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Clinical Studies

Useful parameters for distinguishing nonalcoholic steatohepatitis with mild steatosis from cryptogenic chronic hepatitis in the Japanese population

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Abstract: *Background/Aims:* As detecting mild steatosis is difficult by abdominal ultrasonography (US), nonalcoholic steatohepatitis (NASH) with mild steatosis may sometimes be confused with cryptogenic chronic hepatitis. We aimed to test this possibility and to isolate factors that may indicate NASH. *Methods:* First, 53 Japanese patients diagnosed as having cryptogenic chronic hepatitis by laboratory examination and US were enrolled. These patients were histologically divided into NASH and non-NASH groups, and their clinical features were compared. Second, the diagnostic accuracy of predictors of NASH was examined prospectively. *Results:* Fifteen patients (28%) were histologically diagnosed as having NASH with mild steatosis. Multivariable analysis revealed that body mass index (BMI) and serum ferritin level were independent predictors of NASH. The best cutoff values to detect NASH were assessed by using receiver-operating characteristic curves: BMI $> 25.2\,kg/m^2$ and serum ferritin level $> 142\,ng/ml$. When both markers were concomitantly negative, the negative predictive value to detect NASH was 100%. *Conclusions:* In cases of mild steatosis, US is not a perfect tool for the accurate diagnosis of NASH. BMI and serum ferritin level are useful discriminators of NASH from cryptogenic chronic hepatitis, and might be helpful markers for diagnosing NASH more accurately in Japanese patients.

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Key words: abdominal ultrasonography – body mass index – cryptogenic chronic hepatitis – ferritin – nonalcoholic steatohepatitis

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Chronic hepatitis is histologically characterized by sustained hepatocyte injury with apparent inflammation, and is clinically defined as the persistent elevation of serum aminotransferase levels for more than 6 months. The common causes of chronic hepatitis include persistent viral infection such as that of hepatitis B virus and hepatitis C virus (HCV), autoimmune disorders, and intake of alcohol, drugs, or chemicals. However, patients with cryptogenic chronic hepatitis or unexplained elevation of serum aminotransferase levels still exist whose exact pathogenesis has not been verified. Because cryptogenic chronic hepatitis may progress to cirrhosis, accurate diagnosis and treatment are needed.

Nonalcoholic steatohepatitis (NASH) is defined as a disease entity showing characteristic pathological findings common to alcoholic liver disease, including hepatic steatosis, hepatocellular ballooning, and perisinusoidal/pericellular fibrosis, despite no alcohol consumption. Skelly et al. (1) have reported that 34% of British patients with unexplained abnormal liver function tests are later diagnosed as NASH by liver biopsy, and in the United States, most patients with unexplained aminotransferase elevation are

considered to have nonalcoholic fatty liver disease (NAFLD) (2, 3). Recently, NASH has also been recognized as one of the major causes of chronic hepatitis in Japan, though its prevalence in patients with unexplained aminotransferase elevation or cryptogenic chronic hepatitis remains unclear.

Radiological imaging devices such as ultrasonography (US), computed tomography, and magnetic resonance are indispensable in evaluating hepatic steatosis. When lipid accumulation is observed in more than 33% of hepatocytes, these modalities have a strong ability to accurately diagnose hepatic steatosis (4). Of these, US is the least invasive and thus most preferred method. If fatty infiltration is evident by US examination, it is simple to conclude that unexplained persistent elevation of aminotransferase levels may be caused by NAFLD or NASH. However, mild (accumulation of triglycerides in less than 33% of hepatocytes) or focal steatosis can be underestimated by imaging modalities such as US (5). Additionally, in advanced stages of NASH, it is increasingly difficult to detect hepatic steatosis by imaging modalities as steatosis regresses and becomes focal as fibrosis progresses. Therefore, NASH with mild steatosis or advanced fibrosis may be confused with cryptogenic chronic hepatitis.

Based on these premises, we hypothesized that NASH presenting atypical features such as mild steatosis may be erroneously included with cryptogenic chronic hepatitis, and we retrospectively examined histological findings in patients who were diagnosed as having cryptogenic chronic hepatitis based on biochemical data and abdominal US to reevaluate the prevalence of NASH. We also compared the clinical features between NASH and non-NASH (intrinsically cryptogenic chronic hepatitis) groups and sought to find useful markers to differentiate NASH from cryptogenic chronic hepatitis that can be used in addition to US.

Patients and methods

Study 1

Patients

Chronic hepatitis was clinically defined by the following criteria: (1) elevation of serum aminotransferase levels (>40 IU/l) on two or more occasions during a period of at least 6 months and (2) exclusion of extrahepatic-origin elevation of serum aminotransferase levels such as myopathy and thyroid diseases by measuring serum levels of lactate dehydrogenase, creatine kinase, thyroid hormones, and thyroid-stimulating hormone. Patients who showed evidence as having liver cirrhosis were excluded. One thousand six hundred and ninety-one patients with chronic hepatitis who underwent liver biopsy at Shinshu University Hospital or affiliated hospitals between April 1, 1990 and September 30, 2004 were enrolled in this study. The diagnosis of cryptogenic chronic hepatitis was made according to the exclusion criteria shown in Fig. 1: (1) no consumption of alcohol; (2) negative results for hepatitis B surface antigen (HBsAg), high titer of hepatitis B core antibody (anti-HBc), and anti-HCV antibody (anti-HCV); (3) exclusion of other liver diseases such as drug-induced liver injury, autoimmune hepatitis, primary biliary cirrhosis, primary sclerosing cholangitis, Wilson's disease, hereditary hemochromatosis, and α1-antitrypsin deficiency; and (4) exclusion of obvious hepatic steatosis in abdominal US, that is, positive hepatorenal contrast and blurring of vascular wall and/or profound attenuation of the diaphragm. Patients presenting obvious hepatic steatosis were excluded from this study, as typical NAFLD or NASH is easily distinguishable using US. In total, 53 Japanese patients were clinically diagnosed as having cryptogenic chronic hepatitis (Fig. 1).

At the time of admission for liver biopsy, body mass index (BMI) was calculated. Patients were considered to have hypertension if their systolic/diastolic pressure was greater than 140/90 mmHg,

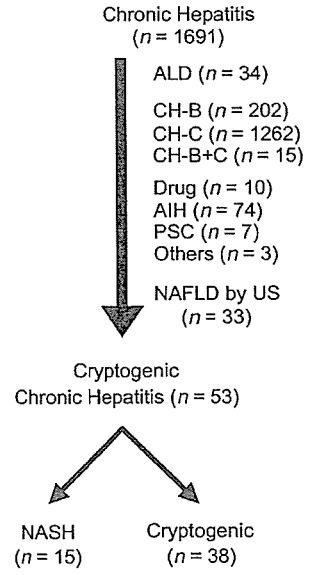

Fig. 1. The number of patients with chronic hepatitis enrolled in Study 1. The number of patients is indicated in parentheses. ALD, alcoholic liver disease; CH-C, chronic hepatitis C; CH-B, chronic hepatitis B; AIH, autoimmune hepatitis; PSC, primary sclerosing cholangitis; NAFLD, nonalcoholic fatty liver disease; US, ultrasonography.

or if they were taking anti-hypertensive drugs. Patients were considered to be diabetic if they had a fasting glucose level equal to or higher than 126 mg/dl, or if they were taking insulin or oral hypoglycemic drugs. Patients were considered to have hyperlipidemia if their fasting serum levels of cholesterol and triglycerides were equal to or higher than 220 and 150 mg/dl, respectively, or if they were taking lipid-lowering drugs.

Laboratory examination
All data were obtained in a fasting state and measured by standard methods. The homeostasis model assessment for insulin resistance (HOMA-IR) was calculated using the following equation: [fasting glucose (mg/dl) × fasting insulin (μU/ml)]/405. A HOMA-IR greater than 2.0 is considered to indicate the presence of insulin resistance. If the patient had a fasting glucose level equal to or higher than 140 mg/dl, or was taking insulin, HOMA-IR was not calculated.

Histological diagnosis of NASH
Before liver biopsy, informed consent was obtained from each patient. Liver biopsy specimens were immediately fixed in 10% neutral formalin. Sections were cut at 4 μm thickness and stained with hematoxylin and eosin and Azan–Mallory methods. Histological diagnosis of NASH was made according to the following criteria: macrovesicular steatosis mainly present in zone 3, hepatocellular ballooning and/or perisinusoidal/pericellular fibrosis. Histological findings were classified using the grading/staging system proposed by Brunt et al. (6) with minor modifications. The degree of hepatic steatosis was expressed as the percentage of steatotic hepatocytes in biopsied specimens. The appearance frequency of hepatocellular ballooning, glycogenated nuclei, and eosinophilic intracytoplasmic inclusion bodies was graded as absent, few, or many based on the number of hepatocytes showing the respective changes. The activity of lobular and portal inflammation was graded as absent, mild, moderate, or severe. Perisinusoidal/pericellular fibrosis was scored as absent, mild, or severe based on the proportion of zone 3 area involved, and portal fibrosis was assessed as absent, periportal, bridging, or cirrhosis. Histological diagnosis was made be three experts (E. T., N. T., K. Y.).

Detection of hepatic steatosis by US
Each patient underwent abdominal US (Hitachi model EUB-525 equipped with a 3.5 MHz convex-type transducer, Hitachi, Medical Corp., Tokyo, Japan) in a fasting state. The presence of hepatic steatosis was assessed independently by three hepatologists according to findings such as hepatorenal contrast, blurring of the vascular wall, and profound attenuation of the diaphragm.

Study 2

To estimate the diagnostic accuracy of the markers found in Study 1, 256 patients with elevated serum aminotransferase levels who underwent liver biopsy between October 1, 2004 and March 31, 2006 were eligible for entry into this prospective study. Exclusion criteria were positive for HBsAg, anti-HBc, anti-HCV, anti-mitochondrial antibody, the presence of the history of alcohol comsumption or hepatotoxic drug intake, and the presence of hepatic steatosis, which was easily detectable by US. The remaining 22 patients were divided into three groups according to the number of positive markers, and the final diagnosis was performed histologically. Diagnostic accuracy was calculated by sensitivity, specificity, and positive and negative predictive values (PPV and NPV, respectively).

Statistical analysis

Statistical analyses were performed using SPSS software 11.5J for Windows (SPSS Inc., Chicago, IL). Comparison between the groups was made using Fisher's exact probability test for the categorical variables, χ^2 test for the histological findings, and Mann–Whitney *U*-test for the continuous variables, respectively. To assess the use of clinical parameters in differentiating NASH from cryptogenic chronic hepatitis, we constructed receiver-operating characteristic (ROC) curves by plotting the sensitivity against the reverse specificity (1 minus specificity) for each value. In this assessment, a larger area under the ROC curve (AUC) corresponds to a more useful marker for diagnosing NASH. The most appropriate cutoff point for the diagnosis of NASH was the point at which the sum of the sensitivity and the specificity was maximized. To identify independent predictors of NASH, multivariable logistic regression analysis was conducted. A probability value of <0.05 was considered statistically significant.

