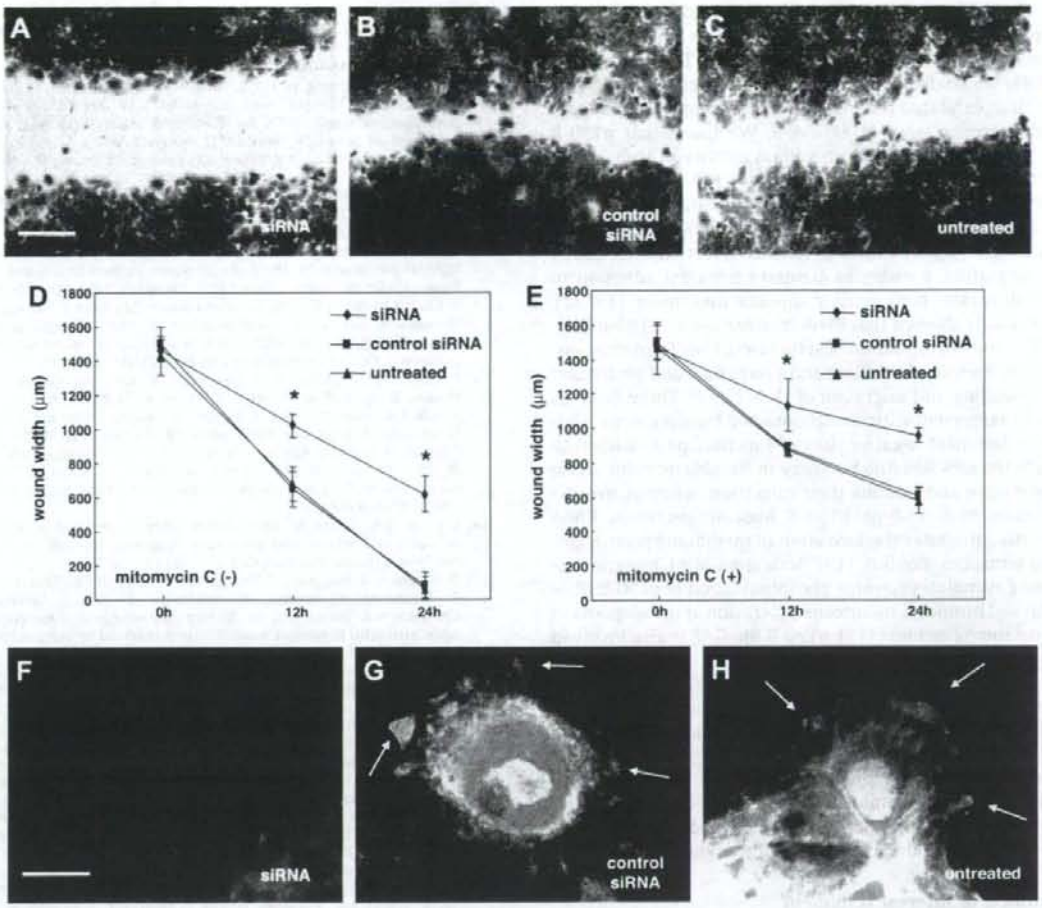

Fig. 5. Knockdown of *ARHGAP5* inhibits migration in Huh-7 cells. Monolayer wound healing assay in Huh-7 cells transfected with siRNA targeting *ARHGAP5* (A, F) or control siRNA (B and G), or left untreated (C and H). Cells were cultured in the absence (A–D, F–H) or presence (E) of mitomycin C. (A–C) Cells were allowed to migrate into a monolayer wound for 24 h and afterward stained with Giemsa stain. Original magnifications: 40×. Scale bar = 500 μm. (D and E) Cells were cultured in the absence (D) or presence (E) of mitomycin C. Wound widths were measured in three randomly chosen regions at the indicated time after wounding. Values are represented as the mean ± SD. Differences were analyzed by ANOVA (*$P < 0.05$). (F–H) Wound edge cells were triple-labeled with anti-p190-B RhoGAP, rhodamine-conjugated phalloidin and DAPI to reveal p190-B RhoGAP (green), actin filaments (red) and nuclei (blue), respectively. Arrows indicate p190-B RhoGAP on membrane protrusions. Scale bar = 10 μm.

NA cells. Taken together, these observations suggest that the inhibition of RhoA activity by p190-B RhoGAP promotes cell movement and formation of membrane protrusions in migrating cells.

4. Discussion

We report here the amplification of *ARHGAP5* in HCC and ESCC cell lines. We undertook a molecular definition of the amplicon at 14q12 that is present in HCC and ESCC cell lines. The amplification at 14q12 has been reported in various types of cancers, including HCC [10], ESCC [7], nasopharyngeal carcinoma [11] and non-squamous cell lung carcinoma [12], although the frequency of 14q12 gain is low in primary HCC (4–6%) [10,13]. The range of the amplicon varies among these tumors, and their boundaries have not been determined in each case. Moreover, the target oncogene(s) in the amplified regions have not been fully identified. Here we defined the amplified regions in one HCC and two ESCC cell lines and narrowed the site of the amplification to a relatively short section. Among the four genes within the smallest region of the amplification, only *HEATR5A* and *ARHGAP5* were overexpressed in all the tested lines exhibiting copy number gains in the region; hence they are thought to be candidate targets in the amplicon. Of the two genes, we chose to focus further analysis on *ARHGAP5* because its protein product, p190-B RhoGAP, is purported to play an important role in dynamic cellular processes by regulating RhoA activity, while little is known about *HEATR5A*. During the preparation of this manuscript, amplification of *ARHGAP5* was reported in Huh-7 cells [14].

Although several studies have suggested an association of p190-B RhoGAP with tumors [15–17], its biological function in cancer cells is poorly understood. Therefore, using siRNA, we studied its function in Huh-7 cells, the HCC cell line that exhibited the most remarkable copy number gain and overexpression of *ARHGAP5*. We found that p190-B RhoGAP negatively regulates RhoA activity in Huh-7 cells cultured in medium containing 10% FCS and plated on fibronectin. Adhesion to fibronectin regulated RhoA activity in a triphasic or biphasic manner, as previously reported in fibroblasts [18,19]. Although some RhoA activity is required for migration, possibly to maintain sufficient adhesion to the substrate, high activity inhibits movement [19–22]. Our results showed that RhoA inactivation by p190-B RhoGAP results in inhibition of actin stress fiber formation, enhanced membrane ruffling and protrusion, and promotion of spreading and migration of Huh-7 cells. These findings are in agreement with results obtained from previous studies. A dominant negative (loss-of-function) p190-B RhoGAP mutation elevates RhoA activity in fibroblasts cultured on fibronectin and inhibits their migration, whereas overexpression of wild-type p190-B RhoGAP decreases RhoA activity, promotes the formation of membrane protrusions and enhances mobility [19]. Activation of β1 integrin signaling stimulates tyrosine phosphorylation of p190-B RhoGAP and promotes membrane protrusion at invadopodia in a melanoma cell line [17]. p190-B RhoGAP is also involved in invasion by breast cancer cells [15].

