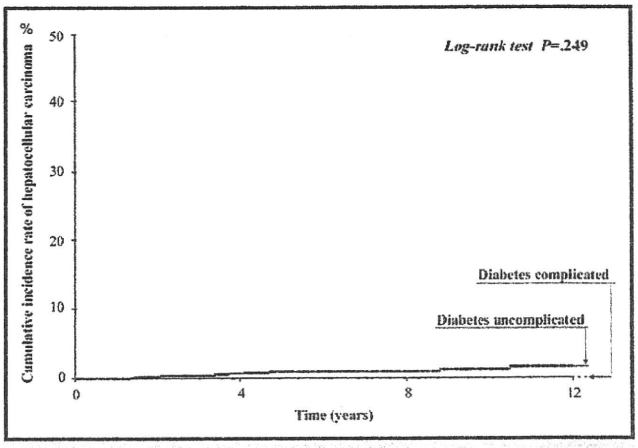

Figure 5 Cumulative rate of development of hepatocellular carcinoma from first interferon therapy in noncirrhotic patients with chronic hepatitis C infection who showed sustained virologic response to interferon therapy according to the presence or absence of diabetes.

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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Table 2 Predictors of Hepatocarcinogenesis in Noncirrhotic, Interferon-Treated Patients with Chronic Hepatitis C Infection

		Univariate Analysis		Multivariate Analysis	
Variables	Category	HR (95% CI)	*P*	HR (95% CI)	*P*
Gender	1: Female	1		1	
	2: Male	2.38 (0.23-0.77)	.005	4.90 (2.47-9.71)	<.001
Age	1: <60	1		1	
	2: ≥60	3.34 (2.01-5.52)	<.001	3.28 (1.88-5.74)	<.001
Histopathologic grade	1: F1-2	1			
	2: F3	2.98 (1.48-6.02)	.002		
Total ethanol intake (kg)	1: <500	1			
	2: ≥500	3.486 (2.02-6.01)	<.001		
Albumin (g/dL)	1: ≥4.0	1			
	2: <4.0	1.73 (0.10-3.00)	.053		
Total bilirubin (mg/dL)	1: <0.5	1			
	2: ≥0.5	1.50 (0.75-3.02)	.256		
AST (IU/L)	1: <50	1		1	
	2: ≥50	5.75 (2.86-11.59)	<.001	3.91 (1.81-8.43)	.001
ALT (IU/L)	1: <100	1			
	2: ≥100	2.22 (1.37-3.60)	.001		
γ-GTP (IU/L)	1: <50	1			
	2: ≥50	2.59 (1.58-4.25)	<.001		
Platelet count ($\times 10^4$/mL)	1: ≥17	1		1	
	2: <17	3.00(1.85-4.88)	<.001	1.96 (1.11-3.48)	.021
AFP (μg/L)	1: <20	1		1	
	2: ≥20	4.71 (2.51-8.85)	<.001	2.89 (1.43-5.84)	.003
Diabetes mellitus	1: No	1		1	
	2: Yes	4.50 (2.54-7.95)	<.001	2.00 (1.05-3.84)	.036
Total cholesterol level (mg/dL)	1: ≥220	1			
	2: <220	1.28 (0.30-5.41)	.735		
Triglyceride level (mg/dL)	1: <150	1			
	2: ≥150	2.221 (0.78-6.20)	.134		
LDL cholesterol level (mg/dL)	1: ≥140	1			
	2: <140	1.19 (0.27-5.21)	.817		
HDL cholesterol level (mg/dL)	1: ≥40	1			
	2: <40	1.98 (0.80-4.93)	.142		
HCV serologic group	1: sero group 2	1			
	2: sero group 1	2.23 (1.22-4.07)	.009		
Viral load	1: Low	1			
	2: High	2.18 (1.29-3.67)	.003		
Effect of IFN therapy acquired viral elimination*	1: Yes	1		1	
	2: No	2.30 (1.03-7.09)	<.001	7.28 (3.28-16.15)	<.001

HR = hazard ration; CI = confidence interval; AST = aspartate aminotransferase; ALT = alanine aminotransferase; γ-GTP = gamma-glutamyl transpeptidase; AFP = alpha-fetoprotein; LDL = low-density lipoprotein; HDL = high-density lipoprotein; HCV = hepatitis C virus; IFN = interferon.