Results

Study 1

NASH in cryptogenic chronic hepatitis
In this study, we addressed the specific group of subjects with unexplained aminotransferase elevation and normal US findings. Of the 53 patients who were clinically diagnosed as having crypto-

Table 1. Comparison of histological findings between NASH and non-NASH groups

	NASH ($n=15$)	nonNASH ($n=38$)	P
Macrovesicular steatosis (%)			0.000
<5	0 (0%)	35 (92%)	
5–20	3 (20%)	2 (5%)	
21–33	12 (80%)	1 (3%)	
>33	0 (0%)	0 (0%)	
Ballooning			0.000
Absent	0 (0%)	37 (97%)	
Few	4 (27%)	1 (3%)	
Many	11 (73%)	0 (0%)	
Glycogenated nuclei			0.000
Absent	0 (0%)	33 (87%)	
Few	9 (60%)	5 (13%)	
Many	6 (40%)	0 (0%)	
Eosinophilic intracytoplasmic inclusion bodies			0.000
Absent	6 (40%)	38 (100%)	
Few	6 (40%)	0 (0%)	
Many	3 (20%)	0 (0%)	
Lobular inflammation			0.627
Absent	2 (13%)	10 (26%)	
Mild	10 (67%)	19 (50%)	
Moderate	3 (20%)	8 (21%)	
Severe	0 (0%)	1 (3%)	
Portal inflammation			0.021
Absent	4 (27%)	2 (5%)	
Mild	9 (60%)	16 (42%)	
Moderate	2 (13%)	11 (29%)	
Severe	0 (0%)	9 (24%)	
Perisinusoidal/pericellular fibrosis			0.000
Absent	0 (0%)	37 (97%)	
Mild	11 (73%)	1 (3%)	
Severe	4 (27%)	0 (0%)	
Portal fibrosis			0.508
Absent	8 (53%)	25 (66%)	
Periportal	2 (13%)	7 (18%)	
Bridging	4 (27%)	4 (11%)	
Cirrhosis	1 (7%)	2 (5%)	

Data are the number positive and prevalence (in parentheses). A P value was calculated using the χ^2 test. NASH, nonalcoholic steatohepatitis.

genic chronic hepatitis, 15 (28%) fulfilled the histological diagnostic criteria of NASH (Fig. 1). The histological features of these 15 patients, who were not diagnosed as having steatosis by US, but were histologically confirmed as having NASH, are shown in Table 1. The degree of hepatic steatosis was generally mild (<33% of hepatocytes in the biopsy involved), so steatosis was hard to detect by US (Figs 2 and 3). Ballooned hepatocytes, glycogenated nuclei, and perisinusoidal/pericellular fibrosis in zone 3 were observed in all NASH patients, and eosinophilic intracytoplasmic inclusion bodies were detected in 60% of these patients. Lobular and portal inflammation was relatively mild, and portal fibrosis was variable. These results suggest that detection of NASH with mild steatosis is clinically unreliable using US only.

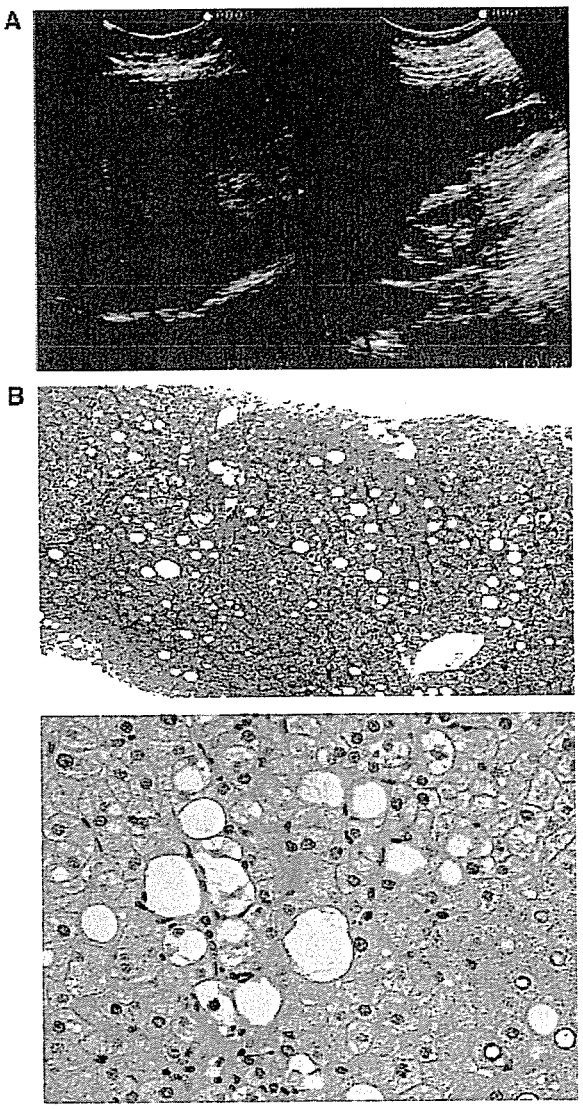

Fig. 2. Ultrasonographic and histological findings of the non-alcoholic steatohepatitis patient, who were clinically diagnosed as having cryptogenic hepatitis. (A) A 61-year-old woman with obesity and hyperlipidemia was diagnosed as having cryptogenic chronic hepatitis because of no obvious steatosis by ultrasonography. (B) Histological evaluation confirmed the presence of mild macrovesicular steatosis, ballooned hepatocytes with eosiniphilic intracytoplasmic inclusion bodies, glycogenated nuclei, and mild perisinusoidal/pericellular fibrosis (upper photograph, Azan–Mallory staining, × 100; lower photograph, hematoxylin and eosin staining, × 400).

Comparison of histological findings between biopsy-proven NASH patients and non-NASH patients

We compared the histological findings between the biopsy-proven NASH patients, i.e. patients having NASH with mild steatosis (NASH group, $n=15$), and non-NASH patients, i.e. patients having intrinsically cryptogenic chronic hepatitis (non-NASH group, $n=38$). As shown in Table 1, the prevalence of macrovesicular steatosis, hepa-

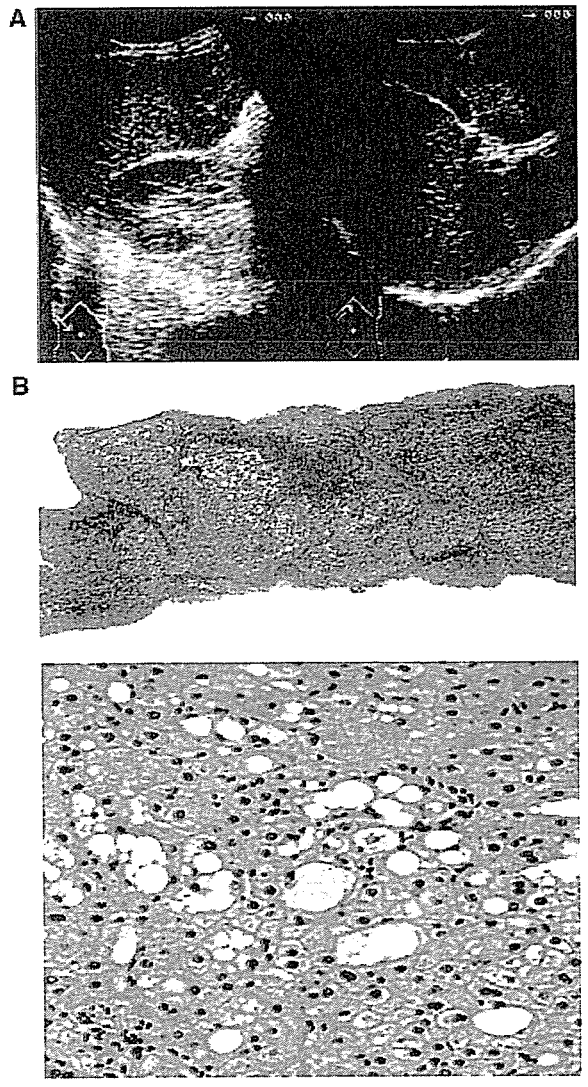

Fig. 3. Ultrasonographic and histological findings of another nonalcoholic steatohepatitis patient. (A) A 72-year-old woman with diabetes was diagnosed as having cryptogenic chronic hepatitis because obvious fatty infiltration was not confirmed by ultrasonography. (B) Histologically, mild and focal macrovesicular steatosis, hepatocellular ballooning, and advanced fibrosis were confirmed (upper photograph, Azan–Mallory staining, × 50; lower photograph, hematoxylin and eosin staining, × 400).

tocellular ballooning, glycogenated nuclei, eosinophilic intracytoplasmic inclusion bodies, and perisinusoidal/pericellular fibrosis was significantly higher in the NASH group. Three patients in the non-NASH group (three in 38 patients, 8%) exhibited mild steatosis but could not be diagnosed as having NASH because of the absence of ballooned hepatocytes or perisinusoidal/pericellular fibrosis. Although the activity of lobular inflammation was similar in both groups, portal inflammation tended to be more severe in the non-NASH group. In NASH livers, lymphocytes and/or polymorphonuclear leukocytes were present in the inflammatory foci, whereas in non-NASH livers, infiltration of plasma cells and/or eosinophils, as well as lymphocytes, was observed. These results demonstrate clear histological differences between the two groups.

Comparison of clinical features between the NASH group and the non-NASH group

To explore other helpful markers for differentiating NASH with mild steatosis from cryptogenic chronic hepatitis, we compared the clinical features and laboratory findings between the two groups. As shown in Table 2, the prevalence of hyperlipidemia ($P = 0.002$), BMI ($P = 0.001$), fasting glucose level ($P = 0.021$), HOMA-IR ($P = 0.009$), and serum ferritin level ($P = 0.001$) were all significantly higher in the NASH group. The prevalence of diabetes and hypertension, serum levels of high-sensitivity C-reactive protein, aminotransferases, and γ-glutamyltransferase, immunoglobulin G and A concentrations, and hemoglobin A1c value were not significantly different between the two groups.

Multivariable analysis

Multivariable logistic regression analysis revealed that BMI and serum ferritin level were independent factors associated with NASH. The odds ratio for BMI was 1.836 [95% confidence interval (CI), 1.063–3.173; $P = 0.029$], and that for serum ferritin level was 1.014 (95% CI, 1.000–1.027; $P = 0.048$).

