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) HBeAg positive (n = 10)	HBeAg negative (n = 17)	Elevated group (n = 87) HBeAg positive (n = 56)	HBeAg negative (n = 31)	P
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	NS
(Genotype: C/other than C)	26/1		72/15		
Period from commencement to emergence of YMDD motif mutation[a]	24 (7–36)	30 (10–63)	13 (5–56)	15 (6–59)	0.0001
	24 (7–63)		14 (5–59)		
Mutant type: I/V/mix	4/5/1	10/6/1	25/12/19	15/9/7	0.02
(Mutant type: I or V/mix)	25/2		61/26		

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

Patient	Sex	Age (years)	Genotype	Histology (staging)	Baseline					Severe hepatitis exacerbation			
					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

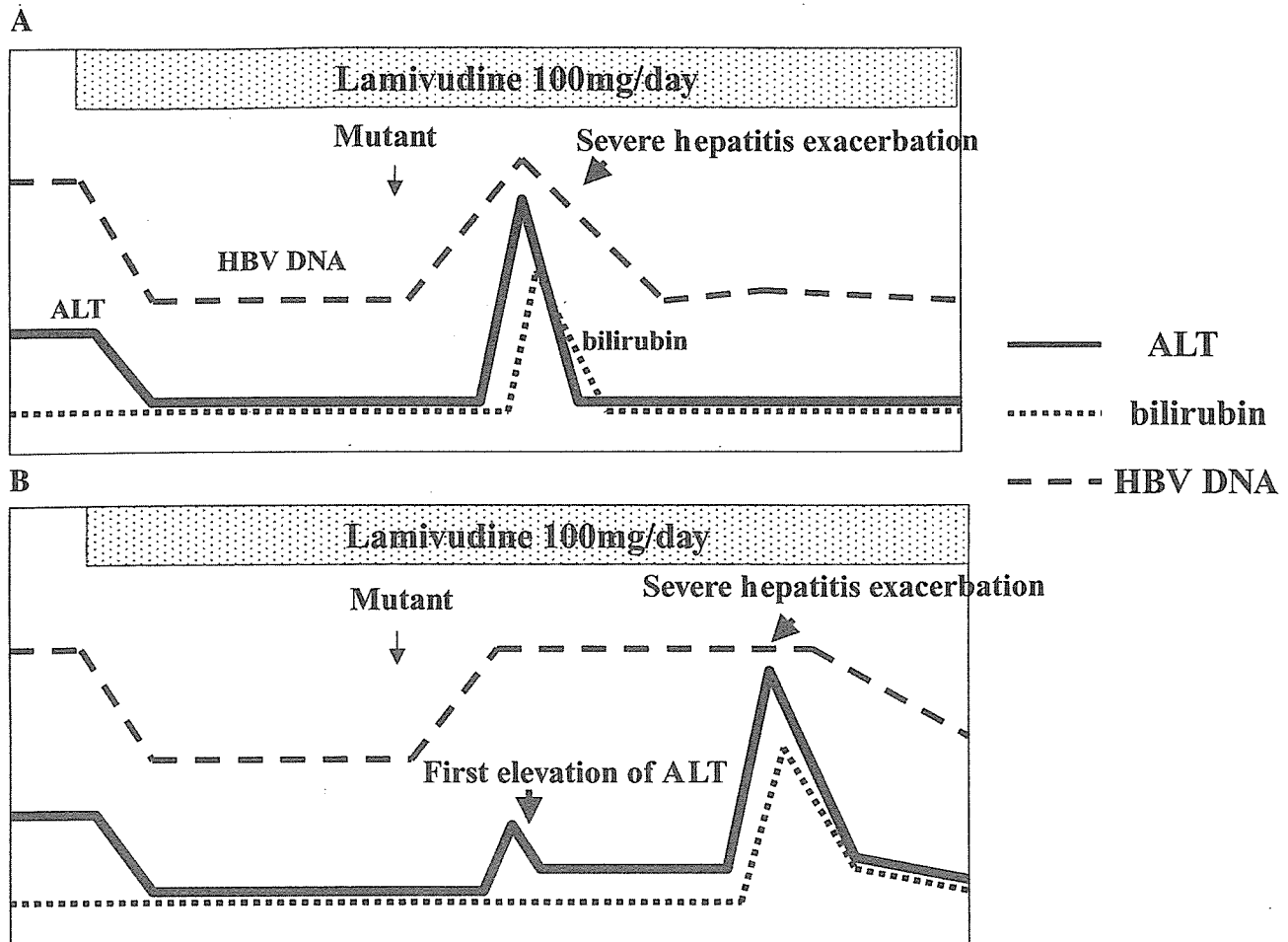F, female; M, male; NA, not available; +, positive; −, negative.

A

B

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
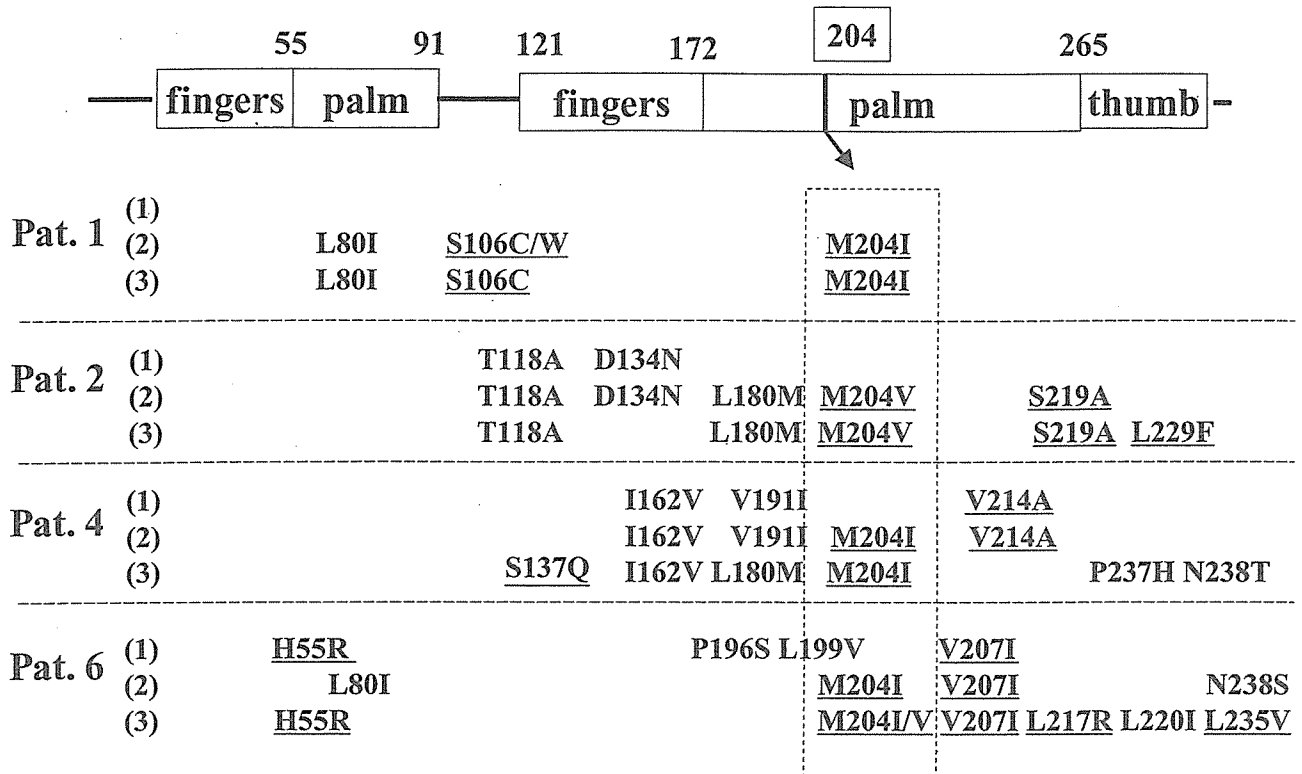
```
        55        91      121       172        [204]              265
  ──┤fingers│ palm ├──────┤  fingers  │      │   palm      │thumb├──
                                                    ↓
```