*Viral elimination means sustained virologic response.

Journal of Medical Virology 82:684–691 (2010)

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.

Grant sponsor: Ministry of Health, Labor and Welfare, Japan (partial support).

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Accepted 3 September 2009

DOI 10.1002/jmv.21681

Published online in Wiley InterScience (www.interscience.wiley.com)

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.

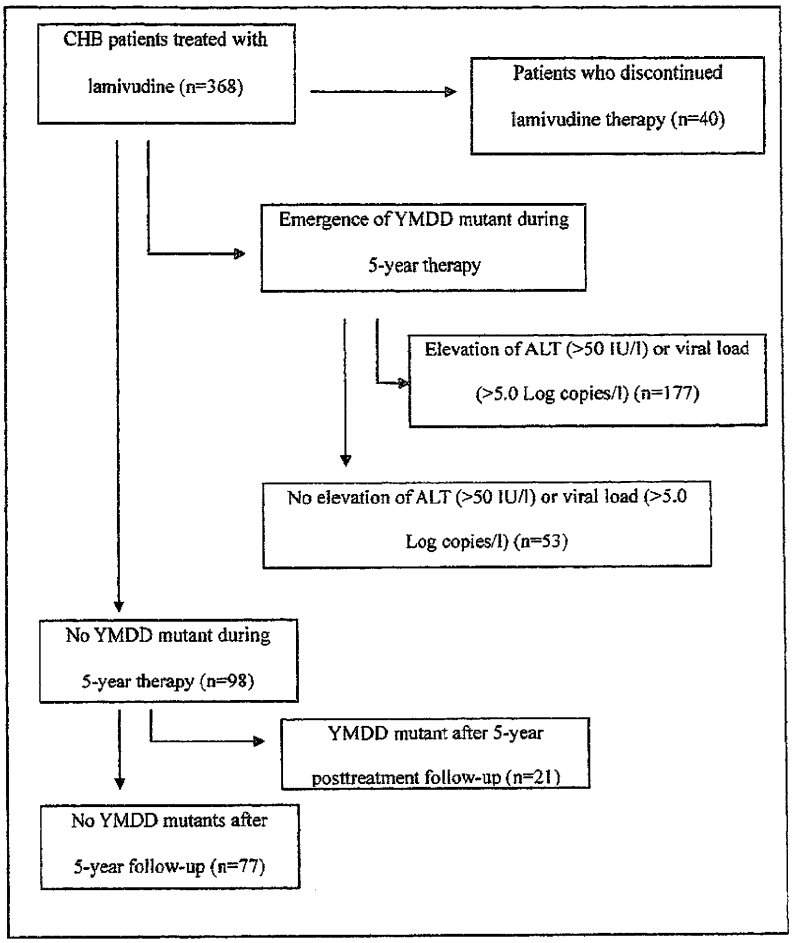

Fig. 1. Outcome of patients with lamivudine therapy. CHB, chronic hepatitis B; YMDD, tyrosine–methionine–aspartate–aspartate; ALT, alanine aminotransferase.

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 ($P = 0.051$).

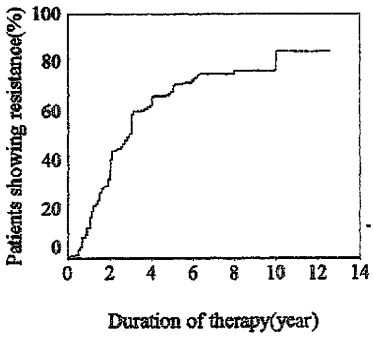

Fig. 2. Cumulative rate of patients who showed emergence of YMDD mutants during lamivudine therapy (Kaplan–Meier method). YMDD, tyrosine–methionine–aspartate–aspartate.

