clearance. These studies indicated that patients with hepatitis C virus have a high tendency for hepatocellular carcinoma.

Survival

Survivals are shown in Figure 3. During the observation period, 15 patients died of various causes; 8 died of malignant tumors (3 with gastric carcinomas, 2 with malignant lymphomas, 1 with tongue carcinoma, 1 with prostatic carcinoma, and 1 with ovarian carcinoma); 2 died of pneumonia; 3 died of heart failure; and 2 died of cerebral infarction. No patients died of liver failure or hepatocellular carcinoma. Hepatic decompensation did not develop in any patients after seroclearance of HBsAg. A Cox proportional hazards model was used to analyze the factors contributing to their survival: factors examined included age, gender, histologic findings, HBV genotype, and interferon administration. By Cox regression analysis, the relative risk of death incidence in patients aged less than 60 years was 3.58 compared with that of patients aged 60 years or more. Survival time was longer in patients aged less than 60 years (P =.017) (Table 5).

DISCUSSION

The results of this study indicate that patients with HBsAg clearance have a good prognosis. None of the patients with liver cirrhosis who had lost serum HBsAg progressed to decompensated liver cirrhosis. Moreover, no patients without liver cirrhosis with HBsAg clearance progressed to liver cirrhosis and/or hepatocellular carcinoma. Our findings agree with the published data by Chen et al[16] (Table 4). However, 2 of 67 patients with liver cirrhosis at the time of HBsAg clearance had hepatocellular carcinoma during follow-up, as Huo et al[15] showed. Fortunately, these 2 patients could be treated with radical resection.

Chen et al[16] reported that the prognosis after spontaneous HBsAg seroclearance is excellent, except in patients with liver cirrhosis or concurrent hepatitis virus infection. On the other hand, Huo et al[15] reported that adverse events were not rare in patients with chronic HBV infection even after HBsAg clearance. The discrepancy among these studies may be attributable to differences in the backgrounds of the patients who were followed up. These discrepancies might depend on concurrent hepatitis, severity of liver disease, ages, and other factors. For example, chronic hepatitis C virus infection is considered to be one of the major causes of hepatocellular carcinoma in many countries, and we suggest a role for hepatitis C virus in the origin of hepatocellular carcinoma in the patients with HBsAg clearance. Patients with hepatitis C virus-RNA after HBsAg seroclearance tend to have occurrences of hepatocellular carcinoma frequently when compared with patients without hepatitis C virus-RNA. Moreover, most asymptomatic carriers with seroclearance of HBsAg have a tendency not to consult a doctor. On the other hand, generally, symptomatic carriers after seroclearance of HBsAg tend to consult a doctor and are followed up. Thus, hepatocellular carcinoma development rates might be high in clinical institutions with a high rate of symptomatic carriers.

In the present study, we assessed the prolonged prognosis in a large number of Japanese patients with HBsAg seroclearance. This article excludes the patients with concurrent hepatitis virus infection. Moreover, most asymptomatic carriers with HBsAg seroclearance and normalization of alanine aminotransferase could be followed up and were included for analysis. This was because most patients in our hospital were civil servants and were frequently examined with liver function tests.

Most patients had a good prognosis after HBsAg clearance, and thus good survival. Moreover, there was no significant difference in survival between the liver cirrhosis and non-liver cirrhosis groups. There was no significant difference in survival between those with HBV genotype B and those with HBV genotype C. Thus, most patients with HBsAg seroclearance also showed clinical improvement and prolonged survival. In fact, all 15 patients died of causes unrelated to liver cirrhosis and/or hepatocellular carcinoma. The Cox proportional hazard model indicated that only age was associated with survival.

Next, it is important to decide how long the patients with seroclearance of HBsAg should be followed up. Our present findings showed the following: (1) The patients with liver cirrhosis at the time of HBsAg seroclearance have a possibility of hepatocellular carcinoma appearance. (2) No patient without liver cirrhosis at seroclearance had hepatocellular carcinoma. Thus, considering cost-effectiveness, it seems reasonable to increase the interval of follow-up after HBsAg seroclearance for patients without liver cirrhosis with chronic HBV infection alone. However, patients with liver cirrhosis should be carefully followed up.

In regard to histologic changes after marked reduction of HBV, most patients showed improvement in liver histology.[10,22-24] However, liver fibrosis resolves significantly less frequently and less quickly than necroinflammation. This may be because slight liver fibrosis can be reduced for a long period, but advanced liver fibrosis cannot be reduced sufficiently.

In regard to the appearance of anti-hepatitis Bs during follow-up, patients treated with interferon and/or CS showed the high cumulative appearance of anti-hepatitis Bs by log-rank test. This result is consistent with other studies that suggest immunomodulation of interferon[25,26] and CS.[27,28] The authors concluded that interferon and CS might stimulate the production of anti-HBs in vivo.

CONCLUSION

The prognosis after HBsAg clearance was excellent except in patients with liver cirrhosis. However, patients

with liver cirrhosis should be closely monitored for predictable complications.

ACKNOWLEDGMENT

The authors acknowledge the editorial assistance of Thomas Hughes.

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Journal of Medical Virology 78:341–352 (2006)

Clinical and Virological Features of Non-Breakthrough and Severe Exacerbation Due to Lamivudine-Resistant Hepatitis B Virus Mutants

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Patients who develop YMDD mutant during lamivudine therapy for hepatitis B virus (HBV) infection exhibit various clinical courses. Some patients show normal ALT levels, whereas others develop severe hepatitis exacerbations (SHEs) due to YMDD mutants. We studied 136 patients with YMDD mutant among 362 Japanese adult patients on lamivudine therapy. Clinical and virological features of patients without elevated HBV DNA after emergence of YMDD mutant (non-elevated group) were investigated. Moreover, virological analysis was also performed in patients with SHE due to YMDD mutants. Patients in the non-elevated group were characterized by HBeAg-negative pretreatment, HBeAg loss during therapy, a longer duration from commencement of therapy until emergence of YMDD mutant, and no mixed-type YMDD mutants. Patients with SHE had more substitutions in the reverse transcriptase (rt) region within the polymerase gene at the time of exacerbation than those without SHE, although no specific substitutions were noted. Sequence analysis of full-length HBV genome showed more substitutions in X, rt, and surface proteins in patients with SHE than in those without elevated HBV DNA level. In conclusion, negativity for HBeAg at commencement of therapy or before emergence of YMDD mutant was an important factor among non-elevated group. More substitutions in the rt region and the other proteins may be related to the emergence of severe hepatitis caused by lamivudine-resistant virus. ***J. Med. Virol. 78:341–352, 2006.*** © 2006 Wiley-Liss, Inc.

KEY WORDS: HBV; breakthrough hepatitis; YMDD mutant; reverse transcriptase

INTRODUCTION

Hepatitis B virus (HBV) infection is a common disease that can lead to a chronic carrier state, and is associated with the risk of development of progressive disease and hepatocellular carcinoma [Beasley et al., 1981]. Several studies have reported the effectiveness of a number of nucleoside analogs, such as lamivudine in the suppression of HBV replication, improvement of transaminase levels and liver histology, and enhancement of the rate of loss of hepatitis B e antigen (HBeAg) [Dienstag et al., 1995, 1999; Lai et al., 1998; Suzuki et al., 1999]. A major problem with the long-term use of lamivudine, however, is the potential development of viral resistance, associated with increases in HBV DNA and serum transaminases. Long-term lamivudine therapy may therefore increase the likelihood of the development of resistance [Nafa et al., 2000; Suzuki et al., 2003].

HBV polymerase can be divided into several functional domains, which have been designated the 'fingers,' 'palm,' and 'thumb' sub-domains by comparison with the reverse transcriptase (rt) of human immunodeficiency virus [Das et al., 2001]. The YMDD motif is located in the palm sub-domain, which is thought to contain the major catalytic nucleus of the polymerase, while the fingers sub-domain contains the region that overlaps the 'a-determinant' of hepatitis surface antigen (HBsAg) [Torresi et al., 2002a]. The polymerase gene completely overlaps the surface gene, resulting in the potential to significantly alter the activity of the

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

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Accepted 7 November 2005
DOI 10.1002/jmv.20546
Published online in Wiley InterScience (www.interscience.wiley.com)

polymerase protein as a consequence of mutations in the overlapping surface gene [Torresi, 2002b].

Although it is generally accepted that the probability of viral resistance or virologic breakthrough increases with the prolongation of lamivudine therapy, the clinical significance of such virologic breakthrough has not been fully elucidated. Previous in vitro and in vivo studies have shown that YMDD mutants are associated with less aggressive liver disease than YMDD wild-type [Fu and Cheng, 1998; Melegari et al., 1998; Ling and Harrison, 1999; Ono-Nita et al., 1999; Leung, 2000; Zollner et al., 2000]. Nevertheless, severe hepatitis exacerbations (SHEs) due to YMDD mutants have been reported, sometimes associated with hepatic decompensation and mortality [Liaw et al., 1999; Kim et al., 2001]. In addition, mutations of the 'a-determinant' of viral envelope gene together with YMDD mutant in liver transplant recipients treated with lamivudine and hepatitis B immunoglobulin (HBIg) have been described by Bock et al. [2002]. In contrast, some patients have persistently normal ALT levels after emergence of YMDD mutant.

In our study of lamivudine resistance in patients with chronic HBV infection, we analyzed the clinical and virological features of patients without virological breakthrough despite emergence of YMDD mutant, and DNA sequences of the polymerase gene in patients with SHE by YMDD mutant. Full-length DNA sequences were also analyzed in two patients with SHE.

PATIENTS AND METHODS

Patients

We studied 362 Japanese adult patients (60 females and 302 males, median age 45 years [range 19–76]) who commenced treatment with lamivudine at the Department of Gastroenterology of Toranomon Hospital between September 1995 and July 2002 and adhered to treatment for more than 6 months. All patients were followed from commencement of therapy at our hospital and were treated continuously until May 2004. Some of these patients have been reported previously [Chayama et al., 1998; Suzuki et al., 1999, 2003]. All patients were negative for hepatitis C serologic markers, but all had detectable HBsAg for at least 6 months prior to commencement of lamivudine therapy. Lamivudine was administered orally at 100 mg/day. Chronic hepatitis or cirrhosis was confirmed by needle biopsy, peritoneoscopy or clinical criteria before treatment [Suzuki et al., 2003]. Chronic hepatitis and cirrhosis were diagnosed in 319 and 43 patients, respectively.

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

Routine biochemical tests were performed using standard procedures before and during therapy at least once every 2 months. Serial blood samples were taken before and during therapy and stored at −80°C until used for HBV molecular analysis. HBV DNA was measured by transcription-mediated amplification and hybridization protect assay (TMA-HPA) (Chugai Diagnostics Science Co., Tokyo, Japan) [Kamisango et al., 1999]. Viral breakthrough was defined as a sustained rise [>5 logarithms of genomic equivalents/ml (LGE/ml)] in HBV DNA levels following a period of undetectable levels using this method.

