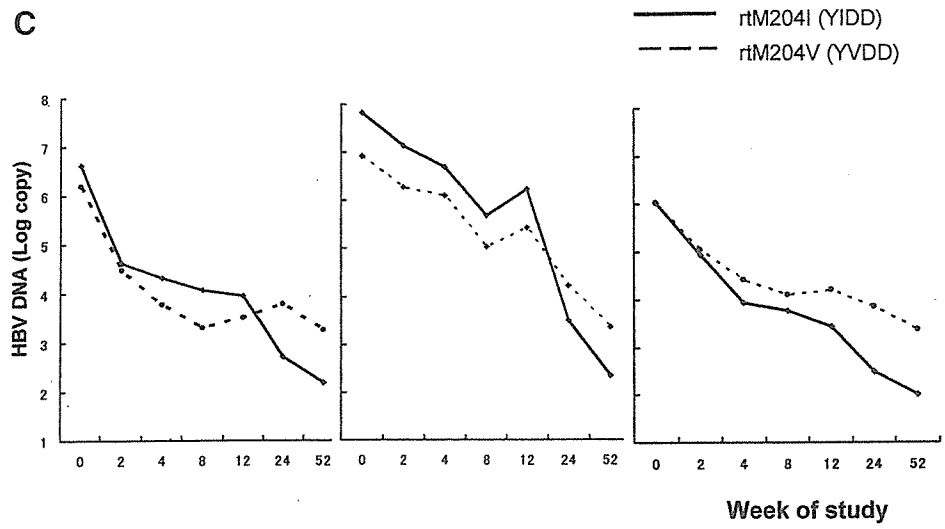

Fig. 3. (*Continued*)

(Fig. 3A). In three patients where the viral load of either rtM204I or rtM204V was major (i.e., viral load of the major mutant was more than 10-fold that of the minor mutant) at commencement of therapy, only the major mutant was detected at 52 weeks (Fig. 3B). However, in three patients where the viral loads of both mutants were similar (i.e., viral load of major mutant was within 10-fold that of minor mutant) at commencement of therapy, rtM204V predominated over rtM204I at 52 weeks (Fig. 3C).

Changes in Precore and Core Promoter Sequences Before and During Therapy

Precore and core promoter sequences in 15 patients were analyzed over 1 year of treatment with coadministered ADV in addition to ongoing lamivudine therapy for lamivudine breakthrough hepatitis. There was no clinical or virological evidence of breakthrough during lamivudine and ADV combination treatment in all 15 patients on ongoing lamivudine therapy. Precore sequences at baseline for lamivudine were the same as those at baseline for ADV in 9 of 10 patients (excluding 5 lacking lamivudine baseline data) (Table II). Analysis of serum samples obtained at ADV baseline revealed a precore stop codon mutation (A1896) in 9 of 15 patients, among whom A1896 occurred as a mixed population with wild-type virus (G1896) in 6 and as a pure population in 3 patients. After coadministration of ADV, A1896 was replaced with wild-type virus in three patients at 1 year and with mixed-type virus in one patient. In particular, among five patients without HBeAg at 1 year, including 2 HBeAg-seronegative patients, A1896 was replaced with wild-type virus in two and by mixed-type virus in one patient. Thus, A1896 was observed in three of eight patients, excluding seven PCR-negative patients, at 1 year.

The core promoter sequences at baseline for lamivudine therapy were the same as those at baseline for ADV in 9 of 10 patients (Table II). Among 15 patients, all had core promoter mutations in samples collected at baseline for ADV therapy. During treatment, the core promoter mutations were replaced with the wild-type in one patient (Patient 6) at 1 year. In this patient, a precore stop codon mutation was also replaced with a wild-type sequence.

Changes in Viral Sequences of Polymerase Reverse Transcriptase Before and During ADV Therapy

Hepatitis B virus DNA levels in all 15 patients coadministered ADV during ongoing lamivudine therapy were below 3,000 copy/ml at 1 year. Analysis of the rt region sequences (amino acid 1–344) of HBV polymerase in seven patients, excluding eight patients who were PCR-negative after ADV for 1 year, showed amino acid substitutions in the rt region in all seven (Fig. 4). Compared with baseline for lamivudine, there were new substitutions at baseline for ADV in all patients. Substitutions after 1 year of ADV, however, were very similar to those at ADV baseline in five patients (Patients 2, 3, 4, 7, 9). Interestingly, the YMDD motif in two patients (Patients 5 and 6) was replaced with wild-type (rt204M/YMDD) after 1 year of ADV. Substitutions in these two patients were fewer and of a different type than those at ADV baseline. Furthermore, Amplicor HBV Monitor assay showed that their HBV DNA levels were negative at 12 weeks after the start of ADV and fell to a greater extent than those of the other patients. This finding suggests that ADV may suppress YMDD mutants more than wild-type virus in some patients.

DISCUSSION

Mutations leading to lamivudine resistance are generally detected by conventional DNA sequencing after PCR amplification of a selected portion of the viral

TABLE II. Serial Precore and Core Promoter Sequences of Patients Treated With Lamivudine and Adefovir Dipivoxil

		Lamivudine					Adefovir dipivoxil									
		Baseline					Baseline					1 Year				
					CP nt					CP nt					CP nt	
Patient	Genotype	eAg	YMDD motif	Precore nt 1896	1762	1764	eAg	YMDD motif	Precore nt 1896	1762	1764	eAg	YMDD Motif	Precore nt 1896	1762	1764
1	C	ND	ND	ND	ND	ND	+	I+V	G/A	T	A	+	I	A	T	A
2	C	+	M	G	T	A	+	I+V	G	T	A	+	I+V	G	T	A
3[a]	C	+	M	G/A	T	A	+	I+V	G/A	T	A	−	V	G	T	A
4	C	+	M	G/A	T	A	+	I+V	G/A	T	A	+	I+V	G	T	A
5	C	+	M	G	T	A	+	I+V	G	T	A	−	M	G/A	T	A
6[a]	C	−	M	G/A	T	A	−	I+V	G/A	T	A	−	M	G	A	G
7	C	−	M	G	T	A	−	I	A	T	A	−	I	G/A	T	A
8	C	+	ND	ND	ND	ND	+	I+V	G	T	A	+	V	N	N	N
9	C	+	M	G	T	A	+	I	G	T	A	+	I	N	N	N
10	A	−	M	G	T	A	−	V	G	T	A	−	N	G	T	A
11	C	ND	ND	ND	ND	ND	+	I+V	G/A	T	A	+	N	N	N	N
12	C	ND	ND	ND	ND	ND	+	I+V	G	T	A	+	N	N	N	N
13[a]	C	+	M	G/A	A/T	G/A	+	I	G/A	T	A	+	N	N	N	N
14[a]	C	+	M	A	T	A	−	I	A	T	A	−	N	N	N	N
15	C	−	ND	ND	ND	ND	−	I+V	A	T	A	−	N	N	N	N

Baseline, time of the beginning of therapy; PCR, core promoter; ND, not done; N, PCR-negative; eAg, HBeAg; YMDD motif; M, rt204M; I, rtM204I; V, rtM204V; I+V, mixed type (rtM204I+rtM204V).
[a]Received lamivudine, adefovir dipivoxil, and interferon therapy.

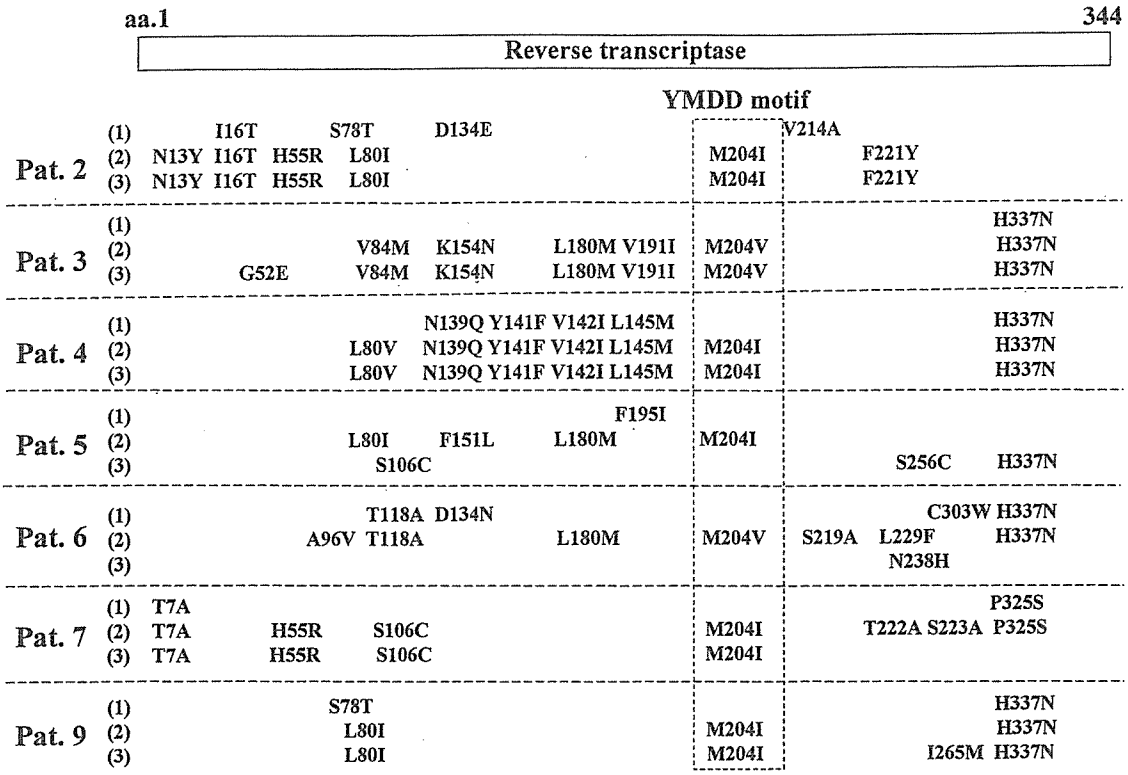

Fig. 4. Changes in viral sequences of polymerase reverse transcriptase before and during therapy. Measurements were taken at three time points: (1) start of lamivudine therapy, (2) start of coadministration of ADV with ongoing lamivudine therapy against breakthrough hepatitis, and (3) after coadministration with ADV for 1 year. Patient numbers are the same as in Table II. L180M denotes the substitution of leucine with methionine at amino acid position 180 in the reverse transcriptase region of HBV polymerase.

polymerase gene. The sensitivity of sequencing for minority quasispecies is low, however, with detection in most cases limited to no more than 20% of the total viral population [Gunthard et al., 1999]. Other molecular techniques developed to detect changes associated with lamivudine resistance include PCR-RFLP, a 5′ nuclease assay, and line probe assay technology [Chayama et al., 1998; Stuyver et al., 2001; Whalley et al., 2001].

