Hepatology Research

Hepatology Research 2010; 40: 477–485 doi: 10.1111/j.1872-034X.2010.00624.x

Original Article

Evaluation for clinical utility of GPC3, measured by a commercially available ELISA kit with Glypican-3 (GPC3) antibody, as a serological and histological marker for hepatocellular carcinoma

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Aims: We evaluated the clinical utility of glypican-3 (GPC3), which has been proposed as a potential novel tumor marker for hepatocellular carcinoma (HCC), as a serological and histological marker for HCC.

Methods: The serum GPC3 level was compared between 200 patients with HCC and 200 patients with chronic liver disease (CLD). In addition, the expression of GPC3 was examined with immunohistochemistry on 38 resected specimens from patients with HCC. A commercially available GPC3 antibody was used for these analyses.

Results: The median values of serum GPC3 in patients with HCC and with CLD were 924.8 pg/mL and 1161.6 pg/mL, respectively. We found no elevation of serum GPC3 level in patients with HCC in comparison with those with CLD; rather the level was higher in patients with CLD ($P < 0.0001$). In immunohistochemical analysis, 14 of 38 (36.9%) HCC tissues were positive for GPC3, whereas no corresponding noncancerous tissue was positive. The positivity for GPC3 tended to increase with pathologic decreased differentiation of HCC.

Conclusions: We did not find serum GPC3 level, measured by a commercially available ELISA kit with GPC3 antibody, to be useful in the diagnosis of HCC. However, we did observe increased GPC3 staining in HCC tissue with moderate or poor differentiation, suggesting that GPC3 is produced by HCC tumors. This lack of utility could have been due to the measuring procedure used in the present study. Further evaluation of GPC3 in HCC with other measuring procedures is needed.

Key words: ELISA, glypican-3, hepatocellular carcinoma, immunohistochemistry, tumor marker

INTRODUCTION

HEPATOCELLULAR CARCINOMA (HCC) is one of the most prevalent malignancies worldwide. It is the sixth most common cancer, and the third most common cause of cancer-related death, in the world.[1] In Japan, HCC is the third most common cause of death from cancer in men, and the fifth most common in women.[2] The most important risk factor for the development of HCC is liver cirrhosis, regardless of etiology.[3] In addition, chronic infection with hepatitis viruses such as hepatitis B virus (HBV) and hepatitis C virus (HCV), as well as high alcohol intake, increase the risk of HCC.[4–7]

Alpha-fetoprotein (AFP),[8–11] Lens culinaris agglutinin-reactive fraction of alpha-fetoprotein (AFP-L3),[12–14] and des-gamma-carboxy prothrombin (DCP)[15–17] have been reported to be useful as serological tumor marker for HCC in cases of HCC surveillance and diagnosis, and in the evaluation of patient prognosis.[18] Nevertheless, all tumor markers have limitations and therefore the identification of additional tumor markers for HCC with high sensitivity and specificity is necessary.

Glypican-3 (GPC3) is a member of the glypican family of glycosyl-phosphatidylinositol-anchored cell-

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Received 18 October 2009; revision 19 November 2009; accepted 23 November 2009.

surface heparan sulfate proteoglycans.[19–21] It has been suggested that GPC3 might be a useful histological[22–24] and serological[25–27] marker for HCC. However, there has not been sufficient agreement on its clinical utility, and the relationship between the expression of GPC3 in tissue and GPC3 level in the serum of patients with HCC has not been fully characterized.

In the present study, we evaluate the clinical utility of GPC3 as a serological and histological marker for HCC, and compare histological results with serological ones. In addition, we compare the utility of GPC3 with other serological markers for HCC, such as AFP, AFP-L3, and DCP.

METHODS

Patients and controls

A TOTAL OF 434 consecutive patients with HCC visited the Department of Gastroenterology at Ogaki Municipal Hospital during the period from January 2000 to December 2004. Two hundred and three patients underwent hepatic resection or radiofrequency ablation (RFA) as treatment for HCC. Stored serum samples that had been obtained before the therapy were available for 200 of these 203 patients; these constituted the subjects of the present study. Written informed consent was obtained from all patients for the analyses of their serum or tissue samples.

Diagnosis of HCC was based on histologic examination of tumor tissue taken from resected specimens in 120 patients who underwent hepatectomy, 29 of the 80 patients (36.3%) treated by RFA were diagnosed with HCC based on specimens by fine-needle biopsy. The remaining 51 patients were diagnosed based on clinical criteria:[28,29] a pertinent clinical background (association with liver cirrhosis or viral hepatitis) and typical imaging findings. Typical imaging features of HCC include a mosaic pattern with a halo observed with B-mode ultrasonography; hypervascularity on angiographic images; and a high-density mass on arterial phase dynamic computed tomography (CT) images together with a low-density mass on portal phase dynamic CT images obtained with a helical or multidetector row CT scanner. When findings typical of HCC were not obtained by means of dynamic CT or angiography, CT during hepatic arteriography and CT during arterial portography or T1- and T2-weighted imaging associated with superparamagnetic iron oxide-enhanced magnetic resonance imaging (MRI) were performed.

Serum samples from 200 HCC patients were obtained at the diagnosis of HCC and before therapy. As controls, serum samples from patients with CLD but without HCC that had been obtained during the same period as the serum samples from HCC patients were selected. We selected samples from patients in whom the lack of HCC development had been confirmed by ultrasonography, CT or MRI at serum sampling and for 3 years after the date of sampling. This was to avoid the inclusion in the control group of patients with occult HCC that could not be detected by imaging modalities at the time of serum sampling. Among them, we made random selection and finally selected 200 samples as controls.