Pat. 1   (1)
         (2)   L80I   S106C/W   M204I
         (3)   L80I   S106C   M204I
- -
Pat. 2   (1)   T118A D134N
         (2)   T118A D134N L180M M204V   S219A
         (3)   T118A   L180M M204V   S219A L229F
- -
Pat. 4   (1)   I162V V191I   V214A
         (2)   I162V V191I M204I   V214A
         (3)   S137Q I162V L180M M204I   P237H N238T
- -
Pat. 6   (1)   H55R   P196S L199V V207I
         (2)   L80I   M204I   V207I   N238S
         (3)   H55R   M204I/V V207I L217R L220I L235V

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
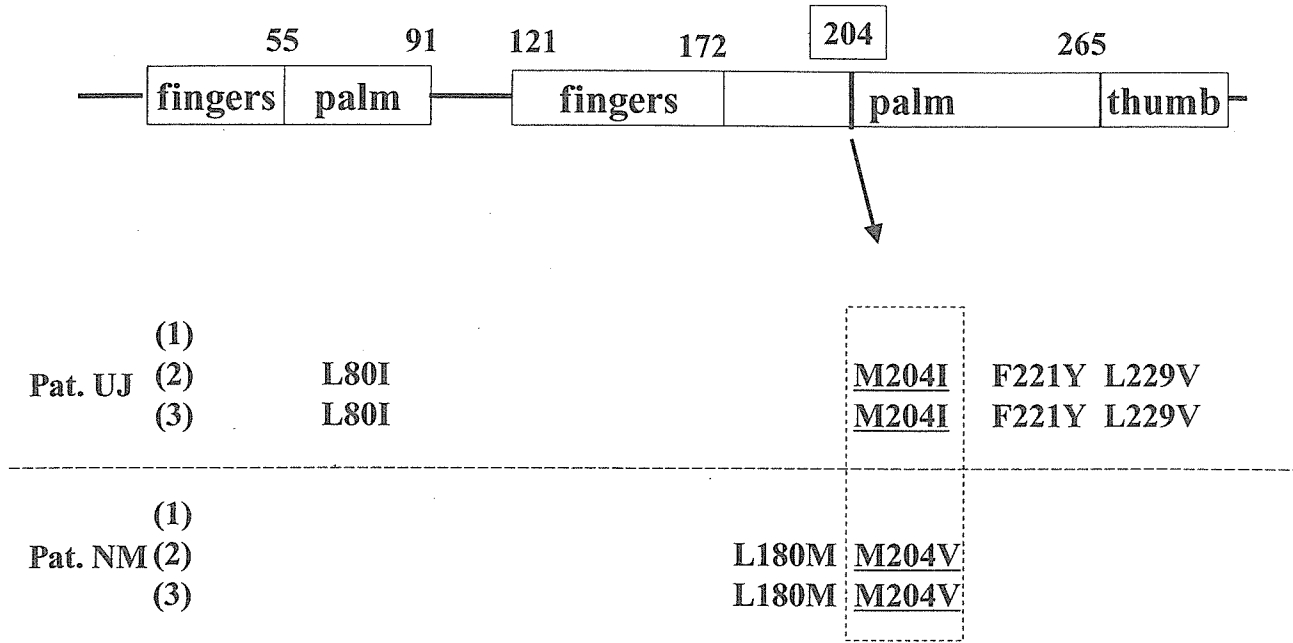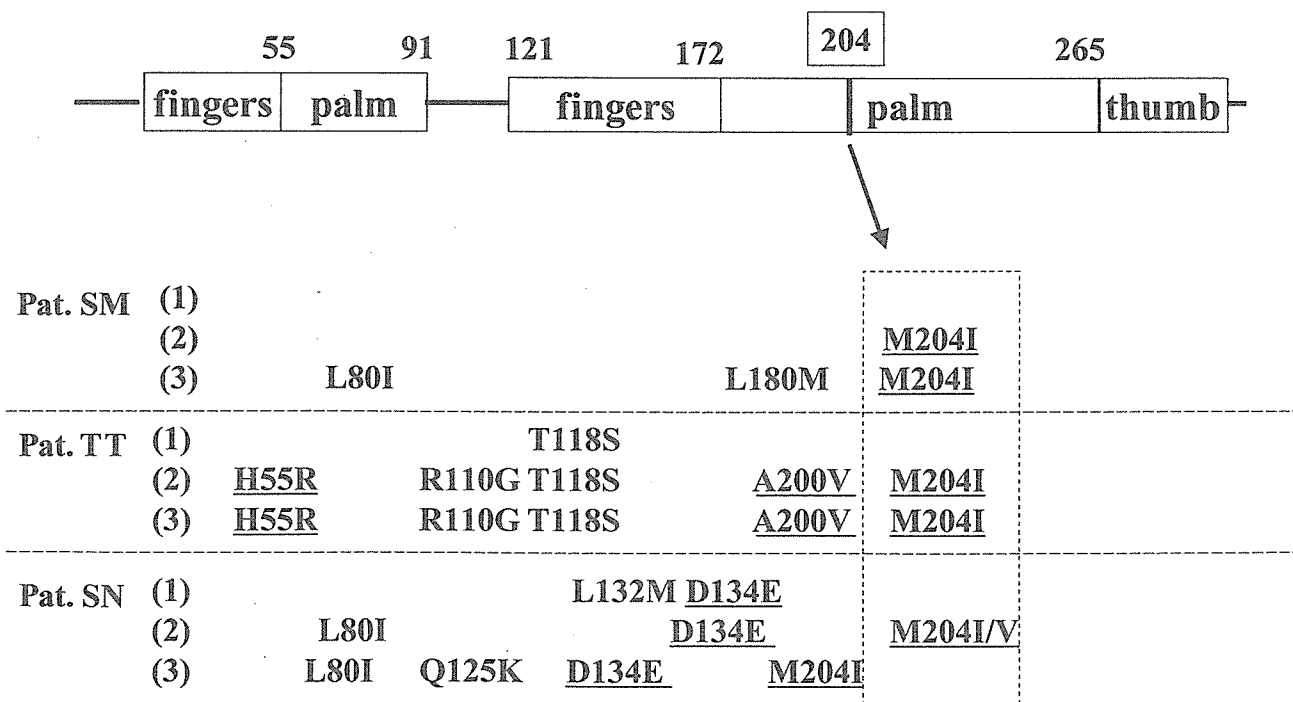
Fig. 3. Substitutions of amino acid sequences in the reverse transcriptase (rt) domain of the HBV polymerase gene in patients with non-elevated HBV DNA levels after emergence of the YMDD mutant. Measurements were conducted at three time points: (1) and (2) were at the same time points, and (3) at >2.5 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.
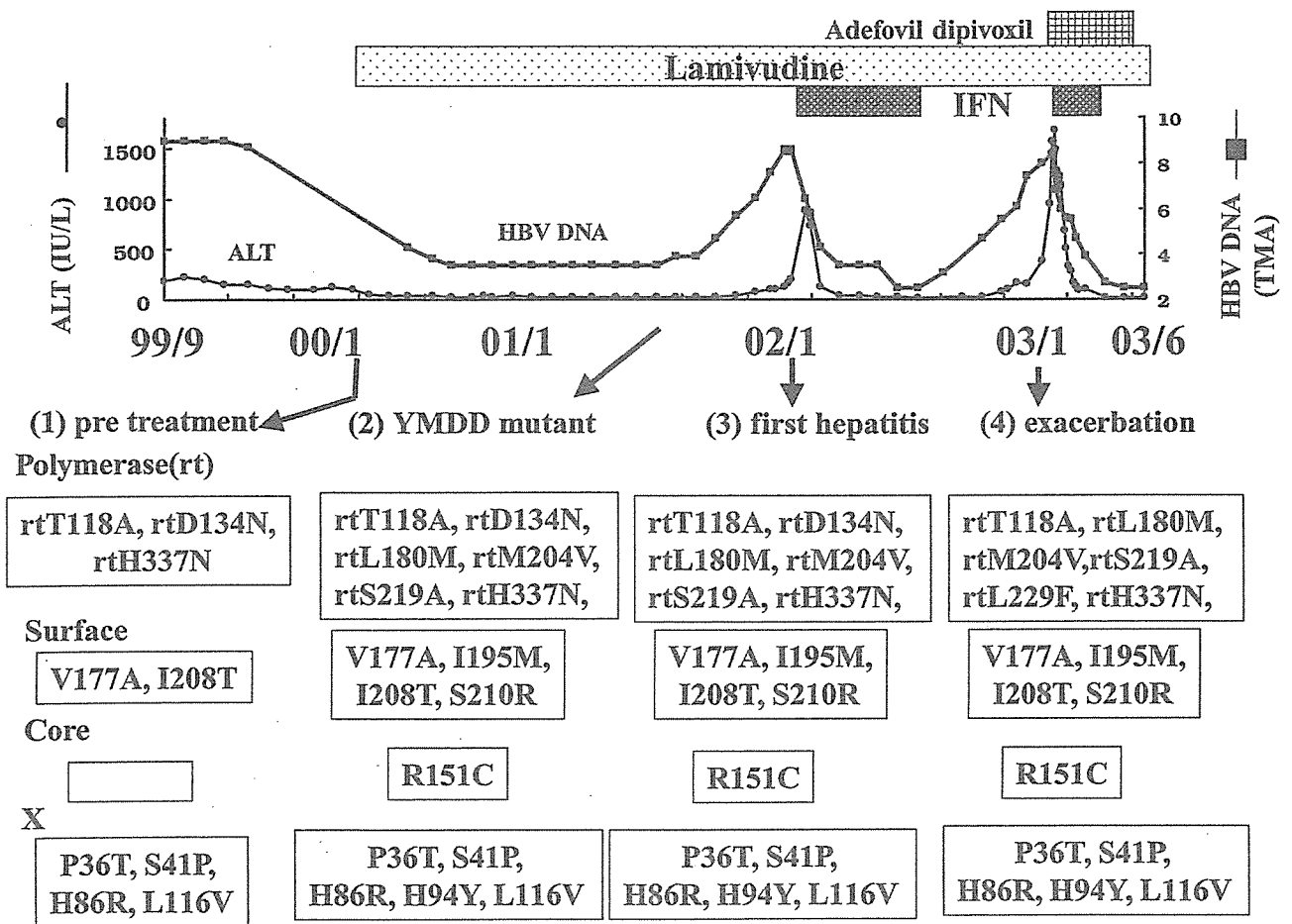
Fig. 5. Correlation between viral load, serum ALT, and accumulation of HBV substitutions during the clinical course in Patient 2, with severe hepatitis exacerbation. Four serial serum samples (arrows) were collected at various time points before and during lamivudine therapy. In addition to biochemical 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
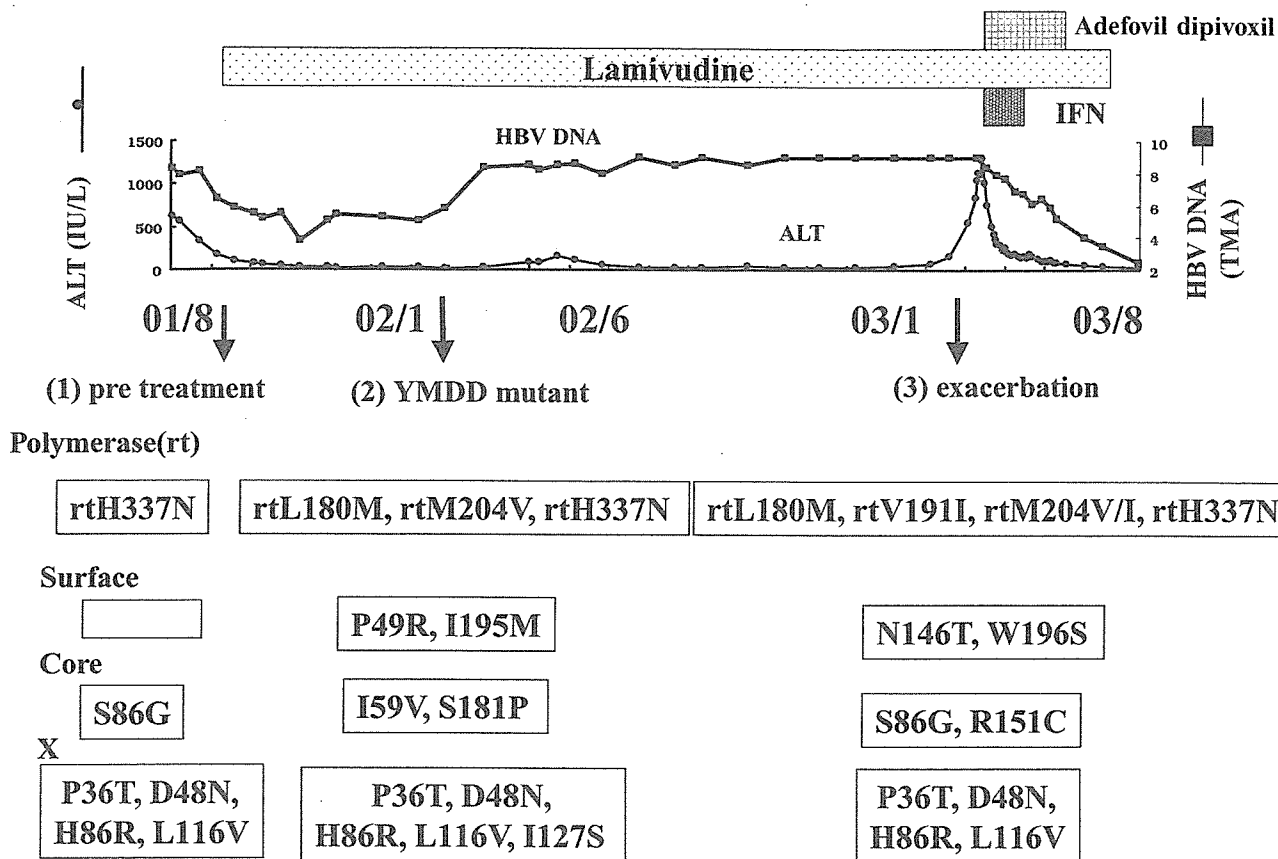
Fig. 6. Correlation between viral load, serum ALT, and accumulation of HBV substitutions during the clinical course in Patient 5, with severe hepatitis exacerbation.