ROC curve analysis

ROC curves were constructed for these two parameters. The AUCs for BMI and serum ferritin level were as great as 0.791 (95% CI, 0.644–0.938; $P = 0.001$) and 0.782 (95% CI, 0.651–0.914; $P = 0.001$), respectively. We next determined the cutoff values of these parameters for the discrimination between NASH with mild steatosis and cryptogenic chronic hepatitis by using the ROC curve. The most appropriate cutoff values were identified as BMI $> 25.2\,kg/m^2$ and serum ferritin level $> 142\,ng/ml$, respectively.

Study 2

We prospectively examined the diagnostic accuracy of the predictors of NASH found in Study 1. In 22 patients with persistent unexplained elevation of serum aminotransferase levels, eight were histologically diagnosed as having NASH (Fig. 4). Most of these NASH patients exhibited severe fibrosis or cirrhosis. When both parameters were concomitantly positive, the sensitivity, specificity, and PPV and NPV to detect NASH were 87.5%, 85.7%, 77.8%, and 92.3%, respectively. On the

Table 2. Comparison of clinical features and biochemical markers between NASH and non-NASH groups

	NASH ($n=15$)	non-NASH ($n=38$)	P
Age (years)	59 (39–77)	56 (14–72)	0.431
Female	57%	85%	0.532
Diabetes	27%	16%	0.143
Hyperlipidemia	47%	5%	0.002
Hypertension	27%	18%	0.275
BMI (kg/m^2)	26.9 (19.7–33.0)	23.1 (17.9–31.6)	0.001
Platelet ($\times 10^4/\mu l$)	18.2 (12.5–35.0)	19.9 (7.9–35.7)	0.552
hsCRP (mg/dl)	0.099 (0.017–0.496)	0.078 (0.004–0.500)	0.103
Albumin (g/dl)	4.5 (3.7–4.9)	4.3 (3.3–4.9)	0.232
AST (IU/l)	64 (19–161)	57 (16–639)	0.809
ALT (IU/l)	75 (30–238)	79 (22–604)	0.713
γGT (IU/l)	51 (37–213)	71 (9–269)	0.742
Total cholesterol (mg/dl)	213 (155–278)	178 (129–353)	0.054
Triglycerides (mg/dl)	122 (66–398)	97 (49–266)	0.088
HDL-cholesterol (mg/dl)	40 (20–61)	40 (21–74)	0.826
Immunoglobulin G (mg/dl)	1368 (677–3012)	1341 (987–2612)	0.750
Immunoglobulin A (mg/dl)	250 (99–487)	255 (80–713)	0.951
Glucose (mg/dl)	96 (77–164)	89 (56–113)	0.021
Hemoglobin A1c (%)	5.8 (5.0–7.8)	5.3 (4.4–7.2)	0.064
HOMA-IR*	2.4 (0.3–8.9)	1.2 (0.2–7.6)	0.009
Iron (μg/dl)	145 (67–325)	102 (20–266)	0.104
Transferrin saturation (%)	44 (29–94)	34 (5–90)	0.076
Ferritin (ng/ml)	229 (62–776)	120 (3–376)	0.001

Qualitative data are expressed as percentages, and quantitative data are written as medians and ranges (in parentheses). A P value for qualitative and quantitative data was calculated using Fisher's exact probability test and Mann–Whitney U-test, respectively. *HOMA-IR was not evaluated in patients receiving insulin therapy or having a fasting glucose level equal to or higher than 140 mg/dl. BMI, body mass index; γGT, γ-glutamyltransferase; HDL, high density lipoprotein; HOMA-IR, homeostasis model assessment for insulin resistance.

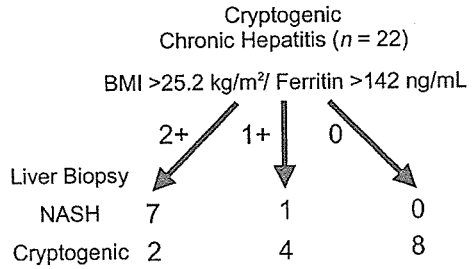

Fig. 4. Flow chart in Study 2. Twenty-two patients with persistent unexplained elevation of aminotransferase levels were enrolled in this prospective study. The number of patients is indicated in parentheses. 2+, body mass index (BMI) >25.2 kg/m^2 and serum ferritin level >142 ng/ml; 1+, BMI >25.2 kg/m^2 or serum ferritin level >142 ng/ml; 0, BMI <25.2 kg/m^2 and serum ferritin level <142 ng/ml.

other hand, when both parameters were concomitantly negative, the sensitivity, specificity, and PPV and NPV were 100%, 71.4%, 71.4%, and 100%, respectively. Although the number of patients enrolled in Study 2 was very limited, these results might suggest the relevance of these parameters as supporting diagnostic markers of NASH for patients with unexplained persistent aminotransferase elevation.

Discussion

The present study demonstrates that sole reliance on abdominal US might overlook NASH with mild steatosis, and that BMI and serum ferritin level are helpful for differentiating NASH with mild steatosis from cryptogenic chronic hepatitis and for diagnosing NASH more accurately.

In NASH, appropriate correction of life style and pharmacological interventions (e.g. insulin sensitizers) can reduce disease activity and prevent the progression (7, 8). These therapeutic strategies are fundamentally distinct from other types of chronic hepatitis, so accurate diagnosis is very important for NASH. US is the most common diagnostic tool for detecting hepatic steatosis, which is an essential component of NASH. Generally speaking, US has a relatively high sensitivity and specificity for the detection of hepatic steatosis. In cases with more than 33% fatty infiltration, the sensitivity and PPV of US are 100% and 62%, respectively (4). However, the diagnostic accuracy of US declines sharply in cases of less than 30% fatty infiltration. This change may be associated not only with mild or focal fatty deposition in livers, but also with interobserver differences in image readings. Although US has been used for the diagnosis of NAFLD in several studies (9–11), there is a possibility that NASH with mild steatosis has been overlooked. Indeed, Hamaguchi et al. (11) have described that US may lead to an incorrect diagnosis of NAFLD in 10–30% of cases analyzed. We found that, regardless of no detection

of steatosis by US, 28% of Japanese patients with unexplained elevation of serum aminotransferase levels have NASH with mild steatosis, suggesting the inadequacy of US to detect certain types of NASH. Therefore, US cannot be considered as a gold standard test for the accurate diagnosis of NASH with mild steatosis, and novel diagnostic markers of NASH, which may compensate for this imperfection, are clearly needed.

We found that BMI and serum ferritin level are highly predictive of NASH. It is well known that NASH is strongly associated with obesity and visceral fat accumulation (11–13). It has also been reported that serum ferritin level, a major determinant of NAFLD in apparently healthy obese individuals (10), is significantly correlated with the amount of visceral fat mass and hepatic steatosis (14). Furthermore, serum ferritin level has been reported to be significantly higher in NASH than that in simple steatosis, which may reflect increased hepatic iron overload and enhanced oxidative stress (15). Therefore, assessment of these markers may be useful not only for discriminating NASH from chronic hepatitis caused by unknown hepatotoxic factors, but also for diagnosing NASH more accurately and efficiently.

As shown in Fig. 3, in patients with NASH in advanced fibrosis or cirrhosis, the accuracy of hepatic steatosis detection is markedly reduced, so the correct diagnosis of NASH becomes increasingly difficult. The results obtained from Study 2 (Fig. 4) suggest that a combination of BMI and serum ferritin level might be helpful parameters for distinguishing NASH from cryptogenic chronic hepatitis, even in advanced stages. Thus, it might be possible to use these markers for the purpose to isolate NASH-derived cirrhosis (burned-out NASH) from intrinsically cryptogenic cirrhosis. Further study is needed to determine whether these markers would be really helpful for identifying the etiology in patients with cryptogenic hepatitis with advanced fibrosis or cirrhosis.

In this study, we could not assess the etiology of the difference between NASH and NASH with mild steatosis. The development of hepatic steatosis depends primarily on the changes of three pathways in fatty acid metabolism: increased influx of circulating nonesterified fatty acids into hepatocytes, increased *de novo* lipogenesis in hepatocytes, and decreased degradation through the mitochondrial β-oxidation system (16). Thus, an imbalance of these pathways might contribute to the difference in the severity of steatosis in NASH.

This study has several limitations. First, the number of patients analyzed was limited in this retrospective study, so a large-scale prospective analysis is needed to confirm these results. Second, although it has been reported that several biomarkers such as adipocytokines (e.g. adiponectin) (17), proinflammatory cytokines (e.g. tumor necrosis factor-α) (18), and oxidative stress markers (e.g. thioredoxin) (15) might be useful predictors of NASH, we could not examine these parameters in this study. In addition to BMI and serum ferritin level, measuring these biomarkers may enable us to more accurately diagnose NASH with mild steatosis. Finally, it is possible that some NASH patients might have been misplaced into the non-NASH group because of biopsy sampling errors. NASH livers show more heterogeneous histological findings than those with chronic hepatitis C (19). Moreover, the sampling variability is more significant in livers of burned-out NASH, so patients with advanced stages of NASH would be classified into the non-NASH group. Indeed, in this study, one obese female patient with hyperlipidemia, hypertension, diabetes, and hyperferritinemia was histologically diagnosed as having cryptogenic cirrhosis because of a lack of histological findings specific to NASH, but her aminotransferase levels normalized only by weight reduction, suggesting a strong likelihood of burned-out NASH. Repeated US-guided biopsy or laparoscopy-assisted biopsy (20) may minimize the possibility of sampling error in patients strongly suspicious of having NASH. To overcome these limitations, it will be mandatory to establish novel biochemical markers of NASH, which are available even in advanced stages of NASH and are independent of the amount of hepatic steatosis.

In conclusion, US is not a perfect tool for the accurate diagnosis of NASH with mild steatosis. Additionally, BMI $>25.2\,kg/m^2$ and serum ferritin level $>142\,ng/ml$ may be good non-US discriminators of NASH from chronic hepatitis of unknown etiology.

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Hepatitis B virus RNA is measurable in serum and can be a new marker for monitoring lamivudine therapy

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Background. Changes in the serum hepatitis B virus (HBV) RNA level during lamivudine therapy were compared to those in the serum HBV DNA and HBV core-related antigen (HBVcrAg) levels in 24 patients with chronic hepatitis B. ***Methods.*** For measurement of HBV RNA, total nucleic acid was extracted from serum samples and treated with RNase-free DNase I. After cDNA synthesis from extracted RNA, HBV RNA was measured by real-time detection polymerase chain reaction. ***Results.*** The peak fraction of HBV RNA in serum samples was consistent with peak fractions of HBV DNA and HBV core protein in a sucrose gradient analysis, indicating that HBV RNA was incorporated into virus particles. All levels of HBV DNA, HBV RNA, and HBVcrAg decreased gradually during lamivudine therapy ($P < 0.001$ for all). The amount of decrease from the start of lamivudine therapy was significantly higher for HBV DNA than for HBV RNA or HBVcrAg during 6 months of lamivudine therapy ($P < 0.001$ for all). However, a similar difference was not seen between HBV RNA and HBVcrAg levels during that period. The HBV RNA level was significantly correlated ($P < 0.001$ for all) with levels of HBV DNA and HBVcrAg both at the beginning and 2 months after the start of lamivudine therapy. ***Conclusions.*** HBV RNA is detectable in serum in a form indicating incorporation into virus particles, and its serum level might serve as a new viral marker with a significance different from that of HBV DNA in lamivudine therapy.