In conclusion, we have identified *ARHGAP5* as a probable target for the amplification at 14q12 detected in a subgroup of HCCs and ESCCs. Our results indicate that p190-B RhoGAP, the protein product of *ARHGAP5*, promotes cell spreading and migration in Huh-7 cells. Further studies are needed to determine the importance of *ARHGAP5* and p190-B RhoGAP in the development and progression of not only HCC and ESCC but also other types of tumors.

Conflicts of interest statement

My co-authors and I declare that we have no proprietary, financial, professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the position presented in the manuscript entitled, "A novel amplification target, ARHGAP5, promotes cell spreading and migration by negatively regulating RhoA in Huh-7 hepatocellular carcinoma cells".

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Original Article

Analysis of hepatic genes involved in the metabolism of fatty acids and iron in nonalcoholic fatty liver disease

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Aims: Hepatic steatosis and iron cause oxidative stress, thereby progressing steatosis to steatohepatitis. We quantified the expression of genes involved in the metabolism of fatty acids and iron in patients with nonalcoholic fatty liver disease (NAFLD).

Methods: The levels of transcripts for the following genes were quantified from biopsy specimens of 74 patients with NAFLD: thioredoxin (Trx), fatty acid transport protein 5 (FATP5), sterol regulatory element-binding protein 1c (SREBP1c), fatty acid synthase (FASN), acetyl-coenzyme A carboxylase (ACAC), peroxisome proliferative activated receptor α (PPARα), cytochrome P-450 2E1 (CYP2E1), acyl-coenzyme A dehydrogenase (ACADM), acyl-coenzyme A oxidase (ACOX), microsomal triglyceride transfer protein (MTP), transferrin receptor 1 (TfR1), transferrin receptor 2 (TfR2) and hepcidin. Twelve samples of human liver RNA were used as controls. Histological evaluation followed the methods of Brunt.

Results: The levels of all genes were significantly higher in the NAFLD patients than in controls. The Trx level increased as the stage progressed. The levels of FATP5, SREBP1c, ACAC, PPARα, CYP2E1, ACADM and MTP significantly decreased as the stage and grade progressed ($P < 0.05$). Hepatic iron score (HIS) increased as the stage progressed. The TfR1 level significantly increased as the stage progressed ($P < 0.05$), whereas TfR2 level significantly decreased ($P < 0.05$). The ratio of hepcidin mRNA/ferritin ($P < 0.001$) or hepcidin mRNA/HIS ($P < 0.01$) was significantly lower in NASH patients than simple steatosis patients.

Conclusions: Steatosis-related metabolism is attenuated as NAFLD progresses, whereas iron-related metabolism is exacerbated. Appropriate therapies should be considered on the basis of metabolic changes.

Key words: fatty acids, iron, NAFLD, oxidative stress

Abbreviations
Trx, thioredoxin; FATP5, fatty acid transport protein 5; SREBP1c, sterol regulatory element-binding protein 1c; FASN, fatty acid synthase; ACAC, acetyl-coenzyme A carboxylase; PPARα, peroxisome proliferative activated receptor α; CYP2E1, cytochrome P-450 2E1; ACADM, acyl-coenzyme A dehydrogenase; ACOX, acyl-coenzyme A oxidase; MTP, microsomal triglyceride transfer protein; TfR1, transferrin receptor 1; TfR2, transferrin receptor 2.

INTRODUCTION

NON ALCOHOLIC FATTY liver disease (NAFLD) is a wide-spectrum liver disease, ranging from simple steatosis to steatohepatitis.[1] Owing to the obesity epidemic, NAFLD is now recognized as a leading health problem worldwide.[1] Since NAFLD has been documented to progress to liver failure[2] and/or hepatocellular carcinoma,[3] various therapeutic studies for NAFLD or nonalcoholic steatohepatitis (NASH) have been conducted to date.[4–8] These studies included weight reduction,[4] use of insulin sensitizers,[5] antioxidants,[6] phlebotomy[7] and hepato-protective drugs,[8] albeit with limited success. Although these treatments are aimed at addressing the pathogenesis of NAFLD, they would not always be efficient at every stage of this "wide spectrum" disease.

NASH is thought to develop through a "two-hit theory".[9] The first hit includes insulin resistance, mostly due to obesity.[9] The second hits include oxidative stress, inflammatory cytokines, and bacterial endotoxin.[9] In particular, the accumulation of fatty acids in the liver results in oxidative stress through oxidation of fatty

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acids.[10] In addition, hepatic iron load, which also induces oxidative stress, has been reported in some groups of patients with NAFLD.[11] Therefore, hepatic metabolism of fatty acids and iron should be the therapeutic target for NAFLD. However, their roles in the development of NAFLD have not yet been studied

In this study, we quantified the expression of genes involved in hepatic metabolism of fatty acids and iron using liver biopsy specimens from patients with NAFLD, and compared them with liver histology. Based on the results, we explored the role of the metabolism of fatty acids and iron in NAFLD. Our study should improve out understanding of the pathogenesis of NAFLD and contribute to the identification of putative therapeutic pathways.

PATIENTS AND METHODS

Patients

NAFLD PATIENTS WHO underwent liver biopsies in our institute between April 2000 and March 2007 were retrospectively selected according to the following criteria: no excessive alcohol intake (more than 20 g/day), as assessed by interview (on at least three occasions); no history of treatment with steatosis-inducing drugs within the 12 months prior to the study; negative serum hepatitis C virus (HCV) antibody; negative for hepatitis B surface antigen or antibodies to human immunodeficiency virus; and an absence of other forms of chronic liver disease, such as autoimmune liver diseases. Anthropometry and laboratory data were collected from all patients at the time of the liver biopsy. All patients had given written informed consent for the analysis of metabolic genes and liver biopsies before the study. The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the Ethics Committee of the Kyoto Prefectural University of Medicine.

Laboratory determinations

After a 12-h overnight fast, venous blood samples were drawn to determine asparatate aminotransferase (AST), alanine aminotransferase (ALT), albumin, total cholesterol, triglyceride, fasting plasma glucose (FPG), glycosylated haemoglobin (HbA_{1c}), insulin and ferritin levels. These parameters were measured using standard techniques from clinical chemistry laboratories. The index of insulin resistance was calculated only in patients without overt diabetes (fasting plasma glucose >126 mg/dL), according to the homeostasis model assessment (HOMA).