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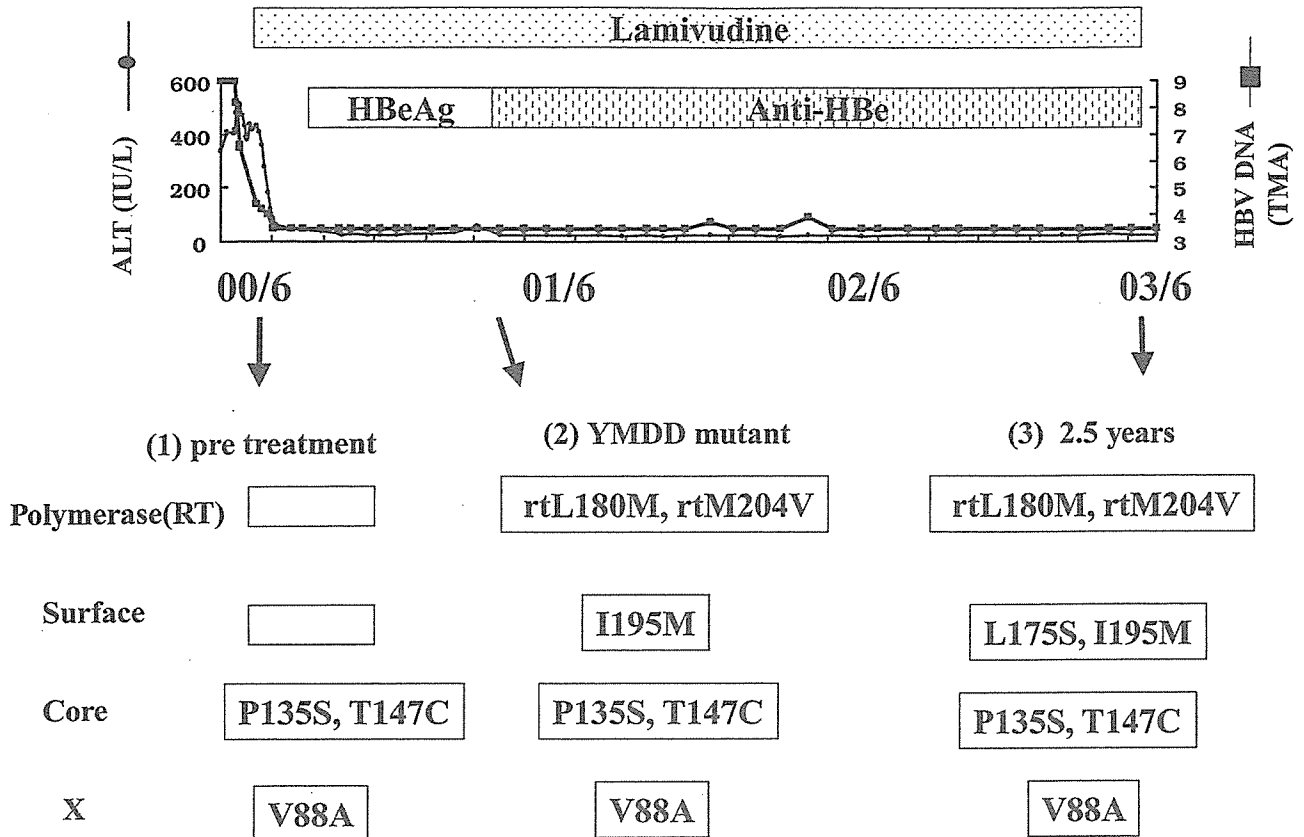
Fig. 7. Correlation between viral load, serum ALT, and accumulation of HBV substitutions during the clinical course in Patient NM, without elevation of HBV DNA.