Measurement of GPC3, AFP, AFP-L3 and DCP

GPC3, AFP, AFP-L3, and DCP were measured from the same serum samples. GPC3 was measured using a commercially available ELISA kit (BioMosaics, Burlington VT) according to the manufacturer's instructions. Total AFP and percentage of AFP-L3 were measured by a liquid-phase binding assay with the Wako LiBASys Autoanalyzer (Wako Pure Chemical Industries, Osaka).[30,31] DCP level was determined by sensitive enzyme immunoassay (Eitest PIVKA-II kit; Eisai Laboratory, Tokyo) according to the manufacturer's instructions.[32]

Immunohistochemical staining

Immunohistochemical staining for GPC3 was performed on 38 resected HCC tissue specimens using a commercially available kit (BioMosaics) according to the manufacturer's instructions. Briefly, 4-μm sections from formalin-fixed, paraffin-embedded tissue blocks were deparaffinized, rehydrated and treated with 3% hydrogen peroxide for 15 min to inhibit endogenous peroxidase. Following water bath-based heat-induced epitope retrieval in 0.1 M citrate buffer at 95°C centidegree and pH 6.0 for 40 min, slides were incubated with blocking solution for 20 min at room temperature. After blocking, slides were incubated with a mouse monoclonal antibody specific for GPC3 (1:200 dilution, clone 1G12; BioMosaics) for 6 hours at room temperature. After washing, detection was performed with biotin-free horseradish peroxidase-labeled polymers using the ChemMate EnVision System (Dako Real EnVision: Dako, Carpinteria CA). Staining was visualized using 3,3′-diaminobenzidine substrate-chromogen solution and a hematoxylin counterstain.

The intensity of staining was graded according to the percentage of the stained area and the intensity of staining as: 0, no staining or partial staining of cytoplasm in <25% of cells; 1+, weak/barely perceptible cytoplasm stain in >25% of cells; 2+, moderate stain of the complete cytoplasm in >25% of cells; or 3+, strong stain of

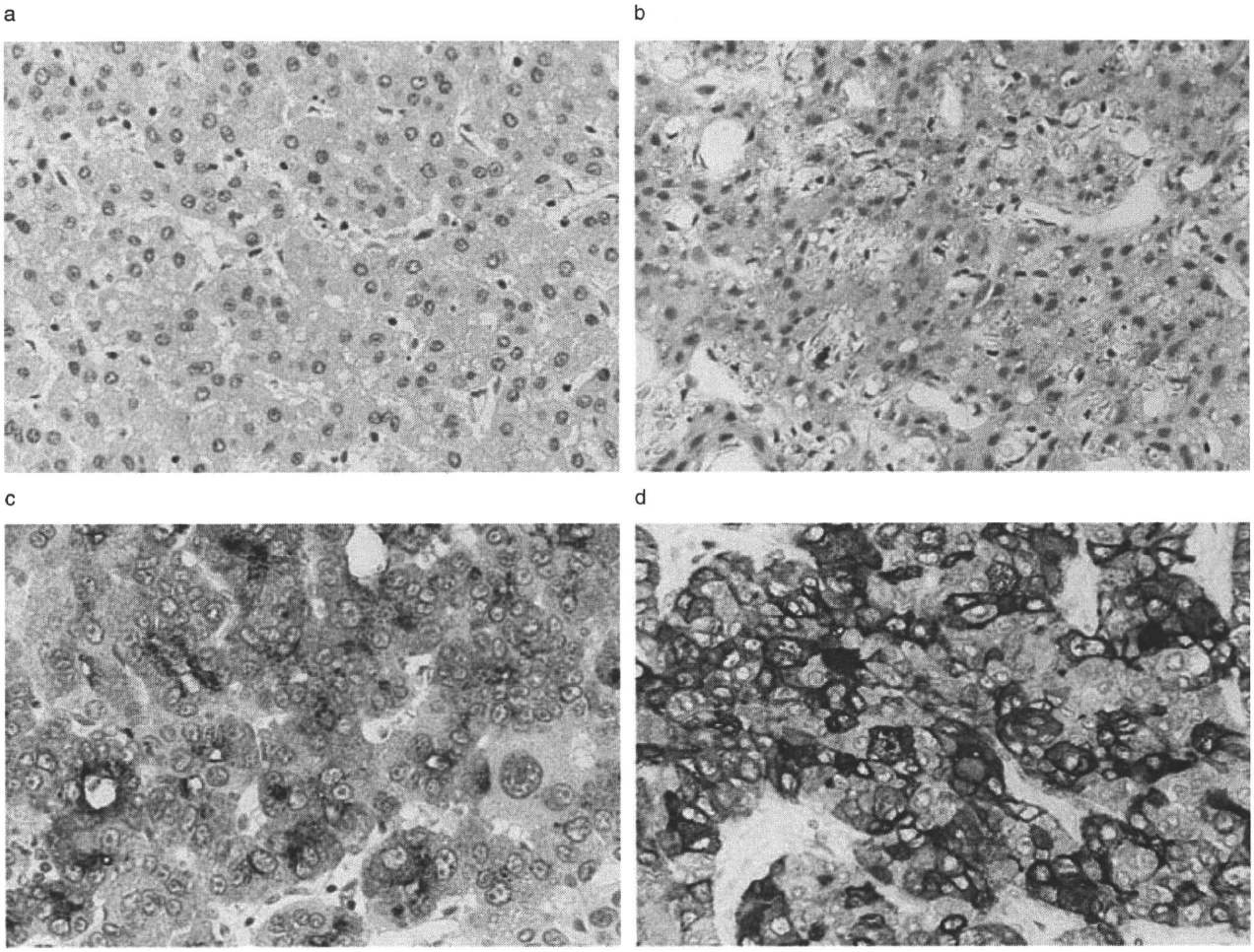

Figure 1 The degree of immunohistochemical staining for glypican-3. (a) No staining, (b) light staining, (c) moderate staining, (d) heavy staining.

the complete cytoplasm in >25% of cells (Fig. 1). HCC with 2+ or 3+ staining was considered to be positive for GPC3. Microscopic findings were evaluated by two authors independently, in comparison with negative and positive controls from the same immunohistochemistry series. Final evaluations of ambiguous cases (fewer than 20% of the samples) were made on a conference microscope with other authors.

