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.

References

1. Bruix J, Barrera JM, Calvet X, et al. Prevalence of antibodies to hepatitis C virus in Spanish patients with hepatocellular carcinoma and hepatic cirrhosis. *Lancet.* 1989;2:1004-1006.
2. Colombo M, Kuo G, Choo QL, et al. Prevalence of antibodies to hepatitis C virus in Italian patients with hepatocellular carcinoma. *Lancet.* 1989;2:1006-1008.
3. Hasan F, Jeffers LJ, De Medina M, et al. Hepatitis C-associated hepatocellular carcinoma. *Hepatology.* 1990;12:589-591.
4. Kew MC, Houghton M, Choo QL, Kuo G. Hepatitis C virus antibodies in southern African blacks with hepatocellular carcinoma. *Lancet.* 1990;335:873-874.
5. Ohkoshi S, Kojima H, Tawaraya H, et al. Prevalence of antibody against non-A, non-B hepatitis virus in Japanese patients with hepatocellular carcinoma. *Jpn J Cancer Res.* 1990;81:550-553.
6. Saito I, Miyamura T, Ohbayashi A, et al. Hepatitis C virus infection is associated with the development of hepatocellular carcinoma. *Proc Natl Acad Sci U S A.* 1990;87:6547-6549.
7. Kiyosawa K, Furuta S. Review of hepatitis C in Japan. *J Gastroenterol Hepatol.* 1991;6:383-391. Review.
8. Alter MJ, Margolis HS, Krawczynski K, et al. The natural history of community-acquired hepatitis C in the United States. The Sentinel Counties Chronic non-A, non-B Hepatitis Study Team. *N Engl J Med.* 1992;327:1899-1905.
9. Tsukuma H, Hiyama T, Tanaka S, et al. Risk factors for hepatocellular carcinoma among patients with chronic liver disease. *N Engl J Med.* 1993;328:1797-1801.
10. Ikeda K, Saitoh S, Koida I, et al. A multivariate analysis of risk factors for hepatocellular carcinogenesis: a prospective observation of 795 patients with viral and alcoholic cirrhosis. *Hepatology.* 1993;18:47-53.
11. Hourigan LF, Macdonald GA, Purdie D, et al. Fibrosis in chronic hepatitis C correlates significantly with body mass index and steatosis. *Hepatology.* 1999;29:1215-1219.
12. El-Serag HB, Tran T, Everhart JE. Diabetes increases the risk of chronic liver disease and hepatocellular carcinoma. *Gastroenterology.* 2004;126:460-468.
13. Mason AL, Lau JY, Hoang N, et al. Association of diabetes mellitus and chronic hepatitis C virus infection. *Hepatology.* 1999;29:328-333.
14. Hu KQ, Kyulo NL, Esrailian E, et al. Overweight and obesity, hepatic steatosis, and progression of chronic hepatitis C: a retrospective study on a large cohort of patients in the United States. *J Hepatol.* 2004;40: 147-154.
15. Hickman IJ, Powell EE, Prins JB, et al. In overweight patients with chronic hepatitis C, circulating insulin is associated with hepatic fibrosis: implications for therapy. *J Hepatol.* 2003;39:1042-1048.
16. Arase Y, Suzuki F, Suzuki Y, et al. Sustained virological response reduces incidence of onset of type 2 diabetes in chronic hepatitis C. *Hepatology.* 2009;49:739-744.
17. Desmet VJ, Gerber M, Hoofnagle JH, et al. Classification of chronic hepatitis: diagnosis, grading and staging. *Hepatology.* 1994;19:1513-1520. Review.
18. Genuth S, Alberti KG, Bennett P, et al. Expert Committee on the Diagnosis and Classification of Diabetes Mellitus. Follow-up report on the diagnosis of diabetes mellitus. *Diabetes Care.* 2003;26:3160-3167.
19. El-Serag HB, Tran T, Everhart JE. Diabetes increases the risk of chronic liver disease and hepatocellular carcinoma. *Gastroenterology.* 2004;126:460-468.
20. Romero-Gómez M, Del Mar Viloria M, Andrade RJ, et al. Insulin resistance impairs sustained response rate to peginterferon plus ribavirin in chronic hepatitis C patients. *Gastroenterology.* 2005;128:636-641.
21. Backus LI, Boothroyd DB, Phillips BR, Mole LA. Predictors of response of US veterans to treatment for the hepatitis C virus. *Hepatology.* 2007;46:37-47.
22. Davila JA, Morgan RO, Shaib Y, et al. Diabetes increases the risk of hepatocellular carcinoma in the United States: a population based case control study. *Gut.* 2005;54:533-539.
23. Inoue M, Iwasaki M, Otani T, et al. Diabetes mellitus and the risk of cancer: results from a large-scale population-based cohort study in Japan. *Arch Intern Med.* 2006;166:1871-1877.
24. Moriya K, Yotsuyanagi H, Shintani Y, et al. Hepatitis C virus core protein induces hepatic steatosis in transgenic mice. *J Gen Virol.* 1997;78:1527-1531.
25. Moriya K, Fujie H, Shintani Y, et al. The core protein of hepatitis C virus induces hepatocellular carcinoma in transgenic mice. *Nat Med.* 1998;4:1065-1067.
26. Shintani Y, Fujie H, Miyoshi H, et al. Hepatitis C virus infection and diabetes: direct involvement of the virus in the development of insulin resistance. *Gastroenterology.* 2004;126:840-848.

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.

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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.