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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Dig Dis Sci (2010) 55:2070–2076
DOI 10.1007/s10620-009-0995-x

ORIGINAL ARTICLE

Association of HLA-DR14 with the Treatment Response in Japanese Patients with Autoimmune Hepatitis

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Received: 30 December 2008 / Accepted: 10 August 2009 / Published online: 22 January 2010

Abstract
Background Influence of human lymphocyte antigen (HLA) on the therapeutic response in autoimmune hepatitis (AIH) is not known.
Aims To evaluate if HLA-DR types influence biological and histological responses to corticosteroids in patients with AIH.
Methods During 28 years from 1979 through 2007, 48 patients with definite diagnosis of AIH received long-term corticosteroid therapy (median 9 years [range: 5–28 years]) in a single Japanese center. They were followed for transaminase levels and received liver biopsy before and after the treatment.
Results DR4 was detected in 32 and DR14 in 11 patients; seven possessed both DR4 and DR14. DR4 was more frequent in AIH patients than in the general population (67% vs. 22%), while DR14 was comparably frequent between them (23% vs. 17%). Overall, biochemical response was achieved in 43 (90%) of the 48 patients. The sustained biochemical response to a maintenance prednisolone dose < 10 mg was gained more frequently in the patients with than without DR14 (10/11 [91%] vs. 10/37 [27%], $P < 0.001$). Marked histological improvement with a decrease in histology activity index (HAI) score by > 2 points was achieved in 31 of the 32 (97%) biochemical responders. Histological aggravation with an increase in HAI score occurred in 4 of the 16 (25%) patients without biochemical response (non-responders and relapsers combined), but in none of the 32 responders.
Conclusion Long-term immunosuppressive treatment can improve the outcome of Japanese patients with AIH, and DR14 is associated with excellent biochemical response.

Keywords Hepatitis · Autoimmune-HLA-DR-corticosteroids-biopsy · Needle

Introduction

Autoimmune hepatitis (AIH) is the inflammation of hepatocytes of unknown etiology and characterized by histological hallmark of interface hepatitis with infiltration of lymphocytes in the portal area [1–3]. Female preponderance, various auto-antibodies and hyper-γ-globulinemia, as well as excellent response to immunosuppressive therapies, are prominent clinical features. AIH is sub-grouped into types 1–3 by the age of onset, severity of disease, and autoantibody profiles [3]. Loss of immunotolerance to self-antigens expressed on hepatocytes is implicated in the pathogenesis of AIH, in the background of major histocompatibility complex (MHC) genes represented by HLA-DR alleles [4].

The disease entity of AIH is not uniform and influenced by geography and ethnicity, in which HLA-DR types play a major role. For the purpose of dealing with a broad clinical spectrum of AIH, diagnostic criteria were proposed by the International Autoimmune Hepatitis Group (IAIHG) in 1993 [5], and they were modified in 1999 [6]. In Japan, an

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indigenous scoring system for defining AIH was established in 1996 [7]. It has allowed to distinguish AIH from other autoimmune liver disease, such as primary biliary cirrhosis and primary sclerosing cholangitis [8]. Although the treatment response differs in AIH patients with distinct DR profiles, aggressive immunosuppressive treatments with precaution to avoid side-effects can prevent histological deterioration toward favorable long-term outcomes [9, 10].

Since by far the most patients with AIH can merit from immunosuppressive treatment, an effective therapy for an appropriate duration is the primary goal of physicians. AIH can run a rapid course accompanied by cirrhosis in some cases, particularly in young male patients [11], when they fail to receive a therapeutic intervention [12]. Some patients relapse after treatment, often accompanied by rapid deterioration in the liver histology [13]; they need utmost care for timely and effective treatment.

In order to examine a long-term prognosis of AIH, 48 patients with the definite diagnosis of AIH were treated with long-term corticosteroid for up to 28 years, and followed for biochemical and histological responses to treatment, with a special reference to their HLA-DR profiles.

Methods

Patients

During 28 years from 1979 to 2007, 118 patients with AIH type-1 visited the Department of Hepatology at the Toranomon Hospital in Metropolitan Tokyo. Of these patients, 78 (66%) fulfilled the definite diagnostic criteria defined by IAIHG [6], while the remaining 40 (34%) did those of probable AIH. All of the patients were negative for antibodies to liver kidney micosome-1 (anti-LKM-1), and they were classified into AIH type-1. They had a median age of 52 years (range: 19–64 years), and included 45 (67%) women. There were four patients who underwent transient and moderate increases in the serum level of alanine aminotransferase (ALT), and they were followed without treatment. The remaining 60 patients received corticosteroid therapy and were followed for biochemical response during the median of 9 years (range: 5–28 years). Of these patients, 48 (70%) were included in this study, and received serial liver biopsies under laparoscopy for the evaluation of histological improvement. None of them had ongoing infection with hepatitis B or C virus, or possessed antibody to human immunodeficiency virus type-1. The study protocol conformed to the 1975 Declaration of Helsinki, and was approved by the Ethics Committee of the Toranomon Hospital. Every patient or his/her next of kin gave an informed consent on the purpose of this study.