Statistical analysis

Data are expressed as the mean ± SD or median and range. Differences in the proportions of patients between groups were analyzed by chi-square test. Differences in quantitative values were analyzed by Mann–Whitney *U*-test and Kruskal–Wallis test. All *P*-values were derived from two-tailed tests, and $P < 0.05$ was accepted as statistically significant. All analyses were performed using JMP6 statistical software (SAS Institute Japan, Tokyo).

RESULTS

THE DEMOGRAPHIC CHARACTERISTICS of the patients included in the analysis are summarized in Table 1. Patient with HCC comprised 153 males (76.5%) and 47 females (23.5%), with a mean age of 67.2 ± 8.5 years. Control patient comprised 112 males (56.0%) and 88 females (44.0%), with a mean age of 61.5 ± 11.8 years. The percentage of patients without cirrhosis, which was clinically evaluated according to typical US findings (e.g. superficial nodularity, a coarse parenchymal echo pattern, and signs of portal

Table 1 Clinical characteristics of the study patients ($n = 400$)

	HCC patients ($n = 200$)	Control ($n = 200$)
Age (years)	67.2 ± 8.5	61.5 ± 11.8
Sex		
Male	153 (76.5)	112 (56.0)
Female	47 (23.5)	88 (44.0)
Etiology of underlying liver disease		
HBV	32 (16.0)	65 (32.5)
HCV	155 (77.5)	132 (66.0)
HBV + HCV	3 (1.5)	3 (1.5)
non-HBV, non-HCV	10 (5.0)	0
Patients without cirrhosis	81 (40.5)	141 (70.5)
Child–Pugh class (in patients with cirrhosis)		
A	86 (72.3)	36 (61.0)
B	33 (27.7)	18 (30.5)
C	0	5 (8.5)
Platelet count (/mm^3)	122 150 ± 57 830	176 830 ± 69 730
Alanine aminotransferase (IU/L)	58.8 ± 39.5	47.4 ± 56.6
Albumin (g/dL)	3.72 ± 0.50	3.87 ± 0.56
Total-bilirubin (mg/dL)	0.84 ± 0.94	0.85 ± 0.92

HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HCV, hepatitis C virus.
Percentages are shown in parentheses.

Table 2 Characteristics of hepatocellular carcinoma ($n = 200$)

Size of largest tumor (cm)	2.76 ± 2.49
<2	99 (49.5)
≥2 to <3	88 (44.0)
≥3	13 (6.5)
Number of tumors	1.37 ± 1.00
Single	158 (79.0)
Multiple	42 (21.0)
Portal vein thrombosis	
Absent	192 (96.0)
Present	8 (4.0)
Tumor stage	
I	86 (43.0)
II	80 (40.0)
III	32 (16.0)
IV	2 (1.0)

hypertension – splenomegaly >120 mm, dilated portal vein diameter >12 mm, patent collateral veins, or ascites), was 27.5% of patients with HCC and 29.5% of control patients. The Child–Pugh class of patients with HCC was class A in 72.3% and class B in 27.7%. The characteristics and the progression of HCC tumor were summerized in Table 2. The percentage of patients at stages I, II, III, and IV were 43.0%, 40.0%, 16.0%, and 1.0%, respectively, according to the TNM Classification of Malignant Tumours of the Liver Cancer Study Group of Japan.[33]

Serum concentration of GPC3, AFP, AFP-L3, and DCP

Serum concentrations of GPC3, AFP, AFP-L3, and DCP are summarized in Table 3. The median GPC3 values

Table 3 Median and quartiles of serological markers for hepatocellular carcinoma ($n = 400$)

	HCC patients ($n = 200$)	Control ($n = 200$)	*P* value
Glypican-3 (pg/mL)	924.8 (495.2, 1335.6)	1161.6 (762.0, 1784.0)	<0.0001
Alpha-fetoprotein (ng/ml)	15.3 (6.3, 78.5)	4.0 (1.6, 7.3)	<0.0001
Lens culinaris agglutinin fraction of AFP	0.5 (0.0, 2.9)	0.0 (0.0, 0.0)	<0.0001
Des-gamma caroxy prothrombin (mAU/mL)	32.5 (18.0, 178.3)	21.0 (16.0, 27.0)	<0.0001

AFP, alpha-fetoprotein; HCC, hepatocellular carcinoma. Median (25%, 75% quarile) are shown.

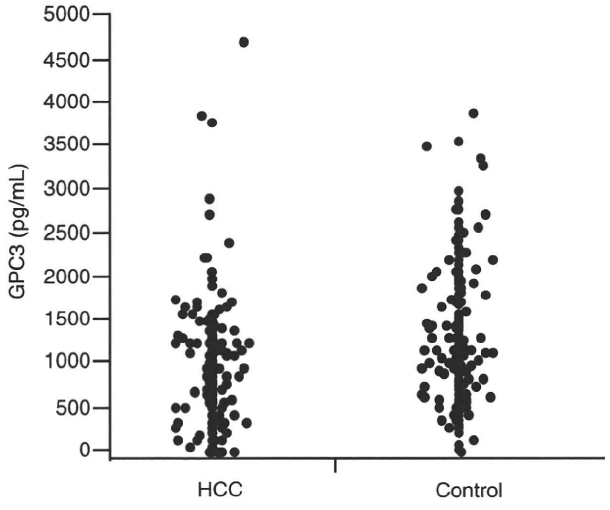

Figure 2 Serum glypican-3 (GPC3) level in patients with hepatocellular carcinoma (HCC) and in patients with chronic liver disease (CLD, control). Serum GPC3 level was higher in patients with CLD (1161.6 pg/mL) than those with HCC (924.8 pg/mL; $P < 0.0001$).

in patients with HCC and those with CLD were 924.8 pg/mL and 1161.6 pg/mL, respectively; patients with CLD showed significantly higher GPC3 concentration than those with HCC (Fig. 2). In contrast, serum concentrations of AFP, AFP-L3, and DCP in patients with HCC were significantly higher than those in patients with CLD (Fig. 3). We found no difference in serum GPC3 level according to the size of the maximal HCC tumor, the number of HCC tumors, or the stage of HCC in 200 patients with HCC (data not shown). Also, we found no difference according to the presence of cirrhosis in 200 control patients (data not shown).