Determination of Nucleotide and Deduced Amino Acid Sequence of Part of the DNA Polymerase Gene (Including YMDD Motif)

Mutation of the HBV DNA polymerase gene (rtM204I/V) was determined using polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP) as described previously [Chayama et al., 1998]. Lamivudine resistance was determined annually before the development of mutations in all patients and if mutation appeared, the time of appearance of resistance was confirmed by monthly measurements.

Determination of Nucleotide Sequences of HBV DNA

DNA was extracted from 100 μl of serum. PCR reactions for detection of the polymerase region (nt 163–864, aa12-aa245 in rt region) of HBV DNA were performed. The first and second PCR reactions for detection of the rt region were performed using primers BGF1-BGR2 and PLF5BamH-BR112 (nucleotide sequences of primers are shown in Table I), respectively, under conditions of initial denaturation for 4 min, 35

TABLE I. Primers Used in the Present Study

Primer	Nucleotide sequence	Nucleotide	Direction
BGF1	5′-CTGTGGAAGGCTGGCATTCT-3′	2757–2776	Sense
BGR2	5′-GGCAGGATAGCCGCATTGTG-3′	1079–1050	Antisense
BGF5	5′-TGCGGGTCACCATATTCTTG-3′	2811–2830	Sense
BGR6	5′-AGAAGTCCACCACGAGTCTA-3′	268–249	Antisense
PLF5BamH	5′-TGTGGATCCTGCACCGAACATGGAGAA-3′	136–162	Sense
BR112	5′-TTCCGTCGACATATCCCATGAAGTTAAGGGA-3′	887–865	Antisense
B11F	5′-GGCCAAGTCTGTACAACATC-3′	759–778	Sense
B14R	5′-GATCCAGTTGGCAGCACACC-3′	1404–1385	Antisense
BXF5	5′-CTTATCGGGACTGACAACTC-3′	1321–1340	Sense
BXR6	5′-AGTTGCATGGTGCTGGTGAA-3′	1821–1802	Antisense
BCS1	5′-ACACCGCCTCTGCTCTGTAT-3′	1995–2014	Sense
BCS2	5′-CTCCCGCTCCTACCTGATTT-3′	3031–3012	Antisense

Nucleotide sequence position numbers are those of AB033550.

cycles of amplification at 94°C for 1 min, 55°C for 2 min, 72°C for 3 min, and 72°C for 7 min. PCR-amplified DNA was purified after agarose gel electrophoresis and cloned into pBluescript plasmid vector (Stratagene, La Jolla, CA). Dideoxynucleotide termination sequencing was performed with the ABI PRISM™ Dye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems, Tokyo, Japan). Sequences of 3 to 10 independent clones for each sample were determined and analyzed.

Nucleotide sequences of the core promoter and precore regions were determined as described previously [Suzuki et al., 2002].

Full-length PCR was performed with previously described primers [Günther et al., 1995]. Amplified full-length HBV genomes were diluted 1:100 and 1 μl thereof was reamplified by primers (Table I) under the same conditions as above. The primers for the second PCR reaction were BGF5-BGR6, PLF5BamH-BR112, B11F-B14R, BXF5-BXR6, and BCS1-BCS2. The amplified PCR products were used for direct sequencing. Because all HBV genomes that were analyzed in detail by sequencing were found to be of genotype C, all sequence alignments were performed in comparison with genotype C wild-type sequences (accession no. AB014378, AB014394, AB033550, AB033551, AB033556, AB042283).

Statistical Analysis

Differences between groups were examined for statistical significance using the χ^2 and Mann–Whitney test (*U*-test) where appropriate. The above calculations were performed using StatView software (Version 4.5J; Abacus Concepts, CA). A two-tailed *P*-value less than 0.05 was considered statistically significant.

Nomenclature

The amino acid positions for the HBV polymerase gene are consistent with the newly established scheme designed to standardize the nomenclature of lamivudine-resistant mutations, rtL180M and rtM204V/I (originally designated as pL528M or pL526M, and pM552V/I or pM550V/I) [Stuyver et al., 2001].

RESULTS

Clinical and Virological Features of Patients Without Virological Breakthrough Despite Emergence of YMDD Mutant

Patients received lamivudine orally for a median duration of 34.3 months [range 6–100 months]. YMDD mutant was detected in 136 (38%) of 362 patients during treatment with lamivudine. Eight (6%), 7 (5%), 116 (85%), 1 (0.7%), and 4 (3%) patients with emergence of YMDD mutant were infected with HBV genotypes A, B, C, F, and unknown, respectively. Among 136 patients with emergence of YMDD mutant, 114 patients were followed for more than 1 year after emergence. In 27 (24%) of these patients, HBV DNA levels were persistently below 5.0 LGE/ml (by the TMA-PHA

TABLE II. Comparison of Patients With and Without Elevated HBV DNA Levels After Emergence of YMDD Motif Mutations During Lamivudine Therapy

Category	Non-elevated group (n = 27)		Elevated group (n = 87)		*P*
	HBeAg positive (n = 10)	HBeAg negative (n = 17)	HBeAg positive (n = 56)	HBeAg negative (n = 31)	
Age[a], year	41 (32–66)	47 (28–67)	42 (23–69)	47 (32–70)	NS
Sex: male/female	7/3	13/4	48/8	27/4	NS
Pretreatment histology: chronic hepatitis/cirrhosis	7/3	15/2	42/14	22/9	NS
Pretreatment bilirubin[a], mg/dL	0.9 (0.4–6.5)	0.6 (0.3–7.1)	0.75 (0.2–16.5)	0.6 (0.3–1.9)	NS
Pretreatment ALT[a], IU/L	159.5 (26–795)	101 (16–2142)	110 (14–1722)	74 (11–1708)	NS
Pretreatment HBV DNA[a], LGE/ml	8.1 (6.7–9)	6.5 (3.5–9)	8.1 (3–9)	6.2 (3–9)	NS
Pretreatment HBeAg: positive/negative	10	17	56	31	0.012
No. of patients with HBeAg loss during therapy	10 (100%)		18 (32%)		<0.0001
Genotype: A/B/C/others	0/1/9/0	0/0/17/0	3/0/52/1	3/6/20/2	
(Genotype: C/other than C)	26/1		72/15		NS
Period from commencement to emergence of YMDD motif mutation[a]	24 (7–36)	30 (10–63)	13 (5–56)	15 (6–59)	
	24 (7–63)		14 (5–59)		0.0001
Mutant type: I/V/mix	4/5/1	10/6/1	25/12/19	15/9/7	
(Mutant type: I or V/mix)	25/2		61/26		0.02

P-values were calculated between non-elevated and elevated groups.
NS, not significant; I, YIDD; V, YVDD; Mix, YIDD + YVDD.
[a]Data are median (range).

method) for more than 1 year (non-elevated group), while serum ALT levels were maintained within normal levels. HBV DNA levels in the remaining 87 patients (76%) were elevated over 5.0 LGE/ml (elevated group). Table II shows the clinical and virological differences between the two groups. The number of HBeAg-negative patients at commencement of therapy in the non-elevated group was greater than in the elevated group. Moreover, all patients of the non-elevated group showed HBeAg loss before emergence of the YMDD mutant. The period from commencement of therapy to emergence of YMDD mutant in the non-elevated group (both HBeAg-positive and -negative) was significantly longer than in the elevated group. The number of mixed type (I + V) at emergence of YMDD mutant in the non-elevated group (both HBeAg-positive and -negative) was less than in the elevated group. No other characteristics related to the non-elevation of HBV DNA levels were seen.

Clinical Features and Sequences of HBV DNA in Patients With Severe Hepatitis Exacerbations Due to YMDD Mutant

SHE was defined as a >8-fold increase in ALT level (>400 IU/L) and a >2-fold increase in bilirubin level (>2.2 mg/dL), the upper limit of normal in chronic hepatitis patients, after excluding other causes of ALT elevation, including other viral hepatitis (A, C, D, E), drug-induced hepatitis, and alcoholic hepatitis. Six patients fulfilled these criteria (Table III). All patients had HBV genotype C, and two were HBeAg-negative. At SHE, ALT levels of five patients were >1,000 IU/L and HBV DNA levels of all patients were >8 LGE/ml. Figure 2 shows a schema of the clinical course of six patients with SHE. Only one patient (Patient 1) had SHE after the first elevation of HBV DNA level (Fig. 1a). The remaining five patients (Patients 2–6) had SHE, which occurred at the second or third elevation of ALT after the first mild elevation and then followed the elevation of HBV DNA levels (Fig. 1b).

We then analyzed the sequences of the rt region (aa12-aa245) of HBV polymerase in four of the six patients with SHE. The sequencing of 3 to 10 independent clones was determined in each sample at three time points: (1) at commencement of lamivudine therapy, (2) at first emergence of YMDD mutant, and (3) at SHE. Figure 1 shows substitutions of amino acids of the rt region in these four patients. One substitution (rtL80I: three of six clones) in the palm subdomain appeared at emergence of YMDD mutant in one patient (Patient 1). This substitution (four of five clones) was sustained at SHE. In the fingers and palm sub-domains, several substitutions were identified at commencement of therapy in the remaining three patients. Different substitutions then appeared at emergence of YMDD mutant and SHE. In Patients 4 and 6, there were more substitutions at the time point of exacerbation than at emergence of YMDD mutant. For example, in Patient 4, the amino acid of rt191 was valine (V) in all five clones at SHE, but was

TABLE III. Clinical Features of Patients With Severe Hepatitis With Exacerbation During Lamivudine Therapy

					Baseline				Severe hepatitis exacerbation			
Patient	Sex	Age (years)	Genotype	Histology (staging)	HBeAg	ALT (IU/L)	Bilirubin (mg/dL)	HBV DNA (LGE/ml)	HBeAg	ALT (IU/L)	Bilirubin (mg/dL)	HBV DNA (LGE/ml)
1	F	70	C	NA	−	78	0.4	6.5	−	1,669	3.1	>8.7
2	M	37	C	2	−	106	0.8	8.6	−	1,671	3.8	8.6
3	M	55	C	1	+	98	1.7	8.4	+	1,230	5.2	>8.7
4	F	54	C	2	+	343	0.5	7.7	+	1,299	5.5	8.5
5	M	37	C	2	+	375	0.4	8.1	+	1,310	6.0	>8.7
6	M	41	C	3	+	64	0.8	>8.7	+	478	5.3	>8.7

F, female; M, male; NA, not available; +, positive; −, negative.