Key words: chronic hepatitis B, viral load, viral replication, cccDNA, sucrose gradient analysis

Introduction

Approximately 350 million people are chronic carriers of hepatitis B virus (HBV) worldwide.[1] In some countries, hepatocellular carcinoma and cirrhosis account for more than 50% of all deaths among HBV carriers.[2] Treatment of patients with hepatitis B has improved remarkably with the advent of oral nucleoside analogs such as lamivudine.[3,4] Lamivudine administration has been shown to cause a rapid decrease in the serum HBV DNA level followed by a decrease in the alanine aminotransferase level and improvement of the liver histology.[5–8] Therefore, measurement of serum HBV DNA is widely used in the clinical setting to monitor the effect of lamivudine.

It has been postulated that measurement of the HBV covalently closed circular (ccc) DNA level in hepatocytes is valuable in a different way than serum HBV DNA for monitoring the effects of antiviral therapy, because cccDNA is a key molecule in HBV replication.[9–12] In practice, the intrahepatic HBV cccDNA level has been reported to be superior to serum HBV DNA for predicting a sustained virologic response to antiviral therapy, including lamivudine.[13] However, the measurement of cccDNA seems ill-suited for clinical use because it requires a liver biopsy. Thus, serum markers that reflect the cccDNA level in the liver are desired.

Recently, an HBV core-related antigen (HBVcrAg) assay developed by our laboratory has been shown to possibly correlate with the cccDNA level, especially during lamivudine therapy.[14–16] This possibility is based on the fact that transcription of messenger RNA from cccDNA and subsequent translation of viral proteins are not inhibited by nucleoside analogs such as lamivudine. The same has been said for synthesis of pregenomic RNA.[7] Therefore, in the present study, we measured serum HBV RNA and analyzed its virologic characteristics. In addition, changes in the serum HBV

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RNA level during lamivudine therapy were compared with those of serum HBV DNA and HBVcrAg levels to clarify whether HBV RNA measurement in serum has any clinical significance.

Patients and methods

Patients

A total of 24 patients with chronic hepatitis B consented to participate in the present study. They were selected from a pool of 32 consecutive patients who underwent lamivudine therapy at Shinshu University Hospital between July 2002 and June 2003. The patients comprised 18 men and 6 women, and their median age was 55 years (range, 39–79 years). Chronic hepatitis B was defined as positive HBV surface antigen for more than 6 months with liver histological findings consistent with chronic hepatitis. All patients had had elevated levels of serum alanine aminotransferase and HBV DNA for at least 6 months. Immediately prior to lamivudine administration, 16 patients were positive for HBV e antigen and 8 were positive for HBV e antibody but negative for HBV e antigen. The HBV genotype was C in all patients. Patients received 100-mg doses of lamivudine daily for at least 6 months. No patient was treated with other antiviral agents, such as interferon, before or during the present study, and all patients were negative for hepatitis C virus and human immunodeficiency virus antibodies. This study was approved by the ethics committee of our institution. Written informed consent was obtained from each patient.

Serum samples were collected at the start of lamivudine therapy, and at 2 and 6 months after commencement. Samples were stored frozen at –20°C or below until assayed.

Routine laboratory tests

HBV surface antigen, HBV e antigen, HBV e antibody, hepatitis C virus antibody, and human immunodeficiency virus antibody were measured by commercially available enzyme-linked immunosorbent assay kits (Abbott Japan, Tokyo, Japan). HBV genotypes were determined by the method reported by Mizokami et al.[17] and classified into six major genotypes, A to F.

The serum level of HBV DNA was determined using an Amplicor HBV Monitor kit (Roche Diagnostics, Tokyo, Japan), which has a quantitative range from 2.6 to 7.6log copies/ml. Sera containing over 7.0log copies/ml HBV DNA were diluted 10- or 100-fold with normal human serum and retested to obtain the end titer.

HBV core and core-related antigen assay

HBV core antigen (HBVcAg) and HBVcrAg in serum were measured using a chemiluminescence enzyme immunoassay as reported previously.[14,15,18] In brief, 100µl serum was mixed with a pretreatment solution containing 15% sodium dodecyl sulfate. After incubation at 70°C for 30min, 50µl of pretreated serum was added to wells coated with monoclonal antibodies against denatured HBV core and e antigens (HB44, HB61, and HB114) and filled with 100µl of assay buffer. The mixture was then incubated for 2h at room temperature. After washing with buffer, either alkaline phosphatase-labeled HB50 monoclonal antibody (specific for denatured HBV core antigen) or a mixture of HB91 and HB110 monoclonal antibodies (against denatured HBV core and e antigens) were added to wells and incubated for 1h at room temperature. After another washing, CDP-Star with Emerald II (Applied Biosystems, Bedford, MA, USA) was added and plates were incubated for 20min at room temperature. The relative chemiluminescence intensity was measured, and HBVcAg and HBVcrAg concentrations were read by comparison to a standard curve generated using recombinant pro-hepatitis B e antigen (amino acids –10 to 183 of the precore/core gene product). The concentrations of HBVcAg and HBVcrAg were expressed as units/ml, and the immunoreactivity of recombinant pro-hepatitis B e antigen at 10fg/ml was defined as 1 unit/ml. The cutoff value of both assays was set at 3log units/ml.[11] Sera containing over 7log units/ml of antigen were diluted 10- or 100-fold in normal human serum and measured again to obtain the end titer.

HBV RNA assay

A High Pure Viral Nucleic Acid kit (Roche Diagnostics) was used for isolation of HBV RNA from serum. Briefly, 200µl of serum was added to 250µl of freshly prepared working solution (6M guanidine-HCl; 10mM urea; 10mM Tris-HCl, pH 4.4; and 20% vol/vol Triton X-100) supplemented with 20µg of poly(A) carrier RNA and 900µg proteinase K. After incubation for 10min at 72°C, 100µl of isopropanol was added and the mixture was transferred into a High Pure filter tube combined with a collection tube. The filter tube was centrifuged for 1min at 3500g in a standard tabletop centrifuge at room temperature and combined with a new collection tube. The inhibitor removal buffer (5M guanidine-HCl, 20mM Tris-HCl, pH 6.6, in ethanol) was added to the upper reservoir and centrifuged for 1min at 3500g. After washing with 250µl of wash buffer (20mM NaCl, 2mM Tris-HCl, pH 7.5, in ethanol), 80µl of RNase-free DNase I solution (QIAGEN, Hilden, Germany) was added and incubated to digest HBV DNA for 15min at room temperature. A volume of

200 µl of wash buffer was added to the filter tube, which was then centrifuged for 15 s at 5000 *g*. After being washed with 450 µl of buffer, the filter was placed in a new collection tube and 50 µl of RNase- and DNase-free water was added to elute the RNA. After centrifugation for 1 min at 3500 *g*, the eluted RNA was stored at –80°C.

Synthesis of cDNA was performed at 42°C for 30 min in a 20-µl reaction mixture containing 10 µl of the extracted RNA; 50 mM Tris-HCl, pH 8.3; 75 mM KCl; 3 mM $MgCl_2$; 1 mM dNTP (1 mM each dATP, dGTP, dCTP, and dTTP); 1 mM dithiothreitol; 100 nM reverse primer for the HBV surface gene (5′-GGTTGG TGAGTGATTGGAGGTT-3′; nt 345 to 324); 40 units of RNasin (TaKaRa, Kyoto, Japan); and 200 units of SuperScript II RNase H—reverse transcriptase (Invitrogen, Carlsbad, CA, USA). The reaction mixture was inactivated by heating to 70°C for 15 min and then cooled to –80°C until the real-time detection polymerase chain reaction (RTD-PCR) assay. A 4-µl aliquot of cDNA solution was used for RTD-PCR, which was performed with a Light Cycler system (Roche Diagnostics) as reported previously.[14] The two primers and TaqMan probe used were designed from a region of the HBV surface gene: forward primer, 5′-ACAACAT CAGGATTCCTAGGAC-3′ (nt 166 to 187); reverse primer as stated above (nt 345 to 324); and TaqMan probe, 5′-FAM-CAGAGTCTAGACTCGTGGTGGA CTTC-TAMRA-3′ (nt 244 to 269). An HBV genome (nt 20 to 1805) that had been subcloned into a pUC vector was used as an internal standard. The lower detection limit for the HBV RNA assay was set at 2.6 log copies/ml. HBV DNA was tested on extracted HBV RNA samples not having undergone the preceding process by RTD-PCR and was confirmed to be negative in all samples.

Sucrose density gradient ultracentrifugation

Serum (0.1 ml) was layered on a linear 10%–60% (wt/wt) sucrose gradient, then centrifuged at 200000 *g* (45000 rpm) for 15 h at 4°C with a Beckman SW50.1 rotor (Beckman Coulter, Fullerton, CA, USA). In total, 24 fractions of 200 µl were collected by micropipette. Each fraction was diluted fivefold and tested for HBV DNA, HBV RNA, and HBVcAg.

Statistical analyses

Statistical analyses with the Mann-Whitney's U test, Friedman's test, and the Spearman rank correlation test were performed using the SPSS 10.0J statistical software package (SPSS, Chicago, IL, USA). A *P* value of less than 0.05 was considered statistically significant.

Results

Serum levels of HBV DNA ($P < 0.001$), HBV RNA ($P < 0.001$), and HBVcrAg ($P < 0.001$) all decreased significantly throughout the course of lamivudine therapy (Fig. 1). The amount of decrease at 2 months following commencement of lamivudine therapy was significantly higher for HBV DNA than for HBV RNA or HBVcrAg (median, 2.45; 25%–75% range, 1.90–3.00 log copies/ml vs median, 0.40, 25%–75% range, 0.00–0.85 log copies/ml, $P < 0.001$, and median, 0.30, 25%–75% range, 0.10–0.65 log units/ml, $P < 0.001$, respectively). Similarly, the amount of decrease after 6 months of treatment was significantly higher for HBV DNA than for HBV RNA or HBVcrAg (median, 3.20; 25%–75% range, 2.00–4.55 log copies/ml vs median, 0.90; 25%–75% range, 0.45–1.90 log copies/ml, $P < 0.001$, and median, 0.90; 25%–75% range, 0.20–1.55 log units/ml, $P < 0.001$, respectively). The amount of decrease did not differ between HBV RNA and HBVcrAg at either 2 ($P > 0.2$) or 6 ($P > 0.2$) months after commencement.