Punia et al. [2004] first reported that rtM204I, rtM204V, and rt180M viral loads could be measured by real-time ARMS-PCR. However, their report included data from only a few cases. Here, we measured sequential viral loads of mutants during coadministration of ADV in addition to established treatment with lamivudine and showed that the viral loads of rtM204I, especially without HBeAg, decreased at the most rapid rate. This finding indicates that ADV therapy has a more suppressive effect against rtM204I. Moreover, when viral loads of both mutants (rtM204I and rtM204V) were similar at commencement of ADV therapy in patients with mixed-type virus, rtM204V predominated over rtM204I at 52 weeks. Considering these findings, the rtM204I may be more sensitive to ADV in vivo. On the other hand, it was reported that ADV was an equally effective inhibitor of rtM204I and rtM204V replication in vitro, and suppressed the rtL180M to an even greater extent [Chin et al., 2001; Ono et al., 2001]. With respect to the effectiveness of ADV against rtM204I and rtM204V, our data (in vivo) differ from that of previous studies (in vitro). Moreover, suppression of the rt180M was linked to that of the rtM204I or rtM204V and the rt180M viral load was influenced by those of rtM204I or rtM204V in vivo. However, it is not clear why ADV was apparently more effective against the rtM204I in vivo, and further studies are necessary to investigate this issue.

On the other hand, the log viral load change for rtM204V was greater than that for rtM204I during IFN coadministration with ongoing lamivudine, although the difference was not statistically significant. However, the number of patients undergoing IFN therapy was small and further studies of larger population samples are necessary to confirm this finding. On the other hand, our previous study showed that the suppression of lamivudine-resistant virus by long-term IFN + lamivudine therapy was clinically insufficient, with only 38% of patients achieving negative HBV DNA status [by branched DNA assay] after 6 months of IFN (unpublished data). On this basis, the long-term clinical efficacy of ADV added to ongoing lamivudine therapy is apparently superior to that of IFN coadministration.

During lamivudine therapy, precore mutants tend to be replaced with wild-type virus at 1 year, and this

change is unrelated to the emergence of YMDD motif mutations [Cho et al., 2000; Suzuki et al., 2002]. On the other hand, patients who showed mutations in the YMDD motif during long-term lamivudine therapy also exhibited the reappearance of precore mutants [Suzuki et al., 2002]. However, the addition of ADV to ongoing lamivudine therapy appeared to result in the preferential selection of wild-type virus, similar to the case of initial lamivudine therapy, although only a few cases were tested. This finding suggests that antiviral nucleoside analogues, such as lamivudine and ADV, selectively suppress precore mutants over wild-type virus. On the other hand, core promoter mutations at 1 year were replaced with wild-type in only one patient (Patient 6). It has been reported that core promoter mutations during lamivudine therapy also tended to be replaced at 1 year by wild-type virus [Cho et al., 2000; Suzuki et al., 2002], and more recently that three of five seroconverters of HBeAg harbored core promoter mutations at baseline that were progressively replaced with wild-type genome during ADV monotherapy [Werle et al., 2004]. However, our present study showed that, compared to initial lamivudine therapy or ADV monotherapy, coadministration of ADV with ongoing lamivudine therapy might be less effective against core promoter mutants than wild-type virus.

With regard to ADV monotherapy, several mutations in the HBV polymerase rt region have been observed during this treatment [Yang et al., 2002; Westland et al., 2003]. Moreover, selection of the rtN236T polymerase mutant was associated with resistance to ADV [Angus et al., 2003; Villeneuve et al., 2003]. Few data are available on sequencing of the rt region during coadministration of ADV with ongoing lamivudine therapy. Mutations after 1 year of coadministration of ADV and lamivudine were very similar to those at coadministration baseline. However, the YMDD motif mutation in two patients was replaced with wild-type (rt204M) at 1 year after coadministration, and another mutation pattern within the rt region was also changed. Moreover, in Patient 6, precore and core promoter mutations were replaced with wild-type at 1 year after coadministration. These findings suggest that ADV may selectively suppress lamivudine-resistant virus, and that wild-type virus may predominate in patients in whom drug efficacy is high, although the status of the rt region in eight patients whose PCR was negative at 1 year could not be ascertained.

In conclusion, we analyzed changes in rtM204I, rtM204V, and rtL180M viral loads in patients with HBV cotreated with lamivudine and ADV for lamivudine-resistant virus. The changes in rtM204I and rtL180M viral loads were greater than that of rtM204V, although the difference was not statistically significant. This finding was also clarified by analysis of serial changes in rtM204I and rtM204V viral loads. Moreover, the change in rtM204I viral load without HBeAg was greatest. Precore wild-type virus was apparently preferentially selected by the coadministration of ADV with lamivudine, in the same way that it was by initial lamivudine therapy at 1 year. Moreover, analysis of the rt region showed that ADV may suppress lamivudine-resistant virus and that wild-type virus may predominate. A better efficacy of ADV was noted against HBeAg-negative (and/or precore mutant) and lamivudine-resistant virus. Further studies are necessary to correlate virological changes and clinical efficacy during longer coadministration of ADV with ongoing lamivudine therapy for lamivudine-resistant virus.

ACKNOWLEDGMENTS

The authors thank Junko Satoh, Sachiyo Watahiki, and Marie Matsuda for their support. This study was supported in part by a Grant-in Aid from the Ministry of Health, Labor and Welfare, Japan.

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Intervirology

Intervirology 2006;49:82–90
DOI: 10.1159/000087268

Anticarcinogenic Impact of Interferon on Patients with Chronic Hepatitis C: A Large-Scale Long-Term Study in a Single Center

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Key Words
Cirrhosis · Fibrosis · Hepatitis C virus · Hepatocellular carcinoma · Interferon

Abstract
Background: The anticarcinogenic capacity of interferon (IFN) was assessed in a cohort of Japanese patients with chronic hepatitis C en masse. ***Patients and Methods:*** The rate of hepatocarcinogenesis was analyzed in 2,166 patients with chronic hepatitis C, of whom 1,654 had received IFN therapy while 512 had not. ***Results:*** Crude rates of hepatocarcinogenesis in treated and untreated patients were 2.6 and 4.6% at the end of the 5th year, 5.8 and 12.7% at the 10th year and 13.9 and 23.9% at the 15th year (after completion of IFN therapy for those treated) ($p < 0.001$). IFN decreased the hazard ratio of carcinogenesis to 0.42 ($p < 0.001$) in multivariate analysis with adjustments for significant covariates including fibrotic stage, γ-glutamyl transpeptidase level, gender, platelet count and age. Among the 1,654 patients treated with IFN, 606 (36.6%) achieved persistent loss of hepatitis C virus (HCV) RNA and an additional 266 (16.1%) gained normal levels of alanine aminotransferase without loss of HCV RNA for 6 months or longer after the completion of IFN therpay. Cumulative rates of hepatocarcinogenesis in sustained virological responders and biochemical responders were 1.4 and 2.0% at the end of the 5th year, 1.9 and 3.6% at the 10th year and 1.9 and 7.5% at the 15th year, respectively. The hazard ratio of sustained virological response was 0.10 ($p < 0.001$), and that of biochemical response was 0.12 ($p < 0.001$). Normalization of aminotransferase levels after IFN therapy without loss of serum HCV RNA decreased hepatocarcinogenesis. ***Conclusion:*** IFN significantly decreased the rate of hepatocarcinogenesis in patients with chronic hepatitis C as a whole in Japan, even in those who fail to clear HCV RNA from serum.

Introduction

In most developed countries, hepatitis B virus (HBV) and hepatitis C virus (HCV) infections account for the great majority of hepatocellular carcinoma (HCC), with incidence rates dependent on the regional prevalence of these hepatitis viruses. HCV-associated HCC typically develops through a sequence of events that progress from chronic inflammation through fibrosis and cirrhosis accompanying dysplasia and ultimately to HCC. In our previous cohort study on Japanese patients with HCV-related cirrhosis [1], cumulative rates of developing HCC at 5, 10 and 15 years were 21.5, 53.2 and 75.2%, respectively. According to our observations of untreated patients with chronic hepatitis C [2], rates of hepatocarcino-

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genesis at 5, 10 and 15 years were estimated to be 4.8, 13.6 and 26.0%, respectively. The life expectancy of patients with HCV-related cirrhosis is largely influenced by the development of HCC in the clinical course. As the efficacy of radically curative therapies for HCC remains limited at best, and since a severe organ shortage does not provide with sufficient chances for liver transplantation, the prevention of HCC in patients with chronic liver disease is of great importance at the present.