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.

REFERENCES

Ayres A, Bartholomeusz A, Lau G, Lam KC, Lee JY, Locarnini S. 2003. Lamivudine and famciclovir resistant hepatitis B virus associated with fatal hepatic failure. J Clin Virol 27:111–116.

Beasley RP, Hwang LW, Lin CC, Chien CS. 1981. Hepatocellular carcinoma and hepatitis B virus. A prospective study of 22,707 men in Taiwan. Lancet ii:1129–1233.

Bock CT, Tillmann HL, Torresi J, Klempnauer J, Locarnini S, Manns MP, Trautwein C. 2002. Selection of hepatitis B virus polymerase mutations with enhanced replication by lamivudine treatment after liver transplantation. Gastroenterology 122:264–273.

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

Chen RY, Edwards R, Shaw T, Colledge D, Delaney WE 4th, Isom H, Bowden S, Desmond P, Locarnini SA. 2003. Effect of the G1896A precore mutation on drug sensitivity and replication yield of lamivudine-resistant HBV in vitro. Hepatology 37:27–35.

Chin R, Shaw T, Torresi J, Sozzi V, Trautwein C, Bock T, Manns M, Isom H, Furman P, Locarnini S. 2001. In vitro susceptibilities of wild-type or drug-resistant hepatitis B virus to (−)-beta-D-2,6-diaminopurine dioxolane and 2′-fluoro-5-methyl-beta-L-arabinofuranosyluracil. Antimicrob Agents Chemother 45:2495–2501.

Colonno RJ, Genovesi EV, Medina I, Lamb L, Durham SK, Huang ML, Corey L, Littlejohn M, Locarnini S, Tennant BC, Rose B, Clark JM. 2001. Long-term entecavir treatment results in sustained antiviral efficacy and prolonged life span in the woodchuck model of chronic hepatitis infection. J Infect Dis 184:1236–1245.

Das K, Xiong X, Yang H, Westland CE, Gibbs CS, Sarafianos SG, Arnold E. 2001. Molecular modeling and biochemical characterization reveal the mechanism of hepatitis B virus polymerase resistance to lamivudine (3TC) and emtricitabine (FTC). J Virol 75:4771–4779.

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

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

Fu L, Cheng YC. 1998. Role of additional mutations outside the YMDD motif of hepatitis B virus polymerase in L(−)SddC(3TC) resistance. Biochem Pharmacol 55:1567–1572.

Günther S, Li B-C, Miska S, Krüger DH, Meisel H, Will H. 1995. A novel method for efficient amplification of whole hepatitis B virus genomes permits rapid functional analysis and reveals deletion mutations in immunosuppressed patients. J Virology 69:5437–5444.

Hadziyannis SJ, Papatheodoridis GV, Dimou E, Laras A, Papaioannou C. 2000. Efficacy of long-term lamivudine monotherapy in patients with hepatitis B e antigen-negative chronic hepatitis B. Hepatology 32:847–851.

Kamisango K, Kamogawa C, Sumi M, Goto S, Hirao A, Gonzales F, Yasuda K, Iino S. 1999. Quantitative detection of hepatitis B virus by transcription-mediated amplification and hybridization protection assay. J Clin Microbiol 37:310–314.

Kim JW, Lee HS, Woo GH, Yoon JH, Jang JJ, Chi JG, Kim CY. 2001. Fatal submassive hepatitis necrosis associated with tyrosine-methionine-asparate-asparate-motif mutation of hepatitis B virus after long-term lamivudine therapy. Clin Infec Dis 33:403–405.

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

Leung N. 2000. Liver disease-significant improvement with lamivudine. J Med Virol 61:380–385.

Liaw YF, Chien RN, Yeh CT, Tsai SL, Chu CM. 1999. Acute exacerbation and hepatitis B virus clearance after emergence of YMDD motif mutation during lamivudine therapy. Hepatology 30:567–572.

Lin CL, Tsai SL, Lee TH, Chien RN, Liao SK, Liaw YF. 2005. High frequency of functional anti-YMDD and mutant cytotoxic T lymphocytes after in vitro expansion correlates with successful response to lamivudine therapy for chronic hepatitis B. Gut 54:152–161.

Ling R, Harrison TJ. 1999. Functional analysis of mutations conferring lamivudine resistance on hepatitis B virus. J Gen Virol 80:601–606.

Melegari M, Scaglioni PP, Wands JR. 1998. Hepatitis B virus mutations associated with 3TC and famciclovir administration are replication defective. Hepatology 27:628–633.

Nafa S, Ahmed S, Tavan D, Pichoud C, Berby F, Stuyver L, Johnson M, Merle P, Abidi H, Trepo C, Zoulim F. 2000. Early detection of viral resistance by determination of hepatitis B virus polymerase mutations in patients treated by lamivudine for chronic hepatitis B. Hepatology 32:1078–1088.

Ono-Nita SK, Kato N, Shiratori Y, Masaki T, Lan KH, Carrilho FJ, Omata M. 1999. YMDD motif in hepatitis B virus DNA polymerase influences on replication and lamivudine resistance: A study by in vitro full-length viral DNA transfection. Hepatology 29:939–945.

Perrillo RP. 2001. Acute flares in chronic hepatitis B: The natural and unnatural history of an immunologically mediated liver disease. Gastroenterology 120:1009–1022.

Stuyver LJ, Locarnini SA, Lok A, Richman DD, Carman WF, Dienstag JL, Schinazi RF. 2001. Nomenclature for antiviral-resistant human hepatitis B virus mutations in the polymerase region. Hepatology 33:751–757.

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

Suzuki F, Suzuki Y, Tsubota A, Akuta N, Someya T, Kobayashi M, Saitoh S, Arase Y, Ikeda K, Kumada H. 2002. Mutations of polymerase, precore and core promoter gene in hepatitis B virus during 5-year lamivudine therapy. J Hepatol 37:824–830.

Suzuki F, Tsubota A, Arase Y, Suzuki Y, Akuta N, Hosaka T, Someya T, Kobayashi M, Saitoh S, Ikeda K, Kobayashi M, Matsuda M, Satoh J,

Takagi K, Kumada H. 2003. Efficacy of lamivudine therapy and factors associated with emergence of resistance in chronic hepatitis B virus infection in Japan. Intervirology 46:182–189.

Tacke F, Gehrke C, Luedde T, Heim A, Manns MP, Trautwein C. 2004. Basal core promoter and precore mutations in the hepatitis B virus genome enhance replication efficacy of lamivudine-resistant mutants. J Virol 78:8524–8535.

Torresi J. 2002b. The virological and clinical significance of mutation in the overlapping envelope and polymerase genes of hepatitis B virus. J Clin Virol 25:97–106.

Torresi J, Earnest-Silveira L, Civitico G, Walters TE, Lewin SR, Fyfe J, Locarnini SA, Manns M, Trautwein C, Bock TC. 2002a. Restoration of replication phenotype of lamivudine-resistant hepatitis B virus mutants by compensatory changes in the "fingers" subdomain of the viral polymerase selected as a consequence of mutations in the overlapping S gene. Virology 299:88–99.