As shown in Fig. 2, the serum level of HBV RNA was significantly correlated with HBV DNA both at the start of lamivudine therapy ($r = 0.801$, $P < 0.001$) and 2 months afterward ($r = 0.837$, $P < 0.001$). Serum HBV

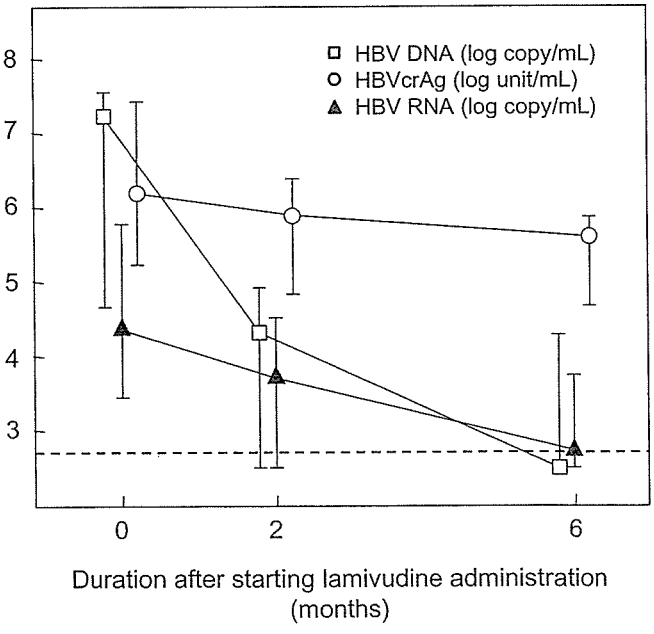

Fig. 1. Changes in serum levels of HBV DNA, HBV RNA, and HBVcrAg during lamivudine therapy in 24 patients with chronic hepatitis B. *Open squares* indicate HBV DNA, *open circles* indicate HBVcrAg, and *closed triangles* indicate HBV RNA. Data are expressed as medians and 25th and 75th percentiles. *HBV*, hepatitis B virus; *HBVcrAg*, HBV core-related antigen

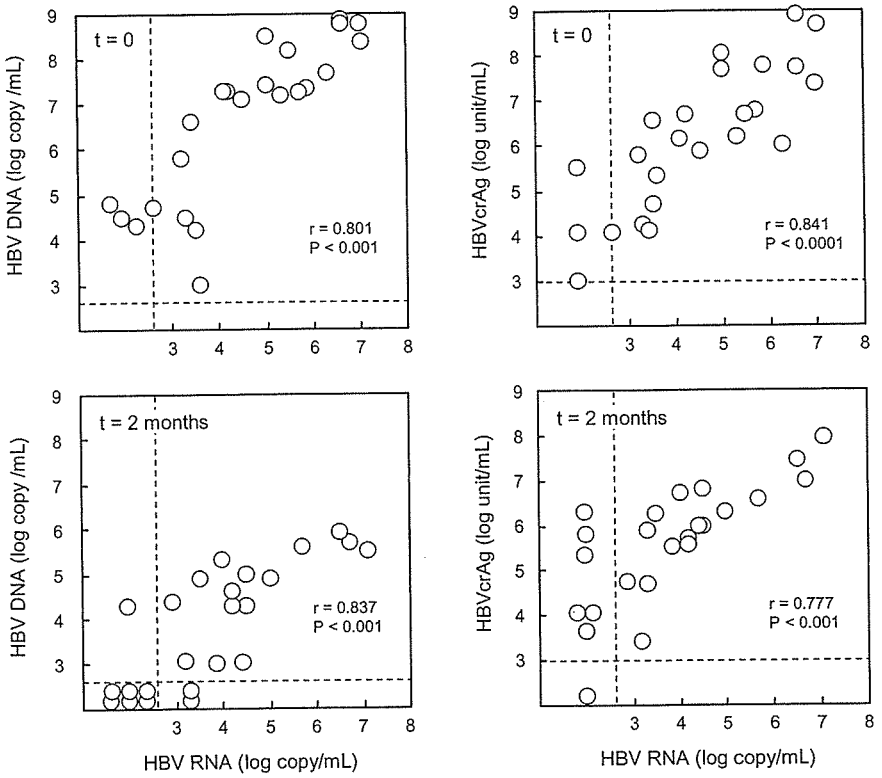

Fig. 2. Correlations between serum levels of HBV DNA and HBVcrAg with HBV RNA at the start of lamivudine therapy and 2 months afterward

RNA was also significantly correlated with that of HBVcrAg both at the start of treatment ($r = 0.841$, $P < 0.001$) and 2 months later ($r = 0.777$, $P < 0.001$). Ratios of HBV DNA and HBVcrAg levels to the HBV RNA level were calculated on log scale in patients who were positive for the above two parameters. The HBV DNA/HBV RNA ratio at the start of lamivudine therapy ($n = 21$; median, 1.36, 25%–75% range, 1.20–1.71) decreased significantly ($P < 0.001$) at 2 months after treatment commencement ($n = 15$; median, 0.98; 25%–75% range, 0.86–1.11). On the other hand, the HBVcrAg per HBV RNA ratio at the start of treatment ($n = 21$; median, 1.32; 25%–75% range, 1.21–1.52) did not change ($P > 0.2$) after 2 months ($n = 17$; median, 1.36; 25%–75% range, 1.15–1.54).

Serial serum samples obtained at the start of lamivudine therapy and at 1 and 2 months afterward were subjected to sucrose density gradient fractionation and tested for HBV DNA, HBV RNA, and HBVcAg. Each of the three viral markers showed a single peak for the same fraction (Fig. 3), suggesting that HBV RNA was incorporated into the virus particles, similarly to HBV DNA. Viral particles containing HBV DNA were dominant at the start of treatment, while those containing HBV RNA became more prevalent 1 and 2 months afterward.

Discussion

Replication of the HBV DNA genome proceeds via pregenomic RNA transcribed from the cccDNA present in the nuclei of infected hepatocytes.[9,11,12] The pregenomic RNA is then packaged into nucleocapsids and reverse transcribed to form minus-strand DNA. Plus-strand DNA synthesis is initiated following degradation of the pregenome. It has been reported that nucleocapsids containing only minus-strand DNA can be enveloped and then secreted from hepatocytes.[19] Thus, HBV virions in circulation have been considered to contain only mature viral genomes. In spite of this, HBV RNA could be detected in serum in the present study. HBV RNA was considered to be incorporated into virus particles because HBV RNA made a single peak for the same fraction where both HBV DNA and HBVcAg made single peaks in sucrose gradient analyses conducted at three different time points during lamivudine therapy. Detection of HBV particles with RNA genome does not necessarily contradict the findings of the previous report,[19] since HBV RNA particles seemed to exist in only a small portion (0.1%–1%) of the HBV virions in patients without lamivudine administration. The possibility that our HBV RNA assay detected HBV DNA left undigested by the DNase was

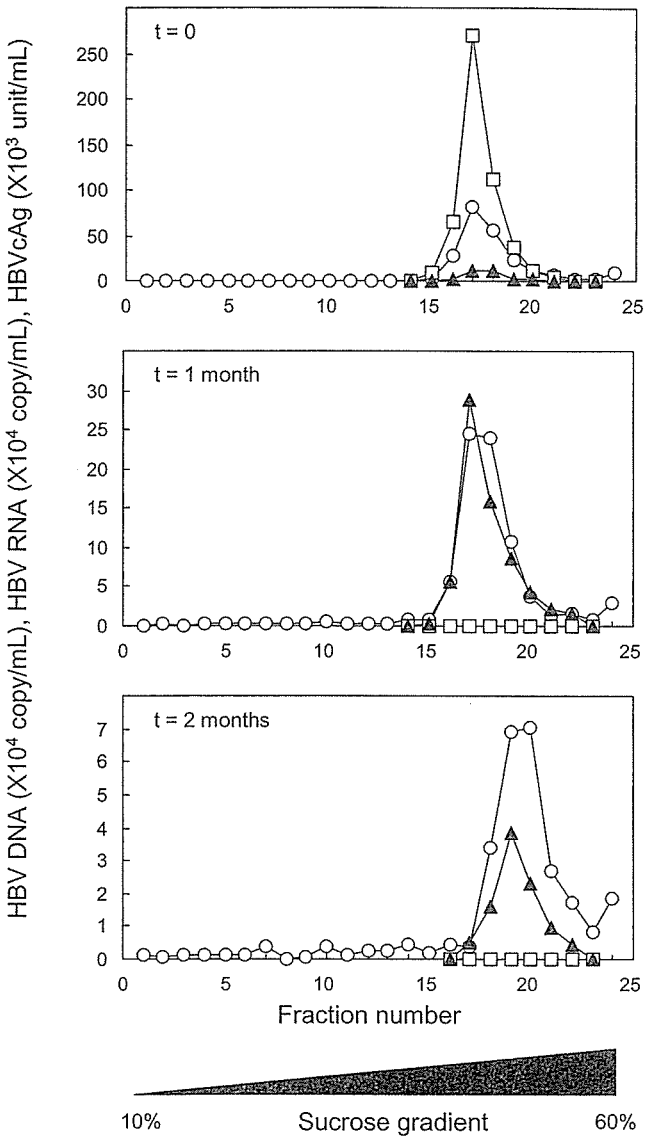

Fig. 3. Distribution of HBV DNA, HBV RNA, and HBV core antigen (*HBVcAg*) in sucrose density gradient fractions. Serum samples obtained at the start of lamivudine therapy and 1 and 2 months after were subjected to ultracentrifugation on a 10% to 60% (wt/wt) sucrose density gradient. *Open squares* indicate HBV DNA, *open circles* indicate HBVcAg, and *closed triangles* indicate HBV RNA

considered to be negligible because HBV DNA was not detected by RTD-PCR in extracted HBV RNA samples, and the change of the HBV RNA level during lamivudine therapy differed significantly from that of HBV DNA.

Detection of HBV RNA in serum has rarely been reported to date. Su et al.[20] reported the presence of full-length and truncated RNAs in serum, though their results seem to be quite different in nature because the HBV RNA that they observed was not incorporated into core particles. Zhang et al.[21] reported the existence of an RNA genome with partially reverse-transcribed minus-strand DNA in serum and speculated that the genome was in a virus particle. Such particles with HBV RNA accounted for about 1% of all HBV virions under untreated conditions, but became a major component under lamivudine administration. Although their analysis was done in a single patient, those observations are consistent with our observations that the rate of decrease of HBV DNA in serum was much faster than that of HBV RNA during lamivudine administration.