Serological Tests

Autoantibodies as well as immunoglobulins of IgG and IgM classes were determined by enzyme immunoassay (EIA). Antinuclear antibodies (ANA) were determined by indirect immuno-fluorescence with Hep-G2 cells, and anti-smooth muscle antibodies as well as anti-LKM-1 by indirect fluorescence on cryostat sections of rat organs by the standard procedure. Hepatitis B surface antigen (HBsAg) was determined by radioimmunoassay, antibody to hepatitis C virus (anti-HCV) by EIA of the third generation, and HCV RNA by reversed-transcription polymerase chain reaction (RT-PCR).

HLA Typing

HLA typing was performed by serological methods, and confirmed by PCR-MPH (microplate hybridization) for patients with inconclusive results [14].

Prednisolone Treatment and Biochemical Response

As soon as the diagnosis of AIH was established, patients received 30–60 mg prednisolone daily and were followed for transaminase levels during a mean follow-up period of 5 years (range: 5–28 years). Aminotransferase levels were monitored monthly, and the dose of prednisolone was reduced by 10–15% for the patients in whom ALT levels were normalized to below 40 U/l for 3 months or longer. The response was judged 6 months after the normalization of ALT. Complete response was defined by the normalization of transaminase levels with a maintenance dose of $\leq$10 mg prednisolone daily; partial response by that with >10 mg prednisolone (up to 20 mg); and no response by the failure in normalizing transaminase levels with a maintenance dose of prednisolone (10–20 mg). Relapse was an exacerbation with increase in ALT levels exceeding 80 U/Ll ($2 \times$ upper limit of normal) after they had been normalized by a maintenance dose.

Laparoscopic and Histological Examinations

Patients received liver biopsy under laparoscopy before and after the treatment with an interval of 5 years with a minor patient-to-patient variation. Biopsied liver specimens were stained for silver for evaluating fibrosis and with D-periodic acid Schiff (PAS) for examining inflammatory changes.

Statistical Analysis

Categorial variables were compared between groups by the χ^2 test and Fisher's exact test, and non-categorial variables by the Mann–Whitney's U test.

Results

Baseline Characteristics of AIH Patients

Table 1 lists the baseline characteristics of the 48 patients with AIH for whom HLA typing was performed and who had received a long-term immunosuppressive therapy (median 9 years [range: 5–28 years]) while they were monitored for biochemical and histological responses. Frequencies of HLA-DR are shown in Fig. 1. DR4 predisposing Japanese patients to AIH [15, 16] was detected in 32 of the 48 (67%) patients, DR8 in nine (19%), DR14 in 11 (23%) and DR15 in 16 (33%) of the 48 AIH patients.

Biochemical Responses of AIH Patients with Reference to HLA Types

Biochemical response with the normalization of aspartate aminotransferase (AST) and ALT levels was achieved in 43 of the 48 (90%) patients after the initial aggressive treatment with corticosteroids (30–60 mg/day of prednisolone) followed by a small maintenance dose (10 mg/day or less). However, 16 of the 43 (37%) responders required occasional increased doses (20 mg/day or more) for the treatment of hepatitis flares. Response differed in patients with distinct HLA-DR types (Table 2). Complete biochemical response was more frequent in the patients with than without DR14 (10/11 [91%] vs. 10/37 [27%], $P < 0.001$).

Table 1 Baseline characteristics of the 48 patients with AIH

Features	Normal range	
Age (years)	Not applicable	52 (22–71)
Men	Not applicable	10 (21%)
AST (IU/l)	11–38	93 (16–1,550)
ALT (IU/l)	6–50	110 (16–2,640)
ALP (IU/l)	117–350	282 (128–949)
γ-GTP (IU/l)	9–109	84 (15–651)
γ-Globulin (g/dl)	0.76–1.76	2.27 (1.36–4.59)
IgG (mg/dl)	870–1,700	2,632 (1,340–2,632)
ANA (x)	<80	640 (0–10,240)
Fibrosis stage	Not applicable	
F_0		0
F_1		19 (40%)
F_2		17 (35%)
F_3		10 (21%)
F_4		2 (4%)