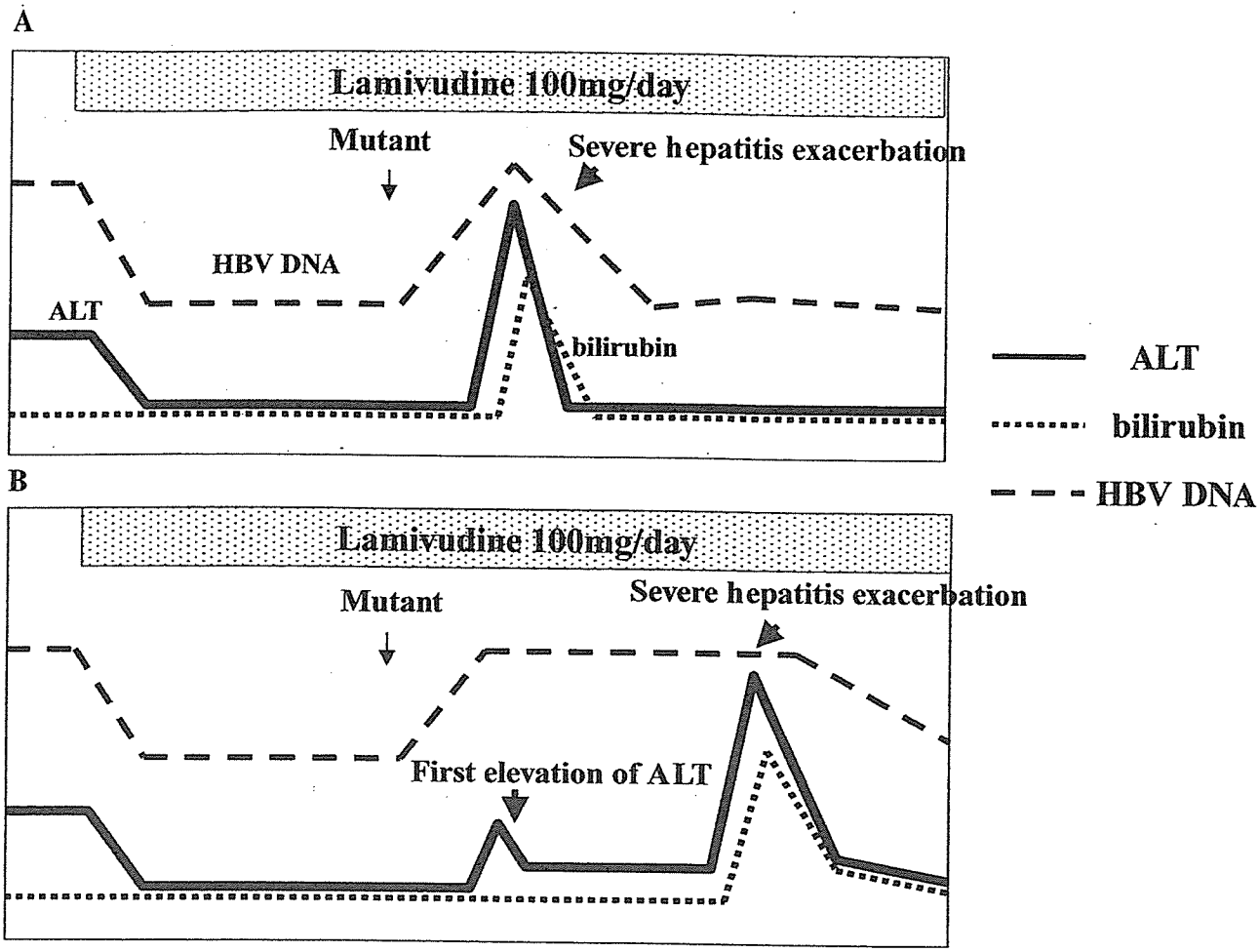

Fig. 1. Schema of transitions of virological, serum alanine aminotransferase (ALT), and serum bilirubin during lamivudine therapy in exacerbation of severe hepatitis. Two types were defined: (A) at first ALT elevation after first elevation of HBV DNA level and (B) at second or third elevation of ALT after first mild elevation of ALT and then followed by elevation of HBV DNA levels.

isoleucine (I) in all eight clones at commencement of therapy and I in six of eight clones at emergence of YMDD mutant. A similar phenomenon was noted in rt214. On the other hand, new amino acid changes appeared in rtS137Q (four of five clones), rtL180M (all five clones), rtP237H (four of five clones) and rtN238T (all five clones) at SHE. In Patient 6, new changes in amino acids appeared in rtL80I (five of six clones), rtM204I (all six clones), and rtN238S (four of six clones) at emergence of YMDD mutant. Moreover, rtH55R (all three clones) reappeared and new changes in amino acids appeared in rtM204I/V (mixed type: I was in two clones, V was in one clone), rtL217R (two of three clones), rtL220I (two of three clones), and rtL235V (two of three clones), although the substitutions in rtL80I and rtN238S disappeared at SHE. Some substitutions also changed the amino acids in the surface genes. However, except for the rtM204I/V mutation, common substitutions were not identified at exacerbation in these four patients. We further investigated the sequences of the same region in two patients without elevated HBV DNA level after emergence of YMDD mutant (Fig. 3). HBV DNA levels remained below the detection level (<3.7 LGE/ml) over 2.5 years. We investigated the changes at three time points using the same methods; (1) and (2) were the same time points as described above and (3) was at >2.5 years (3 and 2.5 years) after emergence of YMDD mutant. There were no substitutions in the rt region at commencement of therapy in either patient. However, three and one substitutions, respectively, appeared at emergence of YMDD mutant and were sustained at a time point >2.5 years thereafter. We also investigated the sequences of the same region in three randomly selected patients with elevated HBV DNA level but no SHE after emergence of YMDD mutant (Fig. 4). The analyses were conducted at three time points using the same methods, with (1) and (2) being the same time points as above and (3) being at the development of hepatitis (time point = about 1 year) after emergence of YMDD mutant. Two and three

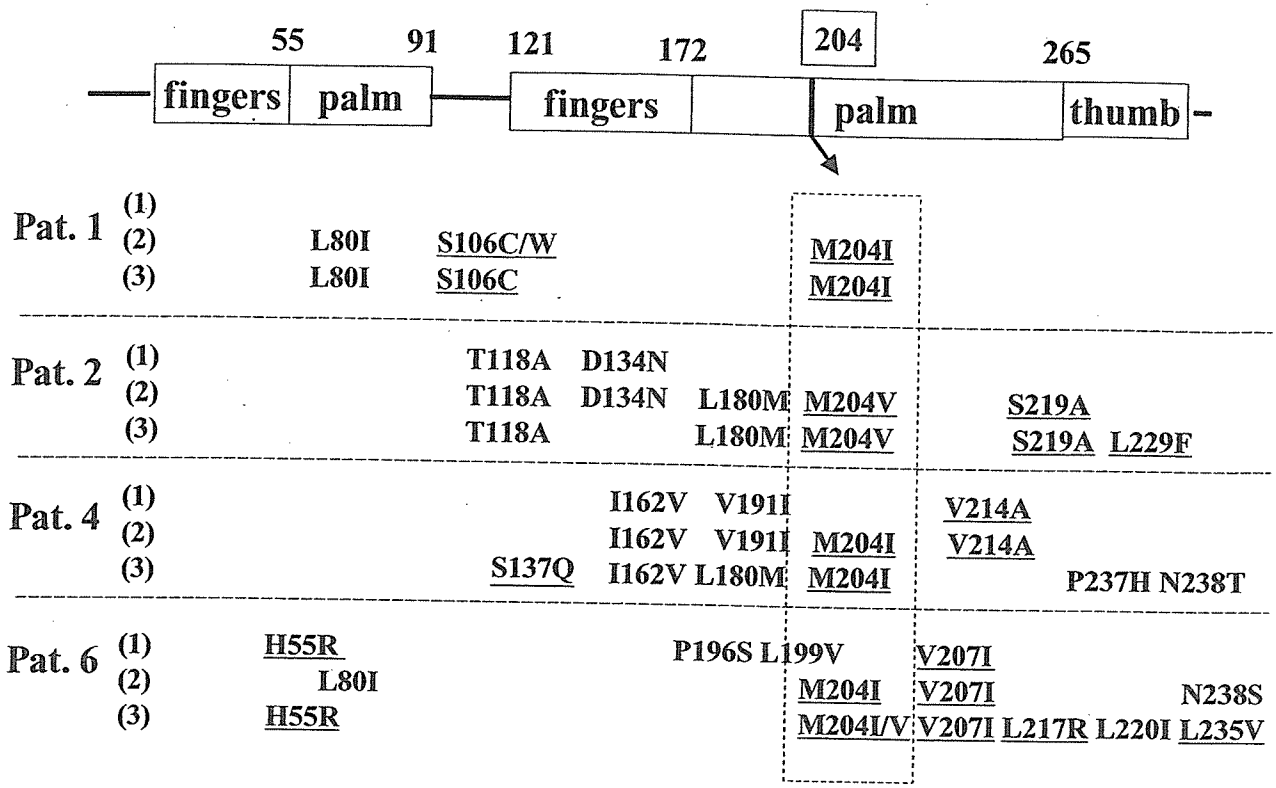

Fig. 2. Substitutions of amino acid sequences in the reverse transcriptase (rt) domain of the HBV polymerase gene are shown in patients with severe hepatitis exacerbation (SHE). The numbers of patients are the same as those in Table III. Underlined *rt* genes show changes introduced in the overlapping surface gene. Measurements were conducted at three time points: (1) at commencement of lamivudine therapy, (2) at first emergence of YMDD motif mutation, and (3) at SHE.

substitutions appeared at emergence of YMDD mutant and hepatitis in the fingers and palm sub-domains, respectively. Although the number of patients analyzed was small, patients with SHE had more substitutions at exacerbation than those without SHE. However, there were no specific substitutions related to SHE.

Analysis of serum samples obtained at baseline identified a precore stop codon mutation (A1896) in three of six patients (Table IV). A1896 occurred as a mixed population with wild-type virus (G1896) in these three patients. At the time of emergence of YMDD mutant and SHE, A1896 was observed in two patients without HBeAg. On the other hand, A1896 was observed in two of four patients with HBeAg at SHE. Five of six patients had core promoter mutations in samples collected at baseline. However, these mutations were persistently detected at times of emergence of YMDD mutant and SHE. In the remaining patient, wild-type (A1762, G1764) was persistently detected at all time points.