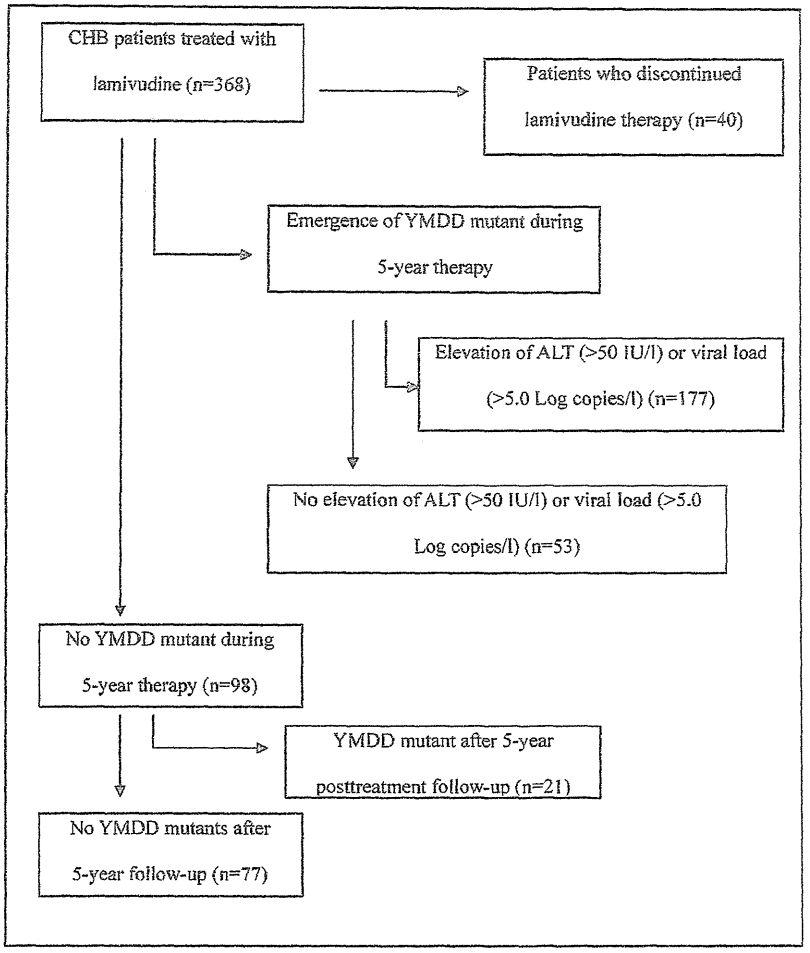

Fig. 1. Outcome of patients with lamivudine therapy. CHB, chronic hepatitis B; YMDD, tyrosine–methionine–aspartate–aspartate; ALT, alanine aminotransferase.

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 ($P = 0.051$).

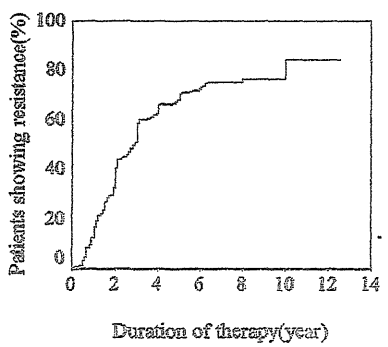

Fig. 2. Cumulative rate of patients who showed emergence of YMDD mutants during lamivudine therapy (Kaplan–Meier method). YMDD, tyrosine–methionine–aspartate–aspartate.

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.

REFERENCES

Adler WH, Nagel JE. 1994. Clinical immunology and aging. In: Hazzard WR, Bierman EL, Blass JP, Ettinger WH, Halter JB, editors. Principles of geriatric medicine and gerontology. 3rd edition. New York: McGraw-Hill, Inc. pp 61–75.

Akuta N, Suzuki F, Kobayashi M, Matsuda M, Sato J, Takagi K, Tsubota A, Suzuki Y, Hosaka T, Someya T, Kobayashi M, Saitoh S, Arase Y, Ikeda K, Kumada H. 2003. Virological and biochemical relapse according to YMDD motif mutant type during long-term lamivudine monotherapy. J Med Virol 71:504–510.

Akuta N, Suzuki F, Kobayashi M, Matsuda M, Sato J, Suzuki Y, Sezaki H, Hosaka T, Someya T, Kobayashi M, Saitoh S, Arase Y, Ikeda K, Kumada H. 2005. Virological and biochemical relapse after discontinuation of lamivudine monotherapy for chronic hepatitis B in Japan: Comparison with breakthrough hepatitis during long-term treatment. Intervirology 48:174–182.

Baldick CJ, Tenney DJ, Mazzucco CE, Eggers BJ, Rose RE, Pokornowski KA, Yu CF, Colonno RJ. 2008. Comprehensive evaluation of hepatitis B virus reverse transcriptase substitutions associated with entecavir resistance. Hepatology 47:1473–1482.

Chang ML, Chien RN, Yeh CT, Liaw YF. 2005. Virus and transaminase levels determine the emergence of drug resistance during long-term lamivudine therapy in chronic hepatitis B. J Hepatol 43:72–77.

Chayama K, Suzuki Y, Kobayashi M, Kobayashi M, Tsubota A, Hashimoto M, Miyano Y, Koike H, Kobayashi M, Koida I, Arase Y, Saitoh S, Murashima N, Ikeda K, Kumada H. 1998. Emergence and takeover of YMDD motif mutant hepatitis B virus during longterm lamivudine therapy and re-takeover by wild type after cessation of therapy. Hepatology 27:1711–1716.

Dienstag JL, Schiff ER, Wright TL, Perrillo RP, Hann HWL, Goodman Z, Crowther L, Condreay LD, Woessner M, Rubin M, Brown MA, for the US Lamivudine Investigator Group. 1999. Lamivudine as initial treatment for chronic hepatitis B in the United States. N Engl J Med 341:1256–1263.