The area under the receiver-operating curve (AUROC) was calculated to compare the clinical utilities of GPC3, AFP, AFP-L3 and DCP (Fig. 4). AUROC values for GPC3, AFP, AFP-L3 and DCP were 0.64, 0.80, 0.77, and 0.66, respectively. The AUROC value for GPC3 was significantly lower than those for AFP and AFP-L3 (both, $P < 0.05$). In addition, patients with HCC were identified by the decreased GPC3 under cut-off level in this ROC analysis; the serum value of GPC3 in patients with HCC was significantly lower than that in patients with

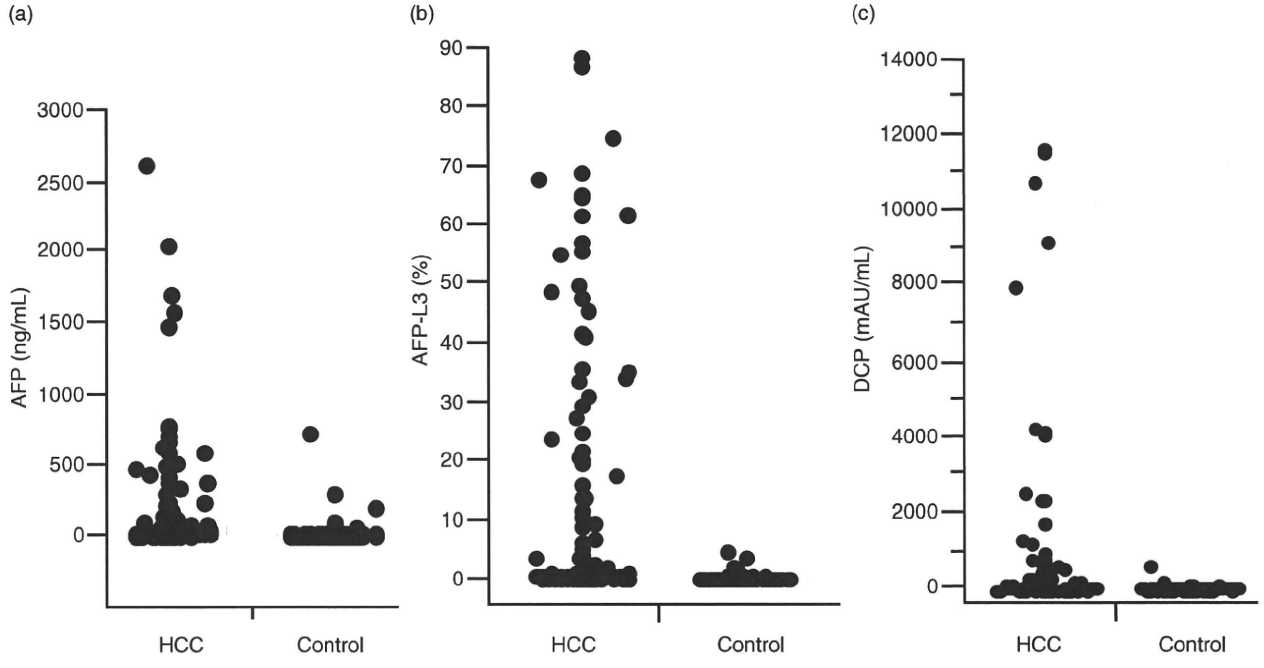

Figure 3 Serum alpha-fetoprotein (AFP), Lens culinaris agglutinin-reactive fraction of AFP (AFP-L3), and des-gamma carboxy prothrombin (DCP) levels in patients with hepatocellular carcinoma (HCC) and in patients with chronic liver disease (CLD, control). Serum AFP, AFP-L3, and DCP levels were significantly higher in patients with HCC (15.3 ng/mL vs. 4.0 ng/mL for AFP; 0.5% vs. 0.0% for AFP-L3; 32.5 mAU/mL vs. 21.0 mAU/mL for DCP; all $P < 0.0001$).

