at the start of treatment and reverse transcribed with random primer and MMLV reverse transcriptase (Takara Syuzo, Tokyo, Japan). Nucleic acids were amplified by PCR using the following primers.

Nucleotide sequences of the core region. Nucleic acids of the Core region were amplified by division into two parts of the N- and C-terminal regions. The first-round PCR in the N-terminal region was performed with 2aC1Fo (sense, 5′-TGCTAGCCGAGTAGCGTTGG-3′) and 2aC1Ro (antisense, 5′-TTCACTTCGGCAGCGGAGAC-3′), and the second-round PCR with 2aC1Fi (sense, 5′-CTTGTGGTACTGCCTGATAG-3′) and 2aC1Ri (antisense, 5′-CAGTGGAGCGCCGATCCTTA-3′). The first-round PCR in the C-terminal region was performed with 2aC2Fo (sense, 5′-CCAGATCGTTGGCGGAGTAT-3′) and 2aC2Ro (antisense, 5′-TCCAGCACCGAGATGTATTC-3′), and the second-round PCR with 2aC2Fi (sense, 5′-TATACTTGTTGCCGCGCAGG-3′) and 2aC2Ri (antisense, 5′-AGTCGTTGGTCACCATGTAG-3′).

Nucleotide sequences of the NS5A. The first-round PCR was performed with 5′ outer primer (sense, 5′-CCAGA(AG)TT(CT)TT(CT)TC(CT)TGGGTGGATGG-3′) and 3′ outer primer (antisense, 5′-GGTT(CG)(AG)TA(GA)(CT)C(CT)GGCCTCTTCCA-3′), and the second-round PCR with 5′ inner primer (sense, 5′-TGTAAAACGACGGCCAGTCAGCTCCCTTGCGATCCTGA-3′ with the sequence of the M13 forward primer underlined) and 3′ inner primer (antisense, 5′-CAGGAAACAGCTATGACC(AT)GG(GA)TTGTA(AG)TC(AT)GG(AC)CG(GT)GCCCA-3′ with the sequence of the M13 reverse primer underlined). All samples were initially denatured at 95°C for 15 min. Forty cycles of amplification were set as follows: Denaturation for 1 min at 94°C, annealing of primers for 1 min at 55°C, and extension for 1 min at 72°C with an additional 7 min for extension. Then, 1 μl of the first PCR product was transferred to the second PCR reaction. Other conditions for the second PCR were the same as the first PCR, except that the second PCR primers were used instead of the first PCR primers. PCR-amplified complementary DNA (cDNA) was purified after agarose gel electrophoresis and used for direct sequencing using each of the second-round PCR primer or M13 primer as the sequencing primer with the Big Dye Deoxy Terminator Cycle Sequencing kit (Perkin-Elmer, Tokyo, Japan). To avoid false-positive results, the procedures recommended by Kwok and Higuchi [1989] to prevent contamination were strictly applied to these PCR assays. No false-positive results were observed in this study.

Statistical Analysis

Non-parametric tests were used to examine the background characteristics of patients and amino acids changes, including the chi-squared test or Fisher's exact probability test, and Mann–Whitney U-test. The cumulative HCV-RNA negative rates by qualitative analysis with PCR were calculated using the Kaplan–Meier technique, and differences between the curves were tested using the log-rank test. Univariate and multivariate logistic regression analyses were used to determine those factors associated with hepatocyte steatosis. All P-values of less than 0.05 by the two-tailed test were considered significant. The odds ratios and 95% CI were also calculated. Variables that achieved statistical significance ($P < 0.05$) or marginal significance ($P < 0.10$) on univariate analysis were entered into multiple logistic regression analysis to identify significant independent factors. Potential factors associated with hepatocyte steatosis included the following 16 variables: sex, age, history of blood transfusion, family history of liver disease, body mass index, body surface area, alanine aminotransferase (ALT), albumin, cholinesterase, hemoglobin, platelet count, serum iron, unsaturated iron binding capacity, ferritin, level of viremia, and pathological staging. Statistical comparisons were performed using the SPSS software (SPSS Inc., Chicago, IL).

RESULTS

Factors Associated With Hepatocyte Steatosis in Multivariate Analysis

Data of patients classified with low-grade steatosis (none [n = 44] to mild [n = 291]) and high-grade (moderate [n = 29] to severe [n = 0]) were examined to determine the factors associated with steatosis. Univariate analysis identified three parameters that significantly influenced hepatocyte steatosis. These included age ($P = 0.001$), serum ferritin level ($P = 0.002$), and body mass index ($P = 0.016$). Of these, multivariate analysis identified serum ferritin level ($P = 0.004$) and body mass index ($P = 0.049$) as two parameters that independently influenced hepatocyte steatosis (Table II).

Treatment Efficacy According to Severity of Steatosis and IFN Regimen

As a whole, 249 of 364 (68.4%) patients achieved sustained virological response, while the remaining 115

TABLE II. Factors Associated With Hepatocyte Steatosis in 364 Patients Infected With HCV Genotype 2a, Identified by Multivariate Analysis

Factor	(Category)	Odds ratio (95% CI)	P
Serum ferritin (μg/L)	1: <200	1	
	2: ≥200	3.48 (1.50–8.07)	0.004
Body mass index (kg/m^2)	1: <25.0	1	
	2: ≥25.0	2.37 (1.00–5.57)	0.049

Variables that achieved statistical significance ($P < 0.05$) on multivariate logistic regression are shown.

TABLE III. Sustained Virological Response Rates Estimated by Interferon Treatment Regimens in 364 Patients Infected With HCV Genotype 2a

		Continuous[a] + intermittent[b]	
Grade of steatosis	8 weeks continuous	24 weeks	>24 weeks
Moderate to severe	25.0% (1/4)	44.0% (11/25)	—
None to mild	61.8% (34/55)	72.1% (191/265)	80.0% (12/15)

[a]2- or 8-week continuous.
[b]Three times a week.

patients (31.6%) failed to respond to therapy. Among 335 patients with the low-grade hepatocyte steatosis (grade none to mild), 237 (70.7%) achieved sustained virological response, while of 29 patients with high-grade steatosis (grade moderate to severe), only 12 (41.4%) showed sustained virological response. The rate of sustained virological response in patients with low-grade steatosis was significantly higher than in those with high-grade steatosis ($P = 0.0017$). The clinicopathological characteristics (16 variables, evaluated as potential risk factors of steatosis) of non-sustained virological response patients (115 patients) at the start of treatment of low-grade (98 patients) and high-grade (17 patients) steatosis groups were not significantly different.

With regard to the efficacy of monotherapy according to IFN regimen in 335 patients with low-grade steatosis, sustained virological response rate was 61.8% in patients who received daily IFN therapy for only 8 weeks. In patients on continuous followed by intermittent therapy, 72.1% achieved sustained virological response after 24 weeks of treatment, and 80.0% showed sustained virological response after >24 weeks of treatment (Table III).

Early Viral Kinetics and Severity of Hepatocyte Steatosis

Table IV shows the features of patients who were evaluated for early viral kinetics. The cumulative HCV-RNA negative rates of the low- and high-grade hepatocyte steatosis were 10.0% and 11.1% at day 1; 10.0% and 13.0% at day 2; 20.0% and 40.7% at week 1; 30.0% and 75.9% at week 2; 60.0% and 94.4% at week 4; and 80.0% and 98.2% at week 8, respectively. Statistical analysis showed that the cumulative HCV-RNA negative rate was significantly higher in the low-grade group than the high-grade steatosis group ($P = 0.0055$; Log-rank test, Fig. 1).

Virological Features of Cases Resistant to IFN According to Severity of Steatosis

Figure 2A,B show the amino acid sequences of HCV core region/NS5A of 9 IFN-resistant patients. In eight patients, the level of viremia was more than 100 kIU/ml, while in the remaining patient (Case 5, who was free of liver steatosis), the level of viremia was 76 kIU/ml. Heterogeneity of two cases or more was found at the following amino acid positions: amino acid 4 (Cases 6

TABLE IV. Clinical and Virological Features of 64 Patients Who Were Evaluated for Early Viral Kinetics, According to the Grade of Hepatocyte Steatosis

	Grade of hepatocyte steatosis		
	None to mild	Moderate to severe	Differences
N	54	10	NS
Age (years)[a]	17–67 (50)	41–66 (52)	NS
Sex (M/F)	31/23	3/7	NS
Body mass index (kg/m^2)	16.8–32.6 (22.2)	19.2–31.1 (25.3)	$P = 0.049$
Level of viremia level (Meq/ml)[b]	<0.5–22.0 (2.2)	<0.5–18.0 (5.8)	NS
Histological staging (F1/F2/F3)[c]	45/6/3	7/1/2	NS
ALT level (IU/l)[a]	15–618 (64)	31–175 (64)	NS
Serum albumin (g/dl)[a]	3.1–4.5 (3.9)	3.3–4.0 (3.6)	$P = 0.024$
Cholinesterase (ΔpH)[a]	0.5–1.8 (1.1)	0.8–1.4 (1.1)	NS
Hemoglobin (g/dl)[a]	9.6–18.3 (14.0)	12.9–15.8 (14.0)	NS
Platelet count (×1,000 μ/L)[a]	89–304 (176)	113–229 (163)	NS
Serum iron (μg/dl)	31–264 (146)	64–283 (196)	NS
Unsaturated iron-binding capacity (μg/dl)	61–484 (198)	24–331 (112)	NS
Serum ferritin (μg/l)	<10–1,417 (102)	<10–698 (243)	NS
IFN dose per day (MU/day)	6–7.5 (6)	6–7.5 (6)	NS

ALT, alanine aminotransferase; IFN, interferon; MU, million units; NS, not significant.
[a]Expressed as range (median).
[b]Viral levels measured by branched DNA assay version 2.0.
[c]Stage of chronic hepatitis by Desmet et al. [1994].

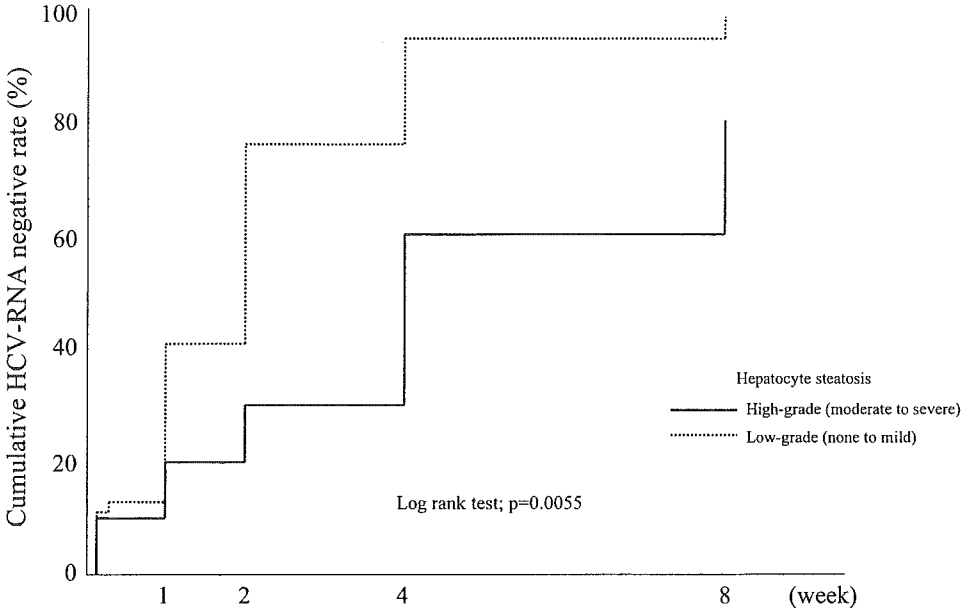

Fig. 1. In early viral kinetic study during IFN monotherapy, the cumulative HCV-RNA negative rate of the low-grade (none to mild) hepatocyte steatosis was significantly higher than that of the high-grade (moderate to severe) steatosis.

and 7; I/N), amino acid 10 (Cases 5 and 9; Q/K), amino acid 78 (Cases 2 and 4; R/K), amino acid 2240 (Cases 1, 2, and 4; V/A, T/A, and V/A, respectively), and amino acid 2243 (Cases 5, 6, and 7; E/R, M/R, and V/R, respectively). These results indicate that the amino acids sequences of four patients resistant to IFN monotherapy with the highest grade of liver steatosis (moderate grade) did not show specific amino acid substitutions, compared with the sequences of five patients also resistant to IFN monotherapy but had no hepatocyte steatosis (none grade).

DISCUSSION

In this study, the factors associated with hepatocyte steatosis were analyzed in HCV genotype 2a. Known factors associated with hepatocyte steatosis include chronic alcohol consumption, high body mass index (obesity), elevated serum concentrations of cholesterol or triglycerides, diabetes mellitus, hepatotoxic drugs, and HCV genotype 3 infection [Wanless and Lentz, 1990; Mihm et al., 1997; Sheth et al., 1997; Czaja et al., 1998; Westin et al., 2002; Castera et al., 2004; Rubbia-Brandt et al., 2004; Sharma et al., 2004]. Recent studies have focused on the mechanism of HCV-related liver damage associated with hepatocyte steatosis. Patton et al. [2004] discussed the possibility of non-inflammatory-mediated mechanism for fibrosis secondary to steatosis. They indicated that one possible mechanism might include lipid peroxidation by HCV, which could result in activation of hepatic stellate cells and subsequent collagen deposition, based on in vitro evidence [Paradis et al., 1997; Okuda et al., 2002; Patton et al., 2004]. Farinati et al. [1995] reported that HCV-related liver damage might be characterized by increased iron storage, which elicits a free-radical-mediated peroxidation, with consequent steatosis and activation of glutathione turnover. In a subsequent study, they also showed that the serum levels 8-hydroxydeoxyguanosine (8-OHdG), a reliable marker of oxidative stress in HCV patients, correlated with serum ferritin levels and the grade of hepatocyte steatosis [Farinati et al., 1999]. In the study of patients infected with HCV genotype 2a, multivariate analysis also identified high serum ferritin level as an independent factor associated with high-grade hepatocyte steatosis. Thus, the results of the present study confirm these early reports that hepatocyte steatosis associated with HCV-related liver damage, including HCV genotype 2 might be characterized by increased iron storage, which elicits a free-radical-mediated peroxidation. Interestingly, steatosis in this study of genotype 2a was not associated with fibrosis. Rubbia-Brandt et al. [2004] reported that liver fibrosis might be associated with steatosis only in genotype 3. Considered together, the results suggest that hepatocyte steatosis might influence progression of liver fibrosis in a viral genotype-specific manner.