Full-length sequencing was performed on serial serum samples collected from two patients with SHE and one with non-elevated HBV DNA levels before and during therapy. In Patient 2, two and four unique substitutions were identified in surface and X proteins, respectively, at commencement of therapy (Fig. 5). At the time of emergence of YMDD mutant, unique substitutions in the core (cR151C) and X (xH94Y) proteins were also detected and sustained at the times of first hepatitis and SHE. Although two substitutions in the surface protein (sI195M, sS210R) were detected at the time of emergence of YMDD mutant, they were related to those in the rt region. In Patient 5, one and four unique substitutions were identified in the core and X genes, respectively, at commencement of therapy (Fig. 6). Three of the four substitutions in X proteins were the same as those in Patient 2. At the time of emergence of YMDD mutant, one unique substitution in the X (xI127S) protein had also emerged. However, this substitution disappeared at the time of SHE. In the core protein, new substitutions (cI59V, cS181P) were detected at the time of emergence of YMDD mutant. However, substitutions (cS86G, cR151C) in core protein at SHE were different from those at the time of emergence of YMDD mutant. The cR151C substitution was identical to that at SHE in Patient 2. In the surface protein, two substitutions (sN146T, sW196S) were detected at the time of SHE. Interestingly, sN146T substitution in the surface protein was identified in "a-determinant." On the other hand, in Patient NM, whose HBV DNA level was maintained below 3.7 LGE/ml during therapy, there were no substitutions in the rt

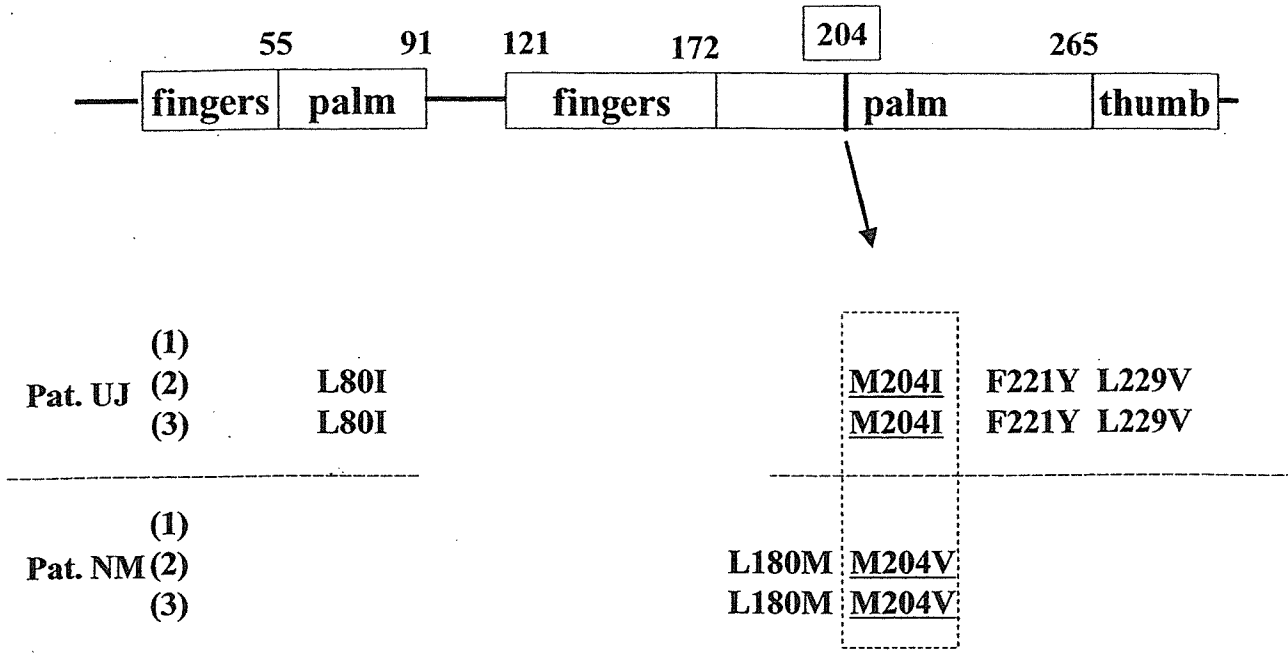

Fig. 3. Substitutions of amino acid sequences in the reverse transcriptase (rt) domain of the HBV polymerase gene in patients with non-elevated HBV DNA levels after emergence of the YMDD mutant. Measurements were conducted at three time points: (1) and (2) were at the same time points, and (3) at >2.5 years (3 and 2.5 years) after emergence of the YMDD mutant.

region or surface protein at commencement of lamivudine therapy (Fig. 7). In the core and X proteins, there were two and one substitutions, respectively, at commencement of therapy. At emergence of YMDD mutant, only mutations related to YMDD mutant emerged in the rt and surface proteins. At 2.5 years after YMDD mutant, only one new substitution had emerged in the surface protein.

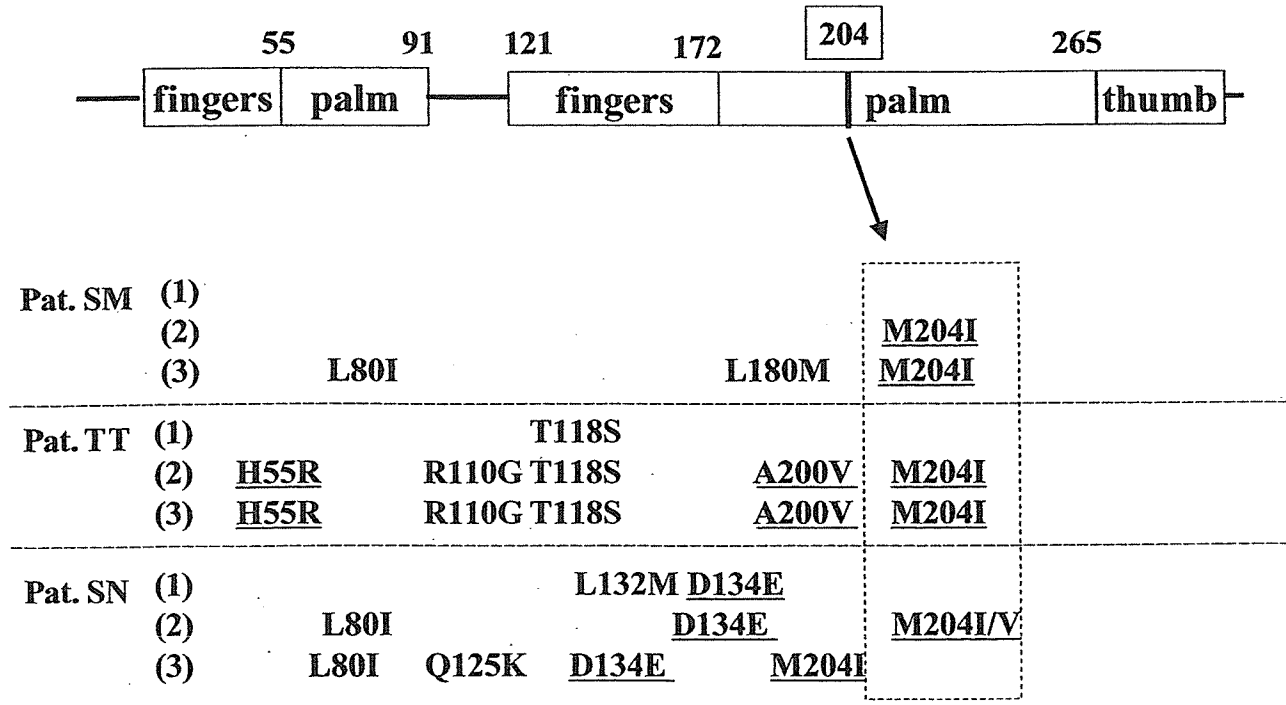

Fig. 4. Substitutions of amino acid sequences in the reverse transcriptase (rt) domain of the HBV polymerase gene in patients with elevated HBV DNA level but no severe exacerbation after emergence of the YMDD mutant. Measurements were conducted at three time points; (1) and (2) were at the same time points, and (3) at the development of hepatitis after emergence of the YMDD mutant.

TABLE IV. Serial Precore and Core Promoter Sequences

Patient	HBeAg	Precore (nt 1,896)			Core promoter (nt 1,762/1,764)		
		Baseline	Mutant	SHE	Baseline	Mutant	SHE
1	−	G	A	A	T/A	T/A	T/A
2	−	G/A	A	G/A	T/A	T/A	T/A
3	+	G	G	G	A/G	A/G	A/G
4	+	G/A	G/A	A	T/A	T/A	T/A
5	+	G/A	G/A	G/A	T/A	T/A	T/A
6	+	G	G	G	T/A	T/A	T/A

Patient numbers are the same as those in Table III.
Baseline, time of commencement of therapy; Mutant, time of emergence of YMDD motif mutation; SHE, time of exacerbation of severe hepatitis.