Dienstag JL, Goldin RD, Heathcote EJ, Hann HWL, Woessner M, Stephenson SL, Gardner S, Gray DF, Schiff ER. 2003a. Histological outcome during long-term lamivudine therapy. Gastroenterology 124:105–117.

Dienstag JL, Cianciara J, Karayalcin S, Kowdley KV, Willems B, Plisek S, Woessner M, Gardner S, Schiff E. 2003b. Durability of serologic response after lamivudine treatment of chronic hepatitis B. Hepatology 37:748–755.

Gish RG, Lok AS, Chang TT, de Man RA, Gadano A, Sollano J, Han KH, Chao YC, Lee SD, Harris M, Yang J, Colonno R, Brett-Smith H. 2007. Entecavir therapy for up to 96 weeks in patients with HBeAg-positive chronic hepatitis B. Gastroenterology 133:1437–1444.

Kawaoka T, Suzuki F, Akuta N, Suzuki Y, Arase Y, Sezaki H, Kawamura Y, Hosaka T, Kobayashi M, Ikeda K, Kumada H. 2007. Efficacy of lamivudine therapy in elderly patients with chronic hepatitis B infection. J Gastroenterol 42:395–401.

Kobayashi S, Shimada K, Suzuki H, Tanikawa K, Sata M. 2000. Development of a new method for detecting a mutation in the gene encoding hepatitis B virus reverse transcriptase active site (YMDD motif). Hepatol Res 17:31–42.

Kobayashi M, Suzuki F, Akuta N, Hosaka T, Sezaki T, Yatsuju H, Yatsuji H, Kobayashi M, Suzuki Y, Arase Y, Ikeda K, Watahiki S, Iwasaki S, Miyakawa Y, Kumada H. 2007. Loss of hepatitis B surface antigen from the serum of patients with chronic hepatitis treated with lamivudine. J Med Virol 79:1472–1477.

Lai CL, Chien RN, Leung NW, Chang TT, Guan R, Tai DI, Ng KY, Wu PC, Dent JC, Barber J, Stephenson SL, Gray DF, for the Asia Hepatitis Lamivudine Study Group. 1998. A one year trial of lamivudine for chronic hepatitis B. N Engl J Med 339:61–68.

Lee WM. 1997. Hepatitis B virus infection. N Engl J Med 337:1733–1745.

Liaw YF, Chien RN, Yeh CT, Tsai SL, Chu CM. 2000. Acute exacerbation and hepatitis B virus clearance after emergence of YMDD motif mutation during lamivudine therapy. Hepatology 30:567–572.

Liaw YF, Sung JJY, Chow WC, Farrell G, Lee CZ, Yuen H, Tanwandee T, Tao QM, Shue K, Keene ON, Dixon JS, Gray DF, Sabbat J, for the Cirrhosis Asian Lamivudine Multicentre Study Group. 2004. Lamivudine for patients with chronic hepatitis B and advanced liver disease. N Engl J Med 351:1521–1531.

Marcellin P, Chang TT, Lim SGL, Sievert W, Tong M, Arterburn S, Borroto-Esoda K, Frederick D, Rousseau F. 2008. Long-term efficacy and safety of adefovir dipivoxil for the treatment of hepatitis B e antigen-positive chronic hepatitis B. Hepatology 48:750–758.

Marcus EL, Tur-Kaspa R. 1997. Viral hepatitis in older adults. J Am Geriatr Soc 45:755–763.

Nafa S, Ahmed S, Tavan D, Pichoud C, Berby F, Stuyver L, Johnson M, Merle P, Abidi H, Trepo C, Zoulim F. 2003. Early detection of viral resistance by determination of hepatitis B virus polymerase mutations in patients treated by lamivudine for chronic hepatitis B. Hepatology 32:1078–1088.

Ooga H, Suzuki F, Tsubota A, Arase Y, Suzuki Y, Akuta N, Sezaki H, Hosaka T, Someya T, Kobayashi M, Saitoh S, Ikeda K, Kobayashi M, Matsuda M, Satoh J, Kumada H. 2004. Efficacy of lamivudine treatment in Japanese patients with hepatitis B virus-related cirrhosis. J Gastroenterol 39:1078–1084.

Patton HM, Lavine JE, Natta MLV, Schwimmer JB, Kleiner D. 2008. Clinical correlates of histopathology in pediatric nonalcoholic steatohepatitis. Gastroenterology 135:1961–1971.

Song B-C, Suh DJ, Lee HC, Chung Y-H, Lee YS. 2000. Hepatitis B e antigen seroconversion after lamivudine therapy is not durable in patients with chronic hepatitis B in Korea. Hepatology 32:803–806.

Suzuki F, Tsubota A, Arase Y, Suzuki Y, Akuta N, Hosaka T, Someya T, Kobayashi M, Saitoh S, Ikeda K, Matsuda M, Satoh J, Takagi K, Kumada H. 2003. Efficacy of lamivudine therapy and factors associated with emergence of resistance in chronic hepatitis B virus infection in Japan. Intervirology 46:182–189.

Suzuki F, Akuta N, Suzuki Y, Sezaki H, Arase Y, Hosaka T, Someya T, Kobayashi M, Saitoh S, Ikeda K, Kobayashi M, Matsuda M, Satoh J, Watahiki S, Kumada H. 2006. Clinical and virological features of non-breakthrough and severe exacerbation due to lamivudine-resistant hepatitis B virus mutants. J Med Virol 78:341–352.