Zollner B, Stoehr A, Plettenberg A, Feucht H, Schroter M, Schafer P, Laufs R. 2000. In vivo dynamics and pathogenicity of wild-type and resistant hepatitis B virus during long-term lamivudine mono-therapy—Clinical note. J Clin Virol 17:183–188.

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CLINICAL RESEARCH STUDY

Long-Term Outcome after Hepatitis B Surface Antigen Seroclearance in Patients with Chronic Hepatitis B

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

[a]Department of Gastroenterology and [b]Hepatic Research Unit, Toranomon Hospital, Tokyo, Japan.

ABSTRACT

PURPOSE: The aim of this study was to elucidate the long-term outcome after hepatitis B surface antigen (HBsAg) seroclearance in a large number of Japanese patients.

METHODS: We studied the biochemical, virologic, histologic, and prolonged prognoses of 231 Japanese patients with HBsAg seroclearance (median follow-up, 6.5 years). Serum alanine aminotransferase, serum hepatitis B virus (HBV) markers, liver histology, and clinical aspects were monitored. HBV-DNA levels were measured with the qualitative polymerase chain reaction assay. The mean age of patients with HBsAg seroclearance was 52 years.

RESULTS: After HBsAg seroclearance, 203 patients (87.9%) had normal alanine aminotransferase levels 1 year after HBsAg seroclearance. HBV-DNA showed positive results in 4 patients (1.7%) 1 year after HBsAg seroclearance. Thirteen patients were examined for histologic changes of the liver after HBsAg seroclearance. All patients showed marked improvement of necroinflammation of the liver, but only 2 of the 13 patients showed no liver fibrosis. Liver cirrhosis and hepatocellular carcinoma did not develop in any of the 164 patients without evidence of liver cirrhosis at the time of HBsAg seroclearance. Hepatocellular carcinoma developed in 2 of the 67 patients with liver cirrhosis at the time of HBsAg seroclearance. During the observation period, 15 patients died. However, the cause of death of these 15 patients was not related to liver disease, such as hepatocellular carcinoma, decompensated liver cirrhosis, and rupture of esophageal varices.

CONCLUSION: Our results suggest that HBsAg seroclearance confers favorable long-term outcomes in patients without hepatocellular carcinoma or decompensated liver cirrhosis at the time of HBsAg seroclearance © 2006 Elsevier Inc. All rights reserved.

KEYWORDS: Chronic hepatitis B; HBV-DNA; Seroclearance of hepatitis B surface antigen

Chronic hepatitis B virus (HBV) is a serious liver disease with significant mortality. In patients with chronic HBV infection, persistent viral replication is associated with ongoing necroinflammation in the liver and progressive liver damage.[1-3] Yang et al[4] reported that the relative risk

of hepatocellular carcinoma was 9.6 among men who were positive for HBsAg alone and 60.2 among men who were positive for hepatitis B surface antigen (HBsAg) and hepatitis B e antigen (HBeAg), compared with men who were negative for both. Epidemiologic studies have shown that positivity for HBsAg is one of the most important risk factors for hepatocellular carcinoma. However, in patients with HBeAg seroclearance and marked reduction of serum HBV-DNA, the prognosis of the disease is generally improved.[5-7] Therefore, marked reduction of HBV replication can possibly prevent hepatocellular carcinoma development. Moreover, HBsAg

seroclearance has been associated with a good prognosis, including liver histology and liver function improvement and even prolonged survival.[8-10]

However, spontaneous remissions occur in a small proportion of patients during the natural history of chronic HBV infections. Seroclearance of HBsAg in patients with chronic HBV infection is unusual (0.4%-2% per year in white patients[11-13] and 0.1%-0.8% per year in Chinese patients[9]). Thus, until now, few studies have dealt with prognosis in Japanese patients with seroclearance of HBsAg.

A few previous studies have had conflicting results, with some suggesting that adverse complications are not rare in patients with HBsAg clearance,[14,15] and others suggesting that spontaneous HBsAg seroclearance is excellent.[16] These discrepancies might depend on concurrent hepatitis infection, age, and other factors. The present study excluded patients with concurrent hepatitis virus infection. Moreover, the main focus of this article is the survival time of patients with HBsAg seroclearance. Thus, we performed this study to elucidate the long-term outcome after HBsAg seroclearance in a large number of Japanese patients.

CLINICAL SIGNIFICANCE

- HBsAg seroclearance confers favorable long-term outcomes in patients without hepatocellular carcinoma or decompensated liver cirrhosis at the time of HBsAg seroclearance.

- Patients with liver cirrhosis at the time of HBsAg seroclearance should be closely monitored for predictable complications such as hepatocellular carcinoma.

- Some patients had a trace of hepatitis B virus DNA at the fifth and/or tenth year after seroclearance of HBsAg and were followed on with the administration of steroids and/or immunosuppressive agents.

MATERIALS AND METHODS

Patients

From 1972 to 2002, a total of 5055 chronic HBsAg carriers, who were known to be seropositive for HBsAg for at least 6 months, were studied at Toranomon Hospital in Tokyo, Japan. After a mean follow-up period of 4 years (range 0.5-30 years), 231 patients were noted to have delayed HBsAg seroclearance, which is defined as persistent absence of HBsAg antigenemia by radioimmunoassay for at least 1 year and until the last examination. We excluded from the study all patients with: concurrent hepatitis C virus and hepatitis D virus; a history of alcohol abuse or autoimmune liver disease; clinical evidence of hepatocellular carcinoma at entry into the study on the basis of ultrasonography, alpha-fetoprotein levels (<200 ng/mL), and/or histology; or history or clinical evidence of complications of decompensated cirrhosis at enrollment (ie, ascites, encephalopathy, or icterus).

A total of 156 of 231 patients had spontaneous seroclearance of HBsAg; 46 patients had been given interferon monotherapy for 1 to 90 months; 14 patients had

been given steroid withdrawal monotherapy; and 12 patients had been treated with both steroids and interferon. The remaining 3 patients had been given 100 mg of lamivudine daily for more than 1 year. The total median dose of interferon monotherapy was 336 MU (range 168-1890 MU). The patients treated with steroids were generally given prednisolone for 4 weeks, given in a single dose of 40 mg/day for 1 week, 30 mg/day for 1 week, 20 mg/day for 1 week, and then 10 mg/day for 1 week until it was abruptly withdrawn (total dose 700 mg). A total of 231 patients were followed up for more than 1 year after HBsAg seroclearance.

Methods

The time of entry into the study was defined as the time of serum HBsAg clearance as measured by radioimmunoassay. After HBsAg seroclearance, patients were followed up every 3 or 6 months or more frequently when their levels of alanine aminotransferase and α-fetoprotein were elevated. Follow-up studies included clinical, biochemical, and virologic aspects, and hepatocellular carcinoma screening with ultrasonography and alpha-fetoprotein. Biochemical tests were measured using routine automated techniques and performed in the clinical pathology laboratories of Toranomon Hospital. HBsAg, anti-HBs, and antibody to hepatitis D virus were

Table 1 Characteristics of subjects at the seroclearance of HBsAg

Characteristic	
N	231
Sex (male/female)	186/45
Age (years)	51 (23-66)
Body weight (kg)	67.5 (46.9-82.4)
HBV genotype (A/B/C/D/F)	5/23/118/2/1
US (non-LC/LC)	164/67
Total protein (g/dl)	7.5 (6.5-9.3)
Albumin (g/dl)	4.2 (3.1-5.1)
Total bilirubin (g/dl)	0.7 (0.1-2.0)
AST (IU/L)	21 (10-219)
ALT (IU/L)	19 (6-946)
Hb (g/dl)	15.2 (12.0-17.4)
Platelet ($\times 10^4$/mm^3)	16.8 (8.4-32.5)
Follow-up period after disappearance of HBs antigen (year)	6.5 (1-23.6)