The HBVcrAg assay is a unique assay that measures the total amount of antigen coded by precore core genes such as HBV core and e antigens.[14,15] During the HBVcrAg assay, core antigen was released from the pretreated HBV virion and denatured, along with free e antigen and e antigen/antibody-complex. The HBVcrAg assay employs monoclonal antibodies that are reactive with the common epitopes of denatured core and e antigens. Under these conditions, the HBVcrAg assay simultaneously measures the core and e antigens within a given sample, irrespective of their source of origin. Serum HBVcrAg levels reflect the viral load in the untreated state because these levels correlate linearly with those of HBV DNA. On the other hand, it has been reported that HBVcrAg levels show different characteristics than HBV DNA levels under lamivudine administration.[15,16] It is noteworthy that HBVcrAg and HBV RNA levels both decreased significantly more slowly than HBV DNA after the beginning of lamivudine administration, but at a similar rate. This phenomenon is quite possible because synthesis of mRNA from cccDNA episomes and subsequent production of viral proteins is not inhibited by lamivudine like the synthesis of pregenomic RNA. Furthermore, it has been reported that the level of cccDNA decreases quite slowly during administration of nucleoside analogs,[22] indicating that serum levels of HBV RNA and HBVcrAg may be markers independent of the serum level of HBV DNA.

Measurement of serum HBV DNA is widely used for predicting and monitoring the effect of lamivudine therapy. However, a negative result of HBV DNA in serum does not necessarily indicate a good subsequent clinical course, because lamivudine-resistant strains often appear during drug administration and reactivation of HBV replication after discontinuation of treatment often occurs, even in patients who test negatively for serum HBV DNA during therapy.[23–25] Sung et al.[13] reported that the intrahepatic HBV cccDNA level at the end of lamivudine monotherapy or peginterferon and lamivudine combination therapy is a better predictor of a sustained virologic response than the serum HBV DNA level. Their conclusion seems quite reasonable because cccDNA in infected hepatocytes, not HBV in circulation, serves as a template for HBV pregenomic and messenger RNA in HBV replication. Although

cccDNA in the liver is a good marker for monitoring the effect of antiviral therapy, it is not easy to measure clinically because it requires a liver biopsy. Thus, serum markers that reflect the cccDNA level in hepatocytes are more suitable for clinical use. We previously reported that the serum HBVcrAg level is an independent marker, different from the serum HBV DNA level, for predicting the appearance of lamivudine resistance, and suggested that the HBVcrAg level reflects the level of cccDNA in hepatocytes.[16] In the present study, serum levels of HBVcrAg and HBV RNA correlated significantly, and the two levels decreased in a similar manner during lamivudine therapy (Figs. 1 and 2). These results further indicate that serum levels of both HBVcrAg and HBV RNA reflect the cccDNA level in hepatocytes since neither the synthesis of only messenger RNA, nor pregenomic RNA synthesized directly from cccDNA, which may be incorporated into viral particles, is inhibited by lamivudine.

In conclusion, HBV RNA that has been incorporated into viral particles is detectable in the serum of chronic HBV carriers. HBV RNA in serum is a new marker, which may reflect the cccDNA level in hepatocytes and may be useful for monitoring lamivudine therapy. We could not clarify the relationship between the HBV RNA level and clinical outcome in the present study. Thus, further studies are required to elucidate the clinical significance of HBV RNA in serum and its relationship to serum HBVcrAg.

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Influence of Genotypes and Precore Mutations on Fulminant or Chronic Outcome of Acute Hepatitis B Virus Infection

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The outcome of acute hepatitis B virus (HBV) infection is variable, influenced by host and viral factors. From 1982 through 2004, 301 patients with acute HBV infection entered a multi-center cross-sectional study in Japan. Patients with fulminant hepatitis (n = 40) were older (44.7 ± 16.3 vs. 36.0 ± 14.3 years, $P < .0017$), less predominantly male (43% vs. 71%, $P = .0005$), less positive for hepatitis B e antigen (HBeAg) (23% vs. 60%, $P < .0001$), less infected with subgenotype Ae (0% vs. 13%, $P < .05$), and more frequently with Bj (30% vs. 4%, $P < .0001$) than those with acute self-limited hepatitis (n = 261). Precore (G1896A) and core-promoter (A1762T/G1764A) mutations were more frequent in patients with fulminant than acute self-limited hepatitis (53% vs. 9% and 50% vs. 17%, $P < .0001$ for both). HBV infection persisted in only three (1%) patients, and they represented 2 of the 23 infected with Ae and 1 of the 187 with the other subgenotypes (9% vs. 0.5%, $P = .032$); none of them received antiviral therapy. In multivariate analysis, age 34 years or older, Bj, HBeAg-negative, total bilirubin 10.0 mg/dL or greater, and G1896A mutation were independently associated with the fulminant outcome. In *in vitro* transfection experiments, the replication of Bj clone was markedly enhanced by introducing either G1896A or A1762T/G1764A mutation. In conclusion, persistence of HBV was rare (1%) and associated with Ae, whereas fulminant hepatitis was frequent (13%) and associated with Bj and lack of HBeAg as well as high replication due to precore mutation in patients with acute HBV infection. *Supplementary material for this article can be found on the HEPATOLOGY website (http://interscience.wiley.com/jpages/0270-9139/suppmat/index.html).* (HEPATOLOGY 2006; 44:326-334.)

Abbreviations: HBV, hepatitis B virus; HBeAg, hepatitis B e antigen; HBc, hepatitis B core antigen; HBsAg, hepatitis B surface antigen; EIA, enzyme immunoassay; PCR, polymerase chain reaction; RFLP, restriction fragment length polymorphism; ALT, alanine aminotransferase.

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Approximately 3 billion people, one half of the world population, have been exposed to hepatitis B virus (HBV), of whom approximately 350 million are persistently infected with it.[1] Acute infection with HBV resolves in the great majority but can induce fulminant hepatitis or go on to become chronic. Host and viral factors may influence fulminant or chronic outcome of acute HBV infection, but they are not fully defined.

Eight genotypes have been detected by a sequence divergence greater than 8% in the entire HBV genome of approximately 3,200 nucleotides (nt), and designated by capital alphabet letters from A (HBV/A) to H in the order of documentation.[2-5] They have distinct geographical distributions associated with severity of liver disease as well as response to antiviral therapies.[6-8] Furthermore, subgenotypes have been reported for HBV/A, B, and C and named Aa/A1 (Asian/African type) and Ae/A2 (European type),[9] Bj/B1 (Japanese type) and Ba/B2 (Asian type),[10] as well as Cs/C1 (Southeast Asian type) and Ce/C2 (East Asian type).[11-13] Increasing lines of evidence indicate that subgenotypes of HBV/A and B influence the replication of HBV and bear clinical relevance.[14-16] Furthermore, genotypes affect mutations in precore region and core promoter, thereby influencing the expression of hepatitis B e antigen (HBeAg).[8,17]

During the 23 years from 1982 to 2004, a multi-center cross-sectional study was conducted throughout Japan on 301 patients with acute hepatitis B. We examined the influence of genotypes/subgenotypes on their fulminant or chronic outcome. Furthermore, the influence of G1896A or A1762T/G1764A on replication of HBV was evaluated in an *in vitro* replication model.

Patients and Methods

Patients With Acute Hepatitis B. During 1982 through 2004, 336 consecutive cases of acute hepatitis B were registered in 16 hospitals throughout Japan. These hospitals were from the following eight areas: Hokkaido (represented by J.-H. K. and S.H.), Tohoku (T.K. and K.S.), Kanto (H.T., Y.A. and K.I.), Koshin (E.T. and S.O), Tokai (A.O., Y.T., E.O., M.S., R.U., M.M., and S.K.), Kinki (T.O.), Honshu/Shikoku (Y.M., K.H., and M.O.), and Kyushu (H.Y. and H.S.). The diagnosis of acute hepatitis B was contingent on a sudden onset of clinical symptoms of hepatitis and detection of high-titered antibody to hepatitis B core antigen (anti-HBc) of IgM class in serum. Patients with initial high-titered anti-HBc (≥90% inhibition by a 1:200 diluted serum) were excluded; they were diagnosed as exacerbation of chronic hepatitis B. Patients with acute hepatitis A, hepatitis C, or human immunodeficiency virus co-infection, and drug- or alcohol-induced acute hepatitis also were excluded; hepatitis D virus infection was not examined because of its extreme rarity in Japan.[18] Most of them were followed for clinical outcomes until the disappearance of hepatitis B surface antigen (HBsAg) during 24 weeks or longer after the presentation. The criteria of fulminant hepatitis are based on the report by Trey et al.,[19] with a slight modification in 1981 (Inuyama symposium, Aichi, Japan): coma of grade II or higher and prothrombin time less than 40% developing within 8 weeks after the onset. Serum samples were collected at the presentation and had been stored at −80°C. HBV genotypes, HBV DNA, and HBeAg were determined, and clinical outcomes of acute hepatitis were analyzed. The study protocol conformed to the 1975 Declaration of Helsinki, and was approved by the Ethics Committees of the institutions. Every patient gave an informed consent for this study.

Serological Markers of HBV Infection. HBsAg was determined by hemagglutination (MyCell; Institute of Immunology Co., Ltd., Tokyo, Japan) or enzyme immunoassay (EIA) (Axsym; Abbott Japan, Tokyo, Japan), and HBeAg by enzyme-linked immunosorbent assay (F-HBe; Kokusai Diagnostic, Kobe, Japan) or chemiluminescent EIA (Fujirebio Inc., Tokyo, Japan). Anti-HBc of IgM and IgG classes were determined by radioimmunoassay (Abbott Japan).

Genotypes and Subgenotypes of HBV. The six major HBV genotypes (A-F) were determined serologically by EIA using commercial kits (HBV GENOTYPE EIA; Institute of Immunology). The method depends on the combination of epitopes on preS2-region products detected by monoclonal antibodies, which is specific for each of them.[20] HBV/G was determined by a slight modification of the polymerase chain reaction (PCR) with specific primers.[21]

Subgenotypes of HBV/A designated Ae prevalent in Europe and Aa frequent in Africa as well as Asia,9 which corresponds to subgroup A′ originally reported by Bowyer et al.,[22] were determined by PCR restriction fragment length polymorphism (RFLP) involving nucleotide conversions in an immediate upstream of the precore region that are specific for each of them.[16,23] HBV/Bj (Japanese type) lacking the recombination with C over the precore region and the core gene and Ba (Asian type) with the recombination were determined by its absence or presence on HBV DNA sequences, as well as RFLP based on specific nucleotide substitutions, after the methods described previously.[15,24]

Subgenotypes of HBV/C, Cs (Southeast Asian type) found only in Southeast Asia, including Vietnam, Myanmar, Thailand, Laos, Bangladesh, Hong Kong, and Southern China, and Ce (East Asian type), found in Far

East Asia, including Japan, Korea, and Northern China, were determined by the PCR-RFLP method described previously.[12]

Quantification of HBV DNA and Sequencing. HBV DNA sequences spanning the S gene were determined by real-time detection PCR according to the method of Abe et al.,[25] with the detection limit of 100 copies/mL. HBV DNA sequences bearing core promoter, precore region, and the core gene were amplified by PCR with hemi-nested primers by the method described previously.[10] Negative samples were tested by another more sensitive second-round PCR with HB7F and HBV1917R (5′-CTC CAC AGT AGC TCC AAA TTC TTT A-3′). Thereafter, PCR products were directly sequenced with Prism Big Dye (Applied Biosystems, Foster City, CA) in the ABI 3100 DNA automated sequencer.