Suzuki F, Akuta N, Suzuki Y, Sezaki H, Sezaki H, Arase Y, Kawamura Y, Hosaka T, Kobayashi M, Ikeda K, Kobayashi M, Watahiki S, Kumada H. 2007. Selection of a virus strain resistant to entecavir in a nucleoside-naïve patient with hepatitis B of genotype H. J Clin Virol 39:149–152.

Tsubota A, Arase Y, Suzuki F, Kobayashi M, Matsuda M, Sato J, Suzuki Y, Akuta N, Sezaki H, Hosaka T, Someya T, Kobayashi M, Saitoh S, Ikeda K, Kumada H. 2004. Severe acute exacerbation of liver disease may reduce or delay emergence of YMDD motif mutants in long-term lamivudine therapy for hepatitis B e antigen-positive chronic hepatitis B. J Med Virol 73:7–12.

Yu MW, Shih WL, Lin CL, Liu CJ, Jian JW, Tsai KS, Chen CJ. 2008. Body-mass index and progression of hepatitis B: A population-based cohort study in men. J Clin Oncol 26:5576–5582.

Yuan JM, Govindarajan S, Arakawa K, Yu MC. 2004. Synergism of alcohol, diabetes, and viral hepatitis on the risk of hepatocellular carcinoma in blacks and whites in the U.S. Cancer 101:1009–1017.

Yuen MF, Sablon E, Hui CK, Yuan HJ, Decraemer H, Lai CL. 2001. Factors associated with hepatitis B virus DNA breakthrough in patients receiving prolonged lamivudine therapy. Hepatology 34:785–791.

Zoulim F, Perrillo R. 2008. Hepatitis B: Reflections on the current approach to antiviral therapy. J Hepatol 48:S2–S19.

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ORIGINAL ARTICLE

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

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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

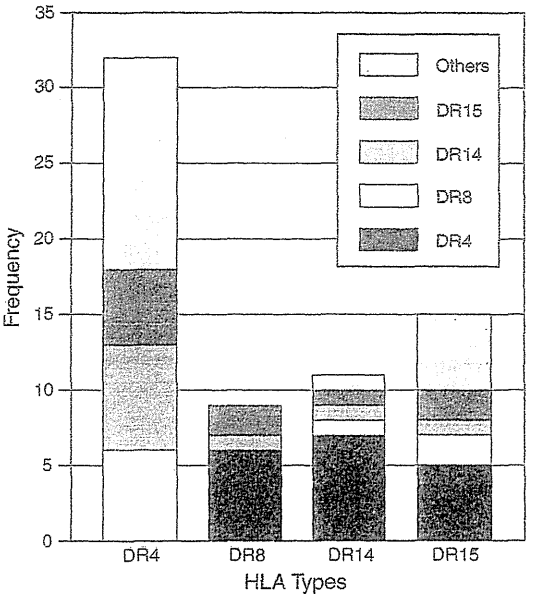

Fig. 1 HLA-DR alleles in the 48 patients with AIH. The allele in the other chromosome is shown in patients with DR4, DR8, DR14, and DR15

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 Response	Number ($n = 48$)	Histological response		
		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

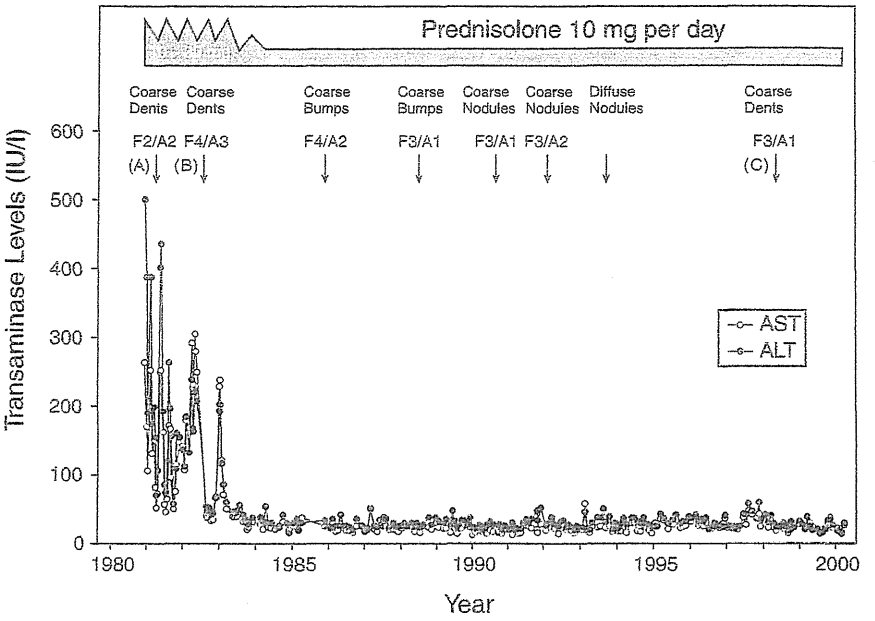

Fig. 2 Clinical course of a patient with AIH (female, 45 years old with HLA-DR4/DR14) who had been followed for 20 years. Doses of prednisolone are indicated at the top, and appearances of the liver surface on laparoscopies, as well as fibrosis stage and inflammation grade on liver biopsies, are shown 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