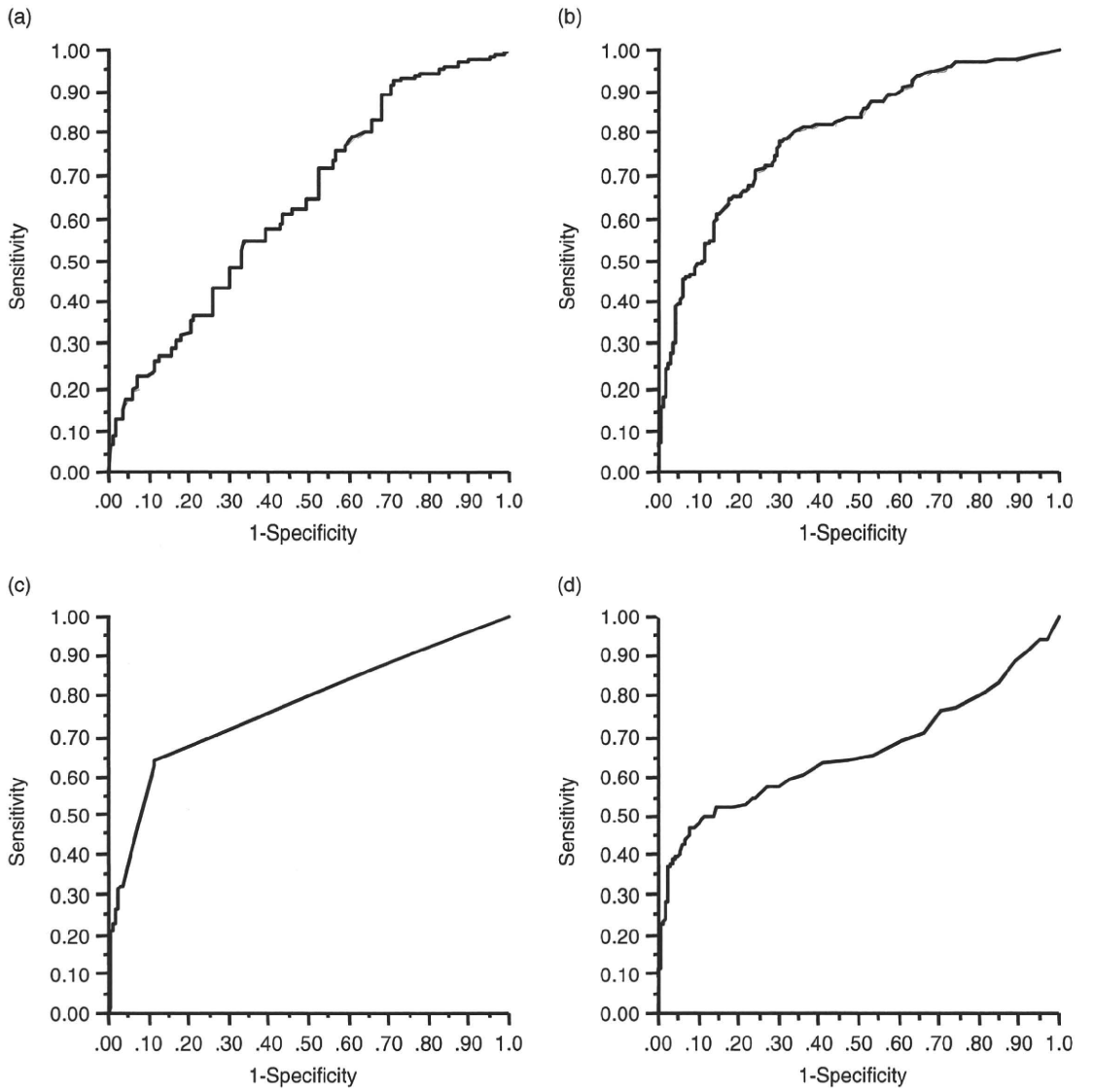

Figure 4 Area under the receiver-operating curve (AUROC) of (a) serum glypican-3 (GPC3), (b) alpha-fetoprotein (AFP), (c) Lens culinaris agglutinin-reactive fraction of AFP (AFP-L3), and (d) des-gamma carboxy prothrombin (DCP) for the diagnosis of hepatocellular carcinoma. AUROC was 0.64 for GPC3, 0.80 for AFP, 0.77 for AFP-L3, and 0.66 for DCP, respectively. AUROC was lowest for GPC3, significantly lower than both AFP and AFP-L3 (both, $P < 0.05$).

CLD. Serum GPC3 level for the diagnosis of HCC in the present analysis therefore was used inversely to the previous report.

GPC3 expression in HCC tissue

Thirty-eight resected liver tissues from patients with HCC were examined by immunohistochemistry for GPC3 expression. Table 4 shows the positivity of GPC3 staining in cancerous and non-cancerous parts of the resected liver tissue. The positivity of GPC3 staining in cancerous parts was 36.8% (14 cases), and that in non-cancerous parts was 0%. When light GPC3 staining was taken to be positive, these values increased to 81.6% (31 cases) and 23.7% (9 cases) for the cancerous and non-cancerous parts, respectively. We found no difference in serum GPC3 concentration according to the degree of staining for GPC3 by immunohistochemistry in these 38 patients (Fig. 5).

Table 4 Immunohistochemical staining of cancerous and non-cancerous parts of hepatocellular carcinoma tissues for glypican-3 ($n = 38$)

	No staining	Light staining	Moderate staining	Heavy staining
Cancerous part	7 (18.4)	17 (44.7)	11 (29.0)	3 (7.9)
Non-cancerous part	29 (76.3)	9 (23.7)	0	0

Percentages are shown in parentheses.

Table 5 shows GPC3 expression in HCC tissue according to the differentiation of HCC. All poorly differentiated HCC showed GPC3 expression, and GPC3 immunoreactivity tended to increase with decreasing differentiation of HCC.

DISCUSSION

RECENT REPORTS HAVE shown significant elevation of GPC3 in the serum of patients with HCC, enabling early detection of HCC with high specificity.[25–27]

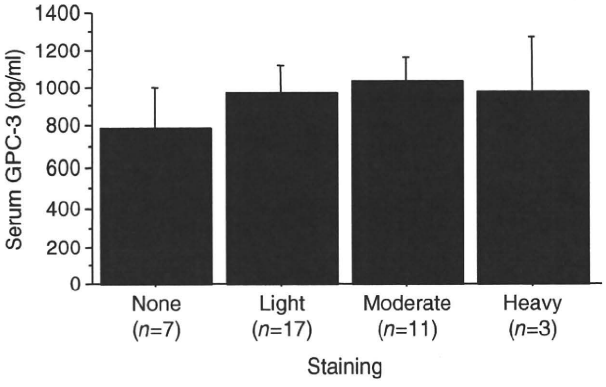

Figure 5 Serum glypican-3 (GPC3) level in 38 patients with hepatocellular carcinoma (HCC) who underwent hepatectomy according to the immunohistochemical staining of GPC3 on the resected HCC specimens. No association was found between serum GPC3 level and immunohistochemical staining of GPC3 on HCC tissues.

Therefore, in the present study we evaluated the usefulness of GPC3 for the diagnosis in comparison with the three standard tumor markers (AFP, AFP-L3, DCP). However, we observed that serum GPC3 concentration showed no increase in patients with HCC; rather, it was higher in patients without HCC. In addition, serum GPC3 did not correlate the stage of HCC, suggesting that the level did not reflect the progression of HCC tumor.