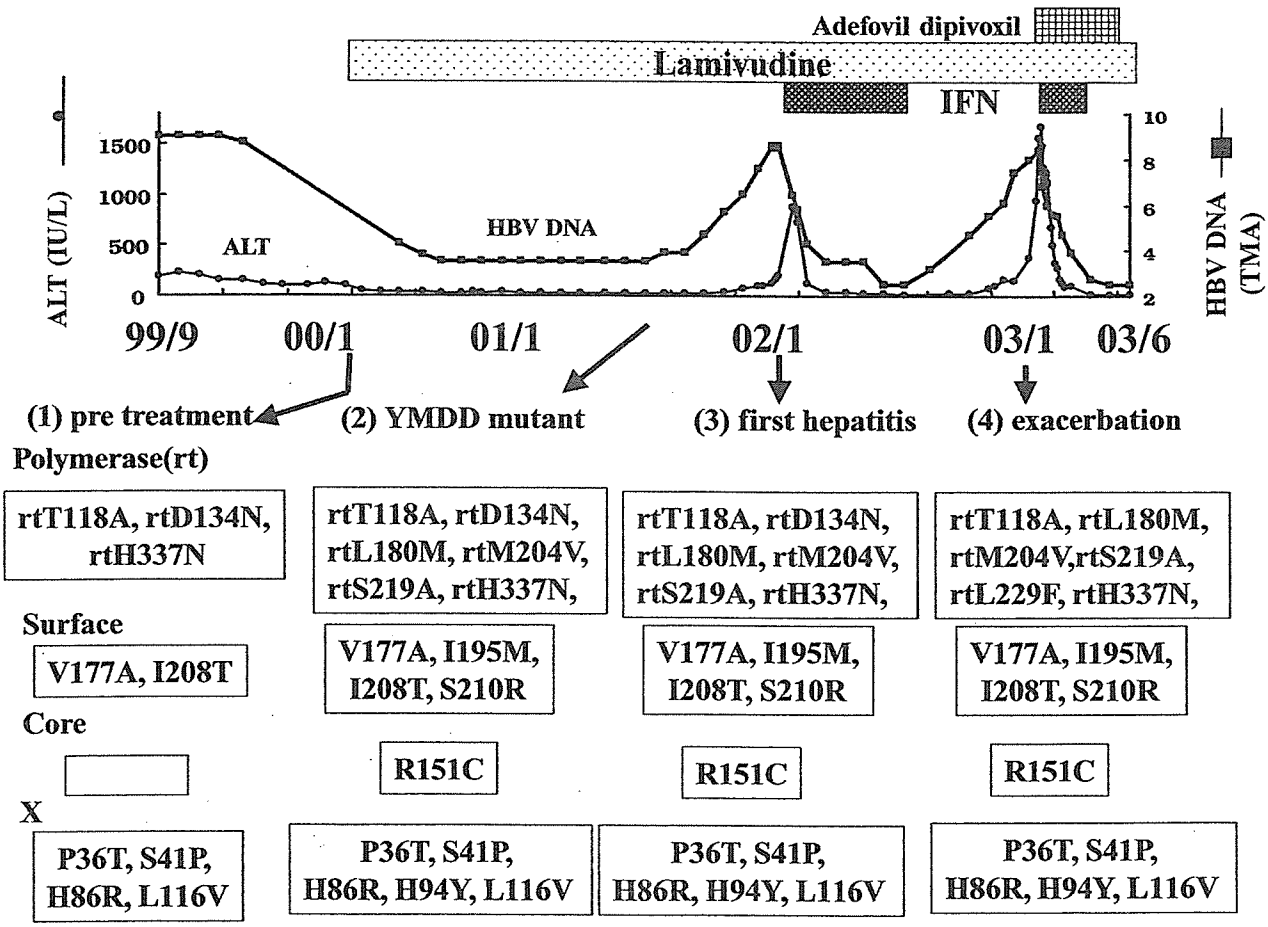

Fig. 5. Correlation between viral load, serum ALT, and accumulation of HBV substitutions during the clinical course in Patient 2, with severe hepatitis exacerbation. Four serial serum samples (arrows) were collected at various time points before and during lamivudine therapy. In addition to biochemical and viral load testing, full HBV genomic sequencing was performed. The progressive appearances of non-consensus genotype C mutations in the polymerase (reverse transcriptase), surface, core, and X proteins are indicated. The letter rt preceding the amino acid substitution denotes reverse transcriptase. rtL180M denotes the substitution of leucine with methionine at amino acid position 180 in the rt region of the HBV polymerase.

DISCUSSION

In patients undergoing treatment with lamivudine for chronic hepatitis B, a high frequency of long-term lamivudine-resistant virus has been reported, and attempts to identify markers that can predict response to treatment have been ongoing. Although previous clinical trials have identified several factors associated with emergence of YMDD mutant [Suzuki et al., 2003], little information is available concerning the clinical features of patients without an increase in HBV DNA after emergence of the mutant. In the present study, 24% of patients showed no rise in HBV DNA after emergence of YMDD mutant. We found several common

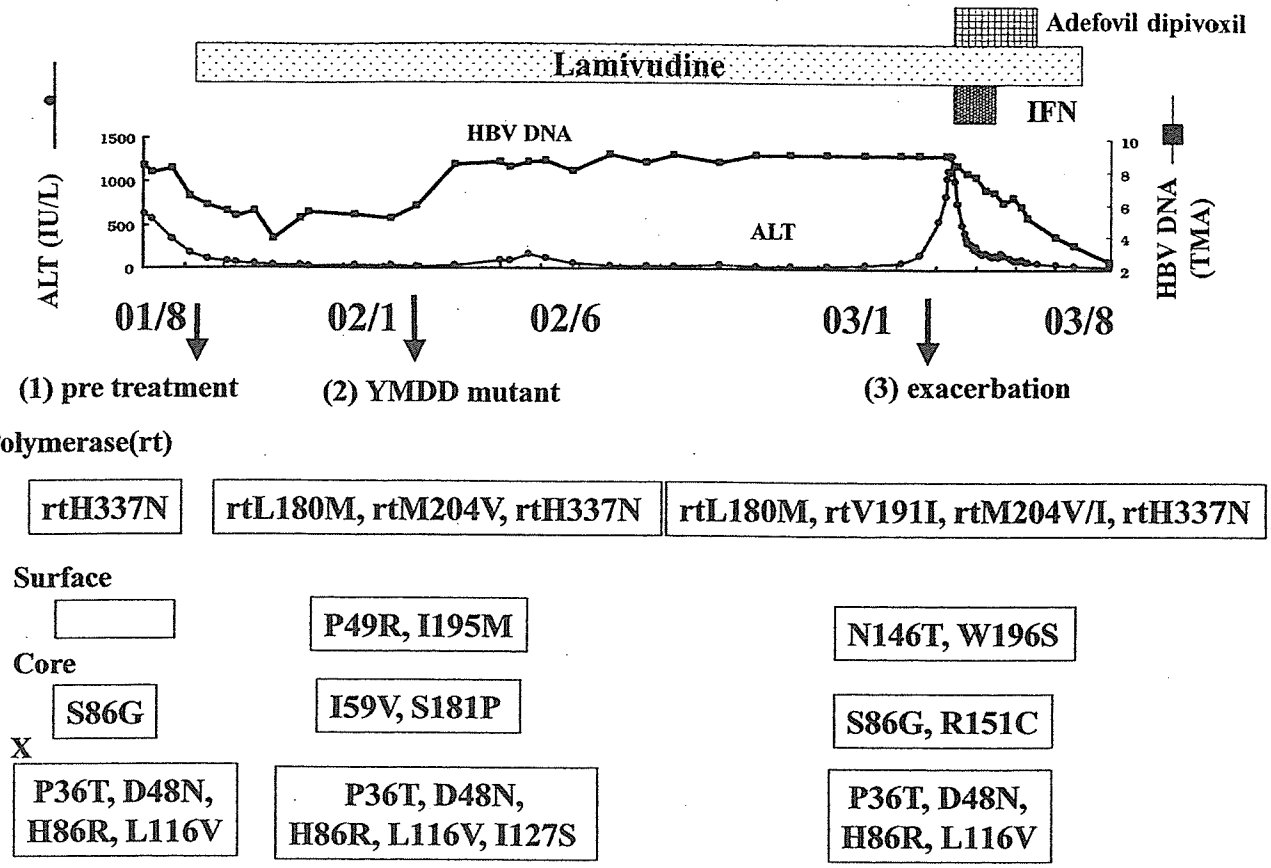

Fig. 6. Correlation between viral load, serum ALT, and accumulation of HBV substitutions during the clinical course in Patient 5, with severe hepatitis exacerbation.

characteristics among this non-elevated group. In particular, negativity for HBeAg at commencement of therapy or before emergence of YMDD mutant was an important factor among this group. These patients benefited from continuous therapy even with emergence of YMDD mutant. On the other hand, a European study showed relatively poor efficacy with long-term lamivudine treatment in HBeAg-negative patients [Hadziyannis et al., 2000]. This difference from our study may suggest that our patients were predominately Genotype C, in contrast to the European study, which mainly involved those of Genotype A or D. Another possibility may be the presence of fewer substitutions in the rt region at commencement of therapy, either alone or with emergence of YMDD mutant, as discussed below. A recent report showed that sustained lamivudine responders with HLA-A2 elicited more potent cytotoxic T-lymphocyte (CTL) immunity against YMDD and its mutant (YIDD and YVDD) [Lin et al., 2005]. Although we do not have HLA type data for the patients in our study, anti-mutant CTLs such as those described above may contribute in suppressing the elevation of mutant virus loads. Further immunological and other investigations into this phenomenon are necessary.

Recently, Bock et al. [2002] reported the occurrence of HBV mutations in liver transplant recipients with severe recurrent hepatitis, reflecting enhanced in vitro replication in the presence of lamivudine. Their patients were treated with HBIg, which is known to be related to mutations in the "a-determinant." Combinations of mutations in the "a-determinant" and YMDD motif (sP120T/rtL180M/rtM204V and sG145R/rtL180M/rtM204V) in patients with severe hepatitis were not only resistant to lamivudine treatment, but also showed enhanced replication in vitro in the presence of lamivudine. Both mutations (rtT128N [=sP120T] and rtW153Q [=sG145R] including finger sub-domain) have uncharged polar amide side chains that may alter the relationship of the deoxynucleotide triphosphates (dNTP) binding pocket to the palm sub-domain of the lamivudine-resistant viral polymerase. However, these changes in the fingers sub-domain, which introduce amide side chains, may also result in a re-positioning of the dNTP binding pocket of the viral polymerase relative to the palm sub-domain, which may in turn result in the partial restoration of replication of lamivudine-resistant HBV mutants [Torresi, 2002b]. We investigated the presence of these mutations in the rt region in our patients with SHE, but did not detect any such mutations. The presence of only a few mutations in the "a-determinant" may be explained by the fact that our patients with SHE did not receive HBIg or

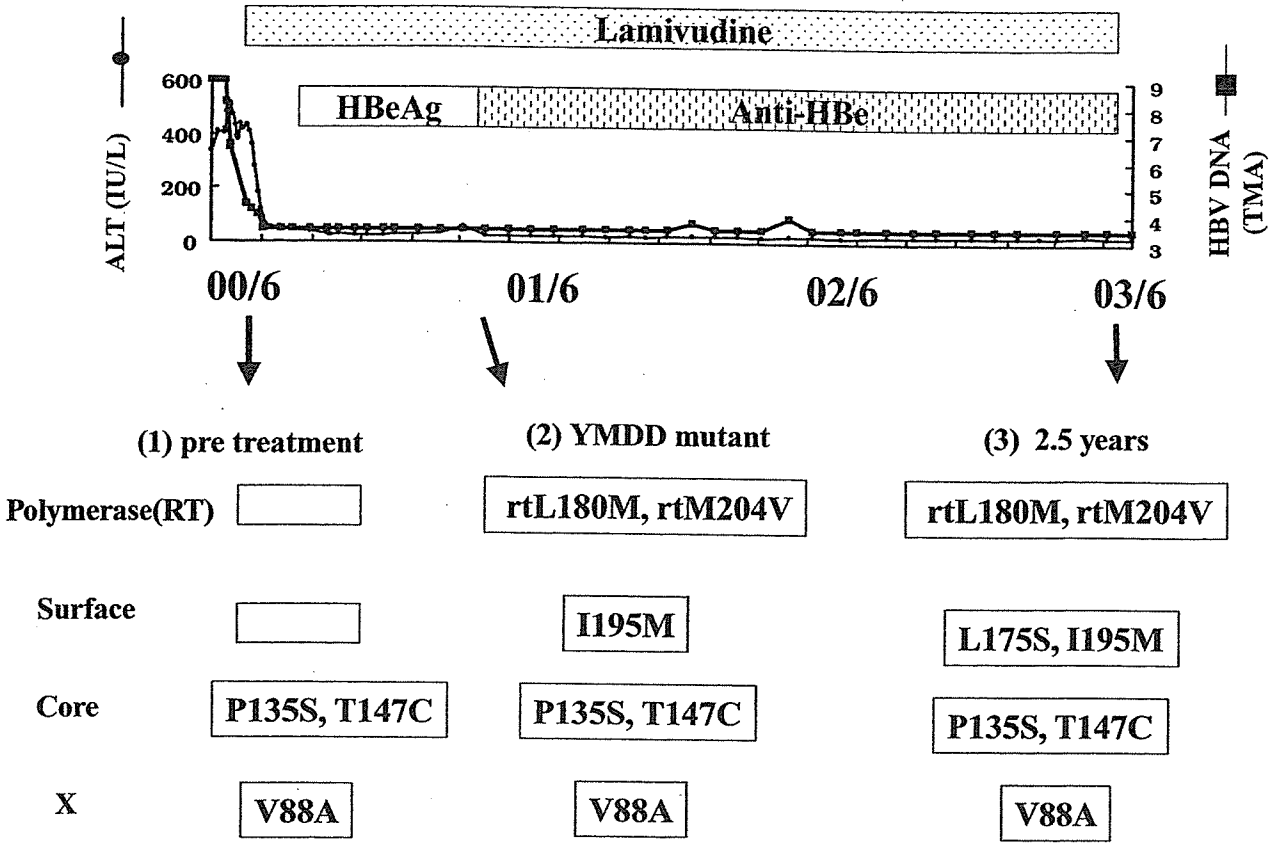

Fig. 7. Correlation between viral load, serum ALT, and accumulation of HBV substitutions during the clinical course in Patient NM, without elevation of HBV DNA.