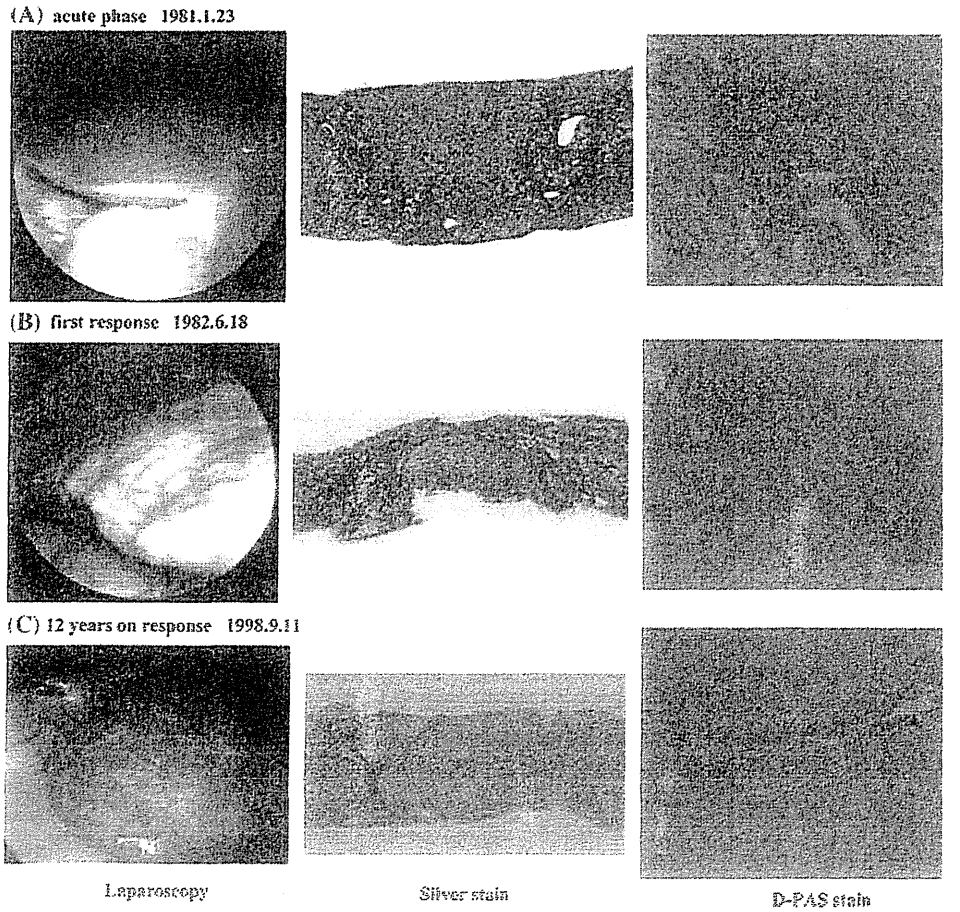

Fig. 3 Laparoscopic findings and histological changes in the patients with AIH. The patient presented in Fig. 2 was examined at three time points (*a*, *b*, and *c* in Fig. 2). Laparoscopic findings were improved since she responded to prednisolone since June, 1982. Histologically, typical submassive 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

susceptibility alleles among Caucasoid Northern Europeans and North Americans, and 84% of adult patients have either or both of these alleles [17, 18]. In contrast, the principal susceptibility allele for AIH in Japan is DR4 [15, 16]; DR3 is detected in none of them, however. DR4 is also frequent in adult patients in Argentina and Mexico [19, 20], while DRB13 prevails in Argentine and Brazilian children with AIH [21, 22]. DR4 is associated with better response and fewer relapse than DR3 in AIH patients from Western countries [1]. However, there have been no reports on the association of HLA types with treatment response in patients with AIH in Japan. In the present study, DR4 was detected in 32 of the 48 (67%) Japanese patients with AIH, with a frequency comparable to those in previous reports [15, 16]; DR4 was more common in AIH patients than in the general Japanese population (67 vs. 22%) [23]. In contrast, DR14 was comparably frequent in AIH patients and the general population of Japan (23 vs. 17%) [23]. Thus, DR4 would predispose the Japanese population to the development of AIH, while DR14 would not, albeit DR14 would increase the response to corticosteroids in AIH patients.

On the basis of DR4 that is more frequent in the individuals with than without DR3, these alleles have been regarded to behave independently and reciprocally toward the susceptibility for AIH. Such a possibility has been evaluated in peripheral blood mononuclear cells and lymphocytes infiltrating in the liver [24]. Liver lymphocytes are sensitized with hepatocytes or hepatic autoantigens. Even among inflammatory cells infiltrating the portal area, CD4+ lymphocytes predominate in the patients with than without AIH. These lines of evidence implicate the class-II MHC in the pathogenesis of AIH, of which DR4 and DR15 would play major roles in Japan. In the patients with AIH who are positive for LKM-1 antibodies, Th1 cells dominate in the cytokine production assay with a T-cell line specific for LKM-1 [25]. Combined, CD4+ lymphocytes would be crucially required in the manifestation of AIH by interacting with class-II MHC antigens.

In conclusion, the association of MHC class-II antigens with biochemical and histological responses to immunosuppressive treatment was evaluated in Japanese patients with AIH, for predicting their long-term outcomes. On the basis of the results obtained, DR14 would be associated with favorable treatment response in Japanese patients with AIH, which needs to be confirmed in an extended series of patients. The validity of such an assumption will be evaluated by in vitro studies, which are underway.