Interferon (IFN) is effective in eliminating HCV and reducing serum levels of alanine aminotransferase (ALT) in some patients with chronic hepatitis C [3–6]. Reduced incidence of HCC in HCV-associated cirrhosis by IFN has been reported by many investigators including ourselves [7–14]; only a few studies have failed to find its benefit [15, 16]. However, many published studies had shortcomings in the study design, in terms of pooling patients who received IFN in diverse regimens, relatively short periods of follow-up despite a long incubation period of HCC, large numbers of dropouts and retrospective studies with historical controls. Moreover, almost all studies evaluated the activity of IFN to prevent HCC by comparing responders and nonresponders to the treatment. Due to difficulties in studying patients with chronic hepatitis C, a number of nonrandomized studies examined the effect of IFN on the incidence of hepatocarcinogenesis [17–20]. With invariable limitations in study design and interpretation of the results, these studies have disclosed useful information as regards the treatment of patients with chronic HCV infection.

In order to evaluate whether IFN can reduce the rate of carcinogenesis in patients with chronic hepatitis C, we compared 1,654 patients with IFN therapy with 512 patients without treatment in a single clinical center, who were adjusted for background features by the multivariate analysis. Therefore, the principal aims of our study were to show the role of IFN in preventing HCC in chronic hepatitis type C en masse and to establish the extent to which IFN decreases the rate of carcinogenesis as a sequel to chronic hepatitis C in a society.

Patients and Methods

Study Population

A total of 2,166 patients with chronic hepatitis were examined, whose initial sera tested negative for hepatitis B surface antigen by radioimmunoassay (Ausria, Dainabot, Tokyo, Japan) and positive for anti-HCV by the second-generation enzyme-linked immunosorbent assay (Dainabot); anti-HCV was tested in sera that had been stored frozen at −80°C. They included 1,421 men and 745 women aged 14–78 with a median of 50 years. They were all diagnosed with chronic hepatitis by liver biopsy with or without peritoneoscopy between 1970 and 2000 at the Department of Gastroenterology in Toranomon Hospital, Tokyo, Japan. Patients who had possibly developed HCC already at the time of diagnosis of hepatitis were strictly excluded from the study. In order to exclusively investigate hepatocarcinogenesis in HCV-related cirrhosis, patients coinfected with HBV were excluded.

Among the 2,166 patients with HCV-related hepatitis, 1,654 (76.4%) received IFN therapy, mostly since 1987 when IFN was available in Japan; new antivirals or anticarcinogenic treatments of viral cirrhosis, except for IFN, were not introduced in 1987 or thereafter in Japan. The remaining 512 patients did not receive IFN or any other antiviral therapies. This is a retrospective cohort study with historical controls composed of patients before 1987 and those who refused or could not receive IFN for various reasons since 1987.

Background and Laboratory Findings

Table 1 shows demographic profiles and laboratory data for the 1,654 patients treated with IFN and the 512 without receiving IFN since they were diagnosed with chronic hepatitis. There were more males, with a median age 3 years lower in treated than in nontreated patients. There were 299 treated patients (18.1%) with a history of alcohol intake ≥ 500 kg until the diagnosis of chronic hepatitis (corresponding to daily consumption of 3,000 ml of beer or 300 ml of whiskey for 20 years) and 113 (22.1%) untreated patients ($p < 0.001$). Because IFN was introduced to our hospital in 1987, the observation period was significantly shorter in the treated than in untreated patients (median 10.4 vs. 12.3 years; $p < 0.0001$).

Although all patients tested positive for HCV RNA during their clinical courses, tests for the concentration of HCV RNA in the initial serum was possible in 1,863 (86.5%) patients. HCV genotypes were analyzed by the serological typing method with a commercial kit (Kokusai Diagnostic Corporation, Kobe, Japan) in which the serological group 1 represented genotypes 1a and 1b, and group 2 stood for 2a and 2b genotypes. HCV in the serological group 2 was significantly more frequent in patients with IFN treatment than in those without. Concentration of HCV RNA was determined in the initial sera from 1,873 (86.5%) patients by the competitive polymerase chain reaction (PCR) method with the HCV probe assay (Chiron Corp., Emeryville, Calif., USA) or by PCR with Amplicor HCV Monitor kits (Roche Diagnostics Japan Co., Tokyo, Japan). High concentration of HCV ($\geq 10^6$ copies/ml by the competitive PCR or $\geq 10^6$ equivalents/ml by the HCV probe assay) was significantly more frequent in untreated than in treated patients ($p < 0.0001$). The stage of hepatic fibrosis was not different between the two groups.

Interferon Treatment and Judgment of the Effect

A total of 1,654 patients underwent IFN therapy in one or more treatment courses: 1,358 patients (82.1%) received IFN once, 240 patients (14.5%) twice, and the remaining 56 patients (3.4%) three times or more. Initial treatment was performed with natural or recombinant IFN-α (n = 1,238), natural IFN-β (n = 386) or both (n = 30). Regimens of IFN were variable: 926 (56.0%) patients received IFN 6–9 million units (MU) daily for 8 weeks, followed by 2 or 3 times per week for 16 weeks; 329 (20.0%) received IFN 6–9 MU daily for 2–4 weeks, followed by 3 times per week for 20–22 weeks; 185 (11.2%) underwent a short-course therapy with IFN

Table 1. Patient profiles and laboratory data at the diagnosis of chronic hepatitis

Factors	Interferon therapy		p value
	yes (n = 1,654)	no (n = 512)	
Male	1,110 (67.1%)	311 (60.7%)	0.024
Age, years	50 (16–72)	53 (21–78)	<0.001
History of transfusion	607 (36.7%)	229 (44.7%)	0.001
Family member with liver disease	426 (25.8%)	140 (27.3%)	0.47
Alcohol intake ≥ 500 kg	299 (18.1%)	113 (22.1%)	0.044
Observation period, year	10.4 (0.1–33.6)	12.3 (0.1–33.6)	<0.001
Laboratory data			
ALT, IU/l	63 (4–1,266)	67 (4–704)	0.098
AST, IU/l	106 (9–1,660)	96 (12–832)	0.0001
γ-GTP, IU/ml	62 (6–1,118)	70 (3–850)	0.39
Platelet counts, $\times 1{,}000/mm^3$	169 (27–433)	165 (35–560)	0.091
ICG R_{15}, %	14 (1–90)	16 (1–95)	0.003
AFP, ng/ml	4 (1–90)	5 (1–1,180)	0.42
HCV serological group			
Group 1, genotypes 1a/1b	1,021 (66.1%)	259 (81.4%)	<0.0001
Group 2, genotypes 2a/2b	488 (31.6%)	48 (15.1%)	
Undetermined	36 (2.3%)	11 (3.5%)	
HCV RNA concentration			
High[a]	937 (58.4%)	191 (71.3%)	<0.0001
Low[b]	668 (41.6%)	77 (28.7%)	
Histological stage of hepatitis			
F1, slight fibrosis	1,029 (62.2%)	298 (58.2%)	0.10
F2/F3, moderate/severe fibrosis	625 (37.8%)	214 (41.6%)	

AST = Aspartate aminotransferase; AFP = α-fetoprotein; ICG R_{15} = retention of indocyanine green at 15 min.

[a] HCV RNA concentration $\geq 10^6$ copies/ml by the competitive PCR or $\geq 10^6$ equivalents/ml by the HCV probe assay.

[b] HCV RNA concentrations less than high concentrations.

daily for 4–8 weeks; 128 (7.7%) were administered with intermittent IFN 3 times per week for 24 weeks; 72 (4.4%) had a prolonged course of IFN for 8–36 months; 8 (0.5%) received IFN-β 6 MU daily for 6–18 months, and the remaining 6 (0.4%) were given IFN-α combined with IFN-β for 4 months. The median dose of 624 MU was administered during the median period of 24 weeks. IFN for 24 weeks or longer was given to 83.2% of the patients. IFN therapy was usually initiated within a few months after the diagnosis of chronic hepatitis, and all patients were started on it within 12 months. The median interval between liver biopsy and initiation of IFN was 9 days.

Almost all the patients given IFN showed varied degrees of fever, chills, myalgia, headache and general malaise after the first injection. Most patients developed leukocytopenia and thrombocytopenia in various degrees. A significant thrombocytopenia $\leq 40{,}000/mm^3$ required a reduction of the IFN dose in 39 patients. IFN therapy was discontinued due to psychosis in 35 patients and ophthalmological symptoms in 12 patients. None of the patients developed decompensated liver disease with ascites, encephalopathy, jaundice or variceal bleeding. Although only 88 (5.3%) patients could not continue injection with IFN, studies for carcinogenesis were analyzed on the intention-to-treat basis.

The efficacy of IFN was judged by the clearance of HCV RNA from serum and ALT levels 12 months after the completion of treatment. Sustained virological response (SVR) was defined as persistent disappearance of HCV RNA after therapy, biochemical response (BR) as normal ALT levels without elimination of HCV RNA for at least 6 months after therapy, and no response (NR) as persistently elevated or transiently normalized ALT levels without loss of HCV RNA lasting for less than 6 months.

Follow-Up of Patients and Diagnosis of HCC

Patients were followed up monthly after diagnosis of chronic hepatitis in our outpatient clinic and monitored for hematological, biochemical and virological parameters. With their admission, during and after the treatment with IFN, weekly or biweekly follow-up was performed in almost all patients who received IFN. Imaging diagnosis was made once or twice per year in the majority of patients with ultrasonography or computed tomography. Angiography was performed only when HCC was highly suspected on imaging by ultrasonography or computed tomography.