vaccination. However, patients with SHE had more substitutions at exacerbation than patients without SHE, although no substitutions specifically related to SHE were seen. Our data allow us to speculate that several changes in the rt region, rather than just one, could increase binding to the primer-template of dNTPs and thereby restore viral replication of lamivudine-resistant mutants. It may be useful to analyze the three-dimensional structure of HBV polymerase in these patients to clarify viral replication. Further, it may also be necessary to identify mutations in the rt region that enhance viral replication in vitro in the presence of lamivudine. As shown in our clinical data in patients with SHE, the load of lamivudine-resistant virus with multiple mutations in the rt region was persistently high (Table III and Fig. 1). However, these flares of hepatitis were thought to be not only due to elevation of HBV DNA levels but also due to result from cytotoxic T-lymphocyte-mediated immune responses against YMDD mutant virus [Liaw et al., 1999]. Future immunological and in vitro analyses using replication-competent HBV clones in patients with SHE are necessary.

Recently, studies using a recombinant HBV baculovirus system or replication-competent HBV vectors showed that a precore stop codon mutation (G1896A) and/or mutation of the basic core promoter increased the replication efficacy of YMDD mutant virus but did not affect in vitro drug sensitivity [Chen et al., 2003; Tacke et al., 2004]. In our study, precore and core promoter mutations were found in four and five of six patients with SHE, respectively. However, both precore and core promoter regions in one patient (Patient 3) were wild-type, suggesting that mutations of these areas may not always be related to emergence of SHE.

One case report described virological factors that contributed to a fatal outcome in a patient who had HBeAg-positive chronic hepatitis B of genotype B and who was on long-term therapy with famciclovir and lamivudine, and compared the full-length HBV genomic sequence comparison between the pre-treatment virus and drug-resistant mutant [Ayres et al., 2003]. The substitutions were different to those in our two patients with SHE, except for rt180, rt204, and s195, although there was a difference in genotype. Interestingly, substitutions in the X protein among the previous and our two cases were numerous at both pretreatment and exacerbation, although it is unclear whether this phenomenon was related to emergence of SHE. On the other hand, there were fewer substitutions in patients with non-elevated HBV DNA. Although the number of patients in whom full-length HBV genomic sequences

were analyzed was insufficient for any conclusive determination, it appears that more numerous substitutions in X, rt, surface, and core proteins may be introduced during persistent elevation of HBV DNA. However, the development of SHE in chronic hepatitis B may be related to an imbalance between viral replication and host immune response [Perrillo, 2001]. In the future, it may be necessary to measure polymorphisms in genes that determine the expression and function of the host immune response among patients with SHE.

In conclusion, we clarified the characteristics of patients who did not show elevations in HBV DNA after emergence of YMDD mutant. In patients with SHE, more substitutions were seen in the palm and fingers sub-domains than in those without SHE; these substitutions might act to restore viral replication of lamivudine-resistant mutants. In analysis of the full-length HBV genomic sequence, a greater number of substitutions in some proteins may be related to emergence of severe hepatitis due to lamivudine-resistant virus. Lamivudine-resistant HBV quasispecies with multiple compensatory changes that can modulate viral replication should be considered of clinical relevance in patients undergoing prolonged therapy. New antiviral agents such as adefovir dipivoxil [Chin et al., 2001] and entecavir [Colonno et al., 2001] may be useful in patients with SHE in conjunction with careful virological monitoring.

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Journal of Medical Virology 78:83–90 (2006)

Predictive Factors of Virological Non-Response to Interferon–Ribavirin Combination Therapy for Patients Infected With Hepatitis C Virus of Genotype1b and High Viral Load

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Patients with high viral load ($\geq 1.0 \times 10^5$ IU/ml) of hepatitis C virus (HCV) genotype 1b do not achieve high sustained virological response rates to interferon (IFN)/ribavirin combination therapy. Previous studies suggested that pretreatment amino acid (aa) substitution patterns in the HCV core region could affect virological non-response especially in patients who could not achieve HCV-RNA negativity during treatment. The present study evaluated 167 consecutive Japanese adults with high HCV genotype 1b viral load who received combination therapy for ≥24 weeks. A case-control study matched for age, sex, genotype, and viral load was conducted to investigate the predictive factors for virological non-response, especially absolute virological non-response (patients who could not achieve >2 log decline of HCV RNA from baseline during the initial 24 weeks of therapy). Virological non-response was identified in 26.3% of patients, and 45.5% of these were absolute virological non-responders. Multivariate analysis identified ribavirin dose <11.0 mg/kg, moderate-to-severe hepatocyte steatosis, and substitutions of aa 70 and/or 91 in the core region as significant independent factors associated with virological non-response. The majority of absolute virological non-responders had such substitutions in the core region (95.0%), as well as substitution of glutamine at aa 70 and/or methionine at aa 91 (90.0%). In the present work, such substitutions significantly affected the viral kinetics in virological non-responders. The results suggest that viral, host, and treatment-related factors determine the response to IFN/ribavirin combination therapy in patients with high HCV genotype1b viral load, and that amino acid substitution patterns in the core region is potentially useful pretreatment predictor of virological non-response. ***J. Med. Virol. 78:83–90, 2006.*** © 2005 Wiley-Liss, Inc.

KEY WORDS: HCV; core region; hepatocyte steatosis; interferon; ribavirin; virological non-response; case-control study

INTRODUCTION

The aims of IFN therapy for chronic hepatitis C virus (HCV) infection include reduction of the risk of development of HCC and liver-related death by viral clearance, and then by normalization of alanine aminotransferase (ALT) even if viral clearance cannot be achieved [Ikeda et al., 1999; Akuta et al., 2005a]. The most effective initial therapy for viral clearance is the combination of interferon (IFN) and ribavirin administered for 48 weeks [Manns et al., 2001; Fried et al., 2002]. However, patients with high load of genotype 1b virus ($\geq 1.0 \times 10^5$ IU/ml), dominant in Japan, do not achieve high sustained virological response rates (less than 50%), even when the most effective combination treatment (pegylated IFN plus ribavirin) is administered for 48 weeks [Manns et al., 2001; Fried et al., 2002]. Furthermore, in genotype 1b, virological non-responders are seen frequently who do not achieve HCV-RNA

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

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Accepted 3 October 2005

DOI 10.1002/jmv.20507

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

negativity, as determined by polymerase chain reaction (PCR), during treatment. The underlying mechanism(s) of the different virological response to treatment in patients with 1b strain infection is still not clear.

Using multivariate analysis, Akuta et al. [2005b] identified hypoalbuminemia, pretreatment substitutions of amino acid (aa) 70 in the core region and pretreatment substitutions of aa 91 as independent and significant pretreatment factors associated with virological non-response, based on 48-week combination therapy of IFN plus ribavirin [Akuta et al., 2005b]. Especially, substitutions of arginine (R) by glutamine (Q) at aa 70, and/or leucine (L) by methionine (M) at aa 91 were significantly more common in virological non-responders. Decline of HCV-RNA levels during treatment in patients with specific substitutions in the core region was significantly less than in those without such substitutions [Akuta et al., 2005b].

The aims of the present study were the following: (1) to investigate the proportion of virological non-responders among a large number of Japanese adult patients who received combination therapy. Especially, to determine the proportion of absolute virological non-responders (i.e., ultimate resistant cases) who did not achieve a log decline of more than 2 from baseline HCV RNA during the initial 24 weeks of therapy, (2) to conduct a case-control study between groups matched for age, sex, genotype, and viral loads, to identify the predictive factors associated with virological non-response, including pretreatment amino acid substitution patterns in the core region, (3) to examine the initial viral kinetics in virological non-responders according to the virological features of the core region.

PATIENTS AND METHODS

Study Population

A total of 323 HCV-infected Japanese adult patients were recruited consecutively into the study of combination therapy with IFN (pegylated [PEG]-IFNα-2b or IFNα-2b) plus ribavirin for 24 weeks or more between 1999 and 2004 at Toranomon Hospital, Tokyo, Japan. Among these, 167 patients were selected in the present study based on the following criteria. (1) They were negative for hepatitis B surface antigen (radioimmunoassay, Dainabot, Tokyo, Japan), positive for anti-HCV (third-generation enzyme immunoassay, Chiron Corp., Emerville, CA), and positive for HCV RNA qualitative analysis with PCR (Amplicor, Roche Diagnostic Systems, California). (2) They were naive to ribavirin therapy. (3) They were infected with HCV genotype 1b alone. (4) Each had a high viral load ($\geq 1.0 \times 10^5$ IU/ml) by quantitative analysis of HCV RNA with PCR (Cobas Amplicor HCV monitor v 2.0 using the 10-fold dilution method, Roche Diagnostics, Tokyo, Japan) at the start of treatment. (5) Each had chronic hepatitis, without cirrhosis or hepatocellular carcinoma (HCC), as confirmed by biopsy examination within the preceding 12 months of enrolment. (6) They had abnormal serum ALT levels (the upper limit of normal for ALT; 50 IU/L) within the preceding 2 months of enrolment. (7) Their body weight was >40 kg. (8) All were free of coinfection with human immunodeficiency virus. (9) None had been treated with antiviral or immunosuppressive agents within the preceding 3 months of enrolment. (10) None was an alcoholic; lifetime cumulative alcohol intake was <500 kg (mild to moderate alcohol intake). (11) None had diabetes, other forms of hepatitis, such as hemochromatosis, Wilson disease, primary biliary cirrhosis, alcoholic liver disease, and autoimmune liver disease. (12) None of the females was pregnant or lactating mother. (13) All accepted treatment for 24 weeks or more as outlined in the study protocol, as well as repeated evaluation of HCV-RNA levels during treatment (at least once every month). (14) Each signed a consent form of the study protocol that had been approved by the Human Ethics Review Committee of Toranomon Hospital.