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References

1. Czaja AJ, Freese DK. Diagnosis and treatment of autoimmune hepatitis. *Hepatology*. 2002;36:479–497.
2. Krawitt EL. Autoimmune hepatitis. *N Engl J Med*. 2006;354:54–66.
3. Manns MP, Strassburg CP. Autoimmune hepatitis: Clinical challenges. *Gastroenterology*. 2001;120:1502–1517.
4. Czaja AJ, Doherty DG, Donaldson PT. Genetic bases of autoimmune hepatitis. *Dig Dis Sci*. 2002;47:2139–2150.
5. Johnson PJ, McFarlane IG. Meeting report: International Autoimmune Hepatitis Group. *Hepatology*. 1993;18:998–1005.
6. Alvarez F, Berg PA, Bianchi FB, et al. International Autoimmune Hepatitis Group Report: Review of criteria for diagnosis of autoimmune hepatitis. *J Hepatol*. 1999;31:929–938.
7. Toda G, Zeniya M, Watanabe F, et al. Present status of autoimmune hepatitis in Japan–correlating the characteristics with international criteria in an area with a high rate of HCV infection. *J Hepatol*. 1997;26:1207–1212.
8. Vergani D, Mieli-Vergani G. Autoimmune hepatitis. *Autoimmun Rev*. 2003;2:241–247.
9. Czaja AJ, Carpenter HA. Decreased fibrosis during corticosteroid therapy of autoimmune hepatitis. *J Hepatol*. 2004;40:646–652.
10. Czaja AJ, Carpenter HA. Progressive fibrosis during corticosteroid therapy of autoimmune hepatitis. *Hepatology*. 2004;39:1631–1638.
11. Al-Chalabi T, Underhill JA, Portmann BC, McFarlane IG, Heneghan MA. Impact of gender on the long-term outcome and survival of patients with autoimmune hepatitis. *J Hepatol*. 2008;48:140–147.
12. Czaja AJ, Bianchi FB, Carpenter HA, et al. Treatment challenges and investigational opportunities in autoimmune hepatitis. *Hepatology*. 2005;41:207–215.
13. Czaja AJ, Menon KV, Carpenter HA. Sustained remission after corticosteroid therapy for type 1 autoimmune hepatitis: a retrospective analysis. *Hepatology*. 2002;35:890–897.
14. Ota M, Seki T, Fukushima H, Tsuji K, Inoko H. HLA-DRB1 genotyping by modified PCR-RFLP method combined with group-specific primers. *Tissue Antigens*. 1992;39:187–202.
15. Miyake Y, Iwasaki Y, Takaki A, et al. Human leukocyte antigen DR status and clinical features in Japanese patients with type 1 autoimmune hepatitis. *Hepatol Res*. 2008;38:96–102.
16. Yoshizawa K, Ota M, Katsuyama Y, et al. Genetic analysis of the HLA region of Japanese patients with type 1 autoimmune hepatitis. *J Hepatol*. 2005;42:578–584.
17. Donaldson PT, Doherty DG, Hayllar KM, McFarlane IG, Johnson PJ, Williams R. Susceptibility to autoimmune chronic active hepatitis: human leukocyte antigens DR4 and A1–B8-DR3 are independent risk factors. *Hepatology*. 1991;13:701–706.
18. Strettell MD, Donaldson PT, Thomson LJ, et al. Allelic basis for HLA-encoded susceptibility to type 1 autoimmune hepatitis. *Gastroenterology*. 1997;112:2028–2035.
19. Pando M, Larriba J, Fernandez GC, et al. Pediatric and adult forms of type I autoimmune hepatitis in Argentina: evidence for differential genetic predisposition. *Hepatology*. 1999;30:1374–1380.
20. Vazquez-Garcia MN, Alaez C, Olivo A, et al. MHC class II sequences of susceptibility and protection in Mexicans with autoimmune hepatitis. *J Hepatol*. 1998;28:985–990.
21. Fainboim L, Marcos Y, Pando M, et al. Chronic active autoimmune hepatitis in children. Strong association with a particular HLA-DR6 (DRB1*1301) haplotype. *Hum Immunol*. 1994;41:146–150.
22. Czaja AJ, Souto EO, Bittencourt PL, et al. Clinical distinctions and pathogenic implications of type 1 autoimmune hepatitis in Brazil and the United States. *J Hepatol*. 2002;37:302–308.

23. Maeda H, Hirata R, Tokunaga K. HLA-DNA typing and HLA in the Japanese: a review (*in Japanese*). *Nihon Ishokugakkai Zasshi*. 1999;34:55–64.
24. Ichiki Y, Aoki CA, Bowlus CL, Shimoda S, Ishibashi H, Gershwin ME. T cell immunity in autoimmune hepatitis. *Autoimmun Rev*. 2005;4:315–321.
25. Schlaak JF, Lohr H, Gallati H, Meyer zum Buschenfelde KH, Fleischer B. Analysis of the in vitro cytokine production by liver-infiltrating T cells of patients with autoimmune hepatitis. *Clin Exp Immunol*. 1993;94:168–173.

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Case Report

Relapse of hepatitis C in a pegylated-interferon-α-2b plus ribavirin-treated sustained virological responder

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A 41-year-old woman with chronic hepatitis C was treated with pegylated-interferon (PEG-IFN)-α-2b plus ribavirin for 24 weeks. She had hepatitis C virus (HCV) genotype 2a (1600 KIU/mL), and her liver histology showed mild inflammation and fibrosis. Four weeks after the start of the therapy, she achieved a rapid virological response (RVR) and then a sustained virological response (SVR). Serum alanine aminotransferase (ALT) levels remained within normal ranges and HCV RNA continued to be negative. However, ALT levels flared with the re-emergence of HCV RNA in the serum 1.5 years after discontinuation of therapy. HCV RNA obtained from sera before therapy and after relapse shared a 98.6% homology with the E2 region, and phylogenetic analyses indicated that they were the same HCV strain. These results eliminated the possibility of a re-infection and strongly indicated a late relapse of the disease. Therefore, follow-up is necessary for chronic hepatitis C patients after SVR, even if they respond well to therapy, including RVR.