Histological evaluation

Formalin-fixed and paraffin-embedded liver biopsy specimens were stained with hematoxylin–eosin, Masson's trichrome, and Perl's Prussian blue. The stage of hepatic fibrosis was scored according to Brunt[12]: 1, zone 3 fibrosis; 2, zone 3 fibrosis with periportal fibrosis; 3, bridging fibrosis; and 4, cirrhosis. The grade of inflammation was scored as follows[12]: 1, mild; 2, moderate; and 3, severe. We considered the scores of stage and grade of simple steatosis as "0". Steatosis was assessed according to the percentage of hepatocytes containing fat droplets. The degree of iron loading was graded using a Perl's score of 0–4, as described previously.[13]

Quantification of the expression of hepatic genes

Liver specimens were immediately frozen after the biopsy and were stored at −80°C until use. Total RNA was isolated from biopsy specimens using the RNeasy kit (Qiagen, Hilden, Germany). First-strand cDNA was obtained from total RNA using the QuantiTect Reverse Transcription kit (Qiagen). PCR was performed using the Light Cycler 2.0 System (Roche, Mannheim, Germany), and the mRNA levels were normalized to those of β-actin. Comprehensive target genes were as follows: thioredoxin (Trx), fatty acid transport protein 5 (FATP5), sterol regulatory element-binding protein 1c (SREBP1c), fatty acid synthase (FASN), acetyl-coenzyme A carboxylase (ACAC), peroxisome proliferative activated receptor α (PPARα), cytochrome P-450 2E1 (CYP2E1), acyl-coenzyme A dehydrogenase, C4 to C12 straight chain (ACADM), acyl-coenzyme A oxidase (ACOX), microsomal triglyceride transfer protein (MTP), transferrin receptor 1 (TfR1), transferrin receptor 2 (TfR2) and hepcidin. Table 1 summarizes the specific primers for these target genes. Twelve samples of human total liver RNA were obtained from commercial sources (Stratagene, CA, USA; Clontech Laboratories, CA, USA; Ambion, TX, USA; Becton, Dickinson, NJ, USA; Cell Applications, CA, USA), and used as controls.

Statistical analysis

Associations between variables were analyzed using the Spearman's correlation coefficient by rank. Differences between variables were analyzed using the Mann–Whitney U-test or Kruskal–Wallis test. All analyses were performed using SPSS software for Windows, version

Table 1 The specific primers used for the target genes

	Sense primers	Antisense primers
Trx	5′-CTGCTTTTCAGGAAGCCTTG-3′	5′-ACCCACCTTTTGTCCCTTCT-3′
FATP5	5′-ACACACTCGGTGTCCCTTTC-3′	5′-CTACAGGGCCCACTGTCATT-3′
SREBP1c	5′-TGCATTTTCTGACACGCTTC-3′	5′-CCAAGCTGTACAGGCTCTCC-3′
FASN	5′-TTCCGAGATTCCATCCTACG-3′	5′-TGTCATCAAAGGTGCTCTCG-3′
ACAC	5′-GAGAACTGCCCTTTCTGCAC-3′	5′-CCAAGCTCCAGGCTTCATAG-3′
PPARα	5′-GGAAAGCCCACTCTGCCCCCT-3′	5′-AGTCACCGAGGAGGGGCTCGA-3′
CYP2E1	5′-CCCAAAGGATATCGACCTCA-3′	5′-AGGGTGTCCTCCACACACTC-3′
ACADM	5′-TTGAGTTCACCGAACAGCAG-3′	5′-AGGGGGACTGGATATTCACC-3′
ACOX	5′-TGATGCGAATGAGTTTCTGC-3′	5′-AGTGCCACAGCTGAGAGGTT-3′
MTP	5′-CATCTGGCGACCCTATCAGT-3′	5′-GGCCAGCTTTCACAAAAGAG-3′
TfR1	5′-ATGCATTTTGCAGCAGTGAG-3′	5′-TCCAAAAGGCCCTACTCCTT-3′
TfR2	5′-GACCCTGCAGTGGGTGTACT-3′	5′-CAGTCGCTCGTCTCTCTCCT-3′
hepcidin	5′-ACCAGAGCAAGCTCAAGACC-3′	5′-AAACAGAGCCACTGGTCAGG-3′

Note: The role of genes analyzed in lipid and iron metabolisms is as follows; oxidative stress-induced, Trx; uptake of fatty acid, FATP5; synthesis of fatty acid, SREBP1c, FASN, ACAC; oxidation of fatty acid, PPARα, CYP2E1, ACADM, ACOX; secretion of triglyceride, MTP; uptake of transferrin-bound iron, TfR1, TfR2; regulation of iron metabolism, hepcidin.
Trx, thioredoxin; FATP5, fatty acid transport protein 5; SREBP1c, sterol regulatory element-binding protein 1c; FASN, fatty acid synthase; ACAC, acetyl-coenzyme A carboxylase; PPARα, peroxisome proliferative activated receptor α; CYP2E1, cytochrome P-450 2E1; ACADM, acyl-coenzyme A dehydrogenase; ACOX, acyl-coenzyme A oxidase; MTP, microsomal triglyceride transfer protein; TfR1, transferrin receptor 1; TfR2, transferrin receptor 2.

14.0 (SPSS, Chicago, IL, USA). A P value of less than 0.05 was considered significant.

RESULTS

The characteristics of patients

TABLES 2 AND 3 summarize the characteristics of patients and the results of liver histology, respectively. Of the 16 diabetic patients, 3 had been treated with metformin, 2 with pioglitazone, 2 with sulfonylurea, and the others had been followed with diet restriction. Serum triglyceride levels were greater in the simple steatosis patients than in the NASH patients. Although the values of HbA_{1c} were comparable in the two groups, those of HOMA-IR [index of insulin resistance (IR)] were significantly higher in the NASH

Table 2 Patients characteristics

	Simple steatosis ($n = 33$)	NASH ($n = 41$)	P value
Age	55.4 ± 15.0	61.2 ± 12.7	0.051
BMI (kg /m^2)	27.5 ± 2.4	26.5 ± 4.4	0.748
Sex (male/female)	24/9	25/16	0.208
Diabetes (yes/no)	7/26	9/32	0.584
Plt	21.6 ± 3.9	19.1 ± 6.3	0.006
AST	43.0 ± 21.4	72.9 ± 30.5	0.0002
ALT	62.3 ± 30.8	89.8 ± 50.3	0.006
Alb	4.7 ± 0.3	4.6 ± 0.3	0.023
T-Cho	231.1 ± 50.5	199.9 ± 44.0	0.006
TG	205.0 ± 105.8	140.9 ± 103.2	0.015
FPG	145.1 ± 68.4	116.7 ± 21.5	0.356
HbA_{1c}	6.6 ± 1.8	6.0 ± 0.6	0.533
HOMA-IR	2.9 ± 1.2	4.6 ± 1.8	0.012
ferritin	223.1 ± 106.0	197.7 ± 160.7	0.227

Note: The value is expressed as either mean ± S.D. or the number of patients.
ALT, alanine aminotransferase; AST, asparatate aminotransferase; Alb, albumin; BMI, body mass index; FPG, fasting plasma glucose; HbA_{1c}, glycosylated haemoglobin; HOMA-IR, homeostasis model assessment-index of insulin resistance; T-Cho, total cholesterol; TG, triglyceride.