Hepatocyte steatosis has been considered recently as an important pretreatment predictor of response to IFN therapy. It is reported to be an important predictive factor of sustained virological response in combination therapy of IFN-ribavirin in patients infected with HCV genotype 1 or 3 [Bjøro et al., 2002; Poynard et al., 2003; Patton et al., 2004; Zeuzem et al., 2004]. However, there is no information on whether hepatocyte steatosis in patients infected with genotype 2 affects the virological response to IFN therapy, a part from a previous report [Akuta et al., 2002]. Akuta et al. [2002] reported previously that hepatocyte steatosis was a negative predictor of sustained virological response to IFN monotherapy in patients infected with genotype 2a, based on

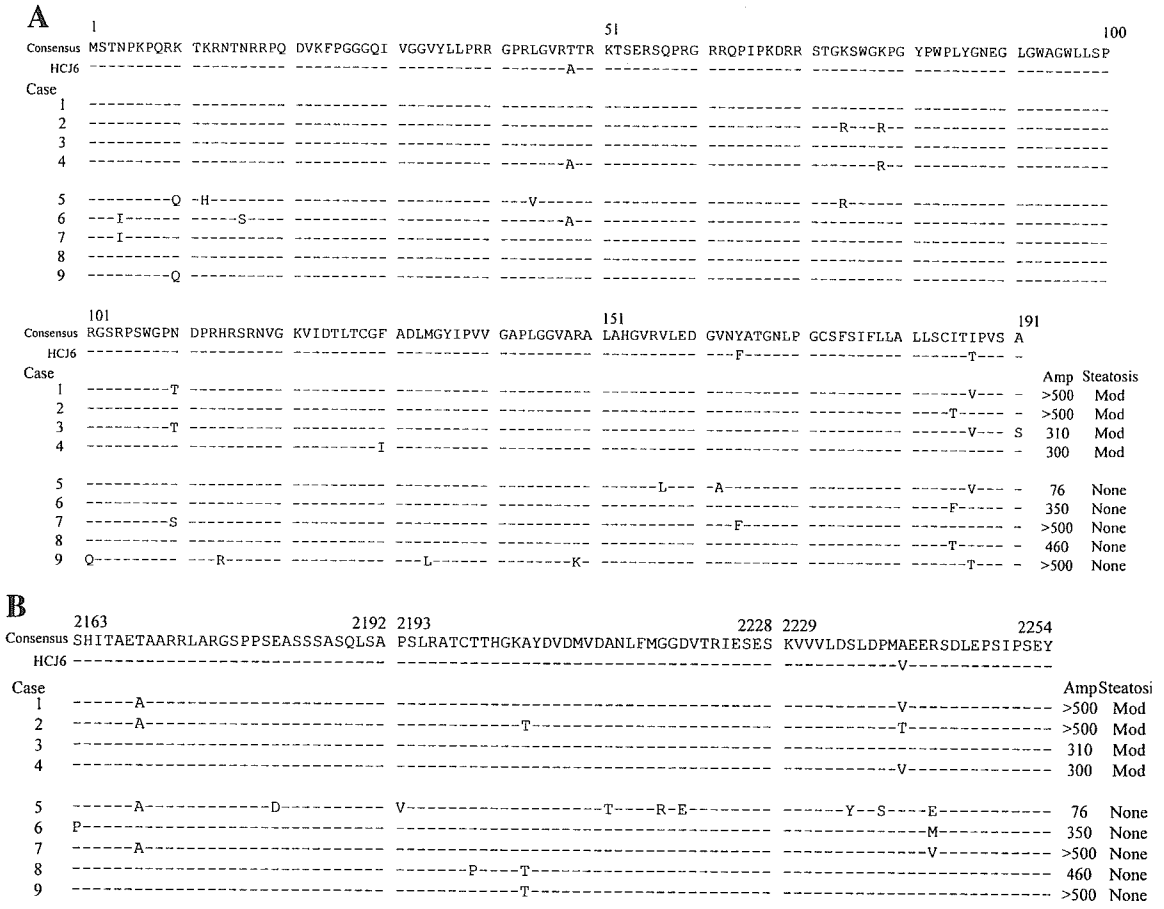

Fig. 2. Sequences of amino acids 1–191 in HCV core region (**A**) and amino acids 2163–2254 in HCV NS5A (**B**) at the start of IFN monotherapy of nine IFN-resistant patients infected with HCV genotype 2a with the highest grade of hepatocyte steatosis (moderate = Mod; Cases 1–4) or without steatosis (none; Cases 5–9). Dashes indicate amino acids identical to the consensus sequence of genotype 2a, and substituted amino acids are shown by standard single-letter codes. Eight IFN-resistant patients had high viremia (HCV more than 100 kIU/ml) while the viral level in the other IFN-resistant case (Case 5), free of steatosis, was 76 kIU/ml. Amp (kIU/ml) = quantitative analysis of HCV-RNA with PCR of Amplicor version 2.0.

multivariate analysis. A recent report [Patton et al., 2004] indicated that patients with genotype 1 who showed an early virologic response (defined as ≥ 2 $\log_{10}$ decline in HCV-RNA at week 12) were more likely to have low-grade steatosis than those who did not show an early response. The results of the present study of early viral kinetics and its relationship to the grade of steatosis in patients infected with genotype 2a were similar to the above study, and is the first report to indicate that the grade of steatosis influences early viral kinetics of HCV-RNA in patients infected with genotype 2a and treated with IFN. Further prospective studies should be performed based on a large number of patients who are matched for factors associated with hepatocyte steatosis (such as alcohol consumption, glucose/lipid metabolism) and pretreatment efficacy predictors (including IFN regimen, viral load, and serum albumin level [Akuta et al., 2002]).

Previous studies that patients infected with genotype 3 who could achieve sustained virological responses by IFN treatment often demonstrated a marked decrease in steatosis as confirmed by repeated posttreatment biopsies, and it was concluded that steatosis of genotype 3 might be considered as a consequence of viral infection [Kumar et al., 2002; Poynard et al., 2003; Castera et al., 2004; Patton et al., 2004]. However, it is not known whether steatosis of genotype 2a is also a consequence of viral infection or other non-viral factors (e.g., metabolic factors). Hence, it is important to investigate the relationship between treatment efficacy and improvement of steatosis grade, based on repeated biopsies in future studies.

IFN-resistance mechanisms specific for hepatocyte steatosis are so far unknown. Steatosis may be associated with reduced liver metabolism probably due to reduced activity of hepatic cytochrome induced by high liver fat content [Leclercq et al., 1998]. Previous studies showed that liver fibrosis correlates with steatosis and obesity [Hourigan et al., 1999; Clouston et al., 2001; Hwang et al., 2001]. Thus, lipid deposits within hepatocytes might cause functional disturbances by increasing the architectural distortion of the hepatic lobule caused by fibrosis and decrease in the contact area between drugs and hepatocytes [Taliani et al., 1995; Giannini

et al., 1999]. Another study reported a close correlation between the level of intrahepatic HCV-RNA and severity of steatosis [Rubbia-Brandt et al., 2000]. In the present study, no significant clinicopathological parameter (including serum HCV-RNA levels, fibrosis, and body mass index) was identified in non-sustained virological response patients with low-grade and high-grade steatosis. Thus, the results failed to establish a link between IFN-resistance and hepatocyte steatosis.

It is also not clear whether the virological characteristics of HCV play any pathogenic role in the derangement of lipid metabolism, and hence contribute to hepatocyte steatosis. Experiments conducted in vitro and in transgenic mice suggested that HCV core protein and NS5A region might be involved in the pathogenesis of lipid accumulation [Barba et al., 1997; Moriya et al., 1997; Shi et al., 2002]. On the other hand, Rubbia-Brandt et al. [2000] reported that steatosis might be a morphological expression of viral cytopathic effect in patients infected with HCV genotype 3, but that analysis of the HCV core protein failed to identify a sequence specially associated with the development of steatosis [Rubbia-Brandt et al., 2000]. No study has investigated the effects of HCV core protein and NS5A region on IFN efficacy in patients with hepatocyte steatosis. In this context, the relationship between amino acid substitutions in HCV core protein/NS5A region and the grade of hepatocyte steatosis was analyzed in the present study in IFN-resistant patients. However, the analysis showed no specific amino acid substitutions in these regions that could establish a role for hepatocyte steatosis in IFN-resistance. It should be noted, however, that the present study was based on a small group of patients and sequence analysis of the defined regions should be investigated in large-scale studies to confirm the present findings.

β IFN is rarely used and is not licensed outside Japan. It was reported previously that the type of IFN (α vs. β) is not a predictor of sustained virological response to IFN monotherapy in 394 patients infected with genotype 2a, based on multivariate analysis [Akuta et al., 2002], and accordingly when the present study was designed, it was thought that the type of IFN should not affect the outcome of patients infected with genotype 2a. Incidentally, based on data from Toranomon Hospital, the frequency of β IFN-related adverse events seems to be lower than those by α IFN, especially in elderly patients (unpublished data). Therefore, the use of β IFN rather than α IFN is recommended at least for elderly patients.

In conclusion, the present study of patients infected with HCV genotype 2a suggested that hepatocyte steatosis is possibly associated with excessive iron storage, and that it might be an important predictor of the efficacy of IFN monotherapy. Further studies should be performed to investigate whether hepatocyte steatosis associated with HCV genotype 2a might be also a predictor of other treatments, including IFN-ribavirin combination therapy and pegylated IFN. In this study, amino acid substitutions associated with IFN-resistance specific for hepatocyte steatosis could not be identified, and large-scale studies should be conducted to confirm the present findings. Further analysis of IFN-resistance mechanisms should be conducted in future studies taking into consideration pharmacokinetic, viral, and host-related factors.

ACKNOWLEDGMENTS

This study was supported in part by a Grant-in-Aid from the Ministry of Health, Labor and Welfare, Japan.

REFERENCES

Akuta N, Suzuki F, Tsubota A, Suzuki Y, Someya T, Kobayashi M, Saitoh S, Arase Y, Ikeda K, Kumada H. 2002. Efficacy of interferon monotherapy to 394 consecutive naive cases infected with hepatitis C virus genotype 2a in Japan: Therapy efficacy as consequence of tripartite interaction of viral, host and interferon treatment-related factors. J Hepatol 37:831–836.

Akuta N, Suzuki F, Tsubota A, Suzuki Y, Hosaka T, Someya T, Kobayashi M, Saitoh S, Arase Y, Ikeda K, Kumada H. 2003. Association of amino acid substitution pattern in nonstructural protein 5A of hepatitis C virus genotype2a low viral load and response to interferon monotherapy. J Med Virol 69:376–383.

Barba G, Harper F, Harada T, Kohara M, Goulinet S, Matsuura Y, Eder G, Schaff Z, Chapman MJ, Miyamura T, Brechot C. 1997. Hepatitis C virus core protein shows a cytoplasmic localization and associates to cellular lipid storage droplets. Proc Natl Acad Sci USA 94:1200–1205.

Bjøro K, Bell H, Hellum KB, Skaug K, Raknerud N, Sandvei P, Døskeland B, Mæland A, Lund-Tønnesen S, Myrvang B. 2002. Effect of combined interferon-α induction therapy and ribavirin on chronic hepatitis C virus infection: A randomized multicentre study. Scand J Gastroenterol 37:226–232.

Bressler BL, Guindi M, Tomlinson G, Heathcote J. 2003. High body mass index is an independent risk factor for nonresponse to antiviral treatment in chronic hepatitis C. Hepatology 38:639–644.

Castera L, Hezode C, Roudot-Thoraval F, Lonjon I, Zafrani ES, Pawlotsky JM, Dhumeaux D. 2004. Effect of antiviral treatment on evolution of liver steatosis in patients with chronic hepatitis C: Indirect evidence of a role of hepatitis C virus genotype 3 in steatosis. Gut 53:420–424.

Chayama K, Tsubota A, Arase Y, Saitoh S, Koida I, Ikeda K, Matsumoto T, Kobayashi M, Iwasaki S, Koyama S, Morinaga T, Kumada H. 1993. Genotypic subtyping of hepatitis C virus. J Gastroenterol Hepatol 8:150–156.

Chayama K, Tsubota A, Kobayashi M, Okamoto K, Hashimoto M, Miyano Y, Koike H, Kobayashi M, Koida I, Arase Y, Saitoh S, Suzuki Y, Murashima N, Ikeda K, Kumada H. 1997. Pretreatment virus load and multiple amino acid substitutions in the interferon sensitivity-determining region predict the outcome of interferon treatment in patients with chronic genotype 1b hepatitis C virus infection. Hepatology 25:745–749.