Key words: chronic hepatitis C, genotype 2a, sustained virological response, relapse, phylogenetic analyses.

INTRODUCTION

HEPATITIS C VIRUS (HCV) is an important cause of chronic liver disease, and more than 170 million people are infected worldwide, including 1.5–2 million people in Japan.[1] Approximately 70% of Japanese chronic hepatitis C patients are infected with genotype 1b, whereas the rest are infected with genotypes 2a or 2b.[2] At present, pegylated-interferon (PEG-IFN)-α plus ribavirin is the optimal therapy for chronic hepatitis C. Sustained virological response (SVR), defined as undetectable serum HCV RNA 24 weeks after therapy completion, is the primary goal of this therapy. Approximately 80% of patients infected with genotypes 2 or 3 achieve SVR after 24 weeks of treatment, whereas approximately 50% patients with genotype 1 achieve SVR after 48 weeks of treatment.

Late relapse, defined as a HCV RNA reappearance in serum after achieving SVR, is rare in SVR patients. Furthermore, distinguishing relapse from re-infection is difficult without comparing the HCV nucleotide sequence before the start of the therapy and after relapse. Here we describe the clinical course of an HCV genotype 2a-infected woman treated with PEG-IFN-α plus ribavirin for 24 weeks. She achieved a rapid virological response (RVR) because HCV RNA was undetectable by a qualitative polymerase chain reaction (PCR) assay 4 weeks after initiating therapy. However, she achieved SVR and suffered a relapse of chronic hepatitis C 1.5 years after therapy discontinuation. We analyzed nucleotide sequences within the E2 region of HCV RNA containing the hypervariable region (HVR)1 and the IFN sensitivity-determining region (ISDR) of nonstructural protein 5A (NS5A), using sera before treatment and after relapse and confirmed that they were the same HCV strain.

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CASE REPORT

A 41-YEAR-OLD WOMAN had elevated serum alanine aminotransferase (ALT; 138 IU/L) and aspartate aminotransferase (AST; 248 IU/L) levels on a routine medical check-up in mid-September 2005. Because of liver dysfunction in October 2005, she visited Aiseikai Yamashina General Hospital for further examination. She had no family history of liver diseases. Her height was 145 cm and weight was 42 kg. No abnormalities were detected on physical examination; her average alcohol intake was less than 20 g/week. She had no history of i.v. drug abuse.

Table 1 shows the laboratory data. On 25 October 2005, transaminase and biliary enzyme levels were elevated. Serum anti-HCV antibody was positive. The HCV RNA load was 2400 KIU/mL (Amplicor Monitor ver. 2.0; Roche Diagnostic Systems, Tokyo, Japan), and she had the 2a HCV genotype. She hesitated to undergo IFN treatment in the beginning. We strictly prohibited her from alcohol and started treating her with 600 mg/day oral ursodeoxycholic acid (UDCA) and 40 mL i.v. glycyrrhizin twice a week (Stronger Neo-Minophagen C, SNMC). Her liver functions improved significantly, but did not normalize following treatment with UDCA and SNMC.

She was admitted to Aiseikai Yamashina General Hospital on 3 February 2006, and PEG-IFN plus ribavirin treatment was initiated for chronic hepatitis C. Abdominal ultrasonography revealed that the liver was almost normal in size, the edge was sharp and the internal echo was slightly coarse. Other tests including hepatitis B virus PCR, HBc antibody, HIV 1/2 antibodies, antinuclear antibody, anti-mitochondrial antibody, serum ceruloplasmin, copper and ferritin were normal. The laboratory test results obtained on 3 February 2006 are presented in Table 1. The liver biopsy specimen before treatment revealed mild fibrosis with mild inflammation, which was graded as A1F1 according to the classification of Ichida *et al.* or Bedossa and Poynard.[3,4] She received combination therapy consisting of PEG-IFN-α-2b (1.5 μg/kg; 60 μg) once a week plus 600 mg ribavirin daily.

After therapy initiation, ALT levels declined rapidly and remained within the normal range after completion of the treatment. Serum HCV RNA levels were measured by a quantitative PCR assay (Amplicor HCV Monitor ver. 2.0) before therapy initiation and after relapse and by a qualitative PCR assay (Amplicor HCV Test ver. 2.0) at 4, 8, 12, 16, 20 and 24 weeks (all during the treatment period) as well as at 4, 8, 12, 16, 20 and 24 weeks after therapy completion. Serum HCV RNA was qualitatively

Table 1 Laboratory findings

	Normal	Initial visit (10/20/2005)	Before PEG-IFN + Rib (2/3/2006)	After relapse (1/25/2008)
White blood cell (μL)	(3900–9300)	9070	8110	8800
Red blood cell ($\times 10^4$/μl)	(425–571)	458	433	412
Platelet ($\times 10^4$/μL)	(12.7–35.6)	29.2	29.7	27.3
PT (%)		85%	82%	92%
Albumin (g/dL)	(4.0–5.0)	4.2	4.2	4.1
T. Bil (mg/dL)	(0.3–1.2)	0.6	0.4	0.4
AST (IU/I)	(<33)	87	35	61
ALT (IU/I)	(<35)	195	38	96
ALP (IU/I)	(115–360)	278	240	247
γ-GTP (IU/I)	(<47)	256	48	88
RPR	(–)	NA	(–)	(–)
HBsAg	(–)	(–)	(–)	(–)
ANA	(<40)	NA	<40	<40
Type IV collagen 7S (ng/mL)	(<5)	NA	3.8	3.2
Serum ferritin (ng/mL)	(5.3–179.7)	NA	50.7	NA
HCV RNA (Amplicor Monitor ver. 2.0) (KIU/mL)	(–)	2400	1600	2600
HCV genotype		2a	2a	2a

ALP, alkaline phosphatase; ALT, alanine aminotransferase; ANA, antinuclear antibody; AST, aspirate aminotransferase; γ-GTP, γ-glutamyltranspeptidase; HBsAg, hepatitis B surface antigen; HCV, hepatitis C virus; NA, not available; PT, prothrombin time; RPR, Rapid Plasma Reagin; T. Bil., total bilirubin.