When angiography pictured a characteristic hypervascular nodule specific for HCC in patients, histological confirmation was not required in the majority of them. Microscopic examinations of liv-

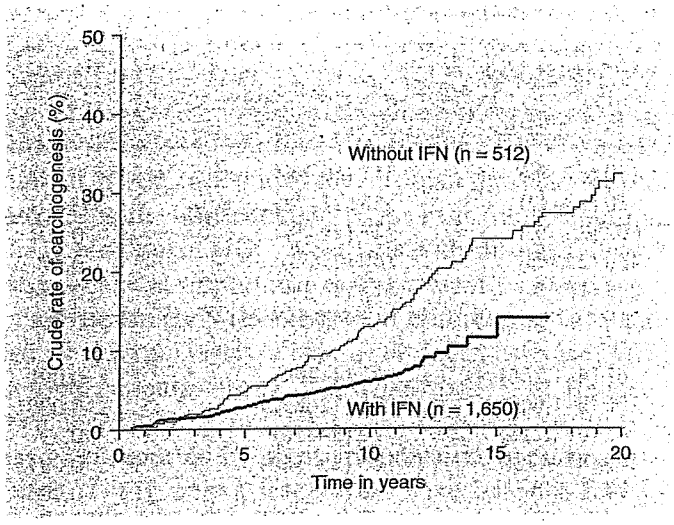

Fig. 1. Crude rates of hepatocarcinogenesis in patients treated with IFN and those untreated. The carcinogenesis rate was significantly lower in treated than in untreated patients (log-rank test, $p < 0.0001$).

er tissues obtained by a fine-needle biopsy were performed in 14 patients whose angiogram could not portray a typical image of HCC. There were 89 patients in whom HCC was confirmed histologically on liver specimens obtained at surgery or autopsy. Detection of serological tumor markers and increase with time were also taken into account in the diagnosis of HCC.

There were 223 (10.3%) patients lost to follow-up, including 164 (9.9%) treated and 59 (11.5%) untreated. Rates of annual dropouts in treated and untreated patients were 0.95 and 0.93%, respectively. In 9 patients, the response to IFN was judged by information on aminotransferase levels determined in other clinics and by persistent HCV RNA, as well as aminotransferase levels at 6 months after the completion of therapy in an additional 3 patients. Therefore, the response to IFN could be judged in all patients including the 12 who were lost to our follow-up early. Since the eventual outcome with respect to the development of HCC was not confirmed in these patients, their data were censored in statistical analyses [21]. Deaths unrelated to liver disease were censored and withdrawn from the analysis. The date of the last follow-up in this study was May 1, 2004, and the median observation period of studied patients was 10.7 years, with a range of 0.1–33.6 years.

Statistical Analysis

Nonparametric Mann-Whitney U test and χ^2 test were used for analysis of background characteristics of patients. The rate of HCC development was calculated by the Kaplan-Meier method [22]; it was based on the duration between diagnosis of chronic hepatitis by liver biopsy and detection of HCC. Differences in slopes of carcinogenesis curves were evaluated by the log-rank test. To gain a robust statistical power for the anticarcinogenic activity of IFN, observation of treated patients was initiated at the commencement of IFN therapy, in lieu of the diagnosis of chronic hepatitis. Independent factors associated with the development of HCC were studied using the stepwise Cox regression analysis [23]. The following 18 variables were analyzed for potential covariates in hepatocarcinogenesis at the time when hepatitis was diagnosed: age, sex, total alcohol intake, family history of liver disease, history of blood transfusion, stage of hepatic fibrosis, aspartic aminotransferase, ALT, albumin, bilirubin, globulin, γ-glutamyl transpeptidase (γ-GTP), platelet count, retention of indocyanine green at 15 min, serological grouping of HCV, HCV RNA level and IFN treatment.

Although continuous variables without conversion of data were evaluated in multivariate analyses, several variables were transformed into categorical data consisting of two or three ordinal numbers in calculating hazard ratios. All factors found to be marginally associated with hepatocarcinogenesis with p values <0.15 were tested by the multivariate Cox proportional hazard model. All analyses of data were performed with the computer program SPSS version 11 [24], and a p value <0.05 was considered significant.

Results

Response to IFN

Response to IFN was judged 12 months after the completion of therapy by both HCV RNA and serial ALT readings. Among the 1,654 patients with IFN treatment, SVR (elimination of HCV RNA) was achieved by 606 (36.6%), BR (ALT normalized for at least 6 months without clearance of HCV RNA from serum) in 266 (16.1%) and NR (elevated or transiently decreased ALT levels without loss of serum HCV RNA) in 782 (47.3%).

Crude Rates of Hepatocarcinogenesis

During the median observation period of 10.7 years, HCC developed in 199 of the 2,166 (9.2%) patients, including 96 of the 1,654 (5.8%) patients treated with IFN and 103 of the 512 (20.1%) patients without IFN (fig. 1). Among the 199 patients with HCC, 140 (70.4%) imaged a typical hypervascular stain on angiography and dynamic computed tomography, while 59 failed to exhibit tumor stains on angiography. HCC in these 59 patients was confirmed histologically on liver specimens obtained at surgery or by fine-needle biopsy.

Crude rates of hepatocarcinogenesis in patients treated with IFN and those untreated were 1.3 and 1.8% at the end of the 3rd year (after the completion of therapy), 2.6 and 4.6% at the end of the 5th year, 5.8 and 12.7% at the 10th year and 13.9 and 23.9% at the 15th year, respectively (fig. 1). The carcinogenesis rate was significantly lower in patients treated with IFN than in untreated patients (log-rank test, $p < 0.0001$).

Impact of IFN on Hepatocarcinogenesis

During the observation period, HCC developed in 96 of the 1,654 (5.8%) patients treated with IFN, including

11 patients (1.8%) with SVR, 10 (3.8%) with BR and 75 (9.6%) with NR to IFN. Rates of hepatocarcinogenesis in patients with SVR, BR and NR were 0.7, 0.8 and 2.0% at the end of the 3rd year, 1.4, 2.0 and 3.8% at the 5th year, 1.6, 2.9 and 6.5% at the 7th year, 1.9, 3.6 and 9.6% at the 10th year and 1.9, 7.5 and 27.6% at the end of 15th year (fig. 2). Hepatocarcinogenesis was significantly less frequent in patients with SVR or BR than in patients with NR and those untreated (log-rank test, $p < 0.0001$).

Factors Influencing Hepatocarcinogenesis

Univariate analysis identified 9 factors significantly associated with carcinogenesis. They were fibrotic stage ($p < 0.001$), age ($p < 0.001$), α-fetoprotein ($p < 0.001$), aspartic aminotransferase ($p = 0.001$), retention of indocyanine green at 15 min ($p = 0.002$), total alcohol intake ($p = 0.002$), γ-GTP ($p = 0.005$) and HCV serotype ($p = 0.045$). IFN therapy ($p = 0.064$), histological activity of hepatitis ($p = 0.069$) and ALT ($p = 0.70$) were marginally associated with carcinogenesis.

In order to prove the role of IFN on carcinogenesis in patients with chronic hepatitis type C en masse, multivariate analysis was performed by non-time-dependent proportional hazard analysis. Fibrotic stage, γ-GTP, gender, IFN therapy, platelet count and age independently influenced the development of HCC in the cohort (table 2). Advanced liver fibrosis in F2/F3 stages imposed a higher risk for carcinogenesis with a hazard ratio of 8.68, 95% confidence interval (CI) 5.08–14.81, compared with the F1 stage. Similarly, higher γ-GTP levels (hazard ratio 2.64), male sex (2.38), low platelet count (2.22) and older age (1.90) posed higher carcinogenesis risks. After adjusting background clinical biases between treated and untreated patients for the 5 significant covariates identified in the multivariate analysis, IFN therapy significantly decreased the hepatocarcinogenesis rate in the entire patients with chronic hepatitis C with a hazard ratio of 0.42 (95% CI 0.29–0.61) in comparison with untreated patients.

Based on the multivariate analysis, curves of carcinogenesis rates were theoretically illustrated in treated and untreated patients with the average histological stage, average γ-GTP value, average ratio of male to female, average platelet count and average age (fig. 3).

Hazard of Hepatocarcinogenesis Stratified by the Response to IFN

Since the carcinogenesis rate in patients with SVR or BR was significantly lower than that of patients with NR or untreated patients by the product limit method, a mul-

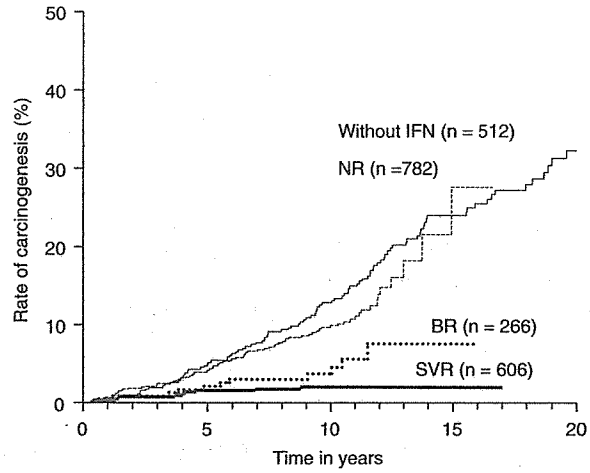

Fig. 2. Rates of hepatocarcinogenesis in patients with SVR, BR and NR to IFN. The rate in patients with NR (persistently elevated ALT or transiently normalized ALT for less than 6 months) was significantly higher than that in patients with SVR or BR.