Data are number of patients or median (range).
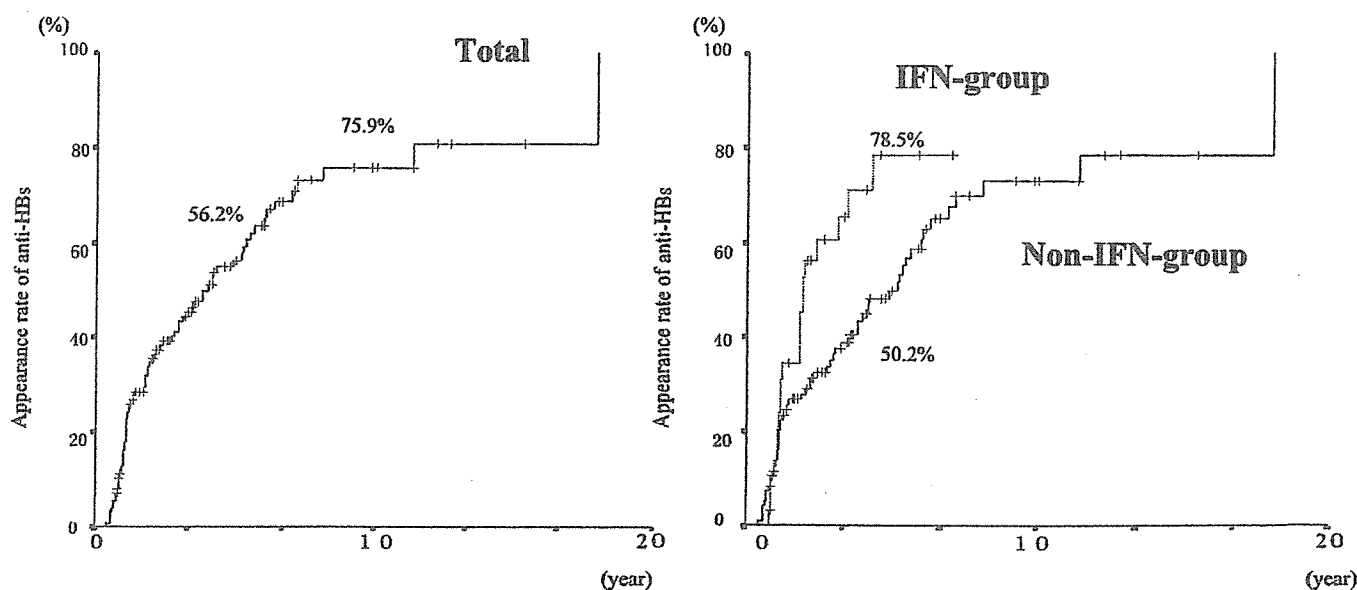ALT = alanine aminotransferase; AST = aspartate aminotransferase; Hb = hemoglobin; US = ultrasonography

Figure 1   Cumulative appearance rate of the antibody to hepatitis B surface antigen (HBsAg) after seroclearance of HBsAg. IFN = interferon.

all assayed with commercially available radioimmunoassay kits. Antibody against HCV was detected with a third-generation enzyme-linked immunoassay (Ortho Diagnostic Japan, Tokyo, Japan). HBV genotype was determined with a previously reported method.[17]

Serum HBV-DNA level was measured with a commercially available quantitative polymerase chain reaction assay (Amplicor HBV, monitor, Roche Diagnostics, GmbH, Mannheim, Germany)[18] 1 year after seroclearance of serum HBsAg. The sensitivity of HBV-DNA according to the manufacturer is approximately 400 copies/mL in quantitative polymerase chain reaction. Serum samples were conserved at −80° until use.

Status of liver cirrhosis was determined on the basis of liver biopsy and/or ultrasonographic findings. Ultrasonography was performed with a high-resolution, real-time scanner (model SSD-2000; Aloka Co., Ltd, Tokyo, Japan: Logic

700 MR; GE-Yokokawa Medical Systems, Tokyo, Japan). The diagnosis of liver cirrhosis was defined as having a score of more than 8 in an ultrasonographic scoring system based on liver surface, liver parenchyma, hepatic vessel, and spleen size, as reported by Lin et al.[19]

The diagnostic accuracy of ultrasonography for liver cirrhosis was at least 80%. This study was approved by the institutional review board of our hospital. The physicians in charge explained the purpose and method of this clinical trial to each patient, who gave their informed consent for participation.

Liver Histology and Ultrasonographic Findings

Liver biopsy specimens were obtained percutaneously or by peritoneoscopy using a modified Vim Silverman needle with an internal diameter of 2 mm (Tohoku University style, Kakinuma Factory, Tokyo, Japan), fixed in

Table 2   Predictive factors for appearance of anti-HBsAg

Factor	Category	Odds ratio	95% CI	P value
Interferon therapy	(−)/(+)	1/1.90	1.13-3.21	.016
Prednisolone withdrawal therapy	(−)/(+)	1/2.25	1.07-4.72	.032
Age (years)	<60/≥60	1/0.500	0.248-1.01	.052
Total protein (g/dl)	<8/≥8	1/1.84	0.90-3.76	.096
US	Non-LC/LC	1/0.71	0.430-1.16	.167
HBV-genotype	B/C	1/1.63	0.58-4.55	.350
Sex	Male/Female	1/1.34	0.72-2.50	.355
AST (IU/L)	≥38/<38	1/1.59	0.57-4.43	.375
Platelet (×10⁴/mm³)	≤20/>20	1/1.20	0.700-2.06	.504
ALT (IU/L)	≥50/<50	1/1.28	0.46-3.55	.634

ALT = alanine aminotransferase; AST = aspartate aminotransferase; CI = confidence interval; Hb = hemoglobin; HCV = hepatitis C virus; US = ultrasonographic findings; LC = liver cirrhosis; HBV = hepatitis B virus.

Table 3 Histological features of the 13 patients with HBsAg seroclearance

Patients	Age of HBsAg clearance, y	Histologic activity index before HBsAg clearance*				Interval-1, mo	Interval-2, mo	Histologic activity index after HBsAg clearance*			
		Periportal bridging necrosis	Intralobular degeneration and focal necrosis	Portal inflammation	Fibrosis			Periportal bridging necrosis	Intralobular degeneration and focal necrosis	Portal inflammation	Fibrosis
1	24	3	3	1	1	16.0	12.1	0	0	0	0
2	29	3	3	3	3	72.5	43.1	0	0	0	3
3	36	5	3	3	3	105.4	52.8	0	0	0	2
4	40	4	3	4	3	47.3	12.0	0	1	0	1
5	42	3	3	3	2	101.6	2.3	1	0	1	1
6	42	5	4	3	2	87.5	12.1	0	0	1	2
7	45	3	3	4	3	183.0	34.5	0	0	1	1
8	46	5	4	4	3	180.2	36.5	0	0	1	2
9	48	4	3	3	4	204.0	69.8	0	1	0	4
10	59	3	4	4	3	207.7	2.3	0	1	1	0
11	61	3	3	4	4	72.1	8.9	1	1	0	1
12	61				2	124.1	18.9	1	0	1	2
13	61					98.6	40.0	0	0	1	2

*Histologic activity index score: 0-10 for periportal bridging necrosis; and 0-4 for intralobular degeneration and focal necrosis; portal inflammation, and fibrosis.
†Interval-1: interval between first biopsy before HBsAg clearance and last biopsy before HBsAg clearance. Interval-2: interval between HBsAg clearance and last biopsy after HBsAg clearance.

10% formalin, and stained with hematoxylin-eosin, Masson's trichrome, silver impregnation, and periodic acid-Schiff after diastase digestion. The size of specimens for examination was more than 6 portal areas. Histopathologic interpretations of these 3- to 4-μm–thick sections were made independently by experienced liver pathologists (YA and HK) who had no clinical information or knowledge of chronologic order of the biopsies in each pair. The biopsy specimens were scored according to the system of Knodell et al.[20]

Patient Follow-Up

Clinical evaluation and biochemical and hematologic tests were performed at 2- to 6-month intervals. Thirty patients were lost to follow-up. Because the appearance of hepatocellular carcinoma was not identified in these 30 patients, they were considered as censored data in statistical analysis.[21] Hepatocellular carcinoma was diagnosed by histology or the typical hypervascular characteristics observed on angiography, in addition to certain features of computed tomography and ultrasonography.

Statistical Analysis

Statistical analysis was performed with Fisher's exact test, Kaplan-Meier estimate, log-rank test, and a Cox proportional hazard model where appropriate. P values less than .05 were considered statistically significant. The SPSS software package (SPSS Inc., Chicago, Ill) was used to perform statistical analysis.

RESULTS

Changes of Liver Biochemistry After HBsAg Seroclearance

Table 1 shows the characteristics of the 231 patients who had seroclearance of HBsAg. These patients were classified into a liver cirrhosis group or a non-liver cirrhosis group by ultrasonographic findings. A total of 67 patients showed a finding of liver cirrhosis. Histologic evidence of liver cirrhosis before HBsAg seroclearance was seen in 47 patients.