We also evaluated the expression of GPC3 in HCC tissue by immunohistochemistry, on the basis of reports that the clinical utility of GPC3 is higher when as a histological tumor marker.[22–25] In our study, the sensitivity of GPC3 in 38 HCC tissues was 36.8% when light staining was considered to be negative, whereas all non-cancerous tissue was negative for GPC3. When light staining was included to be positive, sensitivity was 81.6% in HCC tissue and 23.7% in non-cancerous tissue. Most HCC specimens (13/14, 92.9%) with positive staining were moderately or poorly differentiated HCC. GPC3 staining tended to increase with decreasing differentiation, suggesting that GPC3 production might increase with the progression of HCC. In contrast to the report by Wang *et al.*[34], who suggested that GPC3 was useful in the differential diagnosis of liver cell adenomas and well-differentiated HCC, we found positive staining for GPC3 in only one of seven (14.3%) well-differentiated HCCs. Shirakawa *et al.* recently reported the low rate of staining of GPC3 in well-differentiated HCC in a larger study population.[35] Our results were in accordance with their report. The immunohistochemical staining, not serum level, of GPC3 might be an

Table 5 Association between differentiation and immunohistochemical staining for glypican-3 in hepatocellular carcinoma tissuses ($n = 38$)

	No staining ($n = 7$)	Weak staining ($n = 17$)	Moderate staining ($n = 11$)	Heavy staining ($n = 3$)
Well-differentiated ($n = 7$)	2 (28.6)	4 (57.1)	1 (14.3)	0
Moderately differentiated ($n = 27$)	5 (18.5)	13 (48.1)	7 (25.9)	2 (7.4)
Poorly differentiated ($n = 4$)	0	0	3 (75.0)	1 (25.0)

Percentages are shown in parentheses.

indicator of the progression of HCC tumor and predictor of patient prognosis.[35]

GPC3 is a member of the heparan sulfate proteoglycans and its C-terminal region binds to the cell membrane via glycosilphosphatidylinositol anchors. Therefore, the existence of a soluble form of GPC3 is predicted, which would allow detection of GPC3 in the serum of HCC patients. The cleavage sites of GPC3 were between amino acids 358 and 359, and between amino acids 482 and 483. Hippo *et al.*[27] demonstrated that soluble GPC3 was present in the serum (51% of patients with HCC), and the antibody they used for the measurement of serum GPC3 was the NH_2-terminal portion of GPC3 cleaved at Arg358 (amino acids 25–358). Nakatsura *et al.*[26] reported the elevation of serum GPC3 in 40% of patients with HCC, and they used the antibody with amino acids 303–464. The commercially available kit (BioMosaics) used for the measurement of serum GPC3 in the present study uses the anti-GPC3 monoclonal antibody "clone 1G12" that recognizes the last 70 amino acids of the C-terminal of the core protein (amino acids 491–560).[25] This C-terminal region of GPC3 binds to the cell membrane and might not be released into the serum, although the original study by Capurro *et al.* reported the increase in serum GPC3 using the antibody clone 1G12′ in 53% of patients with HCC.[25] This could explain why we did not observe an increase in the level of soluble GPC3 between patients with HCC in comparison to those without it, or within patients with HCC according to the progression of HCC, despite the staining of GPC3 in many moderately or poorly differentiated HCC specimens. This discrepancy is the reason we found no clinical utility of serum GCP3 for the diagnosis of HCC in the present study. We might have observed an increase in serum GPC3 level in patients with HCC in case of the use of antibody other than monoclonal antibody clone 1G12, such as antibodies by Hippo *et al.*[27] or Nakatsura *et al.*,[26] which recognize another part of GPC3. A recent study by Beale *et al.*,[36] comparing AFP, AFP-L3%, DCP, GPC3 and SCCA-I between patients with HCC and those with cirrhosis, also did not find clinical utility for GPC3 in HCC detection, in agreement with the present study. According to a report by Capurro *et al.*,[37] however, the NH_2-terminal region and C-terminal region of GPC3 are linked despite the cleavage of GPC3 by convertase at Arg358, due to the presence of one or more disulfide bonds in the molecule. This would allow the "clone 1G12" antibody to detect GPC3 in the serum. It seems that further evaluation is needed for GPC3 as a serological marker of HCC, with the most important question being the form of the GPC3 protein in circulating blood.

In conclusion, we found no clinical utility of GPC3 as a serologic marker for detection of HCC in comparison to AFP, AFP-L3, and DCP. Further, high clinical utility of GPC3 as a histological marker was not observed in our study population, although we did observe an increase in GPC3 expression in HCC tissue in association with the progression of HCC. The lack of utility of the measurement of serum GPC3 may be due to the measuring procedure used in the present study. Further evaluation with other measuring procedures will be needed in the future; the clinical utility of GPC3 as a serological marker for HCC will remain unclear until further evaluation with other measuring procedures is undertaken. In addition, identification of a soluble form for GPC3, which could be useful as a serological marker for HCC, will require further study.

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Journal of Medical Virology 82:539–545 (2010)

Incidence of Hepatocellular Carcinoma in Patients With Chronic Hepatitis B Virus Infection Who Have Normal Alanine Aminotransferase Values

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The importance of alanine aminotransferase (ALT) levels in the progression of hepatitis B virus (HBV) infection remains a subject of debate. This study sought to identify independent risk factors involved in development of hepatocellular carcinoma (HCC), particularly in patients with chronic HBV infection who have normal ALT values. Data from 381 consecutive hepatitis B patients were analyzed with average ALT integration values ≤40 IU/L and follow-up periods of >3 years. Integration values were calculated from biochemical tests, and serological markers associated with the cumulative incidence of HCC were analyzed. HCC developed in 17 of the 381 patients (4.5%) during the follow-up period. Male sex (hazard ratio, 6.011 [95% confidence interval: 1.353–26.710], $P=0.018$), high HBV-DNA levels (≥5.0 log copies/ml; 5.125 [1.880–13.973], $P=0.001$), low platelet counts ($<15.0\times10^4/mm^3$; 4.803 [1.690–13.647], $P=0.003$), and low total cholesterol levels (<130 mg/dl; 5.983 [1.558–22.979], $P=0.009$) were significantly associated with greater incidence of HCC development. High HBV-DNA levels and low platelet counts are associated with the development of HCC in patients infected with hepatitis B who have normal ALT values. Therefore, maintenance of low HBV-DNA levels is important for the prevention of HCC in patients with low platelet counts, particularly in patients whose ALT values fall within the current normal range. ***J. Med. Virol. 82:539–545, 2010.*** © 2010 Wiley-Liss, Inc.