Clouston AD, Jonsson JR, Purdie DM, Macdonald GA, Pandeya N, Shorthouse C, Powell EE. 2001. Steatosis and chronic hepatitis C: Analysis of fibrosis and stellate cell activation. J Hepatol 34:314–320.

Czaja AJ, Carpenter HA, Santrach PJ, Moore SB. 1998. Host- and disease-specific factors affecting steatosis in chronic hepatitis C. J Hepatol 29:198–206.

D'Alessandro AM, Kalayouglu M, Sollinger HW, Hoffmann RM, Reed A, Knechtle SJ, Pirsch JD, Hafez GR, Lorentzen D, Belzer FO. 1991. The predictive value of donor liver biopsies for the development of primary nonfunction after orthotopic liver transplantation. Transplantation 51:157–163.

Desmet VJ, Gerber M, Hoofnagle JH, Manna M, Scheuer PJ. 1994. Classification of chronic hepatitis: Diagnosis, grading and staging. Hepatology 19:1513–1520.

Farinati F, Cardin R, De Maria N, Della Libera G, Marafin C, Lecis E, Burra P, Floreani A, Cecchetto A, Naccarato R. 1995. Iron storage, lipid peroxidation and glutathione turnover in chronic anti-HCV positive hepatitis. J Hepatol 22:449–456.

Farinati F, Cardin R, Degan P, De Maria N, Floyd RA, Van Thiel DH, Naccarato R. 1999. Oxidative DNA damage in circulating leukocytes occurs as an early event in chronic HCV infection. Free Radic Biol Med 27:1284–1291.

Giannini E, Ceppa P, Botta F, Fasoli A, Romagnoli P, Cresta E, Venturino V, Risso D, Celle G, Testa R. 1999. Steatosis and bile duct damage in chronic hepatitis C: Distribution and relationships in a group of Northern Italian patients. Liver 19:432–437.

Haydon GH, Jarvis LM, Blair CS, Simmonds P, Harrison DJ, Simpson KJ, Hayes PC. 1998. Clinical significance of intrahepatic hepatitis C virus levels in patients with chronic HCV infection. Gut 42:570–575.

Hino K, Sainokami S, Shimoda K, Iino S, Wang Y, Okamoto H, MIyakawa Y, Mayumi M. 1994. Genotypes and titers of hepatitis C virus for predicting response to interferon in patients with chronic hepatitis C. J Med Virol 42:299–305.

Hourigan LF, Macdonald GA, Purdie D, Whitehall VH, Shorthouse C, Clouston A, Powell EE. 1999. Fibrosis in chronic hepatitis C correlates significantly with body mass index and steatosis. Hepatology 29:1215–1219.

Hwang SJ, Luo JC, Chu CW, Lai CR, Lu CL, Tsay SH, Wu JC, Chang FY, Lee SD. 2001. Hepatic steatosis in chronic hepatitis C virus infection: Prevalence and clinical correlation. J Gastoroenterol Hepatol 16:190–195.

Kanai K, Kako M, Okamoto H. 1992. HCV genotypes in chronic hepatitis C and response to interferon. Lancet 339:1543.

Kumar D, Farrell GC, Fung C, George J. 2002. Hepatitis C virus genotype 3 is cytopathic to hepatocytes: Reversal of hepatic steatosis after sustained therapeutic response. Hepatology 36: 1266–1272.

Kwok S, Higuchi R. 1989. Avoiding false positives with PCR. Nature 339:237–238.

Leclercq I, Horsmans Y, Desager JP, Delzenne N, Geubel AP. 1998. Reduction in hepatic cytochrome P-450 is correlated to the degree of liver fat content in animal models of steatosis in the absence of inflammation. J Hepatol 28:410–416.

Mahaney K, Tedeschi V, Maertens G, Di Bisceglie AM, Vergalla J, Hoofnagle JH, Sallie R. 1994. Genotypic analysis of hepatitis C virus in American patients. Hepatology 20:1405–1411.

Mihm S, Fayyazi A, Hartmann H, Ramadori G. 1997. Analysis of histopathological manifestations of chronic hepatitis C virus infection with respect to virus genotype. Hepatology 25:735–739.

Moriya K, Yotsuyanagi H, Shintani Y, Fujie H, Ishibashi K, Matsuura Y, Miyamura T, Koike K. 1997. Hepatitis C virus core protein induces hepatic steatosis in transgenic mice. J Gen Virol 78:1527–1531.

Murakami T, Enomoto N, Kurosaki M, Izumi N, Marumo F, Sato C. 1999. Mutations in nonstructural protein 5A gene and response to interferon in hepatitis C virus genotype 2 infection. Hepatology 30:1045–1053.

Okamoto H, Okada S, Sugiyama Y, Kurai K, Iizuka H, Machida A, Miyakawa Y, Mayumi M. 1991. Nucleotide sequence of the genomic RNA of hepatitis C virus isolated from a human carrier: Comparison with reported isolates for conserved and divergent regions. J Gen Virol 72:2697–2704.

Okuda M, Li K, Beard MR, Showalter LA, Scholle F, Lemon SM, Weinman SA. 2002. Mitochondrial injury, oxidative stress, and antioxidant gene expression are induced by hepatitis C virus core protein. Gastroenterology 122:366–375.

Paradis V, Mathurin P, Kollinger M, Imbert-Bismut F, Charlotte F, Piton A, Opolon P, Holstege A, Poynard T, Bedossa P. 1997. In situ detection of lipid peroxidation in chronic hepatitis C: Correlation with pathological features. J Clin Pathol 50:401–406.

Patton HM, Patel K, Behling C, Bylund D, Blatt LM, Vallee M, Heaton S, Conrad A, Pockros PJ, McHutchison JG. 2004. The impact of steatosis on disease progression and early and sustained treatment response in chronic hepatitis C patients. J Hepatol 40:484–490.

Poynard T, Ratziu V, McHutchison J, Manns M, Goodman Z, Zeuzem S, Younossi Z, Albrecht J. 2003. Effect of treatment with pegintefreon or interferon alfa-2b and ribavirin on steatosis in patients infected with hepatitis C. Hepatology 38:75–85.

Rubbia-Brandt L, Quadri R, Abid K, Giostra E, Male PJ, Mentha G, Spahr L, Zarski JP, Borisch B, Hadengue A, Negro F. 2000. Hepatocyte steatosis is a cytopathic effect of hepatitis C virus genotype 3. J Hepatol 33:106–115.

Rubbia-Brandt L, Fabris P, Paganin S, Leandro G, Male PJ, Giostra E, Carlotto A, Bozzola L, Smedile A, Negro F. 2004. Steatosis affects chronic hepatitis C progression in a genotype specific way. Gut 53:406–412.

Sharma P, Balan V, Hernandez J, Rosati M, Williams J, Rodriguez-Luna H, Schwartz J, Harrison E, Anderson M, Byrne T, Vargas HE, Douglas DD, Rakela J. 2004. Hepatic steatosis in hepatitis C virus genotype 3 infection: Does it correlate with body mass index, fibrosis, and HCV risk factor? Dig Dis Sci 49:25–29.

Sheth SG, Gordon FD, Chopra S. 1997. Nonalcoholic steatohepatitis. Ann Intern Med 126:137–145.

Shi ST, Polyak SJ, Tu H, Taylor DR, Gretch DR, Lai MM. 2002. Hepatitis C virus NS5A colocalizes with the core protein on lipid droplets and interacts with apolipoproteins. Virology 292:198–210.

Simmonds P. 1997. Clinical relevance of hepatitis C virus genotypes. Gut 40:291–293.

Taliani G, Duca F, Lecce R, Livoli D, Pasquazzi C, De Bac C. 1995. Hepatic Lidocaine metabolism in chronic hepatitis C virus hepatitis with or without steatosis. Hepatology 21:1760–1761.

Tsubota A, Chayama K, Ikeda K, Arase Y, Koida I, Saitoh S, Hashimoto M, Iwasaki S, Kobayashi M, Kumada H. 1994. Factors predictive of response to interferon-α therapy in hepatitis C virus infection. Hepatology 19:1088–1094.

Wanless IR, Lentz JS. 1990. Fatty liver hepatitis (steatohepatitis) and obesity: An autopsy study with analysis of risk factors. Hepatology 12:1106–1110.

Westin J, Nordlinder H, Lagging M, Norkrans G, Wejstal R. 2002. Steatosis accelerates fibrosis development over time in hepatitis C virus genotype 3 infected patients. J Hepatol 37:837–842.

Zeuzem S, Hultcrantz R, Bourliere M, Goeser T, Marcellin P, Sanchez-Tapias J, Sarrazin C, Harvey J, Brass C, Albrecht J. 2004. Peginterferon alfa-2b plus ribavirin for treatment of chronic hepatitis C in previously untreated patients infected with HCV genotypes 2 or 3. J Hepatol 40:993–999.

Original Paper

Intervirology

Intervirology 2005;48:174–182
DOI: 10.1159/000081746

Virological and Biochemical Relapse after Discontinuation of Lamivudine Monotherapy for Chronic Hepatitis B in Japan: Comparison with Breakthrough Hepatitis during Long-Term Treatment

Norio Akuta[a] Fumitaka Suzuki[a] Mariko Kobayashi[b] Marie Matsuda[b] Junko Sato[b] Yoshiyuki Suzuki[a] Hitomi Sezaki[a] Tetsuya Hosaka[a] Takashi Someya[a] Masahiro Kobayashi[a] Satoshi Saitoh[a] Yasuji Arase[a] Kenji Ikeda[a] Hiromitsu Kumada[a]

[a]Department of Gastroenterology and [b]Liver Research Laboratory, Toranomon Hospital, Tokyo, Japan

Key Words

Chronic hepatitis B · Lamivudine monotherapy · Biochemical and virological relapse · Basic core promoter · YMDD motif mutant · Breakthrough hepatitis · Retreatment · HBV genotype

Abstract

Objective: Comparison of virological and biochemical relapse in patients with chronic hepatitis B, based on continuation or discontinuation of lamivudine monotherapy. ***Methods:*** In Japanese genotype C-dominant hepatitis B patients, 25 patients who stopped treatment at normal levels of alanine transferase (ALT) were retrospectively compared with 75 patients who continued treatment. Both groups were matched for age, sex, and observation period after start of treatment. We investigated the relapse rates, and evaluated predictive factors for relapse and efficacy of retreatment of the discontinuous group. ***Results:*** Virological and biochemical relapse occurred significantly earlier in the discontinuous than continuous group, and the peak levels and ratios of peak to pretreatment levels of serum bilirubin and ALT after relapse were not significantly different between the two groups. Multivariate analysis identified three independent factors at discontinuation of treatment associated with early biochemical relapse: HBeAg positivity, presence of liver cirrhosis, detection of basic core promoter mutant. Normalization of ALT levels with retreatment occurred in 62.5% of patients, but 2 HBeAg-positive patients retreated after the emergence of YMDD motif mutant developed severe relapse with hyperbilirubinemia. ***Conclusion:*** Our results in Japanese patients with genotype C-dominant hepatitis B suggest that discontinuation of lamivudine monotherapy, and retreatment after the emergence of YMDD mutant should be given attention.

Introduction

Lamivudine, an oral cytosine nucleoside analog clinically used for the treatment of chronic hepatitis B virus (HBV) infection, potently inhibits HBV replication by interfering with HBV reverse transcriptase activity [1–4],

KARGER

Fax + 41 61 306 12 34
E-Mail karger@karger.ch
www.karger.com

0300–5526/05/0483–0174$22.00/0

Accessible online at:
www.karger.com/int

Norio Akuta, MD
Department of Gastroenterology, Toranomon Hospital
2-2-2 Toranomon, Minato-ku
Tokyo, 105-0001 (Japan)
Tel. +81 44 877 5111, Fax +81 44 860 1623

and results in a marked decrease of HBV DNA and serum transaminase levels, seroconversion of HBe antigen (HBeAg) to anti-HBe, and histopathological improvement [4–9].

The optimal duration of lamivudine administration for HBV-infected patients is still controversial for two main problems; drug resistance and sustainability of the response to treatment. In particular, there is a need to evaluate short-term treatment with respect to post-treatment safety and the sustainability of responses, and long-term treatment with regard to biochemical relapse (breakthrough hepatitis) associated with the emergence of YMDD motif mutant [10–17]. The American Association for the Study of Liver Diseases practice guidelines suggested that lamivudine could be discontinued in patients who had completed one year of treatment and had persistent HBeAg seroconversion on more than one occasion determined 2–3 months apart [18]. However, this is not completely evaluated in Japanese genotype C-dominant hepatitis B patients.

The present study was designed to deal with the following three issues: (1) To compare the virological and biochemical relapse rates according to the continuation or termination of lamivudine monotherapy, and to compare the risk of biochemical relapse after the termination of the treatment and breakthrough hepatitis during long-term treatment; (2) to determine the independent predictive factors at discontinuation of treatment that contributed to early biochemical relapse in discontinuous patients, and (3) to evaluate the efficacy of retreatment with lamivudine monotherapy.

Patients and Methods

Patients

Lamivudine therapy was provided to 394 consecutive patients with chronic hepatitis B who tested positive for HBs antigen at Toranomon Hospital between September 1995 and December 2002. Among these, 269 patients started lamivudine monotherapy at abnormal alanine transferase (ALT) levels (normal for ALT, 6–50 IU/l) and were able to achieve ALT normalization during treatment, and were enrolled in this retrospective study. The latter group consisted of 25 patients who stopped the lamivudine monotherapy during ALT normalization (discontinuous group) and 244 patients who did not stop the lamivudine monotherapy (continuous group), and the discontinuation or not of lamivudine during ALT normalization was selected at their own request. To compare the cumulative virological and biochemical relapse rates between the discontinuous group and continuous group, all 25 patients of the discontinuous group entered this study along with 75 patients of the continuous group. The latter group was selected from among the 244 because they matched patients of the discontinuous group with respect to sex, age, and observation period after the start of lamivudine monotherapy. They had been confirmed to have hepatitis by liver biopsies, were free of decompensated liver cirrhosis and hepatocellular carcinoma. Coinfection and superinfection with hepatitis A, C, and delta viruses, and human immunodeficiency virus were ruled out serologically or genomically using commercially available kits or conventional polymerase chain reaction (PCR)-based assays. None of the patients had a history of other liver diseases, such as autoimmune hepatitis, alcoholic liver disease, and metabolic disease.