The alanine aminotransferase test showed that 203 of 231 patients (87.9%) had normal alanine aminotransferase levels 1 year after seroclearance of HBsAg. Twenty-eight patients had elevated alanine aminotransferase levels (18 with fatty infiltration of liver, 3 with alcohol abuse, and 8 with unknown origin).

Changes of HBV Marker after HBsAg Seroclearance

The cumulative appearance of anti-HBs is shown in Figure 1. A Cox proportional hazards model was used to analyze the factors contributing to the appearance of anti-HBs (Table 2). The patients treated with interferon showed the high cumulative appearance of anti-HBs by
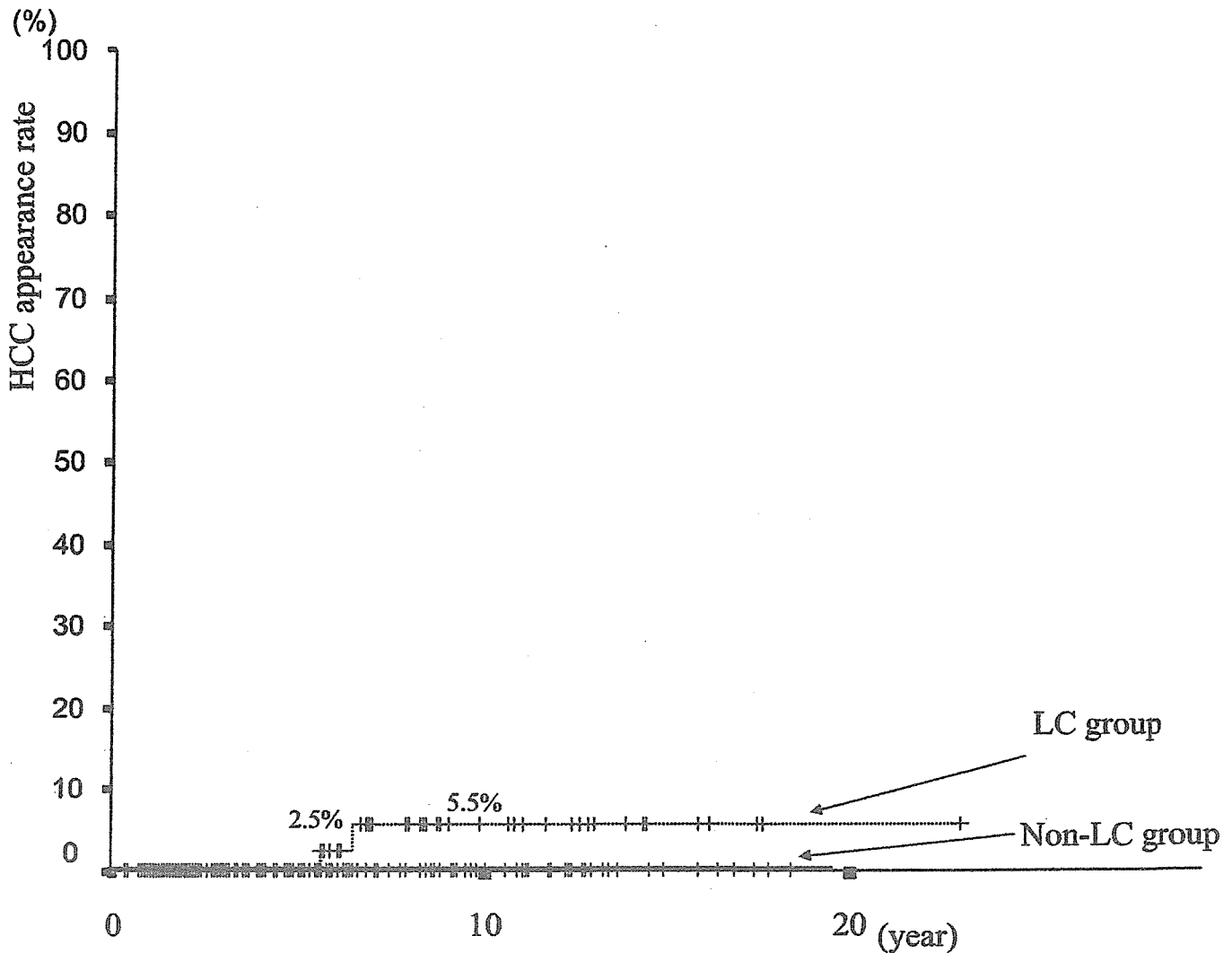
Figure 2   Hepatocellular carcinoma (HCC) appearance rate after seroclearance of HBsAg. LC = liver cirrhosis.

log-rank test. Anti-HBs became detectable in 50.2% of patients with spontaneous seroclearance of HBsAg and in 78.5% of patients treated with interferon at the fifth year after HBsAg seroclearance.

Next, we examined serum HBV-DNA level with the qualitative polymerase chain reaction assay (Amplicor HBV monitor). HBV-DNA showed positive results in 1.7% (3/231) 1 year after seroclearance of HBsAg.

Table 4   Recent studies on the outcomes following HBsAg clearance

Source	Status at clearance	No of cases	No of HBV alone	Follow-up, mo	Mean age, y	Outcomes Decompensated LC	HCC
Fattovich et al[14]	LC	32	30*	55	44	6	1§
Huo et al[15]	Non-LC	55	32*	23	54	6	1§
Chen et al[16]	Non-LC	189	146*	65.4	43	0	28
	LC	29	17*	50.8	54	4†	1§
Yuen et al[10]	LC or non-LC	92	92	51.1	42.6		5
Present	Non-LC	167	167	61.1	51	0	0
	LC	67	67	74.1	52.5	0	2

LC = liver cirrhosis; HCC = hepatocellular carcinoma.
*Remaining patients had concurrent virus of hepatitis C virus and/or HDV.
†Two of 4 patients had concurrent virus.
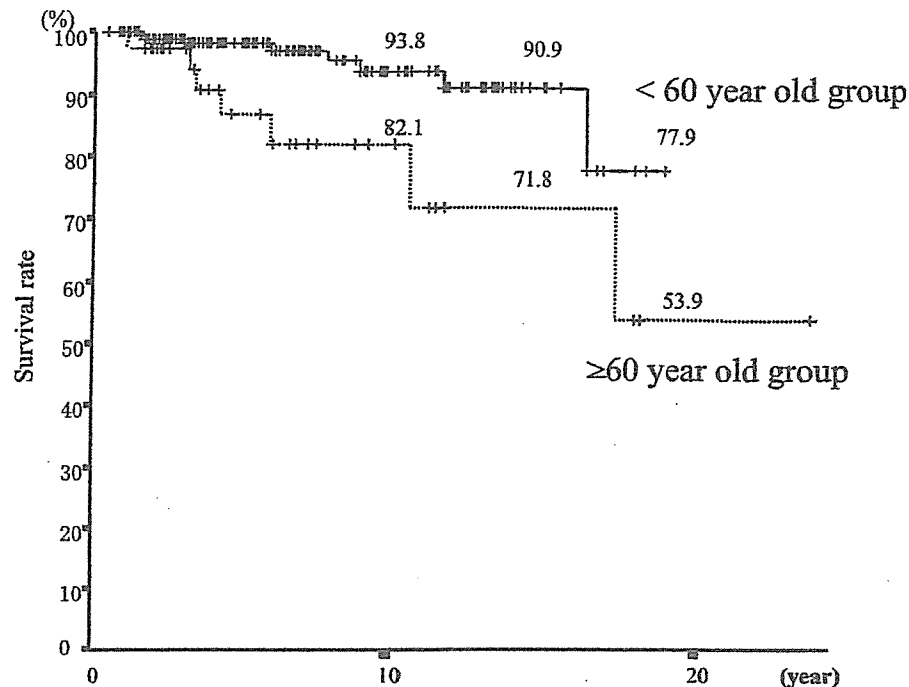§These patients had concurrent virus.

Figure 3   Survival based on difference of age after HBsAg seroclearance.