Construction of Plasmid and Site-Directed Mutagenesis of HBV DNA. Serum samples were obtained from two patients infected with HBV/Bj and a patient with Ce. HBV DNA was extracted from 100 μL serum using QIAamp DNA blood kit (QIAGEN, GmbH, Hilden, Germany). Four primer sets were designed to amplify two fragments covering the entire HBV genome. Amplified fragments were inserted into pGEM-T Easy Vector (Promega, Madison, WI) and cloned in DH5a competent cells (TOYOBO, Osaka, Japan). At least five clones of each fragment were sequenced and the consensus sequence determined. Among them, those containing the consensus sequence were identified and adopted as templates for further construction. Finally, 1.24-fold the HBV genome (nt 1413-3215/1-2185), just enough to transcribe oversized pregenome and precore mRNA, was constructed into pUC19 vector (Invitrogen Corp., Carlsbad, CA). For site-directed mutagenesis, the wild-type HBV was digested by *HindIII* and *EcoO65I* and ligated with the fragment carrying T1762/A1764 to produce 1.24-fold the genome carrying the core-promoter double mutation. Similarly, 1.24-fold the HBV genome with the precore stop-codon mutation (1896A) was generated. Further details are available online at: http://interscience.wiley.com/jpages/0270-9139/suppmat/index.html.

Cell Culture and DNA Transfection. For the standard replication assay, 10-cm-diameter dishes were seeded with 1×10^6 Huh7 cells each. After 16 hours of culture, cells were transfected with 5 μg DNA construct using the FuGENE 6 transfection reagent (Roche Diagnostics, Indianapolis, IN) and harvested 3 days later. Transfection efficiency was measured by cotransfection with 1 μg reporter plasmid expressing secreted alkaline phosphatase and estimating its enzymatic activity in the culture supernatant.

Southern Blot Hybridization. HBV DNA samples from cells at day 3 in culture were separated on 1.2% (wt/vol) agarose gel, transferred to a positive-charged nylon membrane (Roche Diagnostics), and hybridized with full-length HBV DNA labeled with alkaline phosphatase. Detection was performed with CDP-star (Amersham Biosciences, Piscataway, NJ), and signals were analyzed in the LAS-1000 image analyzer (Fuji Photo Film, Tokyo, Japan).

Statistical Analysis. Categorical variables were compared between groups by the chi-squared test and noncategorical variables by the Mann-Whitney *U*-test. A *P* value less than .05 was considered significant. Multivariate analyses with logistic regression were used to determine independent factors for fulminant hepatitis. STATA Software (StataCorp LP, College Station, TX) version 8.0 was employed for analyses.

Results

Demographic and Clinical Differences in Patients Infected With Various HBV Genotypes/Subgenotypes. Genotypes of HBV were not classifiable in 28 (8%), and sufficient clinical data were not available in 7 (2%) of the 336 patients with acute hepatitis B. Exclusive of these 35 patients, 301 (90%) were left for evaluation of HBV genotypes in reference to clinical outcome.

HBV genotypes/subgenotypes were Aa in 10 (3%), Ae in 33 (11%), Ba in 22 (7%), Bj in 22 (7%), Cs in 11 (4%), Ce in 192 (64%), D in 5 (2%), and G in 6 (2%); none of them were infected with F or H (Table 1). All six patients with HBV/G were co-infected with another genotype; Ae in two, Ba in two, and Ce in the remaining two. The mean age was lower in the patients with HBV/Ae than Ba ($P = .0001$), Aa ($P < .01$), Bj or Cs ($P < .05$ for each) and Ce than Ba ($P < .05$). Men predominated in HBV infections with foreign (Ae and Ba) compared with domestic genotypes (Bj and Ce) ($P < .05$).

HBeAg was detected in 79% of patients with HBV/Ae at a frequency much higher than that with Bj ($P < .005$), Ce ($P < .001$) or Ba ($P < .05$). HBeAg in four of the six (67%) patients with HBV/G was coded for by HBV of the other genotypes co-infecting them, because it has two stop codons and an insertion in the core gene that prohibit encoding HBeAg.[21] HBV DNA levels as well as HBeAg-positive rates at the presentation were higher in HBV/Ae than Ce ($P < .005$) or Bj ($P < .05$) infection.

The peak alanine aminotransferase (ALT) level was higher in HBV/Bj than Ae infection ($P < .05$). Fulminant hepatitis was significantly more frequent in patients infected with HBV/Bj (55%) than the other genotypes ($P < .05$); it occurred in two of the five (40%) patients with HBV/D, also. In reflection of severe clinical course,

Table 1. Clinical Characteristics of Patients Acutely Infected With HBV of Distinct Genotypes/Subgenotypes

Features	Genotypes/Subgenotypes							
	Aa (n = 10)	Ae (n = 33)	Ba (n = 22)	Bj (n = 22)	Cs (n = 11)	Ce (n = 192)	D[a] (n = 5)	G[a,b] (n = 6)
Age (years)	42.2 ± 13.1	31.2 ± 10.3[d]	41.5 ± 10.7[e]	43.5 ± 19.1	38.5 ± 11.1	36.3 ± 15.0	38.6 ± 20.8	42.7 ± 17.5
Men	8 (80%)	30 (91%)[f]	19 (86%)[g]	9 (41%)	7 (64%)	122 (64%)	2 (40%)	6 (100%)
HBeAg positive	7 (70%)	26 (79%)[h]	11 (50%)	8 (36%)	8 (73%)	101 (53%)	1 (20%)	4 (67%)
ALT (IU/L)	1875 ± 759	2070 ± 1113[i]	2523 ± 1185	3472 ± 2720	2269 ± 995	2610 ± 1719	2559 ± 1672	2142 ± 722
Duration of elevated ALT (weeks)[c]	7.9 ± 5.8	9.5 ± 6.2	8.8 ± 3.7[j]	6.0 ± 2.5	10.1 ± 7.5	7.7 ± 5.1	5.7 ± 2.1	9.8 ± 1.5
Total bilirubin (mg/dL)	14.1 ± 10.3	9.0 ± 7.2	9.3 ± 5.9	10.9 ± 9.0	11.0 ± 13.8	9.8 ± 10.7	8.2 ± 2.2	13.0 ± 7.8
HBV DNA (log copies/mL)								
Median	4.76	6.08[k]	5.15	4.93	5.61	4.94	5.91	5.97
(range)	(2.90-8.08)	(2.00-8.46)	(2.00-8.19)	(2.00-8.44)	(2.00-8.50)	(2.00-9.06)	(2.00-8.37)	(3.35-7.11)
<2.00 (undetectable)	0 (0%)	1 (3%)	2 (9%)	3 (14%)	2 (18%)	28 (15%)	1 (20%)	0 (0%)
Medication with								
Lamivudine	1 (10%)	9 (27%)	2 (9%)	5 (23%)	2 (18%)	28 (15%)	4 (80%)	2 (33%)
Steroid	0	3 (9%)	0	5 (23%)	1 (9%)	16 (8%)	0	0

[a]Patients with HBV genotype D or G were not included in the analysis.
[b]All patients with HBV genotype G were co-infected with HBV of another genotype; Ae in two, Ba in two, and Ce in two.
[c]Exclusive of the 16 patients who died of fulminant hepatitis, 3 receiving liver transplantation and 10 without clinical data available.
[d]$P = .0001$, Ae vs. Ba. $P < .01$, Ae vs. Aa. $P < .05$, Ae vs. Bj or Cs.
[e]$P < .05$, Ba vs. Ce.
[f]$P = .0001$, Ae vs. Bj. $P < .005$, Ae vs. Ce.
[g]$P < .005$, Ba vs. Bj. $P < .05$, Ba vs. Ce.
[h]$P < .005$, Ae vs. Bj. $P < .01$, Ae vs. Ce. $P < .05$, Ae vs. Ba.
[i]$P < .05$, Ae vs. Bj.
[j]$P < .01$, Ba vs. Bj. $P < .05$, Ba vs. Ce.
[k]$P < .005$, Ae vs. Ce. $P < .05$, Ae vs. Bj.

the peak ALT level tended to be high in patients with HBV/Bj.

Presumed infection routes of 301 patients were sexual transmission in 172 (57%), blood transfusion in 4 (1%), medical accidents in 17 (6%), and unknown in the remaining 108 (36%).

Clinical Outcome of Patients With Acute Hepatitis B. Fulminant hepatitis developed in 40 (13%) patients. To cope with severe acute liver disease, lamivudine and steroid were administered to 53 (18%) and 25 (8%) patients, respectively. Fulminant hepatitis led to death in 16 (5%) patients, and three (1%) received liver transplantation. Exclusive of the 40 patients with fulminant hepatitis who received various treatments and five without clinical data, 256 (85%) were followed for the chronic outcome (Fig. 1). Serum ALT levels stayed elevated for longer than 24 weeks for the diagnosis of chronic hepatitis in eight (3%) of them. Among them, five had cleared HBsAg from serum until then, and therefore, their liver function abnormality was not attributed to persistent HBV infection. Table 2 summarizes persistence of HBV infection in the 256 patients with acute hepatitis; 253 (99%) lost serum HBsAg by 6 months. Hence, HBV infection evolved into chronicity in only 3 of the 256 (1%) patients, representing 2 of the 32 (6%) infected with HBV/Ae and 1 of the 21 (5%) with Ba. All of the three with chronic outcome had low-titered IgG anti-HBc at the presentation, and two of them had been negative for HBsAg before the presentation. None of them had received lamivudine or steroid treatment during their acute phase of illness. Of the patients without antiviral therapy, chronic outcome was significantly more frequent in those infected with HBV/Ae than non-Ae genotypes (9% 2/23 vs. 0.5% 1/187, $P = .032$).