Table 3 Results of liver biopsy

	Simple steatosis	NASH
Stage: 1/2/3/4		13/13/13/2
Grade: 1/2/3		27/10/4
Iron: 0/1/2/3	11/12/3/1	14/8/6/6
Steatosis:		
<30%	14	18
30%–60%	7	13
60% <	2	10

NASH, nonalcoholic steatohepatitis.

patients than in the simple steatosis patients. Neither significant fibrosis nor inflammation was observed in the biopsy specimens from patients with simple steatosis. Six specimens from simple steatosis patients and seven specimens from NASH patients were not available for iron staining.

Hepatic oxidative stress

We evaluated hepatic oxidative stress by the level of hepatic Trx, since Trx is known to be a redox-sensitive molecule.[14] We have previously reported that serum Trx levels are a marker of NASH.[15] We measured hepatic thioredoxin mRNA, because it would reflect the redox status of the liver more precisely than serum thioredoxin levels. Hepatic thioredoxin consists of both reduced and oxidized forms, whereas serum thioredoxin is an oxidized form. Therefore, hepatic thioredoxin levels do not correlate with serum thioredoxin levels. The Trx level increased in the order of controls, then simple steatosis patients with the highest levels in NASH patients (Table 4). The differences among the groups were significant (Table 4). The Trx level tended to increase as the stage progressed; however, it did not show any association with the grade (Table 5).

Fatty acid metabolism

The levels of transcripts for the genes involved in fatty acid metabolism were increased in the order of controls, then NASH patients with the highest levels in simple steatosis patients (Table 4). The differences among the groups were significant (Table 4). When values were compared between simple steatosis and NASH patients by the Mann–Whitney's test, the difference was significant in FATP5 ($P < 0.01$), ACAC ($P < 0.05$), PPARα ($P < 0.05$), CYP2E1 ($P < 0.05$), ACADM ($P < 0.05$), ACOX ($P < 0.05$), MTP ($P < 0.05$). Levels of all these genes were significantly higher in the simple steatosis patients than the NASH patients. When compared with the liver histology, the levels of FATP5, SREBP1c, ACAC, PPARα, CYP2E1, ACADM and MTP significantly decreased as the stage and grade progressed (Table 5). The level of ACOX tended to decrease as the stage and grade progressed (Table 5). The level of FASN was similarly decreased, although the difference between groups

Table 4 The levels of hepatic gene involved in lipid and iron metabolism

	Control	Simple steatosis	NASH	*P* value
Trx	1.0 ± 1.1	2.3 ± 0.9	2.5 ± 1.0	$P < 0.00001$
FATP5	1.0 ± 0.4	6.1 ± 3.6	4.3 ± 2.5	$P < 0.00001$
SREBP1c	1.0 ± 0.6	73.9 ± 74.3	56.0 ± 85.4	$P < 0.00001$
FASN	1.0 ± 1.0	28.2 ± 26.8	17.8 ± 15.1	$P < 0.00001$
ACAC	1.0 ± 0.8	12.2 ± 5.9	8.7 ± 3.4	$P < 0.00001$
PPARα	1.0 ± 0.8	21.1 ± 11.3	15.5 ± 8.1	$P < 0.00001$
CYP2E1	1.0 ± 0.4	8.0 ± 4.2	6.2 ± 3.2	$P < 0.00001$
ACADM	1.0 ± 0.9	17.8 ± 9.7	13.1 ± 6.1	$P < 0.00001$
ACOX	1.0 ± 0.9	16.6 ± 9.2	12.0 ± 5.7	$P < 0.00001$
MTP	1.0 ± 1.0	10.8 ± 3.8	8.8 ± 3.3	$P < 0.00001$
TfR1	1.0 ± 1.1	10.8 ± 11.3	11.8 ± 10.3	$P < 0.00001$
TfR2	1.0 ± 0.4	7.6 ± 3.6	5.6 ± 2.8	$P < 0.00001$
hepcidin	1.0 ± 0.9	11.2 ± 9.6	5.7 ± 3.9	$P < 0.00001$

Note: The value is expressed as folds to mean control values (mean ± S.D.). The deference between the groups was determined using the Kruskal–Wallis test.

Trx, thioredoxin; FATP5, fatty acid transport protein 5; SREBP1c, sterol regulatory element-binding protein 1c; FASN, fatty acid synthase; ACAC, acetyl-coenzyme A carboxylase; PPARα, peroxisome proliferative activated receptor α; CYP2E1, cytochrome P-450 2E1; ACADM, acyl-coenzyme A dehydrogenase; ACOX, acyl-coenzyme A oxidase; MTP, microsomal triglyceride transfer protein; TfR1, transferrin receptor 1; TfR2, transferrin receptor 2.

Table 5 Correlation of the gene levels with liver histology*

	Stage		Grade	
	r	*P* value	*r*	*P* value
Trx	0.209	0.074	0.132	0.266
FATP5	−0.334	0.004	−0.339	0.003
SREBP1c	−0.264	0.024	−0.283	0.015
FASN	−0.158	0.178	−0.182	0.124
ACAC	−0.264	0.024	−0.313	0.007
PPARα	−0.253	0.031	−0.244	0.038
CYP2E1	−0.264	0.024	−0.293	0.012
ACADM	−0.241	0.040	−0.246	0.036
ACOX	−0.213	0.070	−0.213	0.071
MTP	−0.262	0.025	−0.271	0.020
TfR1	0.227	0.037	0.182	0.089
TfR2	−0.307	0.008	−0.318	0.006
hepcidin	−0.251	0.032	−0.221	0.060

*Using Spearman's test. Trx, thioredoxin; FATP5, fatty acid transport protein 5; SREBP1c, sterol regulatory element-binding protein 1c; FASN, fatty acid synthase; ACAC, acetyl-coenzyme A carboxylase; PPARα, peroxisome proliferative activated receptor α; CYP2E1, cytochrome P-450 2E1; ACADM, acyl-coenzyme A dehydrogenase; ACOX, acyl-coenzyme A oxidase; MTP, microsomal triglyceride transfer protein; TfR1, transferrin receptor 1; TfR2, transferrin receptor 2.

did not reach statistical significance (Table 5). In parallel with these findings, the level of hepatic steatosis decreased as the stage and grade progressed (Fig. 1). None of these genes was independently correlated with hepatic steatosis (not shown).

TfR1 and TfR2

The hepatic iron score (HIS) tended to increase as the stage progressed (Table 6). We examined the levels of TfR1 and TfR2, since the uptake of serum iron by hepatocytes is largely through a transferrin-bound form.[16] The levels of both of these genes were significantly higher in the NAFLD patients than in the controls (Table 4). When values were compared between simple steatosis and NASH using the Mann–Whitney's test, the TfR2 level was significantly ($P < 0.01$) higher in the simple steatosis patients than the NASH patients. The TfR1 level significantly increased as the stage progressed, whereas that of TfR2 significantly decreased as the stage and grade progressed (Table 5). Neither TfR1 nor TfR2 were independently correlated with HIS (not shown).