Table 2. Factors associated with hepatocarcinogenesis in patients with chronic hepatitis C[a]

Factors	HR	95% CI	p value
Fibrosis stage			
F1	1		
F2–F3	8.68	(5.08–14.81)	<0.001
γ-GTP, IU/ml			
<50	1		
≥50	2.64	(1.58–4.42)	<0.001
Gender			
Women	1		
Men	2.38	(1.56–3.70)	<0.001
IFN therapy			
No	1		
Yes	0.42	(0.29–0.61)	<0.001
Platelet count, $\times 10^3/mm^3$			
≥100	1		
<100	2.22	(1.47–3.44)	<0.001
Age, years			
<50	1		
≥50	1.90	(1.27–2.85)	0.002

HR = Hazard ratio.
[a] Evaluated by the Cox proportional hazard analysis.

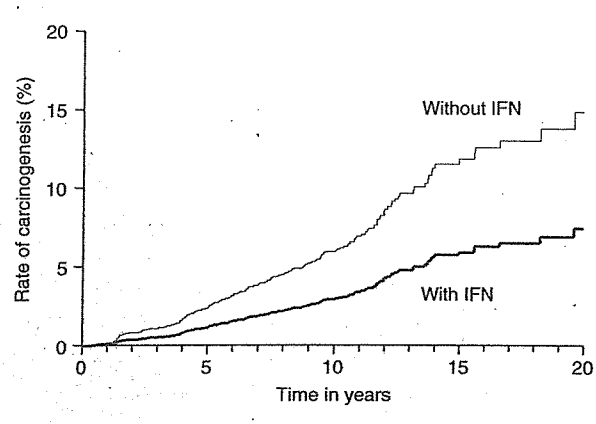

Fig. 3. Theoretical curves of hepatocarcinogenesis in patients treated with IFN and those untreated who have the average histological stage, average γ-GTP value, average ratio of male to female, average platelet count and average age. They are based on the analysis of 1,654 patients treated with IFN and 512 untreated patients.

Table 3. Factors associated with hepatocarcinogenesis in patients with chronic hepatitis C who had distinct responses to IFN therapy[a]

Factors	HR	95% CI	p value
Fibrosis stage			
F1	1		
F2–F3	9.90	(4.19–23.40)	<0.001
Gender			
Women	1		
Men	3.44	(1.89–6.25)	<0.001
γ-GTP, IU/ml			
<50	1		
≥50	2.68	(1.30–5.54)	0.008
Age, years			
<50	1		
≥50	2.56	(1.50–4.38)	0.001
AFP, ng/ml			
<20	1		
≥20	2.32	(1.34–4.02)	0.003
Platelet count, $\times 10^3/mm^3$			
≥100	1		
<100	2.09	(1.14–3.75)	0.013
Response to IFN			
Without IFN	1		<0.001
NR	0.57	(0.13–2.56)	0.46
BR	0.12	(0.04–0.35)	<0.001
SVR	0.10	(0.03–0.30)	<0.001

HR = Hazard ratio; AFP = α-fetoprotein.
[a] Evaluated by the Cox proportional hazard analysis.

tivariate analysis was performed taking into account the response to IFN. Hazard ratios of patients with SVR and BR to IFN therapy were 0.10 (95% CI 0.03–0.30, $p < 0.001$) and 0.12 (95% CI 0.04–0.35, $p < 0.001$), respectively, in comparison with that of untreated patients, when the other 5 factors served as significant covariates (table 3). The hazard ratio of NR at 0.57 (95% CI 0.13–2.56) was less than 1, but fell short of making a significant difference against untreated patients.

Mortality and Causes of Death

During the observation period, 116 of the 2,166 (5.4%) patients died, including 52 of the 1,654 (3.1%) subjects treated with IFN and 64 of the 512 (12.5%) subjects without IFN. Estimated survival rates in the treated and untreated patients were 99.3 and 98.3% at 5 years, 97.8 and 96.0% at 10 years and 93.8 and 86.9% at 15 years, respectively. The survival rate of treated patients was significantly higher than that of untreated patients (log-rank test, $p < 0.0001$).

Discussion

Based on our epidemiological data obtained by long-term observations of patients with chronic hepatitis [2] and patients with cirrhosis [1], the life expectancy of patients with HCV-related chronic liver disease heavily depends on the development of HCC. The possibility of eventually developing HCC in patients with HCV infection and cirrhosis is staggeringly high at 75% [1]. Theoretically, the treatment of chronic HCV infection with IFN can prevent the development of HCC. From the ethical point of view, a prospective randomized trial with control untreated patients is not to be allowed at present when IFN has become the standard radical therapy for chronic hepatitis C; everyone can receive IFN, as expenses are being covered for by the medical insurance in Japan. Another difficulty involves the informed consent in prospective randomized studies. It requires at least 5 years in order that IFN can decrease the incidence of carcinogenesis in chronic hepatitis C, with a statistical difference in the carcinogenesis rate between treated and 'untreated' patients. Since any randomized studies are considered extremely difficult in the future, we attempted to carry out this retrospective study by the multivariate analysis with statistical adjustments for possible covariates.

In the product limit analysis, IFN significantly decreased the crude rate of hepatocarcinogenesis in the

entire cohort of 2,166 patients with chronic hepatitis C. Since there were some background differences between treated and untreated patients, we tried to correct for biases including stage of fibrosis, γ-GTP value, sex, platelet count and age, which significantly affect the carcinogenesis rate. Demographic, histological and biochemical factors having been adjusted, IFN is proven to bring about a significant decrease in the hazard of carcinogenesis in patients with chronic hepatitis C en masse (hazard ratio 0.42, $p < 0.001$ by the non-time-dependent model). Taking into consideration that a significant number of patients without IFN had received anti-inflammatory medicines, which might have contributed to suppression of hepatocarcinogenesis, the actual anticarcinogenic activity of IFN may be higher than the observed. Having published results of a similar study on a cohort of 1,643 patients with a median observation period of 5.4 years in 1999 [18], we could not establish the anticarcinogenic activity of IFN because of a low risk of carcinogenesis in untreated patients (1.2% per year). Nevertheless, we expected a significant statistical difference if we could extend the median observation period to longer than 7 or 10 years in our studied patients. This has been realized in the present study, in which 2,166 patients with and without IFN therapy were observed for a median of more than 10 years. As far as we are aware, it represents the first study that has demonstrated preventive effects of IFN on the carcinogenesis rate in a large cohort of patients in a single center, in correlation with distinct responses to it, such as SVR, BR and NR.

Treatment of patients with chronic HCV infection using IFN-α and ribavirin has led to sustained loss of serum HCV RNA in 40–50% of recipients with HCV genotype 1 and 75–80% with HCV genotype 2 or 3. However, to date, the combination therapy with IFN-α and ribavirin has not been evaluated for its impact on the risk of developing HCC. Monotherapy with IFN-α achieves sustained clearance of serum HCV RNA in only 20–30% of patients; the impact of IFN-α on the development of HCC has been evaluated only in patients who had received IFN-α without ribavirin [17–20, 25–27].

Multivariate analysis definitively demonstrated that IFN lessens the carcinogenesis risk in the patients whose ALT levels decreased after therapy. Furthermore, the anticarcinogenic capacity of IFN was demonstrated not only in the patients with persistent aminotransferase normalization, but also in those with transient normalization of ALT for at least 6 or 12 months. Many authors have already described that the activity of IFN to suppress the development of HCC in patients with HCV RNA clearance (SVR) is similar to that in patients with ALT normalization in the absence of eliminating HCV RNA (BR) [18, 25–27]. Based on these compelling lines of evidence, the anticarcinogenic activity of IFN is ascribed to the suppression of inflammatory and regenerative processes in hepatocytes. Moreno and Muriel [28] reported that IFN reverts liver fibrosis, and therefore, control of the necroinflammatory process can suppress the growth of HCC. Tarao et al. [29] reported that high aminotransferase levels increase the rate of HCC recurrence in patients with cirrhosis. Our results stand in favor of the view that the carcinogenic process in patients with chronic hepatitis C would be enhanced by fluctuating as well as persistently elevated levels of aminotransferases. It does seem that IFN exerts suppressive effects on HCC through reduction or complete remission of inflammatory activity. Recently, a few authors reported that even transient disappearance of HCV RNA during IFN therapy contributed to a low carcinogenesis rate in the clinical course of hepatitis [17, 27]. The significance of transient HCV in decreasing hepatocarcinogenesis should be further explored and confirmed by multicenter clinical studies with rigorous virological assessments.

HCC developed in a few patients with SVR 5 years after the HCV infection had been terminated by IFN, along with normalized ALT levels. These patients would have developed minute HCC in their livers already while receiving IFN which escaped the detection by imaging modalities or screening for serological tumor markers. This would indicate the limitation of IFN in preventing HCC. IFN will not be able to suppress HCC once it has developed, even when it succeeds in eliminating HCV and suppressing necroinflammatory processes in the liver.

With many difficulties in vaccine development, the recent progress in treatment of chronic HCV infection, from IFN monotherapy to combination therapy with ribavirin, is very auspicious. SVR and BR can be achieved in up to 56% of patients with combined IFN and ribavirin [30]. There is evidence that a sustained virological response can lead to decrease in fibrosis and even reversal of cirrhosis [31]. Because HCV-associated HCC occurs almost exclusively in patients with cirrhosis, successful treatment for SVR in patients without cirrhosis is likely to prevent future development of HCC [32]. However, once cirrhosis has been established, a preventive benefit of IFN monotherapy is restricted to the patients who can achieve SVR or BR. In their meta-analysis of 3 randomized and 11 nonrandomized controlled trials, Camma et

al. [33] have reported a low but statistically significant preventive effect.

In conclusion, IFN significantly decreases the rate of hepatocarcinogenesis in patients with chronic hepatitis C, irrespective of the response to it.