With regard to the treatment protocol, 21 (31.8%) patients received PEG-IFNα-2b at a dose of 1.5 μg/kg subcutaneously each week plus oral ribavirin at 600–800 mg/day for 24 weeks or more. The remaining 45 (68.2%) patients received 6 million units of IFNα-2b intramuscularly each day for 24 weeks or more (daily for the initial 2 weeks, followed by three times per week for 22 weeks or more), and oral ribavirin at a dose of 600–800 mg/day for 24 weeks or more.

Table I summarizes the profiles and data of the 167 patients at the commencement of combination therapy of IFN plus ribavirin. They included 119 men and 48 women, aged 22–68 years (median, 54 years). The median total duration of treatment was 24 weeks (range, 24–48 weeks). In 46 (27.5%) patients, the dose of ribavirin was reduced during treatment due to a fall in hemoglobin concentration.

Patients who remained positive for HCV RNA based on quantitative and/or qualitative PCR analyses during and at the end of initial 24 weeks of combination therapy, were defined as virological non-responders. On the other hand, patients who became HCV RNA negative by qualitative PCR analysis during and/or at the end of initial 24 weeks were defined as virological responders. Virological non-responders who could not or could achieve a log decline of more than 2 from baseline of HCV RNA based on quantitative PCR analyses during the initial 24 weeks of combination therapy, were defined as absolute virological non-responders or relative virological non-responders, respectively.

Applying multivariate analysis, previous studies identified substitutions of aa 70 in the core region and substitutions of aa 91 as independent and significant pretreatment factors associated with virological non-response to combination therapy in patients with high viral load of genotype 1b [Akuta et al., 2005b]. Therefore, based on the larger numbers of patients, a case-control study was conducted to compare the substitution patterns in aa 70 and/or aa 91 of the core region, between virological non-responders and virological responders who were matched for age, sex, genotype, and viral load, in the present study.

TABLE I. Patient Profile and Laboratory Data at Commencement of Combination Therapy of Interferon Plus Ribavirin

n	167
Age (years)*	54 (22–68)
Sex (M/F)	119/48
Positive history of blood transfusion	50 (29.9%)
Positive family history of liver disease	52 (31.1%)
Genotype 1b	167 (100%)
High viral load ($\geq 1.0 \times 10^5$ IU/ml)	167 (100%)
Serum alanine aminotransferase (IU/l)*	90 (24–398)
Serum albumin (g/dl)*	3.8 (2.7–4.7)
Hemoglobin (g/dl)*	14.8 (11.1–18.2)
Platelet count ($\times 10^4/mm^3$)*	17.3 (7.1–26.4)
Stage (F1/F2/F3)[a]	94/44/29

Data are number and percentages of patients, except those denoted by *, which represent the median (range) values.
[a]Stage of chronic hepatitis by Desmet et al. [1994]. ALT levels were abnormal in all patients at recruitment. Normal reference ranges: 6–50 IU/L for alanine aminotransferase and 3.9–5.2 g/dl for albumin.

Laboratory Tests

Blood samples were obtained at least once every month before, during, and after treatment, and were analyzed for ALT and HCV-RNA levels. The serum samples were frozen at −80°C within 4 hr of collection and were thawed at the time of measurement. HCV genotype was determined by PCR using a mixed primer set derived from the nucleotide sequences of NS5 region [Chayama et al., 1993]. HCV-RNA levels were measured quantitatively by PCR (Cobas Amplicor HCV monitor v 2.0 using the 10-fold dilution method, Roche Diagnostics, Tokyo, Japan) at least once every month before, during, and after therapy. The dynamic range of the assay was 5.0×10^3 to 5.0×10^6 IU/ml. Samples collected during and after therapy that showed undetectable levels of HCV-RNA ($<5.0 \times 10^3$ IU/ml) were checked also by qualitative PCR (Amplicor, Roche Diagnostic Systems, California), which has a higher sensitivity than quantitative analysis, and the results were expressed as positive or negative. The lower limit of the assay was 50 IU/ml.

Histopathological Examination of Liver Biopsies

Liver biopsy specimens were obtained percutaneously or at peritoneoscopy using a modified Vim Silverman needle with an internal diameter of 2 mm (Tohoku University style, Kakinuma Factory, Tokyo, Japan), fixed in 10% formalin, and stained with hematoxylin and eosin, Masson's trichrome, silver impregnation, and periodic acid-Schiff after diastase digestion. All specimens for examinations contained six or more portal areas. Histopathological diagnosis was confirmed by an experienced liver pathologist (H.K.) who was blinded to the clinical data. Chronic hepatitis was diagnosed based on histological assessment according to the scoring system of Desmet et al. [1994]. Hepatocyte steatosis was graded as either none (absent), mild (less than 1/3 of hepatocytes involved), moderate (greater than 1/3 but less than 2/3 of hepatocytes involved), or severe (greater than 2/3 of hepatocytes involved) [D'Alessandro et al., 1991].

Nucleotide Sequencing of the Core and NS5A Gene

The core amino acids (aa) 1–191 and NS5A aa 2209–2248 (IFN-sensitivity determining region [ISDR]) [Enomoto et al., 1995, 1996] sequences were determined by the direct sequencing method using pretreatment sera of 66 patients. These sequences were compared with the consensus sequence of genotype 1b, which was determined by comparing the sequences obtained in this study and prototype sequence (HCV J) [Kato et al., 1990]. HCV RNA was extracted from serum samples at the start of treatment and reverse transcribed with random primers and MMLV reverse transcriptase (Takara Syuzo, Tokyo, Japan). DNA fragments were amplified by PCR using the following primers. (a) Nucleotide sequences of the core region: The first-round PCR was performed with CC11 (sense, 5′-GCC ATA GTG GTC TGC GGA AC-3′) and e14 (antisense, 5′-GGA GCA GTC CTT CGT GAC ATG-3′) primers, and the second-round PCR with CC9 (sense, 5′-GCT AGC CGA GTA GTG TT-3′) and e14 (antisense) primers. (b) Nucleotide sequences of ISDR in NS5A: The first-round PCR was performed with ISDR1 (sense, 5′-ATG CCC ATG CCA GGT TCC AG-3′) and ISDR2 (antisense, 5′-AGC TCC GCC AAG GCA GAA GA-3′) primers, and the second-round PCR with ISDR3 (sense, 5′-ACC GGA TGT GGC AGT GCT CA-3′) and ISDR4 (antisense, 5′-GTA ATC CGG GCG TGC CCA TA-3′) primers. ([a], hemi-nested PCR; [b], nested PCR). All samples were denatured initially at 95°C for 15 min. The 35 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 PCR product was transferred to the second PCR reaction. The 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. The amplified PCR products were purified by the QIA quick PCR purification kit (Qiagen, Tokyo, Japan) after agarose gel electrophoresis and then used for direct sequencing. Dideoxynucleotide termination sequencing was performed 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.

Viral Kinetic Study of Virological Non-Response

Viral kinetics in the initial 24 weeks was evaluated in the two groups of absolute virological non-responders and relative virological non-responders at three time points (8, 12, and 24 weeks during treatment). Decline of HCV-RNA levels from baseline was expressed using $\log_{10}$ of viral load at each time point, in comparison with the pretreatment viral load. For data analysis, $\log_{10}$ of the cut-off value (5.0×10^3 IU/ml) was used for HCV-RNA values below the limit of detection.

Statistical Analysis

Non-parametric tests were used to compare the characteristics of the groups, including the Mann–Whitney *U* test, Chi-squared test, and Fisher's exact probability test. Multiple comparisons were examined by the Bonferroni test. Univariate and multivariate logistic regression analyses were used to determine the factors that significantly contributed to virological non-response. The odds ratios and 95% confidence intervals (95% CI) were also calculated. All *P* values less than 0.05 by the two-tailed test were considered significant. 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 predictive factors associated with virological non-response included the following variables: sex, age, history of blood transfusion, familial history of liver disease, body mass index, ALT, albumin, hemoglobin, platelet count, indocyanine green retention rate at 15 min (ICG R15), serum iron, serum ferritin, creatinine clearance, viremia level, pathological staging, hepatocyte steatosis, type of IFN, ribavirin dose relative to body weight, dose reduction, and pretreatment amino acid substitution in the core and ISDR of NS5A. Statistical analyses were performed using the SPSS software (SPSS, Inc., Chicago, IL).

RESULTS

The response to IFN/ribavirin combination treatment protocol among the 167 patients included virological non-response in 44 (26.3%) and virological response in 123 (73.7%). Furthermore, the first group of 44 virological non-responders consisted of 20 absolute virological non-responders (45.5%) and 24 relative virological non-responders (54.5%). To compare the pretreatment features between virological non-responders and virological responders, all 44 virological non-responders entered a case-control study along with 22 virological responders. The latter group was selected from among the 123 because they matched patients of the virological non-response group with respect to sex, age, genotype, and viral load. Table II lists the clinical and virological features of patients who entered the matched case-control study.

Predictive Factors Associated With Virological Non-Response in Multivariate Analysis

The clinical and virological data listed in Table II for the whole population sample were analyzed to determine the factors that could predict virological non-response. Univariate analysis identified six parameters that tended to or significantly influenced the virological non-response. These included ribavirin dose according to body weight ($P=0.019$), staging ($P=0.024$), serum albumin ($P=0.062$), hepatocyte steatosis ($P=0.049$), and presence of aa substitution in HCV core in the pretreatment sample (substitution of aa 70, $P=0.030$; and aa 70 and/or 91, $P=0.006$). ISDR amino acid substitutions, which had been reported as one predictor of sustained virological response by IFN monotherapy [Enomoto et al., 1995, 1996], were not identified as a predictor of virological non-response to the combination therapy of IFN/ribavirin.

Multivariate analysis identified three parameters that independently influenced virological non-response; ribavirin dose ($P=0.019$), hepatocyte steatosis ($P=0.040$), and substitutions of aa 70 and/or 91 ($P=0.005$) (Table III).

Treatment Efficacy According to Amino Acid Substitution Patterns in HCV Core Region

Frequencies of the substitution site at aa 70 were 60.0% (12/20), 37.5% (9/24), and 18.2% (4/22) in the three groups of absolute virological non-responders, relative virological non-responders, and virological responders, respectively. The proportion of such substitution site in absolute virological non-responders was significantly higher than that in virological responders ($P=0.015$; Bonferroni test). Frequencies of substitution pattern of glutamine (Q) at aa 70 were 55.0% (11/20), 37.5% (9/24), and 13.6% (3/22) in the three groups of absolute virological non-responders, relative virological non-responders, and virological responders, respectively. The proportion of such substitution pattern in absolute virological non-responders was significantly higher than that in virological responders ($P=0.014$; Bonferroni test).