Table 6 Hepatic iron score and the stage

	Hepatic iron score				
	0	1	2	3	4
Stage 0	11	11	3	0	1
Stage 1	7	1	1	1	0
Stage 2	3	4	3	2	0
Stage 3	4	4	2	2	0
Stage 4	0	0	0	0	1

Note: The value represents the number of patients. Simple steatosis was considered as stage "0", $r = 0.213$, $P = 0.099$, iron score vs stage: Spearman's test.

Hepcidin

Hepcidin is known to be secreted from hepatocytes and regulates systemic iron transport.[16] The hepcidin level was significantly different among the controls, the simple steatosis patients and the NASH patients. The value was higher in the simple steatosis patients than in the NASH patients (Table 4). Hepcidin level decreased significantly as the stage progressed (Table 5). Since the ratio of hepcidin to iron load has been reported to evaluate the appropriateness of the hepcidin response to iron overload,[17] we divided hepcidin mRNA levels by serum ferritin levels or HIS. The ratios of hepcidin mRNA/ferritin and hepcidin mNA/HIS were signifi-

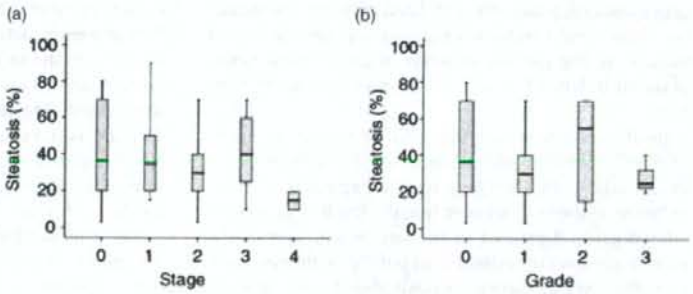

Figure 1 Distributions of the level of hepatic steatosis in association with the stage (a) and grade (b). The level of steatosis decreased as the stage and grade progressed.

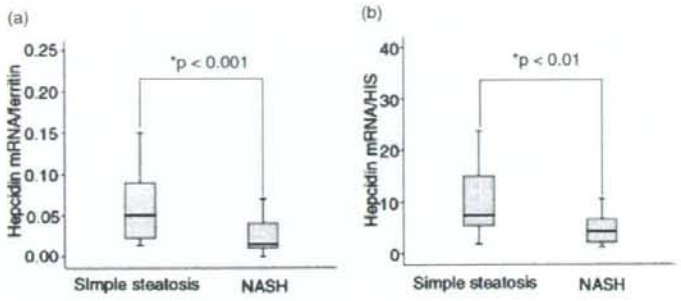

Figure 2 The ratio of hepcidin mRNA levels to serum ferritin levels (a) and that of hepcidin mRNA levels to hepatic iron score (HIS) (b). Hepcidin mRNA levels corrected for iron overload were significantly lower in NASH patients than in simple steatosis patients. *Mann-Whitney U-test.

cantly lower in NASH patients than simple steatosis patients (Fig. 2). The ratio of hepcidin mRNA/ferritin was significantly correlated with stage ($r = -0.523$, $P < 0.00005$) and grade ($r = -0.436$, $P < 0.0005$). The same results were obtained from the ratio of hepcidin mRNA/HIS ($r = -0.424$, $P < 0.01$ vs stage; $r = -0.373$, $P < 0.05$ vs grade). We compared hepcidin mRNA levels with metabolic variables and found that the level of hepcidin was significantly correlated with both total cholesterol ($r = 0.323$, $P < 0.01$) and triglyceride ($r = 0.323$, $P < 0.01$). The ratio of hepcidin mRNA/ferritin was also significantly correlated with total cholesterol ($r = 0.365$, $P < 0.005$).

DISCUSSION

IN THIS STUDY, we investigated the expression levels of hepatic genes that play significant roles in the metabolism of fatty acids and iron. Their roles in hepatocytes include the uptake, synthesis, oxidation, storage and excretion of fatty acids,[10,18,19] the uptake of iron and the regulation of systemic iron transport.[16] We found that the levels of these genes were significantly higher in NAFLD patients than controls. In addition, we found some novel findings. However, none of the individual genes was independently correlated with hepatic steatosis. These results indicated that neither the lack of nor increase in the expression levels of any of these genes plays an independent role in the development of fatty liver.

Insulin resistance is the "first hit" in the development of NASH,[9] which is characterized by an increase in the uptake and synthesis of fatty acids in hepatocytes.[19] Nevertheless, our results showed that the levels of fatty acid-related genes decreased in the later stages despite the presence of insulin resistance. In parallel with these findings, the level of hepatic steatosis also decreased. Considering that fat is the fuel involved in progressive liver injuries,[20] these findings might be associated with "burnout" NASH.[21] Although the underlying reason for this is unclear, some possibilities should be considered. Because hepatic adenosine 5'-triphosphate (ATP) levels tend to be decreased in fatty liver,[22] hepatic adenosine monophosphate-activated protein kinase (AMPK) should be activated.[23] AMPK is known to activate catabolic pathways and switch off protein, carbohydrate and lipid synthesis, such that cellular energy levels remain unchanged.[23] Thus, activated AMPK in hepatocytes might contribute to the decrease in the expression levels of fatty acid-related genes. Anti-diabetic drugs, which ameliorate liver injuries in patients with NASH, have been reported to activate AMPK.[24] Interestingly, the levels of all the genes involved in fatty acid metabolism were lower in the patients treated with insulin sensitizers than in those treated with other agents or followed with diet restriction. Statistical significance was achieved only in FATP5 ($P < 0.05$, Mann–Whitney's test). However, these results may be difficult to evaluate or apply generally, because the numbers of patients were small.

Hepatic iron load has been documented to be another key player in the progression from steatosis to steatohepatitis.[11] Hepatic iron load has been attributed to the Cys282Tyr mutation in the hemochromatosis gene.[11] This mutation decreases hepatic synthesis of hepcidin, resulting in the facilitation of iron absorption from the duodenum.[16] Our results showed that hepatic iron scores tended to correlate with the histological stage of NAFLD. Furthermore, the ratios of hepcidin mRNA/ferritin and hepcidin mRNA/HIS were significantly lower in NASH patients than in simple steatosis patients. This insufficient production of hepcidin may not be attributed to the genetic mutation, since known mutations of hemochromatosis-associated genes have been reported to be rare among Japanese patients.[25]

Interestingly, the hepcidin level was significantly correlated with the levels of total cholesterol and triglycerides. These findings coincide with those recently reported by Barisani *et al.*,[17] who reported that the hepcidin mRNA/ferritin ratio and the hepcidin mRNA/tissue iron score ratio were significantly lower in the NAFLD group with hepatic iron overload than in the NAFLD group without iron overload,[17] and that the level of hepatic hepcidin mRNA was significantly correlated with lipid parameters.[17] Our findings, in concert with those of Barisani *et al.*, suggest that more severe forms of NAFLD are associated with insufficient hepcidin production, and that lipid metabolism might be involved in hepcidin synthesis. Alternatively, the hepatic levels of TfR1 and TfR2 were significantly higher in NAFLD patients than controls. Therefore, TfR1 and TfR2 would be expected to promote hepatic iron load irrespective of iron absorption from the duodenum.