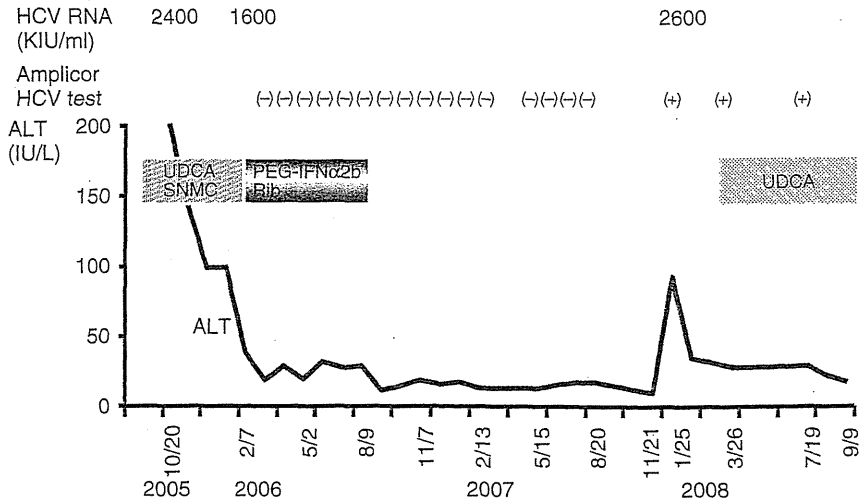

Figure 1 Levels of alanine aminotransferase (ALT) and hepatitis C virus (HCV) RNA load during the clinical course. Pegylated-interferon (PEG-IFN)-α plus ribavirin combination therapy was started in February 2006 and continued for 24 weeks until August 2006. HCV RNA was undetectable within 4 weeks. ALT levels remained within normal ranges until November 2007. Relapse occurred in January 2008. SNMC, Stronger Neo-Minophagen C; UDCA, ursodeoxycholic acid.

undetectable 4 weeks after therapy initiation and remained undetectable 6 months after therapy completion. The patient had few side-effects, and the treatment was completed without reducing either of the drugs.

After achieving SVR, she underwent monthly liver function tests, and a qualitative PCR assay was performed occasionally. In December 2007, her liver function tests deteriorated. AST/ALT levels were elevated, and she tested positive for HCV RNA on 25 January 2008 (Fig. 1, Table 1). A quantitative PCR assay indicated that the HCV RNA titer was 2600 IU/L, and the HCV genotype was 2a. The patient again started taking 600 mg UDCA daily, and ALT returned to low levels (~30–40 IU/L). Repeated tests showed that HCV RNA was persistently positive.

To determine if HCV RNA that appeared 1.5 years after treatment completion was identical to that before therapy, we compared the nucleotide sequences of the two coding regions, namely, the E2 region containing HVR1 and ISDR of NS5A. Informed consent was obtained from the patient before analysis, and the serum samples obtained before treatment and after relapse were stored at −80 °C until use.

Virological analyses proceeded as follows. To reconfirm HCV genotyping, direct sequencing of the 5′-untranslated region was performed, as described previously.[5,6] The genotypes were classified according to the nomenclature proposed in a previous report and were determined to be 2a in both the samples. HCV RNA was amplified by reverse transcription (RT)–PCR to directly sequence the E2 and ISDR regions.

In brief, RNA was extracted from 140 μL sera using a commercially available kit (QIAamp viral RNA kit; QIAGEN, Valencia, CA, USA) and dissolved in 50 μL diethylpyrocarbonate-treated water. This sample was used for RT with random hexamer primers (SuperScript III First-Strand Synthesis System for RT–PCR cDNA synthesis kit; Invitrogen, Carlsbad, CA, USA). The E2 region was amplified by nested PCR, and ISDR regions were amplified by hemi-nested PCR. Each 50-μL PCR reaction contained 100 nM of each primer, 1 ng template cDNA, 5 μL 10× Ex Taq buffer, 4 μL deoxyribonucleotide triphosphate mixture, and 1.25 U of Takara Ex Taq HS (Takara Ex Taq, Otsu, Japan).

The PCR primers were set based on a reference HCV sequence (accession no. AF177036).

The first PCR primer sequences for E2 were: sense (1422, 1441) 5′-ACTTCTCTATGCAGGGAGCG-3′ and antisense (2437, 2418) 5′-GTTTTGGTGGAGGTGGAG AA-3′; and sense (2171, 2190) 5′-TGCCTGATCGACTA CCCCTA-3′ and antisense (2730, 2711) 5′-AGGCC AGTGAGGGAATAGGT-3′. The second PCR primer sequences for E2 were: sense (1453, 1472) 5′-CGTT GTCATCCTTCTGTTGG-3′ and antisense (2261, 2242) 5′-CAACCCCTCCCACATACATC-3′; and sense (2189, 2208) 5′-TACAGGCTCTGGCATTACCC-3′ antisense (2698, 2679) 5′-TACCCGACCCTTGATGTACC-3′.