Patients were given a dose of 100 mg of lamivudine once a day. The median period of treatment in the discontinuous group (0.72 years, range; 0.10–5.6 years) was significantly shorter than that of the continuous group (1.8 years, range; 0.71–7.6 years, $p < 0.0001$). The median observation period after the commencement of lamivudine therapy was not significantly different based on the matching of the two groups, and the periods were 2.1 years (range; 0.68–7.9 years) in the discontinuous group and 1.8 years in the continuous group (range; 0.71–7.6 years). In the discontinuous group, the median observation period after discontinuation of lamivudine therapy was 1.4 years (range; 0.15–6.7 years). With regard to the observation period, patients of the discontinuous group who received another course of lamivudine treatment for biochemical relapse and those of the continuous group who received additional interferon treatment for biochemical relapse, were treated as censored data at the time of lamivudine retreatment and additional interferon treatment in the statistical analysis of cumulative relapse rates. The clinical characteristics of enrolled patients are summarized in table 1, and those of discontinuation are shown in table 2.

Methods

Our study compared virological and biochemical relapse in continuous and discontinuous lamivudine monotherapy groups, and determined the independent predictive factors at discontinuation that contributed to early biochemical relapse in the discontinuous group. Furthermore, we also evaluated the efficacy of retreatment with lamivudine monotherapy. Patients in whom ALT levels became abnormal (>50 IU/l) after a period of ALT normalization were defined as biochemical relapsers. Patients in whom levels of HBV DNA re-elevated after the minimum levels, ignoring undetectable HBV DNA levels, were defined as virological relapsers. Especially, virological relapse during lamivudine treatment associated with the emergence of YMDD motif mutant were defined as DNA breakthrough, and biochemical relapse associated with DNA breakthrough were defined as breakthrough hepatitis. Clinical and laboratory assessments were performed at least once every month before, during, and after treatment. Adverse effects were monitored clinically by a detailed interview and medical examination at least once every month. Patient compliance with treatment was evaluated by a questionnaire.

Blood samples were obtained at least once every month before, during, and after treatment, and were analyzed for various laboratory data including ALT levels, HBV DNA levels, and the presence of YMDD motif mutant. The serum samples were stored in aliquots at −80 °C until use. HBs antigen and HBeAg/eAb were determined by radioimmunoassay (Abbott Diagnostics, Chicago, Ill., USA). HBV DNA was measured by transcription-mediated amplification and hybridization protect assay (TMA-HPA) (Chugai Diagnostica, Tokyo, Japan). The lower and upper limits of detection of TMA-HPA are 5×10^3 and 5×10^8 viral genomic equivalents (GE)/ml, respectively. HBV genotype was determined using a previously reported

Table 1. Clinical characteristics of enrolled patients

	Discontinuous group (n = 25)	Continuous group (n = 75)	p value
Age, years[a]	33 (19–75)	34 (20–75)	matched
Sex, male/female	19/6	57/18	matched
Period of observation[b]	2.1 (0.68–7.9)	1.8 (0.71–7.6)	matched
HBV DNA, LGE/ml[a]	7.6 (<3.7 to >8.7)	7.5 (<3.7 to >8.7)	NS
HBeAg, number of positive	20 (80.0%)	45 (60.0%)	NS
HBV genotype, number of C	22 (88.0%)	61 (81.3%)	NS
Liver cirrhosis[c]	2 (8.0%)	3 (4.0%)	NS
Family history of liver disease[d]	18 (72.0%)	59 (78.7%)	NS
T-Bil, mg/dl[a]	0.7 (0.3–20.7)	0.7 (0.3–10.5)	NS
ALT, IU/l[a]	97 (51–3,168)	150 (53–2,274)	NS
Albumin, g/dl[a]	3.9 (2.8–4.3)	3.8 (2.5–4.8)	NS
Cholinesterase, ΔpH[a]	1.0 (0.6–1.5)	1.1 (0.5–1.7)	NS
Duration of lamivudine therapy, years[a]	0.72 (0.10–5.6)	1.8 (0.71–7.6)	<0.0001

[a] Data expressed as median (range).
[b] Period of follow-up after the start of lamivudine therapy.
[c] Scoring according to the system of Desmet et al. [22].
[d] Family history of positivity for hepatitis B surface antigen including third-degree relatives.
LGE = Logarithm of genome equivalent per millilitre; T-Bil = total bilirubin; ALT = alanine transferase (normal range ≤ 50 IU/l); NS = not significant.

Table 2. Characteristics of patients at discontinuation of lamivudine monotherapy

Number	25
Sex, male/female	19/6
Age, years[a]	34 (19–75)
Number of cirrhosis	2 (8.0%)
HBV genotype, number with genotype C	22 (88.0%)
Family history of liver disease[b]	18 (72.0%)
HBeAg, number of positive	9 (36.0%)
HBV DNA, patients with <3.7 LEG/ml	18 (72.0%)
T-Bil, mg/dl[a]	0.7 (0.3–1.3)
ALT, IU/l[a]	23 (10–50)
BCP nt 1762/1764 (W/M/mi/N)	5/8/2/10
PC nt 1896 (W/M/mi/N)	10/3/2/10
Presence of YMDD motif mutant	3 (12.0%)[c]
Duration of lamivudine therapy, years[a]	0.72 (0.10–5.6)

[a] Data expressed as median (range), or number of patients.
[b] Family history of positivity for hepatitis B surface antigen including third-degree relatives.
[c] One patient was PCR-negative with serum sample at stop of treatment, but had been already detected before stop.
Abbreviations, as in table 1, BCP = Basic core promoter; PC = precore; nt = nucleotide. W = wild type (BCP; $A^{1762}G^{1764}$. PC; G^{1896}); M = mutant type; mi = mixed type of wild and mutant virus; N = PCR-negative.

method [19, 20]. Antibody against HCV was detected with a third-generation enzyme-linked immunoassay (Ortho Diagnostic Japan, Tokyo). YMDD motif mutant was detected using the sensitive PCR-restriction fragment length polymorphism [21].

Liver biopsy specimens were obtained percutaneously or at laparoscopy using a modified Vim Silverman needle of 2 mm internal diameter (Tohoku University style, Kakinuma Factory, Tokyo). Each specimen was scored according to the system of Desmet et al. [22].

This study was conducted in accordance with the guidelines of the Declaration of Helsinki and its subsequent amendments, and informed consent was obtained from each patient. The study was approved by the Human Ethics Committee of Toranomon Hospital.

Nucleotide Sequencing of HBV Basic Core Promoter (nt 1762/1764) and Precore (nt 1896)

Nucleotide sequences of HBV were compared with the prototype sequences of the HBV genotype C [19]. HBV DNA was extracted with a Smitest EX & R kit (Genome Science, Tokyo). Nucleic acids were amplified by nested PCR using the following primers. Nucleotide sequences of basic core promotor (BCP) nt 1762/1764 and precore (PC) nt 1896: The first-round PCR was performed with BCP-F7 [sense, 5′-TGC ACT TCG CTT CAC CTC TG-3′ (nt 1580–1599)] and BCP-R8 [antisense, 5′-TAA GCG GGA GGA GTG CGA AT-3′ (nt 2295–2276)] primers, and the second-round PCR with BCP-F5 [sense, 5′-GCA TGG AAA CCA CCG TGA AC-3′ (nt 1606–1625)] and BCP-R6 [antisense, 5′-ATA CAG AGC AGA GGC GGT AT-3′ (nt 2014–1995)] primers. All samples were initially denatured at 95 °C for 4 min. Thirty-five cycles of amplification were set as follows: denaturation for 1 min at 94 °C, annealing of primers for 2 min

at 55 °C, and extension for 3 min at 72 °C with an additional 7 min for extension. Then 1 μl of the first-round PCR product was transferred to the second-round PCR reaction. Other conditions for the second-round PCR were the same as the first-round PCR, except that the second-round PCR primers were used instead of the first-round PCR primers. The amplified PCR products were purified by the QIA quick PCR purification kit (Qiagen, Tokyo) after agarose gel electrophoresis and then used for direct sequencing. Dideoxynucleotide termination sequencing was performed with the ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems, Perkin-Elmer, Chiba, Japan). To avoid false-positive results, the procedures recommended by Kwok and Higuchi [23] to prevent contamination were strictly applied to these PCR assays. No false-positive results were observed in this study.

Statistical Analysis

The χ^2 test, Fisher's exact probability test, and Mann-Whitney's U test were used to compare the background characteristics between groups. The cumulative virological and biochemical relapse rates were calculated using the Kaplan-Meier technique, differences between the curves were tested using the log-rank test. Statistical analyses of virological and biochemical relapse periods according to the mode of monotherapy (continuous and discontinuous groups) were calculated using the period from the start of lamivudine monotherapy, and those concerned with the characteristics of the discontinuous group were calculated using the period after discontinuation of the treatment. Stepwise Cox regression analysis was used to determine independent predictive factors at discontinuation of lamivudine monotherapy that contributed to early biochemical relapse after discontinuation of the treatment. We also calculated the odds ratios and 95% confidence intervals. Potential predictive factors associated with early biochemical relapse included the following ten variables at discontinuation of treatment: sex, age, histological stage, HBV genotype, levels of HBV DNA, HBeAg, pattern of BCP and PC, presence of YMDD motif mutant, and duration of lamivudine therapy. Each variable was transformed into categorical data consisting of two simple ordinal numbers for univariate and multivariate analyses. Variables that achieved statistical significance ($p < 0.05$) or marginal significance ($p < 0.10$) on univariate analysis were tested by multivariate Cox proportional hazard model to identify significant independent factors. Statistical comparisons were performed using the SPSS software (SPSS, Chicago, Ill., USA). All p values <0.05 by the two-tailed test were considered significant.

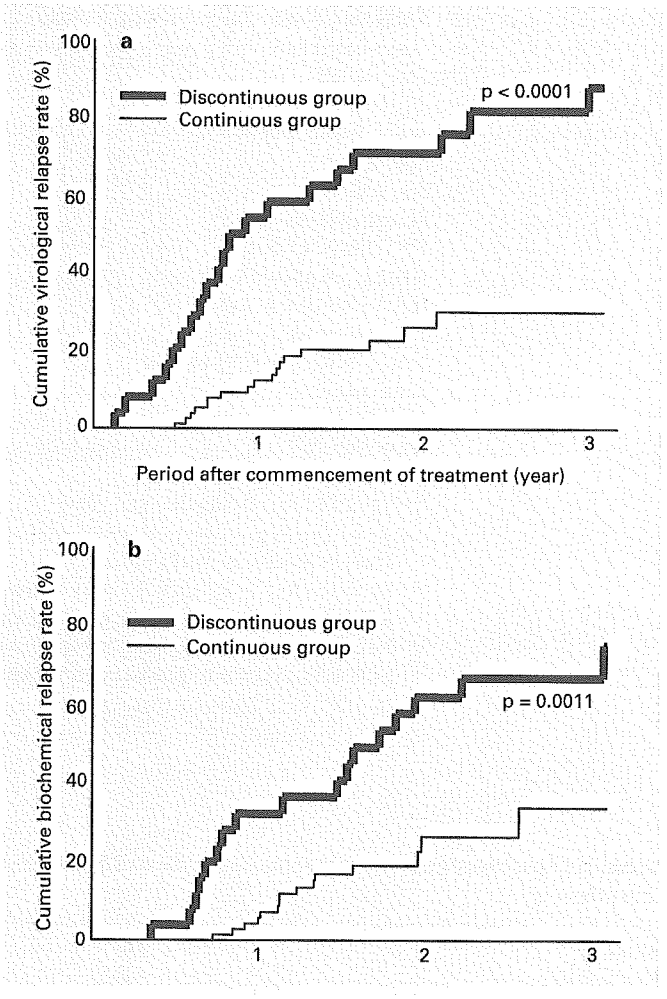

Fig. 1. Virological and biochemical relapse rates according to the continuation or discontinuation of lamivudine monotherapy, in patients matched for age, sex, and observation period after the start of treatment. **a** Cumulative virological relapse rates after the commencement of treatment. **b** Cumulative biochemical relapse rates after the commencement of treatment. Virological and biochemical relapse in the discontinuous group emerged significantly earlier than in the continuous group.

Results

Virological and Biochemical Relapse

Virological relapse occurred in 24.0% (18 of 75 patients) of patients of the continuous group and 84.0% (21 of 25) of the discontinuous group. The cumulative virological relapse rates of the continuous and discontinuous group were 12.3 and 54.1% at the end of one year after the commencement of lamivudine monotherapy; 26.0 and 70.8% at 2 years; and 30.1 and 87.8% at 3 years, respectively. Virological relapse in the discontinuous group emerged significantly earlier than the continuous group ($p < 0.0001$; log-rank test) (fig. 1a).

Biochemical relapse occurred in 22.7% (17 of 75 patients) of patients of the continuous group and 68.0% (17 of 25 patients) of the discontinuous group. The cumulative biochemical relapse rates of the continuous and discontinuous group were 4.27 and 32.2% at the end of one year after commencement of lamivudine monotherapy; 26.5 and 61.9% at 2 years; and 33.9 and 66.7% at 3 years, respectively. Biochemical relapse in the discontinuous group emerged significantly earlier than in the continuous group ($p = 0.0011$; log-rank test) (fig. 1b).