TfR1 is ubiquitously expressed in the human body,[16] while TfR2 is dominantly expressed in specific organs including the liver.[26] TfR1 has a high affinity with transferrin[27] and its expression is regulated by the iron-responsive element (IRE) in the 3′-untranslated regions of mRNAs.[16] In the NAFLD patients, the TfR1 level increased significantly as the stage progressed. Since ROS stabilize TfR1 mRNA via activation of iron regulatory proteins that interact with IRE,[16] hepatic oxidative stress should upregulate TfR1 in NAFLD.

TfR2 was recently identified as a novel transferrin receptor,[26] although the expression mechanisms have not been fully determined.[28] Similarly, neither the physiological nor pathological role of TfR2 in the liver has been documented. The expression level of TfR2 was higher in NAFLD patients than controls. At present, the association between the level of TfR2 and the pathogenesis of NAFLD remains unknown. Regardless of the role of TfR2, we have reported that the TfR2 level is significantly correlated with that of PPARα.[29] It is of much interest to speculate that PPARα might contribute to the regulation of TfR2, since PPARα may be upregulated in NAFLD by intrinsic PPARα ligands. This hypothesis is under investigation in our institute.

In summary, we investigated the metabolism of fatty acids and iron in the livers of NAFLD patients. Steatosis-related metabolism is attenuated as the disease progresses, whereas iron load-related metabolism is exacerbated. Based on these findings, we hypothesize that anti-lipid synthesis should be considered in the early stages and that iron reduction should be considered in the later stages. The former therapies may thus include body weight reduction and insulin-sensitizing drugs, and the latter therapies may include phlebotomy, iron-restriction diets and/or antioxidants.

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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

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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.

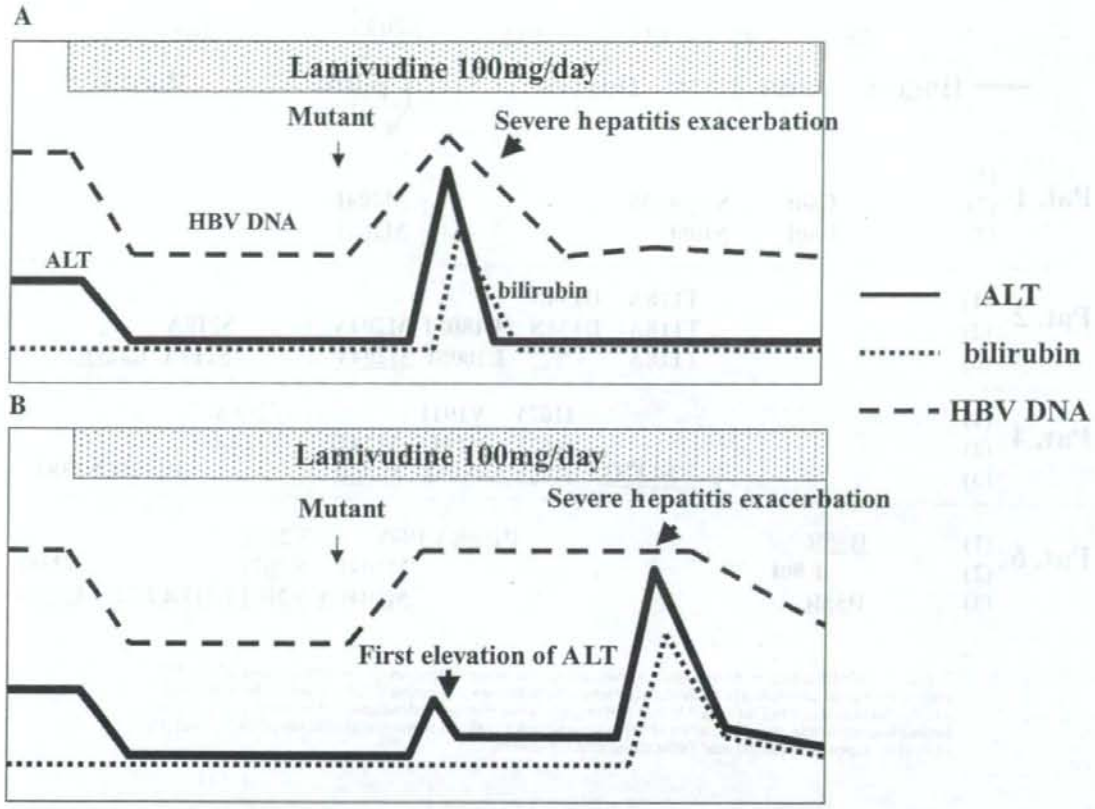

Fig. 1. Schema of transitions of virological, serum alanine aminotransferase (ALT), and serum bilirubin during lamivudine therapy in exacerbation of severe hepatitis. Two types were defined: (**A**) at first ALT elevation after first elevation of HBV DNA level and (**B**) at second or third elevation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

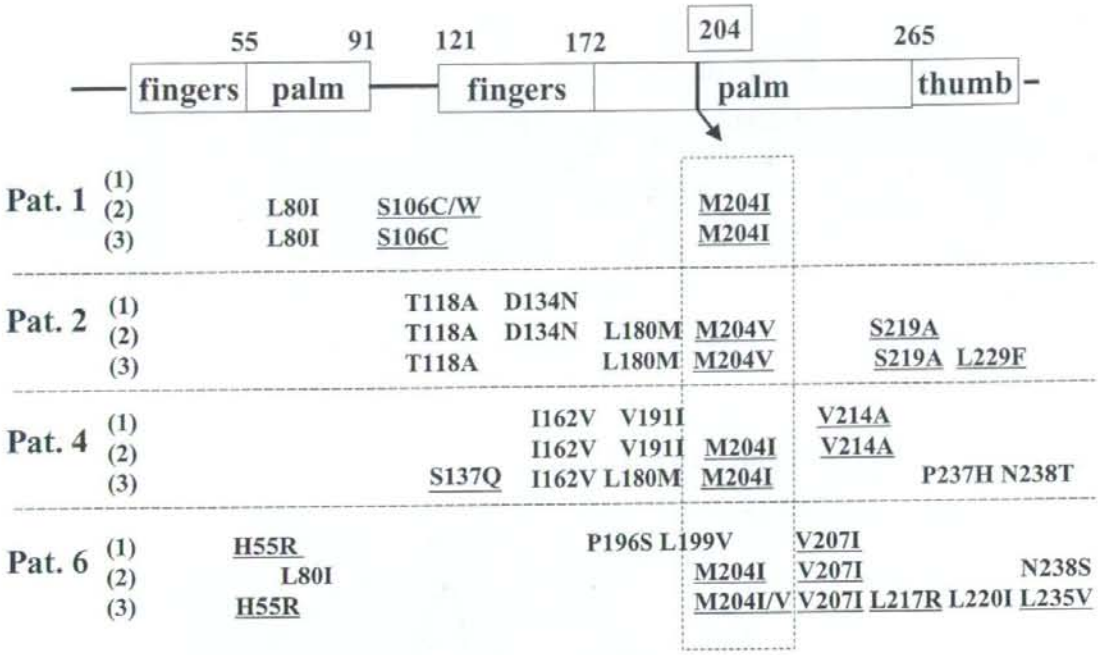

Fig. 2. Substitutions of amino acid sequences in the reverse transcriptase (rt) domain of the HBV polymerase gene are shown in patients with severe hepatitis exacerbation (SHE). The numbers of patients are the same as those in Table III. Underlined *rt* genes show changes introduced in the overlapping 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