KEY WORDS: hepatitis B virus (HBV); HBV-DNA; normal alanine aminotransferase; platelet counts; hepatocellular carcinoma

INTRODUCTION

Worldwide, an estimated 350 million individuals are infected chronically with hepatitis B virus (HBV), and 1 million die each year from HBV-related liver disease [EASL Jury, 2003]. Chronic HBV infection is a major risk factor for the development of hepatocellular carcinoma (HCC) [Beasley, 1988; EASL Jury, 2003]. Patients who test positive for the hepatitis B surface antigen (HBsAg) have a 70-fold greater risk of developing HCC compared with HBsAg-negative patients [Szmuness, 1978; Beasley et al., 1981]. HBV infection is endemic in Southeast Asia, China, Taiwan, Korea, and sub-Saharan Africa, where up to 85–95% of patients with HCC are HBsAg-positive [Rustgi, 1987]. HCC is the third and fifth leading cause of death from malignant neoplasms in Japanese men and women, respectively, and the death rate from HCC has increased markedly in Japan since 1975 [Kiyosawa et al., 2004]. Hepatitis C virus (HCV)-related HCC accounts for 75% of all cases of HCC in Japan, while HBV-related HCC accounts for 15% of such cases [Kiyosawa et al., 2004].

Although an increasing body of epidemiological and molecular evidence suggests that HBV is associated with the development of HCC, the exact role of HBV in carcinogenesis is unclear [Ikeda et al., 2005; Wong et al., 2006]. HBV elicits a chronic necroinflammatory hepatic disease [Yu and Chen, 1994], and liver injury associated with HBV infection is mediated by viral factors in addition to the host immune response. Patients who are positive for the hepatitis B e antigen (HBeAg) commonly have increased hepatic inflammatory activity and an increased risk of developing HCC [Yang et al., 2002]. HBeAg-negative HBsAg carriers who retain high levels of HBV-DNA and show persistent necroinflammation of the liver have an increased risk of acquiring HCC [Yu et al., 2005; Chen et al., 2006].

The authors report no conflicts of interest.

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Accepted 10 September 2009
DOI 10.1002/jmv.21686
Published online in Wiley InterScience (www.interscience.wiley.com)

Alanine aminotransferase (ALT) activity is the most widely used laboratory test for the evaluation of necroinflammatory activity in liver disease [Prati et al., 2002]; however, it is well known that HCC occurs in some HBsAg carriers with normal ALT values. Recently, Chen et al. [2006] conducted a large cohort study in Taiwan and found that elevated serum HBV-DNA levels are strong predictive factors for the development of HCC, independent of the ALT values. It is an important problem for early detection of HCC that general practitioners are sometimes unaware of those patients with normal ALT as high-risk subjects for HCC. There is little information about how many patients with normal ALT develop HCC. It is important that ALT values should be expressed with integration values to ensure a valid analysis, since ALT values fluctuate frequently [Kumada et al., 2007]. Therefore, this study sought to identify the independent risk factors, involving mainly serological markers, associated with the development of HCC in patients infected chronically with HBV with average ALT integration values $\leq$40 IU/L.

MATERIALS AND METHODS

Patient Selection

A total of 1,861 consecutive patients who were positive for HBsAg visited the Department of Gastroenterology at Ogaki Municipal Hospital, Japan, between September 1994 and August 2003. After assessing each patient's long-term prognosis, 381 consecutive patients were selected for further study who (1) were positive for HBsAg for at least 6 months; (2) displayed no evidence of HCV infection; (3) had no other possible causes of chronic liver disease (i.e., alcohol consumption lower than 80 g/day, no history of hepatotoxic drug use, and negative tests for autoimmune hepatitis, primary biliary cirrhosis, hemochromatosis, and Wilson's disease); (4) had a follow-up period of >3 years; (5) had no evidence of HCC for at least 3 years from the start of the follow-up period; (6) had no history of therapy involving interferons, nucleosides, or nucleotide analogues; (7) had ALT measurements taken more than twice in a year; and (8) had average ALT integration values $\leq$40 IU/L (Fig. 1).

Patients were evaluated at the hospital at least every 6 months. During each follow-up examination, platelets, ALT, aspartate aminotransferase (AST), gamma glutamyl transpeptidase (gamma-GTP), total bilirubin, cholinesterase, alkaline phosphatase (ALP), albumin, total cholesterol, HBeAg, anti-HBe, HBV-DNA, and alpha-fetoprotein (AFP) were measured at least every 6 months. Commercial radioimmunoassay kits were used to test blood samples for HBsAg, HBeAg, and anti-HBe (Abbott Japan Co., Ltd, Tokyo, Japan). Before July 2001, serum HBV-DNA concentrations were monitored using the amplification-hybridization protection assay (DNA probe, Chugai-HBV; Chugai Pharmaceutical Co., Ltd, Tokyo, Japan) with a lower detection limit of ~5,000 viral genome copies/ml (3.7 log copies/ml). After August 2001, serum HBV-DNA levels were monitored using the polymerase chain reaction (PCR) (COBAS Amplicor HBV monitor test, Roche Diagnostics K.K., Tokyo, Japan) with a lower detection limit of ~400 viral genome copies/ml (2.6 log copies/ml). HBV genotyping was carried out as described previously [Kato et al., 2001]. ALT, AST, gamma-GTP, ALP, and AFP were expressed as integration values [Kumada et al., 2007]. When ALT was used as an example, the integration value of ALT was calculated as follows: $(y_0+y_1)\times x_1/2+(y_1+y_2)\times x_2/2+(y_2+y_3)\times x_3/2+(y_3+y_4)\times x_4/2+(y_4+y_5)\times x_5/2+(y_5+y_6)\times x_6/2+(y_6+y_7)\times x_7/2+(y_7+y_8)\times x_8/2$ (Fig. 2). The area of a trapezoid with ALT value was calculated and the measurement interval and added the values. The