Acknowledgement

This study was supported in part by a research grant from the Ministry of Health, Labour and Welfare in Japan.

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Journal of Medical Virology 78:1276–1283 (2006)

Response to Long-Term Lamivudine Treatment in Patients Infected With Hepatitis B Virus Genotypes A, B, and C

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Response to lamivudine treatment longer than 1 year was compared in 15 patients persistently infected with hepatitis B virus (HBV) genotype A, 38 with genotype B, and 449 with genotype C. Patients with genotype A were younger (median age 37 [range 24–49] vs. 47 [24–67] or 44 [18–73], $P=0.015$), possessed hepatitis B e antigen (HBeAg) more frequently (73% vs. 21% or 56%, $P<0.001$) and HBV DNA in higher levels (8.6 [6.1–8.7] vs. 6.5 [<3.7–8.7] or 6.5 [<3.7–8.7] log genome equivalents (LGE)/ml, $P=0.024$) than those with genotype B or C. During lamivudine, YMDD mutants (89% vs. 53% or 42%, $P=0.0001$) and breakthrough hepatitis developed more often (47% vs. 21% or 29%, $P=0.023$) in patients with genotype A than B or C. YMDD mutants elicited more frequently in patients with genotype A than B or C who were positive (82% [9/11] vs. 25% [2/8] or 48% [117/245], $P=0.037$) or negative for HBeAg (75% [3/4] vs. 30% [9/30] or 33% [68/204], $P=0.003$). HBeAg (hazard ratio 2.1 [95% confidence interval 1.53–2.92], $P<0.001$) and genotype A (2.78 [1.08–7.12], $P=0.034$) enhanced the emergence of YMDD mutants by the Cox proportional hazard model. The risk for breakthrough hepatitis was increased by the baseline alanine aminotransferase level <500 IU/L (2.56 [1.82–5.50], $P=0.018$), HBeAg (2.11 [1.40–3.16], $P<0.001$), cirrhosis (1.92 [1.24–2.97], $P=0.004$) and HBV DNA ≥8.0 LGE/ml (1.57 [1.04–2.36], $P=0.03$); it was influenced by genotypes only in patients with HBeAg. In conclusion, HBV genotypes help in predicting response to long-term lamivudine treatment and development of YMDD mutants in patients with chronic hepatitis B. ***J. Med. Virol. 78:1276–1283, 2006.*** © 2006 Wiley-Liss, Inc.

KEY WORDS: hepatitis B virus; chronic hepatitis; cirrhosis; genotypes; lamivudine

INTRODUCTION

Lamivudine has been favored in the treatment of patients with chronic hepatitis B since its approval in 1998 [Lai et al., 1997; Nevens et al., 1997; Dienstag et al., 1999; Suzuki et al., 1999]. A major drawback of lamivudine, however, is the development of hepatitis B virus (HBV) mutants resistant to it. They have mutations in the tyrosine-methionine-aspartate-aspartate (YMDD) motif of DNA polymerase/reverse transcriptase, and in the majority of patients emerge increasingly with the duration on lamivudine [Honkoop et al., 1997; Allen et al., 1998; Chayama et al., 1998; Liaw et al., 1999; Suzuki et al., 1999]. These mutants cause breakthrough hepatitis, and therefore, prohibit a long-term administration of lamivudine. Host and viral factors can increase the therapeutic efficacy of lamivudine. These include high serum levels of HBV DNA and the lack of hepatitis B e antigen (HBeAg) in serum at the baseline [Lai et al., 1998; Tassopoulos et al., 1999; Liaw, 2002; Rizzetto, 2002].

Eight genotypes have been determined by the sequence divergence >8% in the entire HBV genome of

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

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Accepted 3 May 2006
DOI 10.1002/jmv.20701
Published online in Wiley InterScience (www.interscience.wiley.com)

approximately 3,200 nucleotides (nt), and named by capital Alphabet letters from A to H in the order of discovery [Okamoto et al., 1988; Norder et al., 1992; Stuyver et al., 2000; Araùz-Ruiz et al., 2002]. It has not been established, as yet, whether or not HBV genotypes influence the response to long-term lamivudine and the emergence of YMDD mutants accompanied by breakthrough hepatitis [Zollner et al., 2001; Akuta et al., 2003; Chan et al., 2003; Yuen et al., 2003b; Moskovitz et al., 2005; Thakur et al., 2005].

As in other Asian counties, genotypes B and C have been prevalent in Japan, probably since the prehistoric era [Orito et al., 1989]. Recently, however, infection with genotype A has been increasing predominantly in young men with promiscuous homo- or hetero-sexual contacts [Kobayashi et al., 2002, 2004; Ogawa et al., 2002]. Infection with HBV genotype A can persist, even if contracted in adulthood, in about 10% of cases [Sherlock, 1987; Kobayashi et al., 2006]. These circumstances provided an opportunity to compare the efficacy and side effects of long-term lamivudine, among patients infected with HBV genotypes A, B, and C, in a single Hepatology Center in Metropolitan Tokyo.

MATERIALS AND METHODS

Patients

During almost 10 years from September 1995 through July 2004, 502 patients infected persistently with HBV and diagnosed with chronic liver disease received oral lamivudine 100 mg per day for longer than 1 year. Genotypes were A in 15 (2.6%) patients, B in 38 (7.6%) and C in the remaining 449 (89.4%). The median age was 44 years (range: 18–73 years) and included 407 (81%) men were included. Of these, 426 (84.9%) had chronic hepatitis and the remaining 73 (14.5%) possessed cirrhosis. Chronic hepatitis was diagnosed by liver biopsies performed under laparoscopy, and cirrhosis by liver biopsy and/or ultrasonographic images plus laparoscopic findings. The median serum level of HBV DNA was 7.2 log genome equivalents (LGE)/ml, and HBeAg was positive in 264 (52.6%) of them. They were given lamivudine for a median of 6.9 years (range: 1–10.2 years) and followed for a median of 6.9 years (0.1–31.2); lamivudine was discontinued in only 62 (12.4%) patients.

During and after treatment, the 502 patients were followed monthly for liver function and serum markers of HBV infection. YMDD mutants were determined at the baseline, and monitored yearly as well as at the development of breakthrough hepatitis. The study design conformed to the 1975 Declaration of Helsinki, and was approved by the Ethic Committee of the institution. Every patient gave an informed consent for this study.

Serological Markers of HBV Infection

HBsAg was determined by hemagglutination (MyCell; Institute of Immunology Co. Ltd., Tokyo, Japan) or enzyme-linked immunosorbent assay (ELISA) (ELISA, F-HBsAg; Sysmex, Kobe, Japan), and HBeAg by ELISA (ELISA, F-HBe; Sysmex). HBV DNA was determined by transcription-mediated amplification and hybridization assay (TMA; Chugai Diagnostics, Tokyo, Japan) and the results were expressed in LGE/ml over a detection range from 3.7 to 8.7.

Determination of HBV Genotypes

The six major genotypes (A–F) were determined serologically by ELISA (HBV GENOTYPE EIA; Institute of Immunology). The method is based on the combination of epitopes on preS2-region products that is specific for each genotype [Usuda et al., 1999, 2000]. Genotype G was determined by preS2 serotype for genotype D and HBsAg subtype adw, and H by those for C and adw, respectively; these combinations are specific for genotypes G and H, respectively [Kato et al., 2001, 2004]. Thus, all the eight HBV genotypes were determined serologically.

Determination of YMDD Mutants

YMDD mutants were determined by restriction fragment length polymorphism (RFLP) [Chayama et al., 1998] and Enzyme-Linked Mini-sequence Assay with commercial assay kits (PCR-ELMA; Genome Science).

Statistical Analysis

Categorial variables were compared between groups by the Mann–Whitney *U* test and Fisher's exact test, and noncategorial variables by the Wilcoxon signed rank test. Loss of HBeAg, HBsAg or HBV DNA, emergence of YMDD mutants and development of breakthrough hepatitis were compared in the Kaplan-Meier life table, and differences were evaluated by the log-rank test with use of the production limit method. Factors independently influencing emergence of YMDD mutants and development of breakthrough hepatitis were evaluated in the Cox proportion hazard model. A *P*-value less than 0.05 was considered significant. Analysis of data was performed with the computer program SPSS software (SPSS, Inc., Chicago, IL).

RESULTS

Baseline Characteristics of Patients Treated by Long-Term Lamivudine

Patients infected with HBV genotype A, B, or C were compared before they were placed on long-term lamivudine therapy (Table I). Patients with genotype A were significantly younger, had higher levels of HBV DNA and HBeAg more frequently than those with genotype B or C. Men predominated in the patients infected with genotypes A, B, or C. There were no differences in the duration of treatment with lamivudine or severity of liver disease among patients infected with the three HBV genotypes.

TABLE I. Baseline Characteristics of Patients With Chronic Hepatitis B Who Received Lamivudine for Longer Than 1 Year

Features	Genotypes of HBV			Differences (*P*-value)
	A (n = 15)	B (n = 38)	C (n = 449)	
Age (years)	37 (24–49)	47 (24–67)	44 (18–73)	0.015
Men	14 (93%)	34 (91%)	359 (80%)	NS
Treatment duration (years)	2.7 (1.2–5.2)	2.3 (1.0–5.7)	3.6 (1.0–9.6)	NS
Chronic hepatitis	13 (87%)	33 (87%)	383 (85%)	NS
Cirrhosis	2 (13%)	5 (13%)	66 (15%)	NS
HBV DNA (LGE/ml)	8.6 (6.1–8.7)	6.5 (<3.7–8.7)	6.5 (<3.7–8.7)	0.024
HBeAg	11 (73%)	8 (21%)	245 (56%)	0.0001

Clearance of HBV Markers in Patients Treated by Long-Term Lamivudine

Time courses of clearance of HBeAg and HBsAg during long-term lamivudine are compared among patients infected with HBV genotypes A, B, and C in Figure 1a and 1b, respectively. The clearance of HBeAg or HBsAg was no different among patients infected with HBV of three genotypes during the first five years on lamivudine (Fig. 1a,b).