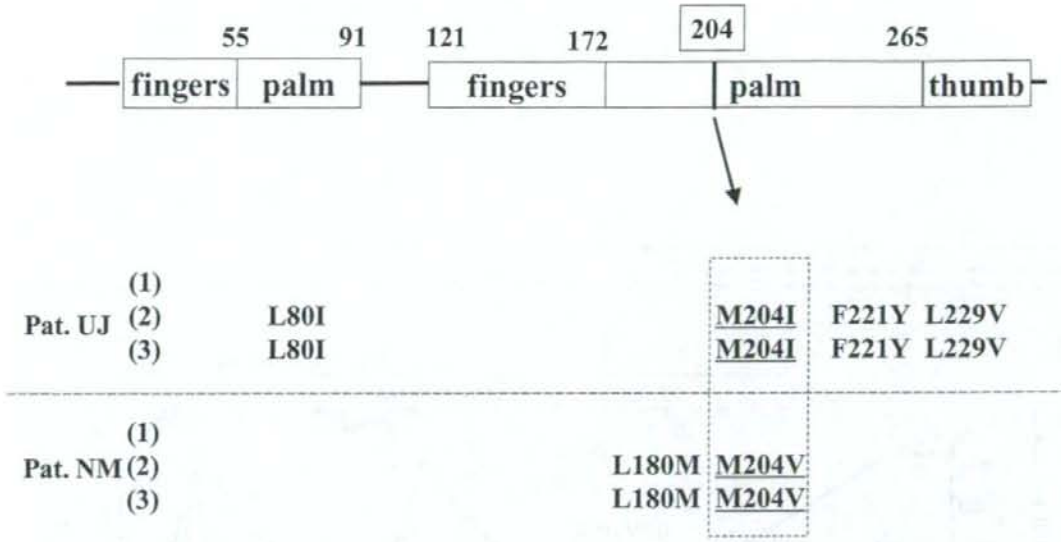

Fig. 3. Substitutions of amino acid sequences in the reverse transcriptase (rt) domain of the HBV polymerase gene in patients with non-elevated HBV DNA levels after emergence of the YMDD mutant. Measurements were conducted at three time points: (1) and (2) were at the same time points, and (3) at >2.5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

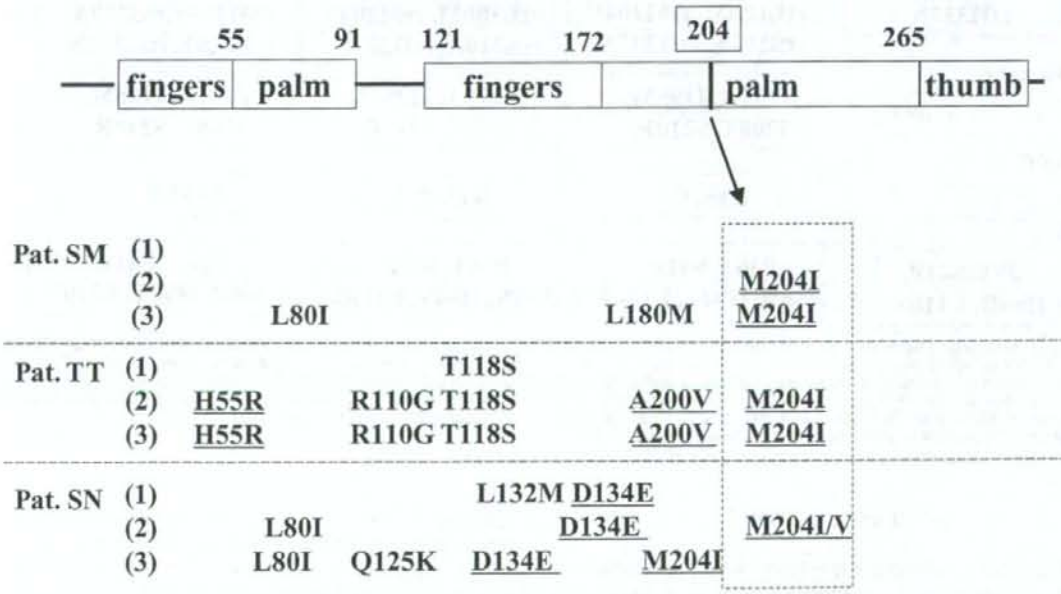

Fig. 4. Substitutions of amino acid sequences in the reverse transcriptase (rt) domain of the HBV polymerase gene in patients with elevated HBV DNA level but no severe exacerbation after emergence of the YMDD mutant. Measurements were conducted at three time points; (1) and (2) were at the same time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