Table 3. Comparison of ALT and T-Bil levels after biochemical relapse and the emergence of YMDD motif mutant between patients who continued and those who discontinued lamivudine monotherapy

	Discontinuous group	Continuous group	p value
Biochemical relapse cases (n = 34)	17	17	
Peak T-Bil, mg/dl[a]	0.9 (0.5–3.8)	1.1 (0.5–4.9)	NS
Peak ALT, IU/l[a]	384 (191–1,480)	538 (67–1,736)	NS
T-Bil ratio[b]	1.5 (0.3–3.5)	2.0 (0.2–12.3)	NS
ALT ratio[b]	3.9 (1.0–18.7)	3.5 (0.1–10.8)	NS
YMDD motif mutant cases (n = 25)	3	22	
Peak T-Bil, mg/dl[a]	2.1 (0.7–3.8)	1.1 (0.5–4.9)	NS
Peak ALT, IU/l[a]	441 (50–638)	236 (26–1,736)	NS
T-Bil ratio[b]	1.0 (0.3–3.5)	1.8 (0.1–12.3)	NS
ALT ratio[b]	5.9 (0.6–11.2)	1.7 (0.1–10.8)	NS

[a] Data expressed as median (range). [b] The ratios of peak levels to pretreatment. For abbreviations, see table 1.

Table 4. Predictors of early biochemical relapse after lamivudine monotherapy, determined by multivariate analysis

Factors	Category	Odds ratio (95% confidence interval)	p
Histology	1: no cirrhosis	1	
	2: cirrhosis	16.1 (2.30–113)	0.0052
HBeAg	1: negative	1	
	2: positive	5.61 (1.77–17.8)	0.0035
Basic core promoter (A1762G1764)	1: undetectable mutant	1	
	2: detectable mutant	3.93 (1.31–11.8)	0.015

Variables that achieved statistical significance ($p < 0.05$) on multivariate Cox proportional hazard model are shown.

YMDD mutants were not detected in any of the pretreatment serum samples. Emergence of YMDD motif mutant was noted in 29.3% (22 of 75 patients) of patients of the continuous group and 12.0% (3 of 25 patients) of the discontinuous group. In the continuous group, all of 18 virological relapsers showed DNA breakthrough associated with the emergence of YMDD motif mutant, and all of 17 biochemical relapsers showed breakthrough hepatitis associated with DNA breakthrough.

ALT and Bilirubin Levels after Biochemical Relapse or Emergence of YMDD Motif Mutant

The peak levels of serum ALT and bilirubin after biochemical relapse, and the ratios of peak levels to pretreatment were not significantly different between continuation or discontinuation groups (table 3). Likewise, the peak levels of serum ALT and bilirubin after the emergence of YMDD motif mutant, and the ratios of peak levels to pretreatment were also not significantly different between the two groups (table 3).

Factors Associated with Early Biochemical Relapse after Discontinuation of Lamivudine Monotherapy

The cumulative biochemical relapse rates of the discontinuous group were 48.0, 64.8, 69.2, and 69.2% at the end of 0.5, 1, 2, and 3 years after discontinuation of lamivudine monotherapy, respectively. Potential predictive factors associated with early biochemical relapse after discontinuation of treatment were explored in 25 patients of the discontinuation group. In univariate analyses, the following six factors tended to or significantly influenced the early biochemical relapse: HBeAg ($p = 0.0048$), levels of HBV DNA ($p = 0.039$), pattern of BCP ($p = 0.026$), pattern of PC ($p = 0.033$), age ($p = 0.083$), and liver cirrhosis ($p = 0.096$). In multivariate analysis using these factors, HBeAg ($p = 0.0035$), liver cirrhosis ($p = 0.0052$), and pat-

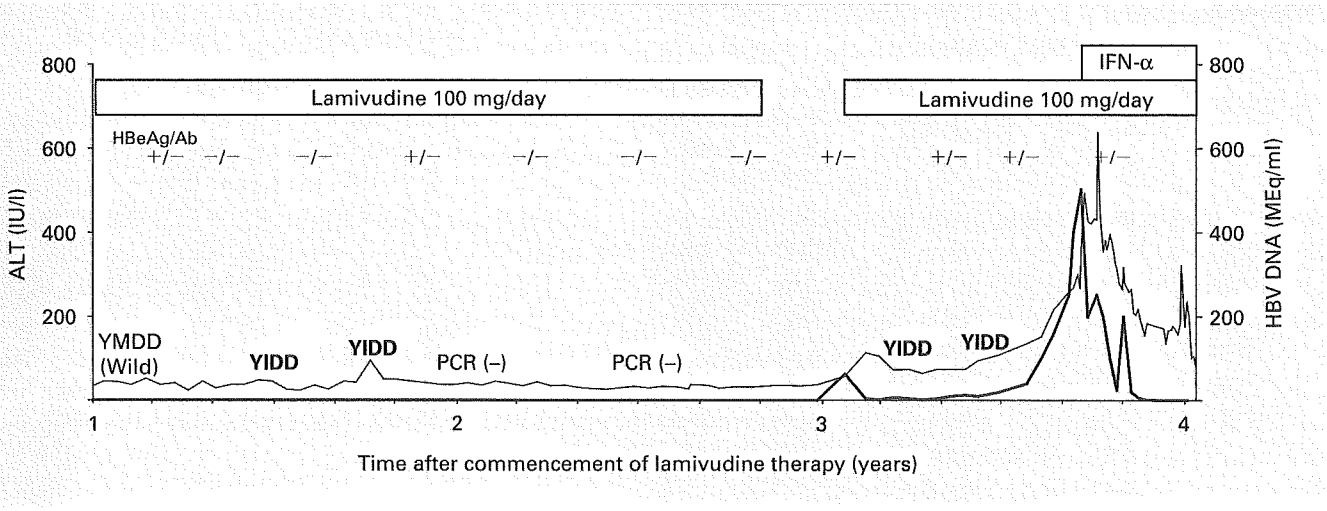

Fig. 2. Clinical summary of a 34-year HBeAg-positive male patient infected with HBV-genotype C complicated with liver cirrhosis. The patient was treated with lamivudine monotherapy for 2.8 years, which resulted in HBeAg negativity and a decrease in HBV DNA to undetectable levels as measured by TMA-HPA for one year or more, despite the emergence of YMDD motif mutant (YIDD type). After the discontinuation of lamivudine, however, the patient developed severe biochemical relapse, during retreatment of lamivudine monotherapy. The case was later controlled when combination therapy of lamivudine and IFN-α was used. HBV DNA was indicated by branched DNA signal amplification technology (Chiron Corp., Emeryville, Calif., USA) to show the viral loads of higher ranges, and the results were expressed as 10^6 genomic equivalents per millilitre (MEq/ml). Thick line = HBV DNA level, thin line = ALT level.

tern of BCP (p = 0.015) were independent significant predictors of early biochemical relapse after discontinuation of the treatment (table 4). The odds ratio of liver cirrhosis was 16.1 compared with the absence of cirrhosis. The odds ratio of HBeAg-positive was 5.61 compared with HBeAg-negative. The odds ratio of detectable BCP mutant virus was 3.93 compared with undetectable BCP mutant virus.

Retreatment for Biochemical Relapse after Discontinuation of Lamivudine Monotherapy

Eight of 17 patients, who showed relapse after the termination of the treatment, received another course of lamivudine monotherapy at the same dose after a median stop (no treatment) period of 0.61 years (range, 0.15–1.8 years). The median period of retreatment was 1.1 years (range, 0.14–2.7 years). Six of these patients were HBeAg-positive, and the remaining 2 were HBeAg-negative at the commencement of retreatment. Five of 8 (62.5%) patients successfully showed normalization of ALT level and disappearance of HBV-DNA after retreatment; of whom 2 were HBeAg-negative (100%) and 3 were HBeAg-positive (50%). The other 3 patients, who did not show normalization of ALT, were HBeAg-positive, and especially 2 patients showed HBeAg reversion. Furthermore, in 2 of the latter 3 nonresponders, lamivudine therapy was terminated following the emergence of YMDD motif mutant, and both developed severe biochemical relapse (a rise in ALT level to ≥ 300 IU/l, accompanied by the elevation of total bilirubin level to ≥ 2.0 mg/dl) during retreatment. In particular, one of them developed severe relapse despite HBeAg seronegative conversion and was HBV DNA undetectable for one year (fig. 2). In summary, 5 of 6 patients (83.3%) without YMDD motif mutant at discontinuation could achieve ALT normalization again with retreatment, but 2 of 2 patients with YMDD motif mutant developed severe biochemical relapse during retreatment. Hence, retreatment with lamivudine monotherapy was effective, but tended to be not very effective for HBeAg-positive patients retreated after the emergence of YMDD motif mutant.

Discussion

Previous studies reported that the estimated half-life of hepatocytes infected with HBV was 10–100 days, suggesting that prolonged administration of lamivudine for a period longer than one year might be needed to clear HBV in the liver by turning over most of cccDNA-containing

hepatocytes [24, 25]. However, a recent report by Ryu et al. [26] showed that HBV DNA and HBeAg reappeared in 31 and 16% of their patients, respectively at 2 years after the termination of lamivudine, even when HBV DNA and HBeAg had been persistently negative for 2 years or more. Based on these findings, they suggested that long-term additional administration of lamivudine might enhance the durability of lamivudine-induced HBeAg seroconversion [26]. Our results of the discontinuous group also indicated higher cumulative biochemical relapse rates of 64.8 and 69.2% at 1 and 2 years after discontinuation, similar to the Korean report (relapse rates, 37.5 and 49.2% at 1 and 2 years) [27], although this might be due to the criteria used for the definition of the discontinuous group, regardless of HBeAg seroconversion and inclusion of subjects who were HBeAg-negative at the start of the treatment.

With regard to long-term treatment, while continued disease suppression, or even HBeAg seroconversion, still occurred in some patients, in others, breakthrough hepatitis associated with the appearance of YMDD mutant may occur. Severe breakthrough hepatitis has been reported despite the continuation of lamivudine [28–32], even though previous studies showed that YMDD mutants are less replication-competent compared with the wild-type, and are associated with lower HBV DNA levels compared with pretreatment HBV DNA levels [4, 5, 33–37]. We have recently reported that 3-year lamivudine therapy induced histopathological improvement regardless of the appearance of YMDD mutants, associated with DNA breakthrough and breakthrough hepatitis, and suggested the benefit of long-term treatment [38].

In our study based on patients matched for age, sex, and observation period, the cumulative virological and biochemical relapse rates were compared according to the continuation or not of lamivudine monotherapy. Our results showed that the relapse rates in the discontinuous group emerged significantly earlier than the continuous group. Furthermore, the peak levels of serum ALT and bilirubin and the ratios of peak to pretreatment levels were not significantly different between the continuation and discontinuation groups, regardless of the emergence of YMDD motif mutant. To our knowledge, this is the first report based on matched patients' backgrounds that compares the virological and biochemical relapse rates according to continuation or discontinuation of lamivudine monotherapy.

One limitation of our study is the small number of patients, the use of various treatment periods, and differences in the discontinuation criteria regardless of HBeAg seroconversion in the discontinuous group. Large-scale prospective studies of each group should be conducted in the future to confirm these findings.

Previous studies showed that HBeAg-positivity, old age, high pretreatment viral loads, and the presence of PC mutant at the start of the treatment might affect the biochemical relapse after treatment [39–41]. Our study based on multivariate analysis-evaluated various aspects of clinicopathological characteristics at the termination of treatment, and identified HBeAg-positivity, liver cirrhosis, and detectable BCP mutant virus as independent significant determinants of early biochemical relapse. Mutations in BCP, increase viral replication and enhance disease activity [42, 43], and are also associated with HBV genotype C and a longer duration of infection (including the higher age, and more advanced liver disease) [44]. These results suggest that the presence of BCP mutant and liver cirrhosis might indicate the more active state of disease, and might be the responsible factors of an early relapse. In our study, the majority of patients of the discontinuous group were Japanese patients infected with HBV genotype C and were positive for a family history of HBV infection (namely, genotype C patients with the longer duration of infection), and thus the presence of BCP mutant together with genotype C and a longer duration of infection might explain the higher viral replication and biochemical relapse after treatment in endemic areas of HBV genotype C infection, such as Japan and Korea, where most HBV infection is considered to be transmitted vertically [27]. To our knowledge, this is the first report of early post-treatment biochemical relapse based on characteristics at discontinuation of lamivudine monotherapy. Previous reports in the United States indicated that viral suppression was maintained after the termination of treatment [45]. The discrepancy between the USA reports and our results are probably due to the differences in HBV genotypes, duration of infection, and follow-up period after the termination of treatment. Further studies of a large group of patients are required to clarify whether the patients' characteristics including HBV genotype and duration of infection affect the early virological and biochemical relapse after the termination of lamivudine monotherapy.

Reinstitution of lamivudine monotherapy is usually effective in controlling exacerbations in patients who have not experienced breakthrough and may result in subsequent HBeAg seroconversion [39], but the benefits of retreatment are usually transient in patients with breakthrough since YMDD mutant rapidly reappears (often within weeks) when lamivudine is resumed [46, 47]

because of possible persistence of YMDD mutant over long periods after the cessation of therapy [48]. In the present study, 83.3% of patients without YMDD motif mutant at discontinuation achieved ALT normalization again with retreatment, but all (100%) patients with YMDD motif mutant developed severe biochemical relapse during retreatment. These results suggest that care should be exercised in the management of patients in whom lamivudine is first discontinued then used again, especially those who show the emergence of YMDD motif mutants.