Histologic Changes of Liver after HBsAg Seroclearance

Thirteen patients with normal alanine aminotransferase levels after HBsAg seroclearance were examined for histologic changes of the liver before and after HBsAg seroclearance (Table 3). The median age of HBsAg seroclearance for the 13 patients with assessable liver biopsy was 45 years (range 24-61 years). The median interval between liver biopsies before and after HBsAg seroclearance was 101.6 months (range 16.0-207.7 months). Moreover, the median interval between HBsAg seroclearance and liver biopsies after HBsAg seroclearance was 18.9 months (range 2.3-69.8 months). The histologic changes of liver biopsies before and after HBsAg seroclearance are listed in Table 3. All patients showed marked improvement of necroinflammation of the liver, but only 2 of the 13 patients showed no liver fibrosis.

Cirrhosis-related Complications and Hepatocellular Carcinoma Appearance Rates

Liver cirrhosis and hepatocellular carcinoma did not develop in any of the 164 patients without evidence of liver cirrhosis at the time of HBsAg seroclearance. Cumulative hepatocellular carcinoma appearance rates are shown in Figure 2 by the Kaplan-Meier method. Two patients with liver cirrhosis at the time of HBsAg seroclearance had an occurrence of hepatocellular carcinoma at 5 and 5.8 years after seroclearance of HBsAg. Cumulative hepatocellular carcinoma appearance rates were 2.5% in the 5th year and 5.5% in the 10th year in the liver cirrhosis group. Pathologic confirmation using fine-needle biopsy or surgically resected specimens showed hepatocellular carcinoma. On the other hand, none of the patients without liver cirrhosis had hepatocellular carcinoma. Table 4 shows recent studies on the outcomes after HBsAg sero-

Table 5 Predictive factors for death after HBsAg seroclearance				
Factor	Category	Odds ratio	95% CI	P value
Age (years)	<60/≥60	1/3.42	1.24-9.45	.018
US	Non-LC/LC	1/2.12	0.80-6.12	.127
Sex	Female/Male	1/1.34	0.42-4.21	.620
Platelet (×10⁴/mm³)	>20/≤20	1/1.33	0.357-4.97	.670
AST (IU/L)	≥38/<38	1/0.437	0.19-12.40	.680
Total protein (g/dl)	<8/≥8	1/0.657	0.082-5.26	.693
ALT (IU/L)	≥50/<50	1/0.420	0.19-12.18	.693
HBV-genotype	B/C	1/1.03	0.20-5.27	.971

ALT = alanine aminotransferase; AST = aspartate aminotransferase; CI = confidence interval; Hb = hemoglobin; HBV = hepatitis B virus; US = ultrasonography

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.

References

1. Liaw YF, Tai DI, Chu CM, et al. The development of cirrhosis in patients with chronic type B hepatitis: a prospective study. *Hepatology.* 1988;8:493-496.
2. De Franchis R, Meucci G, Vecchi M, et al. The natural history of asymptomatic hepatitis B surface antigen carriers. *Ann Intern Med.* 1993;118:191-194.
3. Viola LA, Barrison IG, Coleman JC, et al. Natural history of liver disease in chronic hepatitis B surface antigen carriers. *Lancet.* 1981; 2:1156-1159.
4. Yang HI, Lu SN, Liaw YF, et al. Hepatitis B e antigen and the risk of hepatocellular carcinoma. *N Engl J Med.* 2002;347:168-174.
5. Wong DK, Cheung AM, O'Rourke K, et al. Effect of alpha-interferon treatment in patients with hepatitis B e antigen-positive chronic hepatitis B. A meta-analysis. *Ann Intern Med.* 1993;119:312-323.
6. Niederau C, Heintges T, Lange S, et al. Long-term follow-up of HBeAg-positive patients treated with interferon alfa for chronic hepatitis B. *N Engl J Med.* 1996;334:1422-1427.
7. Hsu YS, Chien RN, Yeh CT, et al. Long-term outcome after spontaneous HBeAg seroconversion in patients with chronic hepatitis B. *Hepatology.* 2002;35:1522-1527.
8. Lindsay KL, Redeker AG, Ashcavai M. Delayed HBsAg clearance in chronic hepatitis B viral infection. *Hepatology.* 1981;1:586-589.
9. Liaw YF, Sheen IS, Chen TJ, et al. Incidence, determinants and significance of delayed clearance of serum HBsAg in chronic hepatitis B infection: a prospective study. *Hepatology.* 1991;13:627-631.
10. Yuen MF, Ho Wong DKH, Sahlon E, et al. HBsAg seroclearance in chronic hepatitis B in the Chinese: virological, histological, and clinical aspects. *Hepatology.* 2004;39:1694-1701.
11. Sampliner RE, Hamilton FA, Iseri QA, et al. The liver histology and frequency of clearance of the hepatitis B surface antigen (HBsAg) in chronic carriers. *Am J Med Sci.* 1979;277:17-22.
12. Alward WJ, McMahon BJ, Hall DB, et al. The long-term serological course of asymptomatic hepatitis B virus carriers and the development of primary hepatocellular carcinoma. *J Infect Dis.* 1985;151: 604-609.
13. Da Silva LC, Madruga CL, Carrilho FJ, et al. Spontaneous hepatitis B surface antigen clearance in a long-term follow-up study of patients
   with chronic B hepatitis: lack of correlation with hepatitis C and D virus infection. *J Gastroenterol.* 1996;31:696-701.
14. Fattovich G, Glustina G, Sanchez-Tapias J, et al. Delayed clearance of serum HBsAg in compensated cirrhosis B: relation to interferon alpha therapy and disease prognosis. European Concerted Action on Viral Hepatitis. *Am J Gastroenterol.* 1998;93:896-900.
15. Huo TI, Wu JC, Lee PC, et al. Sero-clearance of hepatitis B surface antigen in chronic carriers does not necessarily imply a good prognosis. *Hepatology.* 1998;28:231-236.
16. Chen YC, Sheen IS, Chu CM, et al. Prognosis following spontaneous HBsAg seroclearance in chronic hepatitis B with or without concurrent infection. *Gastroenterology.* 2002;123:1084-1089.
17. Usuda S, Okamoto H, Iwanari H, et al. Serological detection of hepatitis B virus genotypes by ELISA with monoclonal antibodies to type-specific epitopes in the preS2-region product. *J Virol Methods.* 1999;80:97-112.
18. Kessler HH, Pierer K, Dragon E, et al. Evaluation of a new assay for HBV DNA quantitation in patients with chronic hepatitis B. *Clin Diagn Virol.* 1998;9:37-43.
19. Lin DY, Sheen IS, Chiu CT, et al. Ultrasonographic changes of early liver cirrhosis in chronic hepatitis B: a longitudinal study. *J Clin Ultrasound.* 1993;21:303-308.
20. Knodell RG, Ishak KG, Black WC, et al. Formulation and application of a numerical scoring system for assessing histological activity in asymptomatic chronic active hepatitis. *Hepatology.* 1981;1:431-435.
21. Harrington DP, Fleming TR. A class of rank test procedures for censored survival data. *Biomedaica.* 1983;62:205-209.
22. Arase Y, Ikeda K, Murashima N, et al. Time course of histological changes in patients with a sustained biochemical and virological response to corticosteroid withdrawal therapy for chronic hepatitis B. *Am J Gastroenterol.* 1999;94:3304-3309.
23. Perrillo RP, Brunt EM. Hepatic histologic and immunochemical changes in chronic hepatitis B after prolonged clearance of hepatitis B e antigen and hepatitis B surface antigen. *Ann Intern Med.* 1991;115:113-115.
24. Chung HT, Lai CL, Lok AS. Pathogenic role of hepatitis B virus in hepatitis B surface antigen-negative decompensated cirrhosis. *Hepatology.* 1995;22:25-29.
25. Korenman J, Baker B, Waggoner J, et al. Long-term remission of chronic hepatitis B after alpha-interferon therapy. *Ann Intern Med.* 1991;114:629-634.
26. Hoofnagle JH, Di Bisceglie AM. The treatment of chronic viral hepatitis. *N Engl J Med.* 1997;336:347-356.
27. Nair PV, Tong MJ, Stevenson D, et al. A pilot study on the effects of prednisolone withdrawal on serum hepatitis B virus DNA and HBeAg in chronic active hepatitis B. *Hepatology.* 1986;6: 1319-1324.
28. Muller R, Vido I, Schmidt FW. Rapid withdrawal of immunosuppressive therapy in chronic active hepatitis B infection. *Lancet.* 1981;1: 1323-1324.

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

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

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

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.

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

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