The frequencies of substitution sites at aa 70 and/ or 91, which were a significant predictor of virological non-response based on multivariate analysis, were 95.0% (19/20), 62.5% (15/24), and 40.9% (9/22) in the three groups of absolute virological non-responders, relative virological non-responders, and virological responders, respectively. The proportion of such substitution sites in absolute virological non-responders was significantly higher than that in relative virological non-responders ($P=0.049$; Bonferroni test) and virological responders

TABLE II. Clinical and Virological Features of Patients Infected With HCV Genotype1b With or Without Virological Response to Combination Therapy of Interferon Plus Ribavirin (Matched Case-Control study)

	Virological non-responders (case; n = 44)	Virological responders (control; n = 22)
Matching data		
Age (years)*	53 (24–67)	53 (20–64)
Sex (M/F)	33/11	17/5
Genotype 1b	44 (100%)	22 (100%)
High viral load ($\geq 1.0 \times 10^5$ IU/ml)[b]	44 (100%)	22 (100%)
Demographic data		
Positive history of blood transfusion	8 (18.2%)	6 (27.3%)
Positive family history of liver disease	11 (25.0%)	7 (31.8%)
Body mass index (kg/m^2)*	23.5 (17.3–32.3)	22.9 (19.3–28.8)
Laboratory data*		
Serum alanine aminotransferase (IU/L)	78.5 (24–247)	100.5 (43–276)
Serum albumin (g/dl)	3.7 (3.3–4.7)	3.9 (3.4–4.2)
Hemoglobin (g/dl)	14.7 (12.0–17.0)	15.0 (12.2–17.4)
Platelet count ($\times 10^4$/mm^3)	16.2 (7.1–26.6)	15.7 (10.1–30.9)
ICG R15 (%)[a]	18 (7–49)	12 (7–26)
Serum iron (μg/dl)	149 (51–253)	142 (52–308)
Serum ferritin (μg/L)	158 (19–696)	136 (<10–644)
Creatinine clearance (ml/min)	95.7 (42.6–174.6)	106.3 (45.7–131.0)
Viral load (KIU/ml)	1,650 (160–5100)	1,700 (650–4900)
Histological findings		
Stage (F1/F2/F3)[b]	19/15/10	15/7/0
Hepatocyte steatosis (none-mild/ moderate-severe)	33/11	21/1
Treatment		
PEG-IFNα-2b/IFNα-2b	11/33	10/12
Ribavirin dose (mg/kg)*	10.8 (7.3–14.2)	11.4 (9.7–13.0)
Virological features		
Number of amino acid substitutions in ISDR (0/1–3/≥4/ND)	26/11/3/4	10/10/2/0
Presence of amino acid substitutions sites in the core region		
aa 70	21 (47.7%)	4 (18.2%)
aa 91	22 (50.0%)	7 (31.8%)
aa 70 and/ or 91	34 (77.3%)	9 (40.9%)

Data are number and percentages of patients, except those denoted by *, which represent the median (range) values.
[a]ICG R15: indocyanine green retention rate at 15 min.
[b]Stage of chronic hepatitis by Desmet et al. [1994]. ALT levels were abnormal in all patients at recruitment. Normal reference ranges: 6–50 IU/L for alanine aminotransferase and 3.9–5.2 g/dl for albumin.

($P < 0.001$; Bonferroni test). Frequencies of substitution patterns of glutamine (Q) at aa 70 and/or methionine (M) at aa 91 were 90.0% (18/20), 62.5% (15/24), and 40.9% (9/22) in the three groups of absolute virological non-responders, relative virological non-responders, and virological responders, respectively. The proportion of such substitution patterns in absolute virological non-responders was significantly higher than in virological responders ($P = 0.002$; Bonferroni test). Figure 1 shows the association of aa substitution patterns at aa 70 and/or 91 and response to combination therapy. There were no significant differences in other substitution sites, patterns and treatment efficacy among the three groups.

Viral Kinetics in Virological Non-Responderstpb

The decline of HCV-RNA levels at 8, 12, and 24 weeks relative to baseline was evaluated in absolute virological non-responders and relative virological non-responders. The decline at each time point was significantly lower in

TABLE III. Factors Associated With Virological Non-Response to Combination Therapy of Interferon Plus Ribavirin in 66 Patients Infected With HCV Genotype 1b, Identified by Multivariate Analysis

Factor	Category	Odds ratio (95% confidence interval)	P
Ribavirin dose (mg/kg)	1: <11.0	1	
	2: ≥11.0	0.195 (0.050–0.765)	0.019
Hepatocyte steatosis	1: None, mild	1	
	2: Moderate, severe	14.299 (1.127–181.344)	0.040
Substitution of aa 70 and/or 91	1: Absent	1	
	2: Present	7.343 (1.841–29.285)	0.005

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

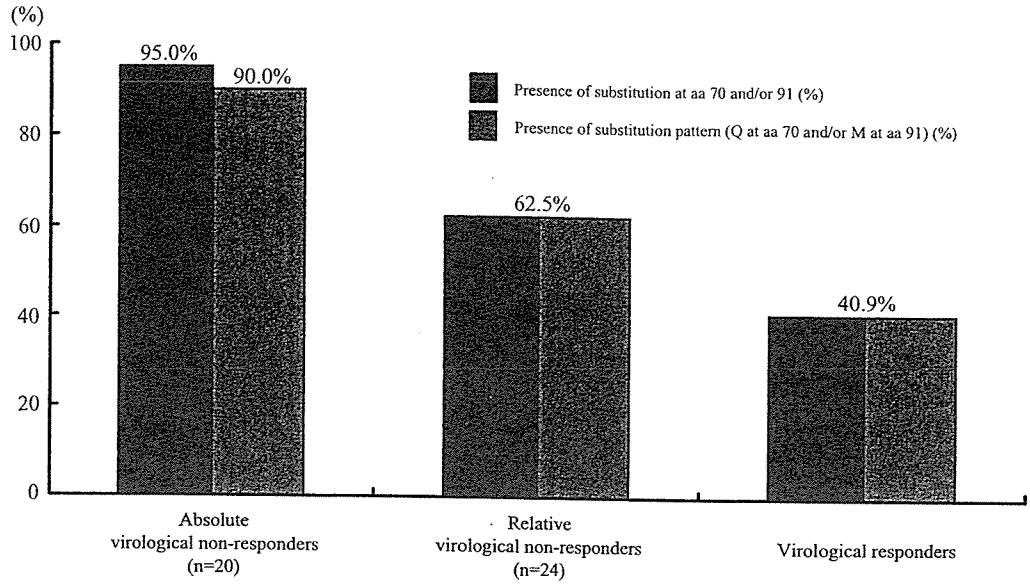

Fig. 1. Frequencies of substitutions at amino acid sites 70 and/or 91 and substitution patterns (glutamine [Q] at aa 70 and/or methionine [M] at aa 91) in HCV core region are evaluated in three groups of absolute virological non-responders, relative virological non-responders, and virological responders. The proportion of such substitution sites in absolute virological non-responders was significantly higher than that in relative virological non-responders ($P = 0.049$; Bonferroni test) and virological responders ($P < 0.001$; Bonferroni test). The proportion of such substitution patterns in absolute virological non-responders was significantly higher than that in virological responders ($P = 0.002$; Bonferroni test).

absolute virological non-responders than in relative virological non-responders (8 weeks, $P = 0.001$; 12 weeks, $P < 0.001$; 24 weeks, $P < 0.001$). Figure 2 shows the decline of HCV-RNA levels in virological non-responders, according to aa substitutions of the core region. The decline at each time point was significantly lower in patients with substitution sites of aa 70 and/or 91 than in those without them (8 weeks, $P = 0.004$; 12 weeks, $P = 0.005$; 24 weeks, $P = 0.013$), and with substitution patterns of Q at aa 70 and/or M at aa 91 than in those without them (8 weeks, $P = 0.008$; 12 weeks, $P = 0.015$; 24 weeks, $P = 0.011$).

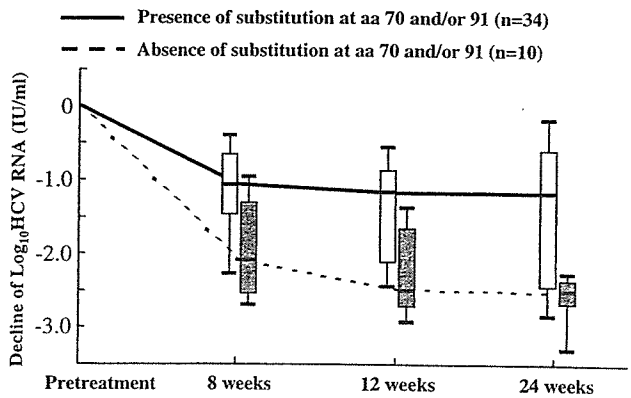

Fig. 2. Log changes in viral load from baseline at 8, 12, and 24 weeks during treatment, according to amino acid substitutions of the HCV core region. Bars within the boxes indicate the median value of log changes in viral load. The boxes denote the 25th to 75th centiles, the lower and upper bars the 10th and 90th centiles, respectively. The decline of HCV-RNA levels at each time point was significantly lower in patients with substitution sites of aa 70 and/or 91 than in those without them (Mann–Whitney U test).

DISCUSSION

Using multivariate analysis, Akuta et al. [2005b] identified pretreatment substitutions of aa 70 in the core region and substitutions of aa 91 as independent and significant pretreatment factors associated with virological non-response to 48-week combination therapy of IFN plus ribavirin. Substitutions of R by Q at aa 70 and/or L by M at aa 91, were significantly more common in virological non-responders. Furthermore, decline of HCV-RNA levels during treatment in patients with specific substitutions in the core region was significantly less than in those without such substitutions [Akuta et al., 2005b]. Using the same analysis, the present study based on a larger number of patients has also identified substitution patterns in aa 70 and/or aa 91 as independent and significant pretreatment factors associated with virological non-response to combination therapy, by a case-control study matched for age, sex, genotype, viral loads. Especially, most absolute virological non-responders, as ultimate resistant cases, were found to have such specific substitution sites (95.0%), and also had substitution patterns of glutamine (Q) at aa 70 and/or methionine (M) at aa 91 (90.0%).

Furthermore, such specific substitutions also significantly affected the viral kinetics in absolute virological non-responders and relative virological non-responders. Hence, we propose that the aa substitution pattern in the core region is useful as a pretreatment predictor of virological non-response to IFN/ribavirin combination therapy.

IFN-α and IFN-β bind to type I IFN receptor, and one major pathway in type I IFN signaling involve the Jak-STAT signaling cascade [Song and Shuai, 1998; Stoiber