In conclusion, the present study indicates that the discontinuation of lamivudine monotherapy for Japanese genotype C-dominant hepatitis B should be followed carefully for virological and biochemical relapses. Further prospective studies are necessary to determine the true risk of post-treatment relapse by discontinuation and breakthrough hepatitis by continuation of long-term treatment. However, it should be stated here that it would be difficult to perform such studies based on ethical grounds. Interferon therapy and new nucleotide analogs (for example, adefovir dipivoxil and entecavir) have been recently shown to be effective in patients with YMDD mutants induced by long-term lamivudine administration [49–52]. Thus, new combination therapies of antiviral drugs or alternative drugs are expected to appear in the future.

References

1 Doong SL, Tsai CH, Schinazi RF, Liotta DC, Cheng YC: Inhibition of the replication of hepatitis B virus in vitro by 2′,3′-dideoxy-3′-thiacytidine and related analogues. Proc Natl Acad Sci USA 1991;88:8495–8499.

2 Dienstag JL, Perrillo RP, Schiff ER, Bartholomew M, Vicary C, Rubin M: A preliminary trial of lamivudine for chronic hepatitis B infection. N Engl J Med 1995;333:1657–1661.

3 Nevens F, Main J, Honkoop P, Tyrrell DL, Barber J, Sullivan MT, Fevery J, De Man RA, Thomas HC: Lamivudine therapy for chronic hepatitis B: A six-month randomized dose-ranging study. Gastroenterology 1997;113: 1258–1263.

4 Lai CL, Chien RN, Leung NWY, Chang TT, Guan R, Tai DI, Ng KY, Wu PC, Dent JC, Barber J, Stephenson SL: A one-year trial of lamivudine for chronic hepatitis B. N Engl J Med 1998;339:61–68.

5 Dienstag JL, Sciff ER, Wright TL, Perrillo RP, Hann HW, Goodman Z, Crowther L, Condreay LD, Woessner M, Rubin M, Brown NA: Lamivudine as initial treatment for chronic hepatitis B in the United States. N Engl J Med 1999;341:1256–1263.

6 Suzuki Y, Kumada H, Ikeda K, Chayama K, Arase Y, Saitoh S, Tsubota A, Kobayashi M, Koike M, Ogawa N, Tanikawa K: Histological changes in liver biopsies after one year of lamivudine treatment in patients with chronic hepatitis B infection. J Hepatol 1999;30:743–748.

7 Liaw YF, Leung NWY, Chang TT, Guan R, Tai DI, Ng KY, Chien RN, Dent J, Roman L, Edmundson S, Lai CL: Effects of extended lamivudine therapy in Asian patients with chronic hepatitis B. Gastroenterology 2000; 119:172–180.

8 Schalm SW, Heathcote J, Cianciara J, Farrell G, Sherman M, Willems B, Dhillon A, Moorat A, Barber J, Gray DF: Lamivudine and alpha interferon combination treatment of patients with chronic hepatitis B infection: A randomised trial. Gut 2000;46:562–568.

9 Leung NW, Lai CL, Chang TT, Guan R, Lee CM, Ng KY, Lim SG, Wu PC, Dent JC, Edmundson S, Codreay LD, Chien RN: Extended lamivudine treatment in patients with chronic hepatitis B enhances hepatitis B e antigen seroconversion rates: Results after 3 years of therapy. Hepatology 2001;33:1527–1532.

10 Balzarini J, Wedgwood O, Kruining J, Pelemans H, Heijtink R, De Clercq E, McGuigan C: Anti-HIV and anti-HBV activity and resistance profile of 2′,3′-dideoxy-3′-thiacytidine (3TC) and its arylphosphoramidate derivative CF 1109. Biochem Biophys Res Commun 1996;2:363–369.

11 Ling R, Mutimer D, Ahmed M, Boxall EH, Elias E, Dusheiko GM, Harrison TJ: Selection of mutations in the hepatitis B virus polymerase during therapy of transplant recipients with lamivudine. Hepatology 1996;3:711–713.

12 Naoumov NV, Smith C, Williams R: Emergence and characterization of lamivudine resistant hepatitis B virus variant. Hepatology 1996;24:282.

13 Tipples GA, Ma MM, Fischer KP, Bain VG, Kneteman NM, Tyrrell DL: Mutation in HBV RNA-dependent DNA polymerase confers resistance to lamivudine in vivo. Hepatology 1996;24:714–717.

14 Bartholomew MM, Jansen RW, Jeffers LJ, Reddy KR, Johnson LC, Bunzendahal H, Condreay LD, Tzakis AG, Schiff ER, Brown NA: Hepatitis-B-virus resistance to lamivudine given for recurrent infection after orthotopic liver transplantation. Lancet 1997;349:20–22.

15 Honkoop P, Niesters HG, de Man RA, Osterhaus AD, Schalm SW: Lamivudine resistance in immunocompetent chronic hepatitis B. J Hepatol 1997;26:1393–1395.

16 Schalm SW: Clinical implications of lamivudine resistance by HBV. Lancet 1997;349:3–4.

17 Allen MI, Deslauriers M, Andrews CW, Tipples GA, Walters KA, Tyrrell DL, Brown N, Condreay LD: Identification and characterization of mutations in hepatitis B virus resistant to lamivudine. Lamivudine Clinical Investigation Group. Hepatology 1998;27:1670–1677.

18 Lok ASF, McMahon BJ: Chronic hepatitis B. Hepatology 2001;34:1225–1241.

19 Okamoto H, Tsuda F, Sakugawa H, Sastrosoewignjo RI, Imai M, Miyakawa Y, Mayumi M: Typing hepatitis B virus by homology in nucleotide sequence: Comparison of surface antigen subtypes. J Gen Virol 1988;69:2575–2583.

20 Usuda S, Okamoto H, Iwanari H, Baba K, Tsuda F, Miyakawa Y, Mayumi M: Serological detection of hepatitis B virus genotypes by ELISA with monoclonal antibodies to type-specific epitopes in the preS2-region product. J Virol Methods 1999;80:97–112.

21 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: Emergence and takeover of YMDD motif mutant hepatitis B virus during long-term lamivudine therapy and re-takeover by wild type after cessation of therapy. Hepatology 1998;27: 1711–1716.

22 Desmet VJ, Gerber M, Hoofnagle JH, Manns M, Sheuer PJ: Classification of chronic hepatitis: Diagnosis, grading, and staging. Hepatology 1994;19:1513–1520.

23 Kwok S, Higuchi R: Avoiding false positives with PCR. Nature 1989;339:237–238.

24 Nowak MA, Bonhoeffer S, Hill AM, Boehme R, Thomas HC, McDade H: Viral dynamics in hepatitis B virus infection. Proc Natl Acad Sci USA 1996;93:4398–4402.

25 Zeuzem S, de Man RA, Honkoop P, Roth WK, Schalm SW, Schmidt JM: Dynamics of hepatitis B virus infection in vivo. J Hepatol 1997;27: 431–436.

26 Ryu SH, Chung YH, Choi MH, Kim JA, Shin JW, Jang MK, Park NH, Lee HC, Lee YS, Suh DJ: Long-term additional lamivudine therapy enhances durability of lamivudine-induced HBeAg loss: A prospective study. J Hepatol 2003;39:614–619.
27 Song BC, Suh DJ, Lee HC, Chung YH, Lee YS: Hepatitis B e antigen seroconversion after lamivudine therapy is not durable in patients with chronic hepatitis B in Korea. Hepatology 2000; 32:803–806.
28 Liaw YF, Chien RN, Yeh CT, Tsai SL, Chu CM: Acute exacerbation and hepatitis B virus clearance after emergence of YMDD motif mutation during lamivudine therapy. Hepatology 1999;30:567–572.
29 Leung N: Treatment of chronic hepatitis B: Case selection and duration of therapy. J Gastroenterol Hepatol 2002;17:409–414.
30 Akuta N, Suzuki F, Kobayashi M, Tsubota A, Suzuki Y, Hosaka T, Someya T, Kobayashi M, Saitoh S, Arase Y, Ikeda K, Kumada H: The influence of hepatitis B virus genotype on the development of lamivudine resistance during long-term treatment. J Hepatol 2003;38:315–321.
31 Akuta N, Tsubota A, Suzuki F, Suzuki Y, Hosaka T, Someya T, Kobayashi M, Saitoh S, Arase Y, Ikeda K, Kumada H: Long-term prognosis by lamivudine monotherapy for severe acute exacerbation in chronic hepatitis B infection: Emergence of YMDD motif mutant and risk of breakthrough hepatitis – An open-cohort study. J Hepatol 2003;38:91–97.
32 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: Virological and biochemical relapse according to YMDD motif mutant type during long-term lamivudine monotherapy. J Med Virol 2003;71:504–510.
33 Fu L, Cheng YC: Role of additional mutations outside the YMDD motif of hepatitis B virus polymerase in L(–)SddC(3TC) resistance. Biochem Pharmacol 1998;55:1567–1572.
34 Melegari M, Scaglioni PP, Wands JR: Hepatitis B virus mutants associated with 3TC and famciclovir administration are replication defective. Hepatology 1998;27:628–633.
35 Ling R, Harrison TJ: Functional analysis of mutations conferring lamivudine resistance on hepatitis B virus. J Gen Virol 1999;80:601–606.
36 Ono-Nita SK, Kato N, Shiratori Y, Masaki T, Lan KH, Carriho FJ, Omata M: YMDD motif in hepatitis B virus DNA polymerase influences on replication and lamivudine resistance: A study by in vitro full-length viral DNA transfection. Hepatology 1999;29:939–945.
37 Leung N: Liver disease – significant improvement with lamivudine. J Med Virol 2000;61: 380–385.
38 Suzuki Y, Arase Y, Ikeda K, Saitoh S, Tsubota A, Suzuki F, Kobayashi M, Akuta N, Someya T, Miyakawa Y, Kumada H: Histological improvements after a three-year lamivudine therapy in patients with chronic hepatitis B in whom YMDD mutants did not or did develop. Intervirology 2003;46:164–170.
39 Honkoop P, de Man RA, Niesters HG, Zondervan PE, Schalm SW: Acute exacerbation of chronic hepatitis B virus infection after withdrawal of lamivudine therapy. Hepatology 2000;32:635–639.
40 Lee CM, Ong GY, Lu SN, Wang JH, Liao CA, Tung HD, Chen TM, Changchien CS: Durability of lamivudine-induced HBeAg seroconversion for chronic hepatitis B patients with acute exacerbation. J Hepatol 2002;37:669–674.
41 Huang YH, Wu JC, Chang TT, Sheen IJ, Lee PC, Huo TI, Su CW, Wang YJ, Chang FY, Lee SD: Analysis of clinical, biochemical and viral factors associated with early relapse after lamivudine treatment for hepatitis B e antigen-negative chronic hepatitis B patients in Taiwan. J Viral Hepatol 2003;10:277–284.
42 Lok AS, Akarca U, Greene S: Mutations in the pre-core region of hepatitis B virus serve to enhance the stability of the secondary structure of the pregenome encapsidation signal. Proc Natl Acad Sci USA 1994;91:4077–4081.
43 Hunt CM, McGill JM, Allen MI, Condreay LD: Clinical relevance of hepatitis B virus mutations. Hepatology 2000;31:1037–1044.
44 Orito E, Mizokami M, Sakugawa H, Kojiro M, Ishikawa K, Ichida T, Okanoue T, Yotsuyanagi H, Iino S: A case-control study for clinical and molecular biological differences between hepatitis B viruses of genotype B and C. Hepatology 2001;33:218–223.
45 Dienstag JL, Schiff ER, Mitchell M, Casey DE, Gitlin N, Lissoos T, Gelb LD, Condreay L, Crowther L, Rubin M, Brown N: Extended lamivudine retreatment for chronic hepatitis B: Maintenance of viral suppression after discontinuation of therapy. Hepatology 1999;30: 1082–1087.
46 Tisdale M, Kemp SD, Parry NR, Larder BA: Rapid in vitro selection of human immunodeficiency virus type 1 resistant to 3′ thiacytidine inhibitors due to a mutation in the YMDD region of reverse transcriptase. Proc Natl Acad Sci USA 1993;90:5653–5656.
47 Lau DTY, Khokhar MF, Doo E, Ghany MG, Herion D, Park Y, Kleiner DE, Schmid P, Condreay LD, Gauthier J, Kuhus MC, Liang TJ, Hoofnagle JH: Long-term therapy of chronic hepatitis B with lamivudine. Hepatology 2000; 32:828–834.
48 van der Eijk AA, Niesters HGM, Pas SD, de Man RA: Persistence of YMDD variants after withdrawal of lamivudine (letter). J Hepatol 2002;36:304–305.
49 Suzuki F, Tsubota A, Akuta N, Someya T, Kobayashi M, Suzuki Y, Saitoh S, Arase Y, Ikeda K, Miyakawa Y, Kumada H: Interferon for treatment of breakthrough infection with hepatitis B virus mutants developing during long-term lamivudine therapy. J Gastroenterol 2002;37:922–927.
50 Perrillo R, Schiff E, Yoshida E, Statler A, Hirsch K, Wright T, Gutfreund K, Lamy P, Murray A: Adefovir dipivoxil for the treatment of lamivudine-resistant hepatitis B mutants. Hepatology 2000;32:129–134.
51 Peters M, Hann HW, Martin P, Heathcote E, Buggisch P, Moorat AE, Sullivan M, Kleber K, Ebrahimi R, Xiong S, Brosgart C: Adefovir dipivoxil alone and in combination with lamivudine suppresses lamivudine-resistant hepatitis B virus replication: 16 week interim analysis. J Hepatol 2002;36:S6–S7.
52 Tassopoulos N, Hadziyannis S, Cianciara J, Rizzetto M, Schiff ER, Pastore G, Rutkiewicz V: Entecavir is effective in treating patients with chronic hepatitis B who have failed lamivudine therapy. Hepatology 2001;34:340A.