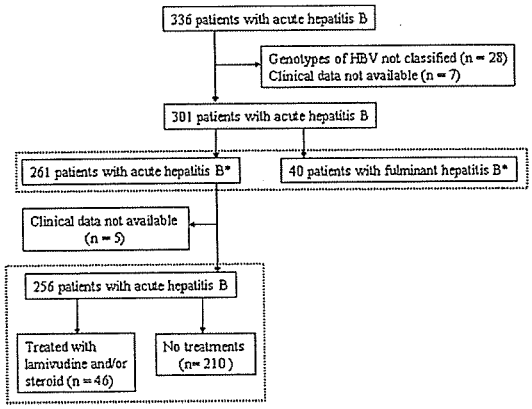

Fig. 1. A flow diagram of 336 patients studied. Comparison was made between patients with fulminant and acute self-limited hepatitis (upper dotted area), and the chronicity was compared between patients with and without treatments (lower dotted area). *Of 301 patients, 37 were negative for HBV DNA, including 27 with acute and 10 with fulminant hepatitis.

Table 2. Persistence of HBV Infection in the Patients With Acute Hepatitis Who Did or Did Not Receive Lamivudine or Steroid

Treatment	Total	Genotypes/Subgenotypes							
		Aa (n = 8)[a]	Ae (n = 32)[a]	Ba (n = 21)[a]	Bj (n = 10)[a]	Cs (n = 10)[a]	Ce (n = 167)[a]	D (n = 3)[a]	G (n = 5)[a]
Total (n = 256)	3/256 (1.2%)	0	2/32 (6%)c	1/21 (5%)	0	0	0	0	0
Lamivudine (n = 36)[b]	0/36 (0%)	0/1 (0%)	0/9 (0%)	0/2 (0%)	0	0/1 (0%)	0/19 (0%)	0/2 (0%)	0/2 (0%)
Steroid (n = 16)[b]	0/16 (0%)	0	0/3 (0%)	0	0	0/1 (0%)	0/12 (0%)	0	0
Neither	3/210 (1.4%)	0/7 (0%)	2/23 (9%)c	1/19 (5%)	0/10 (0%)	0/8 (0%)	0/139 (0%)	0/1 (0%)	0/3 (0%)

[a]Exclusive of 40 patients with fulminant hepatitis and 5 without clinical data available.
[b]Six patients received steroid along with lamivudine.
[c]$P < .05$, Ae vs. non-Ae.

Comparison Between Patients With Fulminant and Acute Self-Limited Hepatitis. Table 3 compares demographic, clinical, and virological characteristics between the 40 patients with fulminant and the 261 with acute self-limited hepatitis for whom analysis was feasible. Patients with fulminant hepatitis were significantly older (44.7 ± 16.3 vs. 36.0 ± 14.3 years, $P = .0017$), less predominantly male (43% vs. 71%, $P = .0005$) and less often positive for HBeAg (23% vs. 60%, $P < .0001$) than those with acute hepatitis. Peak ALT and total bilirubin levels were higher for fulminant than acute hepatitis ($P < .0001$), reflecting severe hepatic lesions. Notably, the median HBV DNA level was lower in patients with fulminant than acute hepatitis (4.89 vs. 5.19 log copies/mL, $P = .0178$); the frequency of undetectable HBV DNA at the presentation was higher in fulminant hepatitis (25% vs. 10%, $P = .0086$). Lamivudine or steroid was given significantly more often to patients with fulminant hepatitis.

There were marked differences in the distribution of genotypes between patients with fulminant and acute hepatitis. HBV/Ae was less frequent (0% vs. 13%, $P = .0121$), whereas Bj was more often (30% vs. 4%, $P < .0001$) in patients with fulminant than acute hepatitis. Although HBV/Ce tended to be less frequent in patients with fulminant than acute hepatitis (55% vs. 65%), the difference fell short of being significant.

Precore stop-codon mutation (G1896A) and core-promoter double mutation (A1762T/G1764A) were more

Table 3. Comparison Between Patients With Fulminant and Acute Self-Limited Hepatitis Who Were Infected With HBV

Features	Fulminant (n = 40)	Acute (n = 261)	P Value
Age (years)	44.7 ± 16.3	36.0 ± 14.3	.0017
Men	17 (43%)	186 (71%)	.0005
HBeAg positive	9 (23%)	157 (60%)	<.0001
ALT (IU/L)	4315 ± 2889	2284 ± 1221	<.0001
Total bilirubin (mg/dL)	20.5 ± 16.4	8.3 ± 7.3	<.0001
HBV DNA (log copies/mL)			
Median	4.89	5.19	.0178
(range)	(2.00-8.44)	(2.00-9.06)	
<2.00 (undetectable)	10 (25%)	27 (10%)	.0086
Treatment			
Lamivudine	16 (40%)	37 (14%)	.0003
Steroid	9 (23%)	16 (6%)	.0022
Genotypes/subgenotypes			
Aa	1 (2.5%)	9 (3%)	NS
Ae	0 (0%)	33 (13%)	.0121
Ba	1 (2.5%)	21 (8%)	NS
Bj	12 (30%)	10 (4%)	<.0001
Cs	1 (2.5%)	10 (4%)	NS
Ce	22 (55%)	170 (65%)	NS
D	2 (5%)	3 (1%)	NS
G	1 (2.5%)	5 (2%)	NS
Mutations[a]			
nt 1753 and/or nt1754[b]	11/30 (37%)	28/234 (12%)	.0003
A1762T/G1764A	15/30 (50%)	39/234 (17%)	<.0001
G1896A	16/30 (53%)	21/234 (9%)	<.0001
G1899A	7/30 (23%)	8/234 (3%)	<.0001

[a]Exclusive of 37 patients in whom precore region and core-promoter could not be amplified by PCR.
[b]T1753C/A/G and/or T1754C/A/G.

Table 4. Multivariate Analysis for Factors Independently Associated With Fulminant Hepatitis

Factors	Odds Ratio	95% Confidence Interval	*P* Value
Age (yr)			
<34[a]	1		
≥34	3.472	1.094-11.023	.0347
Sex			
Male	1		
Female	2.272	0.780-6.613	.1323
HBeAg			
Positive	1		
Negative	3.344	1.065-10.506	.0387
ALT (IU/L)			
<2200[a]	1		
≥2200	2.094	0.683-6.414	.1957
Total bilirubin (mg/dL)			
<10.0[a]	1		
≥10.0	18.818	4.320-81.980	<.0001
HBVDNA (log copies/mL)			
<5.00[a]	1		
≥5.00	1.042	0.367-2.961	.9383
Treatment			
Lamivudine (−)	1		
Lamivudine (+)	2.650	0.814-8.625	.1056
Steroid (−)	1		
Steroid (+)	2.515	0.668-9.472	.1728
Genotypes/Subgenotypes			
Non-Bj	1		
Bj	7.001	1.737-28.228	.0062
Mutations			
nt 1753 and/or 1754[b]			
Absent	1		
Present	2.316	0.698-7.683	.1700
A1762T/G1764A			
Absent	1		
Present	1..013	0.295-3.478	.9841
G1896A			
Absent	1		
Present	4.157	1.265-13.657	.0189
G1899A			
Absent	1		
Present	2.525	0.534-11.949	.2427

[a]Median values.
[b]T1753C/A/G or T1754C/A/G.

frequent in patients with fulminant than acute hepatitis (53% vs. 9% and 50% vs. 17%, respectively, $P < .0001$ for each). Likewise, mutations in core-promoter at nt 1753 or nt 1754, and G1899A mutation were more frequent in patients with fulminant than acute hepatitis ($P = .0003$ and $P < .0001$, respectively).

Factors Independently Associated With the Development of Fulminant Hepatitis. Various factors found in association with fulminant hepatitis were evaluated for the independence in multivariate analysis (Table 4). Age 34 years or older (odds ratio 3.47 [95% confidence interval 1.09-11.02], $P = .035$), HBV/Bj (7.00 [1.74-28.23], $P = .006$), HBeAg-negative (3.34 [1.07-10.51], $P = .039$), total bilirubin ≥10.0 mg/dL (18.82 [4.32-81.98], $P < .0001$) and G1896A (4.16 [1.27-13.66], $P = .019$) were independent risk factors for the development of fulminant hepatitis.

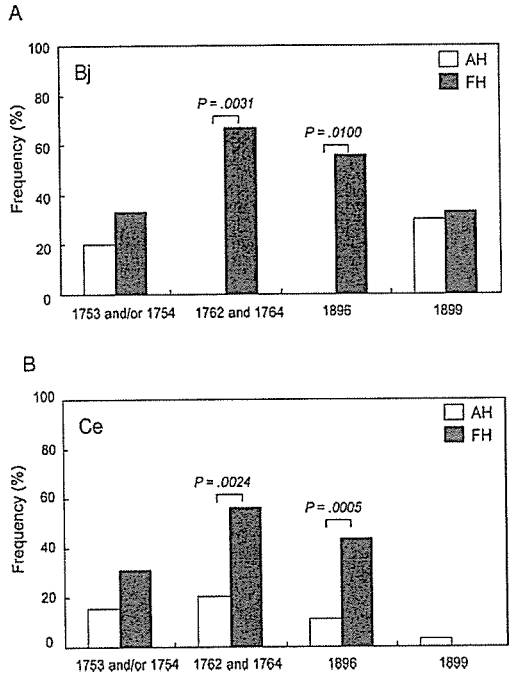

Fig. 2. Frequencies of precore and core-promoter mutations compared between patients with fulminant and acute self-limited hepatitis who were infected with HBV/Bj (A) or Ce (B).

In view of the majority of Japanese patients who were infected with Bj or Ce, mutations in the precore region and core-promoter were compared between those with fulminant and acute self-limited hepatitis for each subgenotype (Fig. 2). G1896A and A1762T/G1764A were significantly more frequent in patients with fulminant than acute hepatitis infected with either HBV/Bj or Ce (56% vs. 0% and 67% vs. 0% for Bj or 44% vs. 11% and 56% vs. 22% for Ce, respectively, $P \leq .01$ for all). For the patients infected with HBV/Bj, in particular, precore and core-promoter mutations were highly frequent in those with fulminant hepatitis (56% and 67%, respectively), whereas they occurred in none of those with acute hepatitis. G1899A was equally frequent in both patients with fulminant and acute hepatitis infected with HBV/Bj; it was rarely seen in those with Ce. Mutations involving nt 1753 or nt 1754 tended to be more frequent in patients with fulminant than acute hepatitis.

***Replication of the Wild-Type HBV as Well as Precore and Core-Promoter Mutants* In Vitro.** Full-length HBV DNA of the wild-type HBV/Bj from a patient with chronic hepatitis B was incorporated with G1896A or A1762T/G1764A mutation *in vitro*. Another plasmid was constructed with HBV/Bj_58 carrying G1896A from a fulminant patient. Figure 3 compares