During lamivudine treatment for longer than 192 weeks (>3.7 years), HBV DNA disappeared from serum only in less than half patients with genotype A, significantly less frequently than in patients with genotype B or C; more than three quarters of them lost it (Fig. 2).

Emergence of YMDD Mutants and Development of Breakthrough Hepatitis During Long-Term Lamivudine Therapy

Emergence of YMDD mutants and development of breakthrough hepatitis were compared among patients infected with HBV of the three genotypes. During the first 4 years on lamivudine therapy, YMDD mutants emerged in 89% of patients with genotype A, significantly more often than in those with genotype B or C; such mutants elicited in only less than half of them

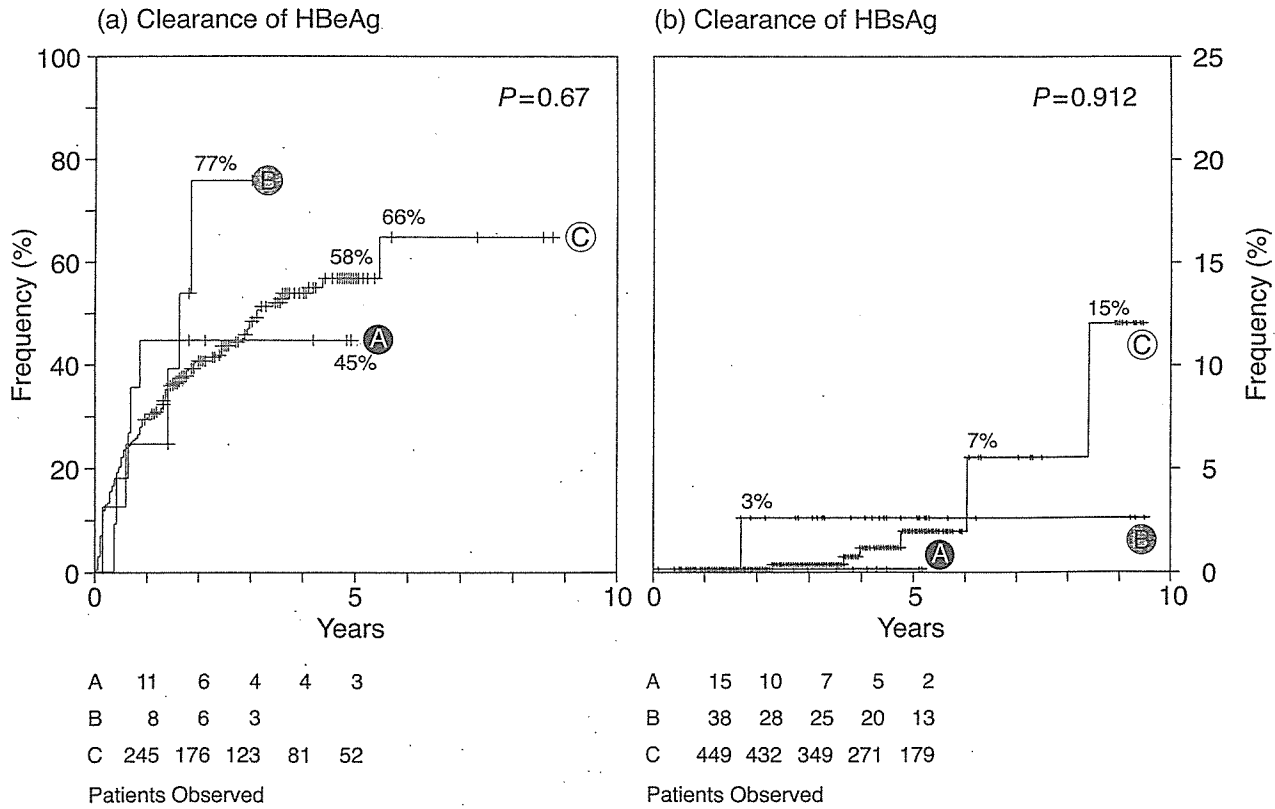

Fig. 1. Serum markers for HBV infection in patients on long-term lamivudine therapy. Patients infected with HBV genotypes A, B, or C are compared for the clearance of HBeAg (**a**) and HBsAg (**b**). Numbers of patients observed at each year are shown below for those infected with genotypes A, B, and C.

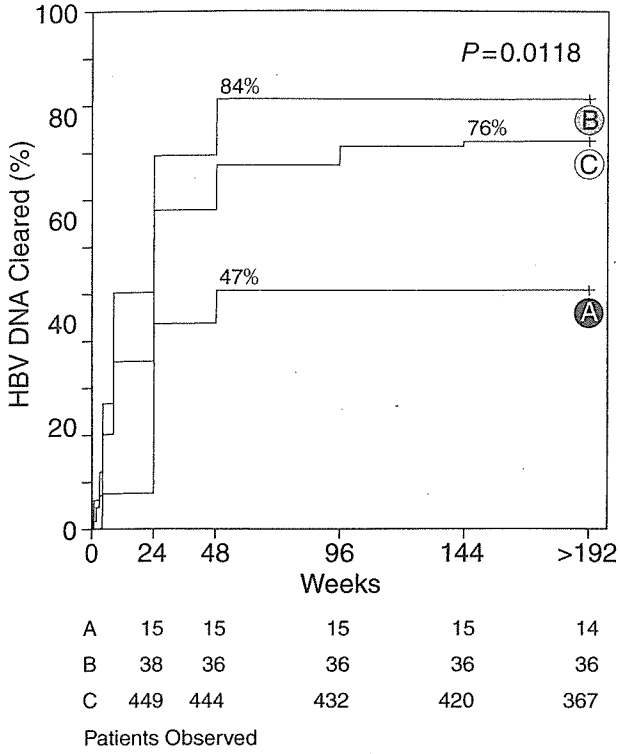

Fig. 2. Clearance of HBV DNA from serum of patients on long-term lamivudine therapy. Patients infected with HBV genotypes A, B, or C are compared. Numbers of patients observed at each year are shown below for those infected with genotypes A, B, and C.

(Fig. 3a). Reflecting the emergence of lamivudine-resistant mutants, breakthrough hepatitis developed twice more frequently in patients with genotype A than B or C (Fig. 3b).

YMDD mutants elicited more often in patients with genotype A than B or C who were positive (82% [9/11] vs. 25% [2/8] or 48% [117/245], $P = 0.037$) or negative for HBeAg (75% [3/4] vs. 30% [9/30] or 33% [68/204], $P = 0.003$).

Factors Influencing YMDD Mutants and Breakthrough Hepatitis

Risks for YMDD mutants and breakthrough hepatitis were evaluated on the nine variables. They included age, gender, liver pathology, cholin esterase, ALT, aspartic transaminase, HBV DNA, HBV genotypes, and HBeAg. In multivariate analysis, only HBeAg at the baseline and genotype A were independent factors significantly increasing the emergence of YMDD mutants (Table II).

Likewise, factors influencing the development of breakthrough hepatitis were evaluated by multivariate analysis (Table III). ALT <500 U/L, HBeAg, cirrhosis (present in about 15% of patients infected with any genotype (Table I)), and HBV DNA > 8.0 LGE/ml at the baseline independently increased the development of breakthrough hepatitis; genotypes did not make significant differences, however.

DISCUSSION

Long-term lamivudine therapy is beneficial for patients with chronic hepatitis B [Lok and McMahon, 2001; Dienstag et al., 2003; Kumada, 2003; Lok et al., 2003], and can retard the progression of fibrosis [Lai et al., 1998; Dienstag et al., 2003; Suzuki et al., 2003b]. Remarkably, treatment decreased the incidence of hepatocellular carcinoma from 7.4% to 3.9% during the median of 2.7 years [Liaw et al., 2004] and from 13.3% to 1.1% during 2.7 years or longer in a multicenter retrospective study [Matsumoto et al., 2005]. Emergence of YMDD mutants and breakthrough hepatitis, however, prohibit long-term treatment with lamivudine [Honkoop et al., 1997; Allen et al., 1998; Chayama et al., 1998; Liaw et al., 1999; Suzuki et al., 1999]. Such adverse events, however, can be managed by timely intervention with other antiviral drugs [Suzuki et al., 2002, 2003b]. Due to merits far outweighing its drawbacks, long-term lamivudine therapy has been favored for the treatment of patients with chronic hepatitis B.

Viral factors can influence the efficacy of lamivudine. Thus pretreatment low HBV DNA levels and absence of serum HBeAg enhance response to lamivudine [Lai et al., 1998; Tassopoulos et al., 1999; Liaw, 2002; Rizzetto, 2002]. Insofar as HBV genotypes make differences in the severity of liver disease and the development of hepatocellular carcinoma [Kao et al., 2000; Orito et al., 2001; Chu and Lok, 2002], they may affect the response to lamivudine, as well. There have been conflicting views, however, on the influence of HBV genotypes on the response to lamivudine [Kao et al., 2002; Chan et al., 2003; Yuen et al., 2003b; Moskovitz et al., 2005]. Geographical distribution of HBV genotypes hampers comparison among three or more genotypes in any single country. Mostly only two genotypes prevail, typified by B and C in Asia, and A and D in Western countries [Lindh et al., 1997; Miyakawa and Mizokami, 2003]. Even when four or more HBV genotypes were compared for response to lamivudine therapy, patients had been assorted from many countries with diverse ethnic backgrounds and distinct modes of transmission [Janssen et al., 2005].