Journal of Medical Virology 75:491–498 (2005)

Favorable Efficacy of Long-Term Lamivudine Therapy in Patients With Chronic Hepatitis B: An 8-Year Follow-Up Study

Norio Akuta,[1]* Fumitaka Suzuki,[1] Yoshiyuki Suzuki,[1] Hitomi Sezaki,[1] Tetsuya Hosaka,[1] Takashi Someya,[1] Masahiro Kobayashi,[1] Satoshi Saitoh,[1] Yasuji Arase,[1] Kenji Ikeda,[1] Mariko Kobayashi,[2] and Hiromitsu Kumada[1]

[1]*Department of Gastroenterology, Toranomon Hospital, Tokyo, Japan*
[2]*Liver Research Laboratory, Toranomon Hospital, Tokyo, Japan*

The long-term efficacy of lamivudine therapy in patients with hepatitis B virus (HBV) infection is still not clear. In this study, 20 non-cirrhotic Japanese patients infected with HBV received lamivudine therapy for more than 1 year and were followed for a median period of 8.5 years (range, 6.7–8.7 years). The rates of HBe antigen (HbeAg) negative, HBV-DNA undetectable, and alanine aminotransferase (ALT) normal level at the start of lamivudine were 55%, 25%, and 20% and 85%, 80%, and were 80%, respectively, at the last visit, including patients who received additional treatment. The values at the last visit tended to and were significantly higher than those at the start. The values improved at the last visit regardless of the emergence of YMDD motif mutant and continuation of lamivudine. YMDD mutant and biochemical relapse with mutant virus (breakthrough hepatitis) appeared in 65% and 45% during follow-up, respectively, but severe breakthrough hepatitis occurred in only 5%. Furthermore, 80% of patients who received additional treatment for breakthrough hepatitis, regardless of continuation of lamivudine, were ALT normal level at the last visit, in contrast to 25% untreated. HBsAg clearance occurred in two patients of the discontinuous lamivudine group with non-vertical transmission, who were relatively young. One was infected with HBV genotype C with breakthrough hepatitis and the other had no YMDD mutant and was infected with genotype D, a rare type in Japan. None developed cirrhosis or hepatocellular carcinoma (HCC) during follow-up. Our results suggest that long-term lamivudine therapy improves long-term prognosis, especially when additional treatment for breakthrough hepatitis is used. ***J. Med. Virol. 75:491–498, 2005.*** © 2005 Wiley-Liss, Inc.

KEY WORDS: YMDD motif mutant; HBV genotype; breakthrough hepatitis; HBsAg clearance; hepatocellular carcinoma

INTRODUCTION

Lamivudine, an oral cytosine nucleoside analog clinically used for the treatment of chronic hepatitis B virus (HBV) infection, potently inhibits HBV replication by interfering with HBV reverse transcriptase activity [Doong et al., 1991; Dienstag et al., 1995; Nevens et al., 1997; Lai et al., 1998], and results in marked decrease of HBV-DNA and alanine aminotransferase (ALT) levels, seroconversion of HBe antigen (HBeAg) to anti-HBe (HBeAb), and histopathological improvement [Lai et al., 1998; Dienstag et al., 1999; Suzuki et al., 1999; Liaw et al., 2000; Schalm et al., 2000; Leung et al., 2001; Akuta et al., 2003a]. However, lamivudine-resistant HBV strains (YMDD motif mutant) have been reported in long-term lamivudine therapy, and the emergence of such mutant virus results in re-elevation of HBV-DNA (DNA breakthrough) and ALT (breakthrough hepatitis) [Tipples et al., 1996; Bartholomew et al., 1997; Lai et al., 1998; Dienstag et al., 1999; Liaw et al., 2000; Schalm et al., 2000; Leung et al., 2001; Yuen et al., 2001; Akuta et al., 2003a,b].

The optimal duration of lamivudine therapy for HBV-infected patients is still controversial for two main reasons; drug resistance and sustainability of the response to treatment. In particular, there is a need to evaluate short-term treatment with respect to post-treatment safety and the sustainability of the response

Grant sponsor: Ministry of Health, Labor and Welfare, Japan.

*Correspondence to: Norio Akuta, MD, Department of Gastroenterology, Toranomon Hospital, 2-2-2 Toranomon, Minato-ku, Tokyo, 105-0001, Japan. E-mail: akuta-gi@umin.ac.jp

Accepted 13 December 2004

DOI 10.1002/jmv.20305

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

to such treatment, and long-term treatment with regard to breakthrough hepatitis [Balzarini et al., 1996; Ling et al., 1996; Naoumov et al., 1996; Tipples et al., 1996; Bartholomew et al., 1997; Honkoop et al., 1997; Schalm, 1997; Allen et al., 1998]. Furthermore, the long-term prognosis of lamivudine-treated patients also remains unknown. Recently, Lok et al. [2003] reported the long-term safety and efficacy of continuous lamivudine treatment based on a median follow-up period of 4 years. However, the efficacy of long-term lamivudine treatment based on a longer follow-up period of more than 5 years is still not clear, especially when associated with or without continuation of lamivudine and with or without the emergence of YMDD mutant.

The present study included 20 consecutive non-cirrhotic Japanese patients with chronic hepatitis B, in whom more than 8 years had elapsed since the induction of lamivudine monotherapy. The aims of the present study were the following: (1) To evaluate the long-term efficacy and safety of more than 1-year lamivudine treatment in Japanese patients with genotype C-dominant hepatitis B; (2) to evaluate HBeAg status, HBV-DNA levels, and ALT levels according to continuation or discontinuation of lamivudine, and according to the emergence or not of YMDD motif mutant; and (3) to evaluate the efficacy of any additional treatment for breakthrough hepatitis.

MATERIALS AND METHODS

Patients

We studied 20 consecutive Japanese adult patients with chronic hepatitis B who agreed to enter a long-term lamivudine trial between September 1995 and July 1996 at the Department of Gastroenterology of Toranomon Hospital [Chayama et al., 1998]. The entry criteria included a positive test for HBV-DNA by dot hybridization and elevated ALT levels (greater than twice the upper limit of normal value [50 IU/L]) within 3 months before the start of therapy. A liver needle biopsy was performed in all patients just before the start of the trial, which confirmed the presence of chronic hepatitis. Individuals, who had hepatocellular carcinoma (HCC), apparent cirrhosis, or signs of hepatic decompensation, were excluded from the study. Also excluded were patients positive for serum markers of hepatitis C virus and human immunodeficiency virus. None of the participating patients received immunosuppressive or antiviral therapy at least 6 months before lamivudine therapy, and none had been previously treated with any nucleoside analogs. Each patient was treated with a single oral dose of 100 mg of lamivudine every day.

The patient characteristics at the start of lamivudine monotherapy are summarized in Table I. The follow-up period represented the time from the start of lamivudine monotherapy until the last visit. The median period of follow-up was 8.5 years (range, 6.7–8.7 years). During follow-up, 6 patients discontinued lamivudine treatment at their own request, and the other 14 patients continued until the last visit. The median period of lamivudine treatment was 1.4 years (range, 1.0–5.6 years) for the discontinuation group, and 8.5 years (range, 6.7–8.7 years) for the continuous patients. As a whole, the median period of lamivudine treatment was 8.4 years (range, 1.0–8.7 years). In some patients, lamivudine therapy was supplemented with other additional treatments.

TABLE I. Patient Characteristics at the Start of Lamivudine Monotherapy

Number	20
Sex (male/female)	16/4
Age (year)[a]	44 (25–65)
HBeAg (no. of positive)	9 (45.0%)
HBV-DNA (gEq/ml)[a]	3.2×10^7 ($7.0 \times 10^5 - 3.2 \times 10^9$)
HBV genotype (no. of B/C/D/F)	3/15/1/1
Histology (no. of F1/2)[b]	12/8
Family history of liver disease[c]	13 (65.0%)
T-Bill (mg/dl)[a]	0.8 (0.2–1.7)
AST (IU/l)[a]	59 (24–247)
ALT (IU/l)[a]	100 (11–371)
Albumin (g/dl)[a]	4.1 (3.4–4.7)
Cholinesterase (ΔpH)[a]	1.0 (0.8–1.3)

T-Bill, total bilirubin; AST, aspartate transferase; ALT, alanine transferase.
[a]Data are expressed as median (range).
[b]Scoring according to the system of Desmet et al. [1994].
[c]Family history of positivity for hepatitis B surface antigen including third-degree relatives.

Methods

As indicators of lower activity of hepatitis, the rates of HBeAg negative, HBV-DNA undetectable (undetectable DNA levels by branched DNA signal amplification technology), and normal ALT level, were evaluated at two points; the start of lamivudine and last visit. The rates at the last visit were evaluated by including those patients who received additional treatment. Furthermore, the cumulative appearance rates of YMDD mutant by polymerase chain reaction (PCR)-based methods and breakthrough hepatitis (ALT becoming abnormal after a period of ALT normalization, accompanied by the emergence of YMDD mutant and re-elevation of HBV-DNA levels) were also evaluated. Clinical assessment and laboratory tests were performed at least once every month before, during, and after treatment. Adverse effects were monitored clinically by careful interviews and medical examination at least once every month. Patient compliance with treatment was evaluated with a questionnaire. All patients underwent abdominal ultrasonography every 6 months at least to exclude the development of cirrhosis and HCC.

Blood samples were obtained at least once every month before, during, and after treatment, and were analyzed for laboratory data including ALT levels, HBV-DNA levels, HBsAg, HBeAg/eAb, and YMDD motif mutant at various time periods. The serum samples were stored in aliquots at −80°C until use. HBsAg and HBeAg/eAb were determined by radioimmunoassay

(Abbott Diagnostics, Chicago, IL). HBV-DNA was measured by the branched DNA signal amplification technology (Chiron Corp., Emeryville, CA), and the results were expressed as genomic equivalents per milliliter (gEq/ml). The lower limit of the assay is 7.0×10^5 gEq/ml. HBV genotype and subgroups of genotype B were determined using the previously reported method [Okamoto et al., 1988; Usuda et al., 1999; Sugauchi et al., 2002]. Antibody against HCV was detected with chemiluminescent enzyme immunoassay (Lumipulse® II Ortho HCV, Ortho Diagnostic Japan, Tokyo). The YMDD motif mutant was detected using sensitive PCR-restriction fragment length polymorphism (PCR-RFLP) [Chayama et al., 1998].

Liver biopsy specimens were obtained percutaneously or at laparoscopy using a modified Vim Silverman needle of 2-mm internal diameter (Tohoku University style, Kakinuma Factory, Tokyo). Each specimen was scored according to the system of Desmet et al. [1994].

This study was conducted in accordance with the guidelines of the Declaration of Helsinki and its subsequent amendments, and informed consent was obtained from every patient. The study was approved by the Local Ethics Committee of Toranomon Hospital.

Statistical Analysis

Fisher's exact probability test was used to compare the rates of HBeAg negative, HBV-DNA undetectable, and ALT normal level between the start point of lamivudine and the last visit. The cumulative appearance rates of YMDD mutant and breakthrough hepatitis during lamivudine treatment were calculated using the Kaplan–Meier technique, and were evaluated from the start point of lamivudine until the last visit or discontinuation. Statistical comparisons were performed using the SPSS software (SPSS, Inc., Chicago, IL). All P-values of less than 0.05 by the two-tailed test were considered significant.

RESULTS

Efficacy Measures, Lamivudine Resistance, and Safety For the Whole Group

The clinical course of 20 patients is shown in Figure 1. For the whole group, the rates of HBeAg negative, HBV-DNA undetectable, and ALT normal level were 55% (11/20), 25% (5/20; HBV-DNA levels of five patients were detectable directly before treatment, but undetectable at the start by chance), and 20% (4/20) at the start of lamivudine treatment, respectively. Five patients received additional treatment for breakthrough hepatitis before or until the last visit. The rates of HBeAg negative, HBV-DNA undetectable, and ALT normal level were 85% (17/20), 80% (16/20), and 80% (16/20) at the last visit, respectively. The rates of HBeAg negative ($P = 0.082$), HBV-DNA undetectable ($P = 0.0012$), and ALT normal level ($P = 0.00036$) at the last visit tended to be or were significantly higher than those at the start of lamivudine treatment.

YMDD mutant was not detected in any of the pretreatment serum samples. For the whole group, YMDD motif mutant and breakthrough hepatitis appeared in 65% (13/20) and 45% (9/20) during lamivudine monotherapy, respectively. Additional treatment was provided to 55.6% (5/9) of patients who developed breakthrough hepatitis, while the other 15 patients received lamivudine monotherapy until the last visit or the time of discontinuation. The cumulative appearance rates of YMDD mutant and breakthrough hepatitis were 70.2% and 46.4% at the end of 5 years; 70.2% and 52.3% at the end of 8 years, respectively (Fig. 2). None developed severe adverse events during long-term lamivudine treatment, apart from the development of breakthrough hepatitis.