Data are expressed by the median with the range in parentheses or the number with percentage in parentheses

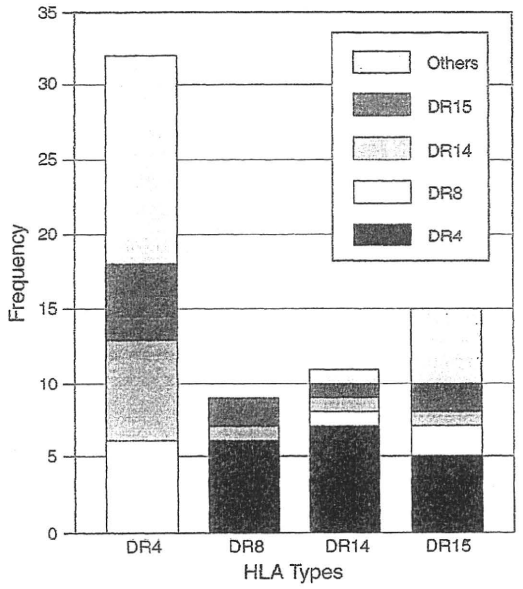

Fig. 1 HLA-DR alleles in the 48 patients with AIH. The allele in the other chromosome is shown in patients with DR4, DR8, DR14, and DR15

Table 2 Biochemical response in AIH patients with or without the DR14 allele

HLA-DR	Number	Biochemical response			Relapse
	($n = 48$)	Complete	Partial	None	
DR14	11 (23%)	10 (91%)*	1 (9%)	0	0
Non-DR14	37 (77%)	10 (27%)	11 (29%)	5 (14%)	11 (28%)
DR4	32 (67%)	11 (34%)	7 (22%)	3 (9%)	11 (34%)
DR8	9 (19%)	3 (33%)	2 (22%)	1 (11%)	3 (33%)
DR15	15 (31%)	6 (40%)	7 (47%)	1 (7%)	1 (7%)
Others	3 (6%)		1 (33%)	2 (67%)	

Transaminase levels were normalized with a maintenance dose of ≤10 mg prednisolone in complete responders and with that of >10 mg in partial responders. Relapse was an exacerbation of transaminase levels after they had been normalized by a maintenance dose

* $P < 0.001$ vs. non-DR14

Relationship Between Biochemical and Histological Responses to Prednisolone Therapy in the 48 Patients with AIH

Histological follow-ups were performed in the 48 patients, and the HAI score markedly improved in 42 (88%), moderately improved in two patients (4%) and worsened in the remaining four (8%) (Table 3). Marked histological

Table 3 Relationship between biochemical and histological responses to prednisolone therapy in the 48 patients with AIH

Biochemical	Number	Histological response		
Response	($n = 48$)	Marked	Moderate	Worsened
Response	32	31 (97%)	1 (3%)	0
Complete	20	19 (95%)	1 (5%)	0
Partial	12	12 (100%)	0	0
No Response	5	2 (40%)	1 (20%)	2 (40%)
Relapse	11	9 (82%)	0	2 (18%)

Histology activity index (HAI) score decreased by ≥2 points in marked response and by 1 point in moderate response

improvement was accomplished in 31 of the 32 (97%) responders, while it was achieved in two of the four (50%) non-responders and nine of the 11 (82%) relapsers. Histology worsened in four of the 16 (25%) patients without biochemical response (non-responders and relapsers combined), but in none of the 32 responders. Changes in the total HAI score as well as respective scores for specific histological parameters (periportal with/without bridging necrosis; intralobular degeneration with focal necrosis; portal inflammation; and fibrosis) are shown in Table 4. The gain in total HAI score was due to an increase in inflammation and not attributed to aggravation of fibrosis in each of them.