As in the majority of Asian countries, genotypes B and C are common in Japan. Infection with genotype A, however, has increased predominantly in the Metropolitan areas [Kobayashi et al., 2002; Ogawa et al., 2002; Yotsuyanagi et al., 2005]. Genotype A infection tends to persist even when it is contracted in the adulthood [Kobayashi et al., 2002; Suzuki et al., 2005]. These backgrounds gave us the opportunity to compare response to long-term lamivudine treatment and development of YMDD mutants, along with breakthrough hepatitis, among patients of a single ethnicity and infected with HBV genotypes A, B, or C. As a result, some differences surfaced among infections with the three genotypes.

At the baseline, patients with genotype A were younger, and more often positive for HBeAg than those with B or C. Frequent HBeAg in patients with

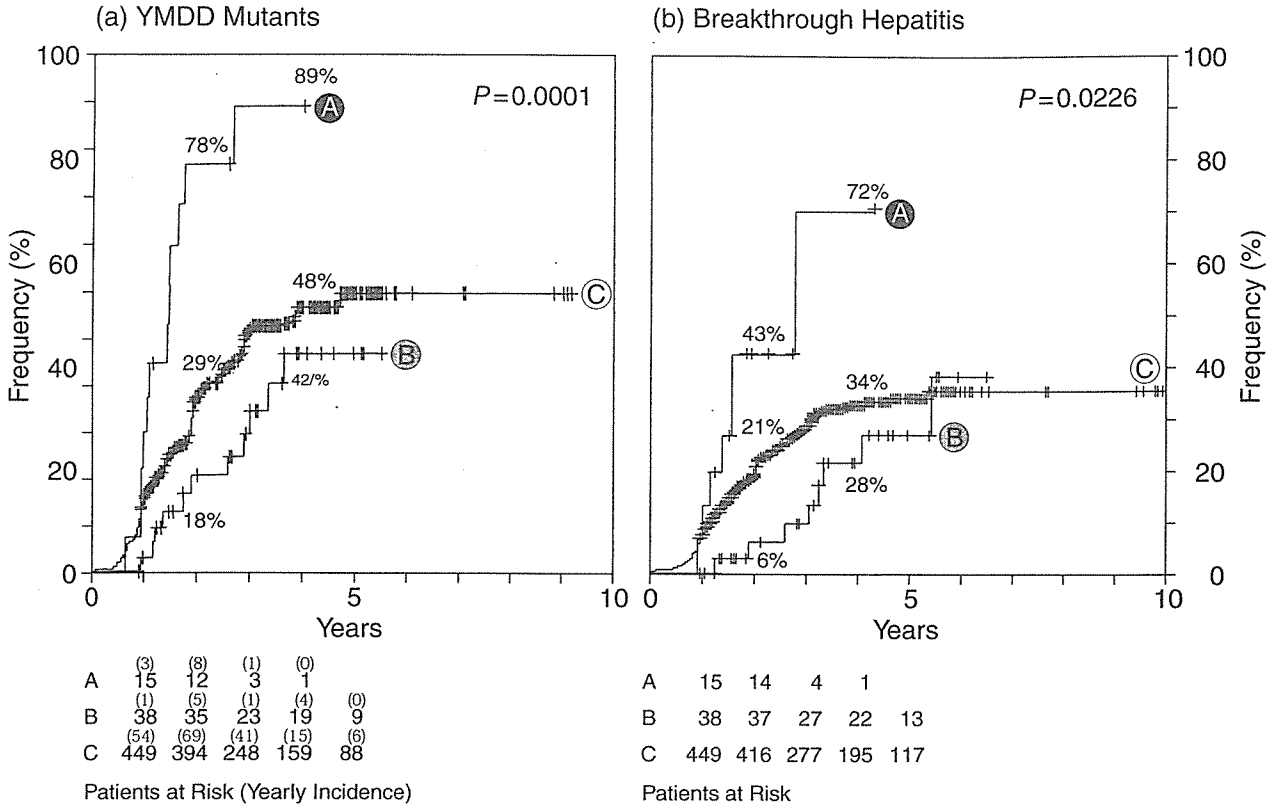

Fig. 3. Adverse events during long-term lamivudine therapy. Patients infected with HBV genotypes A, B, or C are compared for the development of YMDD mutants (**a**) and breakthrough hepatitis (**b**). Numbers of patients at risk at each year are shown below for those infected with genotypes A, B, and C. Development of YMDD mutants during each year is indicated in parentheses.

genotype A may be due to rare precore stop-codon mutation (G1896A) that is unacceptable for HBV DNA of this genotype [Li et al., 1993]. Nucleotide (nt) at the position 1896 is G in the wild-type HBV strains of any genotype, and makes a pair with nt 1858 of T in most of them. Exceptionally, nt 1858 is C in HBV of genotype A. Since a point mutation of G for A at nt 1896 breaks the Watson-Crick pair (C–G) between nt 1858 and 1986 and destabilizes stem-loop structures conforming the 'ε' encapsidation signal, it prohibits the replication of HBV genotype A. In addition, the duration of infection can make differences in the HBeAg status; it is much shorter in patients with genotype A infected in the adulthood than in those with genotype B or C who have been transmitted with HBV perinatally.

TABLE II. Factors Influencing the Emergence of YMDD Mutants*

Factor	Category	Hazard ratio (95% confidence interval)	*P*-value
HBeAg	1: –	1	
	2: +	2.11 (1.53–2.92)	<0.001
HBV genotype	1: B	1	
	2: C	1.23 (0.62–2.42)	0.56
	3: A	2.78 (1.08–7.12)	0.034

*Evaluated by the Cox proportion hazard model.

During long-term lamivudine therapy, HBV DNA was cleared less often in patients with genotype A than B or C (Fig. 2). In accordance with a poor virological response, YMDD mutants developed more frequently in patients with genotype A than B or C, both in those with (82% [9/11] vs. 25% [2/8] or 48% [117/245], $P=0.037$) and without baseline HBeAg (75% [3/4] vs. 30% [9/30] or 33% [68/204], $P=0.003$).

In multivariate analysis, pretreatment HBeAg and genotype A were significant predictive factors for the emergence of YMDD mutants. In confirmation of previous results [Chien et al., 1999; Liaw, 2002;

TABLE III. Pretreatment Variables Influencing the Development of Breakthrough Hepatitis*

Factor	Category	Hazard ratio (95% confidence interval)	*P*-value
ALT	1: ≥500 U/L	1	0.018
	2: <500 U/L	2.56 (1.82–5.56)	
HBeAg	1: –	1	
	2: +	2.11 (1.40–3.16)	<0.001
Pathology	1: Chronic hepatitis	1	
	2: Cirrhosis	1.92 (1.24–2.97)	0.004
HBV DNA	1: <8.0 LGE/ml	1	
	2: >8.0 LGE/ml	1.57 (1.04–2.36)	0.03

*Evaluated by the Cox proportion hazard model.

Kumada, 2003], low ALT levels, HBeAg, severe liver disease, and high HBV DNA at the baseline independently enhanced the development of breakthrough hepatitis (Table III). Breakthrough hepatitis was not influenced by HBV genotypes, however, probably because of the patients with genotype A were fewer than those with genotype B or C (15 vs. 38 or 449). Such great differences in number might have caused a statistical bias in comparison among the three genotypes.

The influence of HBV genotypes on the emergence of lamivudine-resistant mutants has been controversial. Previous studies failed to find differences between infection with genotypes B and C [Yuen et al., 2003a,b; Sun et al., 2005]. The risk of lamivudine resistance is reported to increase in infection with genotype A (represented by HBsAg subtype adw) compared to genotype D (ayw) [Zollner et al., 2001, 2002]; patterns of YMDD mutants differ between infection with genotypes A and D [Zollner et al., 2004]. No differences have been reported on emergence of lamivudine-resistant HBV mutants among infections with genotypes A, B, and C [Akuta et al., 2003; Suzuki et al., 2003a; Moskovitz et al., 2005], although such mutants are more frequent in infection with subgenotype Ba than Bj [Akuta et al., 2003]. The influence of genotype A on the emergence of YMDD mutants found in the present study would be ascribable to larger numbers of patients in comparison or longer duration of lamivudine, or both. Taken together with the report by Zollner et al. [2002, 2001], it does seem that lamivudine resistance occurs more frequently in infection with genotype A than with the other genotypes of HBV.

It has to be pointed out that observed genotype-dependent differences are not readily attributed to genotypes by themselves. Immigration of people and transmission by sexual contact or intravenous drugs have removed national borders in the epidemiology of HBV genotypes, although these are still maintained by perinatal or childhood transmission. Hence the duration of HBV infection differs markedly between imported and domestic genotypes. Even in the present study in patients of a single ethnicity, the duration of infection is much shorter in infection with genotype A than B or C, which would make differences in the response to lamivudine. The exact influence of genotypes on the response to lamivudine can only be evaluated in studies in patients with known duration of infection.

A therapeutic option for patients with genotype A who respond poorly to long-term lamivudine treatment may include adefovir dipivoxil that has a high efficacy unaccompanied by drug-resistant mutants in patients with or without HBeAg [Marcellin et al., 2003; Hadziyannis et al., 2005]. No differences were found, however, in the response to adefovil devoxivil in patients with genotypes A, B, C, and D [Westland et al., 2003]. Patients with genotype A infection may be changed to tenofovir disoproxil fumarate [Kuo et al., 2004] or pegylated interferon that induces a better response patients with genotypes A or B than C or D [Janssen et al., 2005].

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