Clinical Course of 13 Patients With YMDD Motif Mutant

During lamivudine monotherapy, 69.2% (9/13) patients with emergence of YMDD mutant developed breakthrough hepatitis. In these patients, seven continued lamivudine therapy after the development to breakthrough hepatitis, and additional therapy was provided to three of the seven patients (two patients received interferon [IFN], and one received glycyrrhizin [Stronger Neo-Minophagen C®]). The remaining two patients discontinued lamivudine therapy after the development of breakthrough hepatitis, and instead received IFN therapy. At the last visit, ALT normal level and DNA undetectable were detected in 55.5% (5/9) and 66.7% (6/9) of patients who developed breakthrough hepatitis, respectively. Especially, 80% (4/5) of patients who received suitable additional treatment for breakthrough hepatitis, irrespective of lamivudine therapy, were ALT normal level at the last visit in contrast to 25% (only 1/4 patients) of patients untreated for breakthrough hepatitis.

In patients with emergence of YMDD mutant, 30.8% (4/13) did not develop breakthrough hepatitis. At the last visit, 100% (all 4 patients) and 75.0% (3/4) of patients, who did not develop breakthrough hepatitis, were ALT normal level and DNA undetectable, respectively, irrespective of continuous lamivudine therapy.

Clinical Courses of Seven Patients Without YMDD Motif Mutant

All seven patients free of YMDD mutant were HBeAg negative, DNA undetectable, and ALT normal level at the last visit, irrespective of continuous lamivudine therapy. Three patients who transiently discontinued the treatment showed ALT relapse after the cessation of lamivudine therapy, but their ALT levels became stable spontaneously without any additional treatment.

Efficacy Measures According to Patients' Background

The rates of HBeAg negative, HBV-DNA undetectable, and ALT normal level at the two points of the start of lamivudine and the last visit are shown in Figure 3,

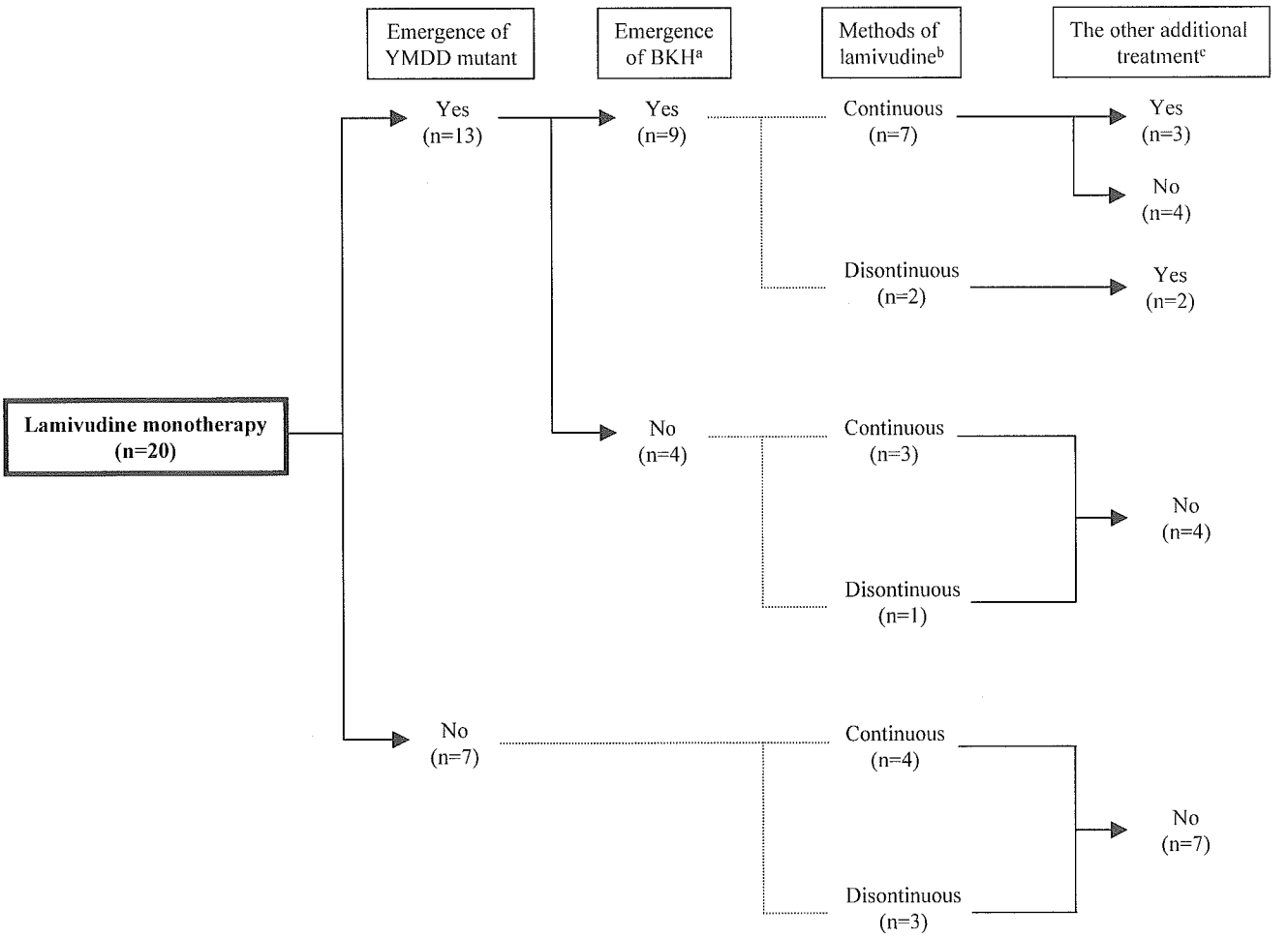

Fig. 1. Clinical course of 20 patients. YMDD motif mutant and breakthrough hepatitis (ALT becoming abnormal after a period of ALT normalization, accompanied by emergence of YMDD mutant and re-elevation of HBV-NA levels) appeared in 65% and 45% during lamivudine monotherapy, respectively. [a]BKH, breakthrough hepatitis. [b,c]The methods of lamivudine treatment and the other additional treatment for BKH were decided at patients' own requests. For the whole group, 30% discontinued lamivudine treatment during follow-up. Additional treatment was provided to 55.6% of patients who developed breakthrough hepatitis.

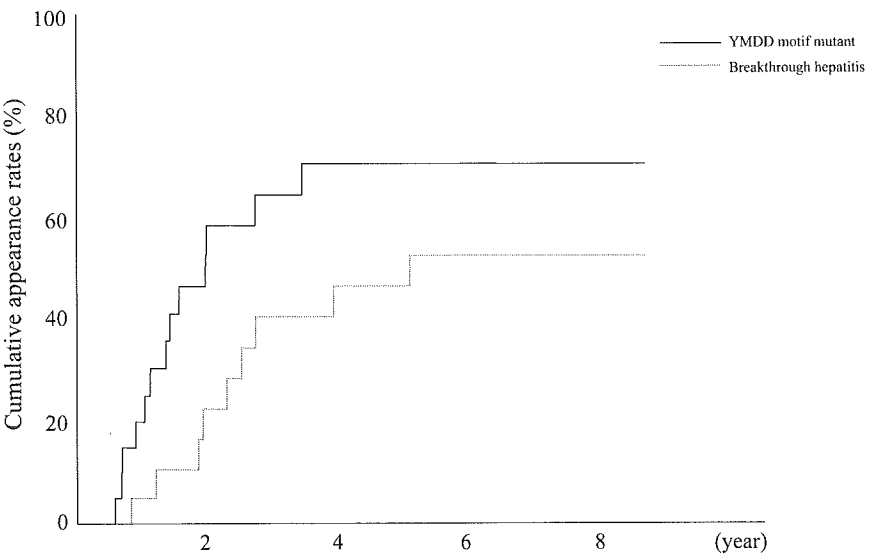

Fig. 2. Cumulative appearance rates of YMDD motif mutant and breakthrough hepatitis throughout follow-up.

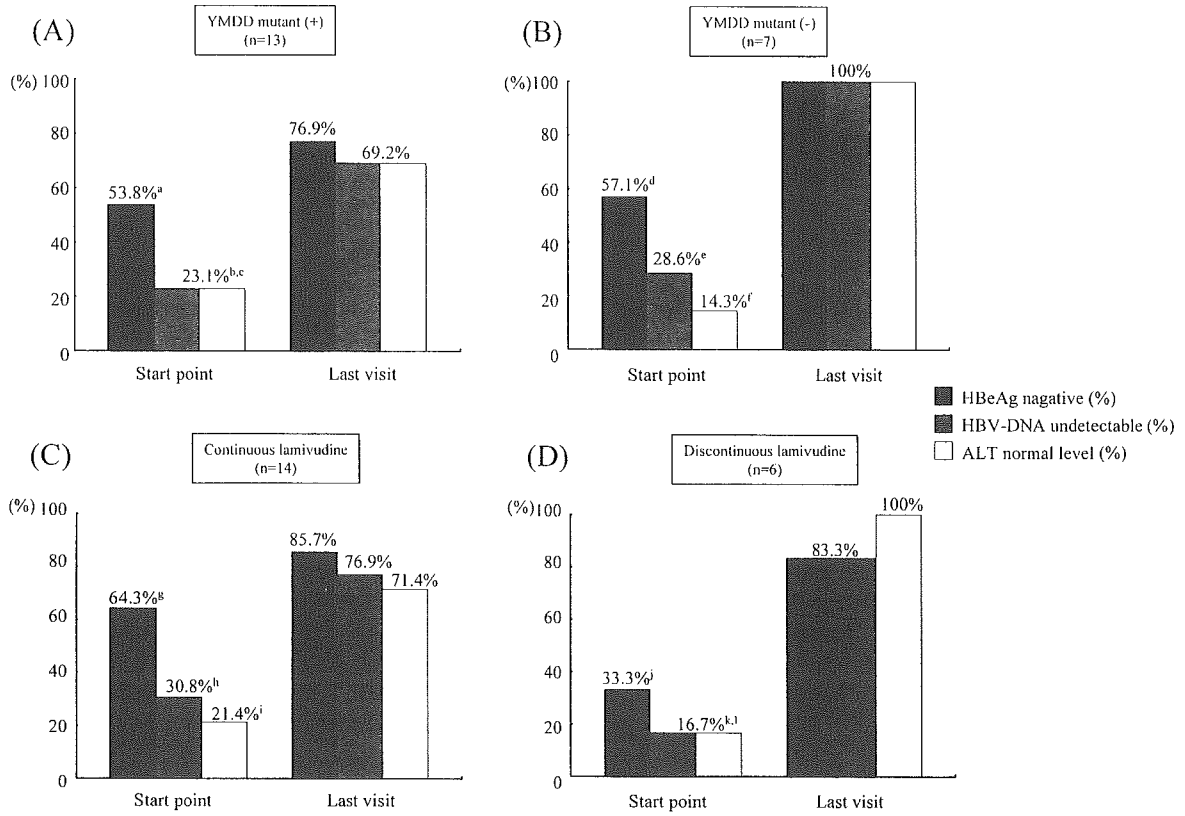

Fig. 3. The rates of HBeAg negative, HBV-DNA undetectable, and ALT normal level at the start of lamivudine treatment and last visit. The results at the last visit were evaluated by including five patients who received additional treatment. [a,d,g,j]Not significant, compared with HBeAg negative rates at each last visit. Fisher's exact probability test. [b]$P = 0.047$, [e]$P = 0.021$, [h]$P = 0.047$, [k]$P = 0.080$, compared with HBV-DNA undetectable rates at each last visit by Fisher's exact probability test. [c]$P = 0.047$, [f]$P = 0.0047$, [i]$P = 0.021$, [l]$P = 0.015$, compared with ALT normal rates at each last visit by Fisher's exact probability test.

according to the emergence of YMDD mutant and lamivudine therapy. The rates of HBV-DNA undetectable and ALT normal level at the last visit tended to be or were significantly higher than those at the start, regardless of the emergence of YMDD mutant and continuous lamivudine therapy. The rate of HBeAg negative at the last visit was also higher than at the start, even when there was no significance based on the relatively small numbers of HBeAg-positive patients at the start of lamivudine therapy.

In five patients who received additional treatment for breakthrough hepatitis, the rates of HBeAg negative, HBV-DNA undetectable, and ALT normal level were 20 (1/5), 0 (0/5), and 0% (0/5) at the start of lamivudine; and 60 (3/5), 60 (3/5), and 80% (4/5) at the last visit, respectively. The rates of ALT normal level at the last visit were significantly higher than at the start ($P = 0.048$). The rates of HBeAg negative and HBV-DNA undetectable at the last visit were also higher than at the start, though statistically insignificant due to the small number of patients.

HBsAg Clearance With Lamivudine Therapy

HBsAg clearance by radioimmunoassay was noted in 10% (2 males of 20 patients) of the whole group during follow-up, and they showed undetectable HBV-DNA levels using a sensitive quantitative PCR assay (Amplicor HBV Monitor Test, Roche Molecular Systems, Inc., NJ). The lower limit of this assay is 2.6 log copies/ml. The characteristics of these two male patients who could achieve HBsAg clearance by radioimmunoassay are shown in Table II. Furthermore, HBsAg clearance occurred in 33.3% (2/6) cases of the discontinuation group and none of the continuous (0/14). The discontinuation group tended to achieve higher rates of HBsAg clearance than the continuous group ($P = 0.079$).

One patient with HBsAg clearance was infected with HBV genotype C (HBV/C), the major type in Japan, and was a relatively young adult under 40 years of age who developed HBV by non-vertical transmission (i.e., patients whose mothers did not suffer from chronic HBV infection). He developed breakthrough hepatitis, especially according to continuous YVDD type [Akuta et al., 2003c] of YMDD motif mutant, and developed HBsAg clearance at about 5 years after the cessation of long-term lamivudine treatment followed by IFN treatment for breakthrough hepatitis.

The other patient with HBsAg clearance was infected with HBV/D; a rare type in Japan, and the infection was considered not to be transmitted vertically. The YMDD motif mutant did not emerge during long-term