Histological Responses of AIH Patients with Reference to HLA

Table 5 compares histological responses between the patients with and without DR4. Although the pretreatment HAI score was somewhat higher in the patients with than without DR14 (9.8 ± 3.5 vs. 7.9 ± 3.3, $P = 0.092$), it improved to comparable extents in both of them after treatment (4.5 ± 0.9 vs. 4.7 ± 2.5). Thus, the marked histological response with a decrease in HAI score ≥2 was no different between the patients with and without DR14

Table 4 Changes in the total HAI score and those in respective parameters in the four patients in whom histology worsened after prednisolone treatment

	Total HAI score (scores for each parameter[a])	
	Before treatment	After treatment
Patient 1	6 (1, 1, 1, 3)	8 (1, 3, 1, 3)
Patient 2	3 (0, 1, 1, 1)	6 (1, 3, 1, 1)
Patient 3	6 (1, 1, 1, 3)	8 (1, 3, 1, 3)
Patient 4	13 (3, 3, 3, 4)	15 (4, 4, 3, 4)

[a] Four histological parameters were graded, including periportal with/without bridging necrosis; intralobular degeneration with focal necrosis; portal inflammation; and fibrosis

Table 5 Histological response in AIH patients with or without DR14

HLA-DR	Number	Histological improvement		
		Marked	Moderate	Worsened
DR14	11 (23%)	10 (91%)	1 (9%)	0
Non-DR14	37 (77%)	32 (86%)	1 (3%)	4 (11%)
DR4	32 (67%)	27 (84%)	1 (3%)	4 (13%)
DR8	9 (19%)	8 (89%)	1 (11%)	0
DR15	15 (31%)	3 (93%)	0	1 (7%)
Others	3 (6%)	2 (67%)	0	1 (33%)

(10/11 [91%] vs. 32/37 [86%], $P = 0.697$). Improvement in the histology was mostly due to changes in the necroinflammatory grade; there were few changes in the fibrosis grade from the baseline values.

Figure 2 illustrates clinical and histological courses of a representative patient (female, 50 years old, HLA-DR4/DR14) who received eight laparoscopes and seven liver biopsies during the follow-up for 20 years. Before she received corticosteroid therapy, liver histology had already progressed to cirrhosis, and she had to undertake sclerotherapies for the treatment of esophageal varices. She had to receive 10–30 mg prednisolone during initial few years for the treatment of several hepatitis flares. Thereafter, her liver function improved remarkably and had remained within normal limits by a maintenance dose of ≤ 10 mg prednisolone through 17 years until the last follow-up. Remarkably, she gained improvement not only in the inflammation grade but also in the fibrosis stage. Serial laparoscopic and histological findings of her liver are demonstrated in Fig. 3. In other AIH patients, also, aggressive immnosuppressive therapy prevented histological progression and gained improvement in their long-term outcomes, even though their responses to prednisolone differed.

Discussion

In the present study, HLA typing was performed in 48 of the 78 (62%) patients with the definite diagnosis of AIH type-1. They had been followed-up during a long-term corticosteroid treatment, with liver biopsies performed as frequently as possible, and histological and biochemical responses were correlated with HLA types. DR14, which has not gained attention in AIH, was detected in 11 of the 48 (23%) patients. Remarkably, the sustained biochemical response was achieved more frequently in the AIH patients with than without DR14 (10/11 [91%] vs. 10/37 [27%], $P < 0.001$).

The association of HLA types and AIH are under regional influence. DR3 and DR4 are the main HLA