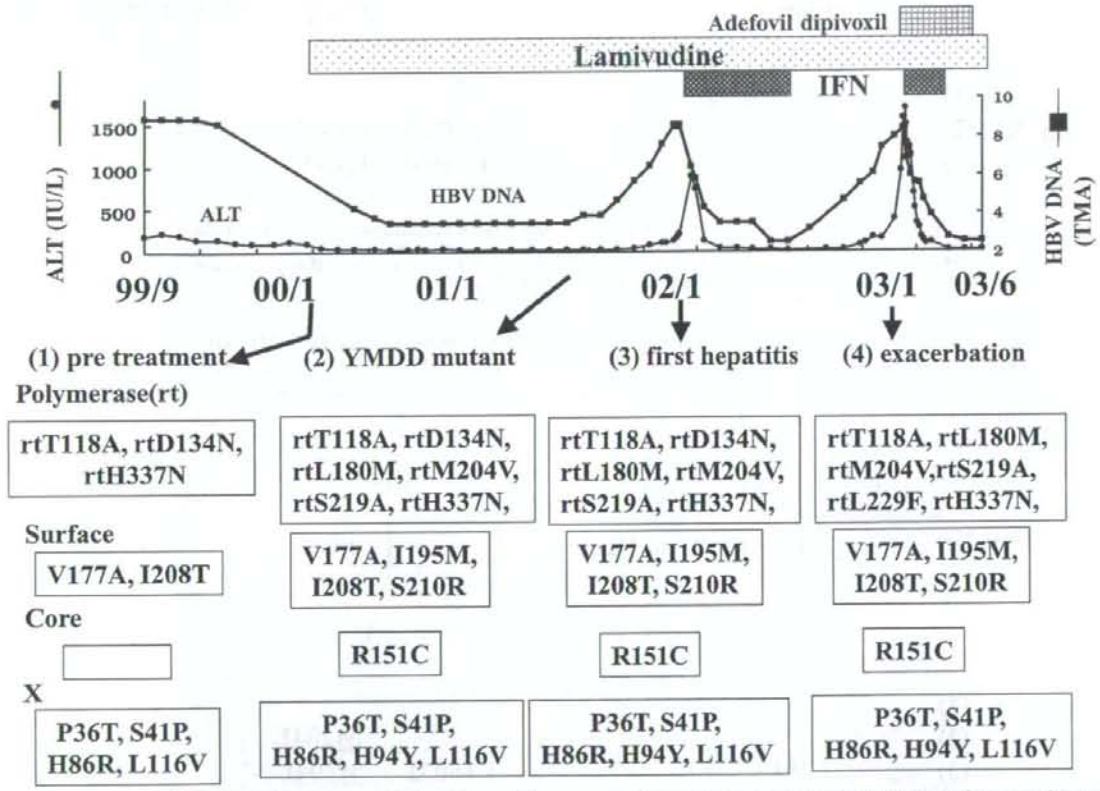

Fig. 5. Correlation between viral load, serum ALT, and accumulation of HBV substitutions during the clinical course in Patient 2, with severe hepatitis exacerbation. Four serial serum samples (arrows) were collected at various time points before and during lamivudine therapy. In addition to biochemical 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

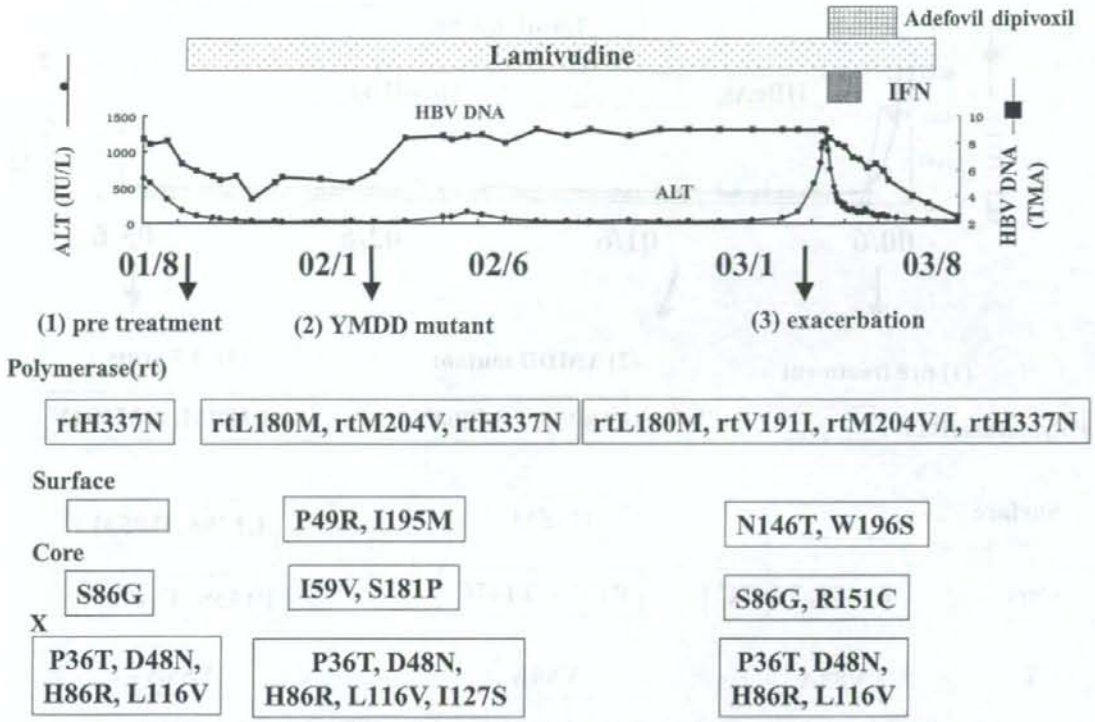

Fig. 6. Correlation between viral load, serum ALT, and accumulation of HBV substitutions during the clinical course in Patient 5, with severe hepatitis exacerbation.

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

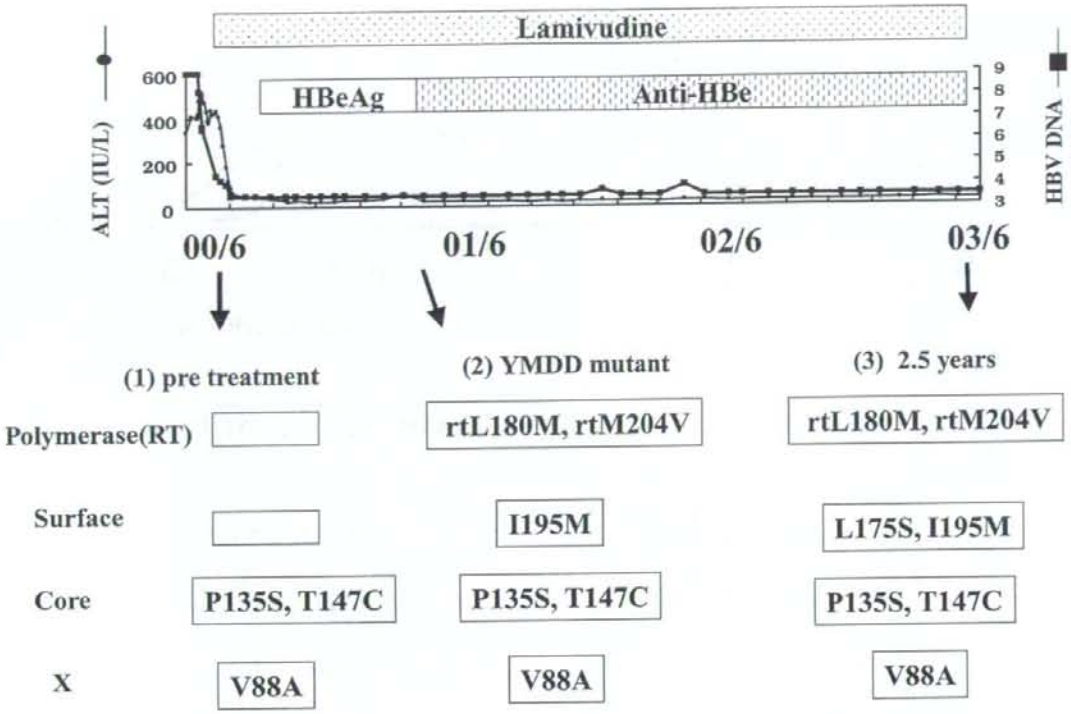

Fig. 7. Correlation between viral load, serum ALT, and accumulation of HBV substitutions during the clinical course in Patient NM, without elevation of HBV DNA.

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