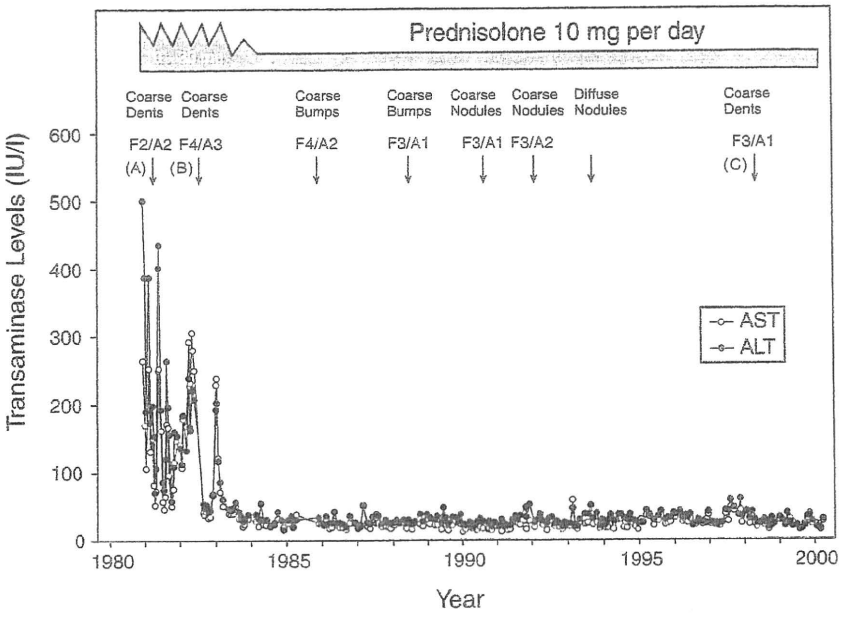

Fig. 2 Clinical course of a patient with AIH (female, 45 years old with HLA-DR4/DR14) who had been followed for 20 years. Doses of prednisolone are indicated at the top, and appearances of the liver surface on laparoscopies, as well as fibrosis stage and inflammation grade on liver biopsies, are shown in the middle. During the initial few years, she received up to 30 mg prednisolone per day for treatment of several hepatitis flares. Thereafter, her liver function improved remarkably and had stayed within normal limits through 17 years with a maintenance prednisolone dose ≤ 10 mg

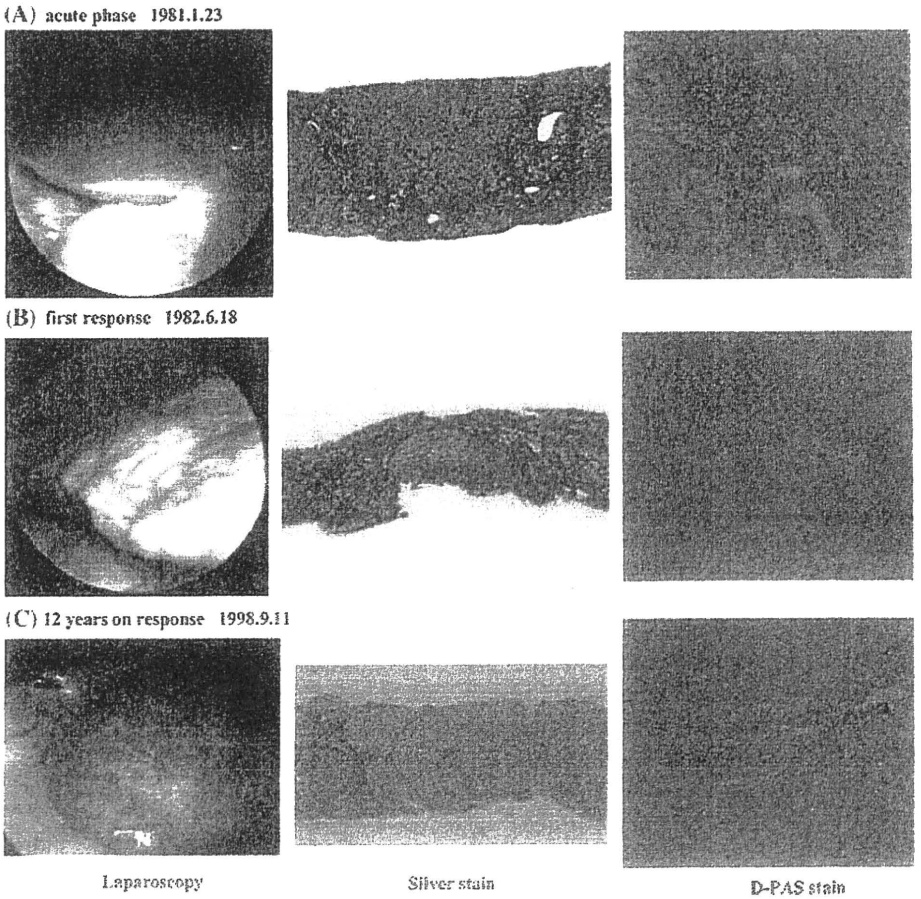

Fig. 3 Laparoscopic findings and histological changes in the patients with AIH. The patient presented in Fig. 2 was examined at three time points (*a*, *b*, and *c* in Fig. 2). Laparoscopic findings were improved since she responded to prednisolone since June, 1982. Histologically, typical submassive necrosis and interface-hepatitis were found in the first biopsy (**a**). Since she responded, necroinflammatory changes improved, however (**b** and **c**). Laparoscopic findings are shown in right row, low-power fields (×20) by silver staining in the middle row; and high-power fields (×200) by D-PAS staining