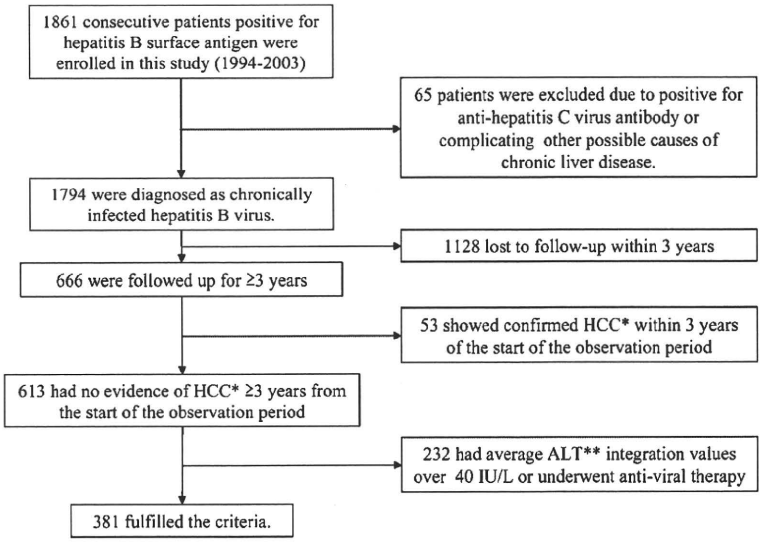

Fig. 1. Schematic flowchart of enrolled patients. *, hepatocellular carcinoma (HCC); **, alanine aminotransferase (ALT).

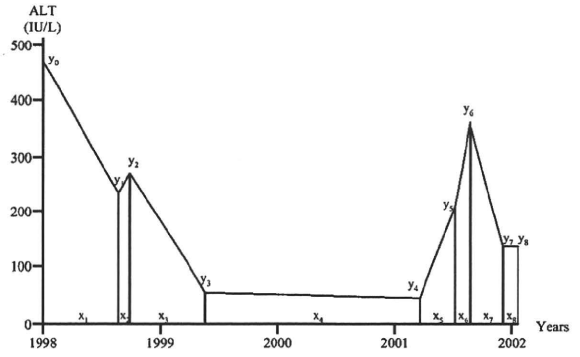

Fig. 2. Integration value of alanine aminotransferase (ALT). The integration value of ALT was calculated as follows: $(y_0+y_1)\times x_1/2+(y_1+y_2)\times x_2/2+(y_2+y_3)\times x_3/2+(y_3+y_4)\times x_4/2+(y_4+y_5)\times x_5/2+(y_5+y_6)\times x_6/2+(y_6+y_7)\times x_7/2+(y_7+y_8)\times x_8/2$. The integration value of ALT was divided by the observation period and expressed as an average integration value.

integration value of ALT was divided by the observation period to obtain the average integration value (Fig. 3). In addition, patients were classified into two groups according to the change of pattern of ALT: persistently normal ALT group and intermittently normal ALT group. The persistently normal ALT group included patients with persistently normal ALT values ≤40 IU/L during follow-up period. The intermittently normal ALT group included patients with temporary ALT fluctuations but the average integration value was ≤40 IU/L. Platelet counts, total bilirubin, cholinesterase, albumin, total cholesterol, HBeAg, anti-HBe, and HBV-DNA were analyzed at the time of entry into the study.

Ultrasonograpy was performed in all patients at the start of the follow-up period for the evaluation of liver fibrosis. The diagnosis of cirrhosis was made according to typical ultrasound findings, for example, superficial nodularity, a coarse parenchymal echo pattern, and

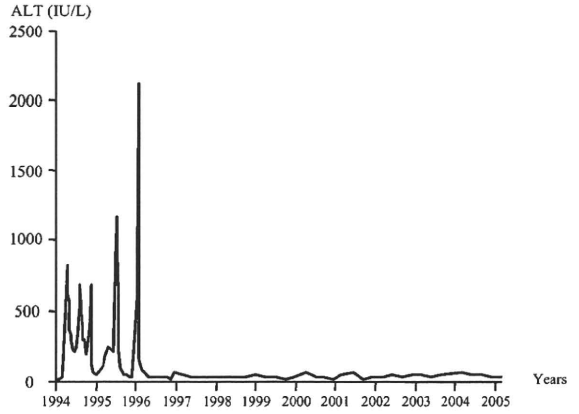

Fig. 3. Average integration value and arithmetic mean value of alanine aminotransferase (ALT) in a 26-year-old patient with hepatitis B virus (HBV). The patient was followed-up for 11.2 years. The number of ALT examinations was 96. The integration value of ALT was 955.2 IU/L × years. The average integration value was 85.3 IU/L, whereas the arithmetic mean value was 255.6 IU/L. This difference is due to the number of ALT measurements between a period of high ALT level and low ALT level.

signs of portal hypertension (splenomegaly >120 mm, dilated portal vein diameter >12 mm, patent collateral veins, or ascites) [Caturelli et al., 2003; Iacobellis et al., 2005; Shen et al., 2006].

To detect early-stage HCC, ultrasonography, computed tomography, magnetic resonance imaging, and/or measurement of tumor markers (i.e., AFP, *Lens culinaris* agglutinin-reactive AFP, and des-γ-carboxyprothrombin) were performed for all patients, at least every 6 months. Blood biochemistry data used in this study were obtained over 1 year prior to HCC development. The study ended in December 31, 2007 or on the date of HCC identification, whichever was earlier. The diagnosis of HCC was based on histological examination (n = 9). In the remaining eight patients, the diagnosis was based on clinical criteria [Kudo, 1999; Torzilli et al., 1999].

Statistical Analysis

Statistical analyses were performed using the Statistical Program for Social Science (SPSS version 17.0 for Windows; SPSS Japan, Inc., Tokyo, Japan). Continuous variables are expressed as median (range). The Kruskal–Wallis test was used to assess continuous variables with a skewed distribution, and the chi-square test was used to assess categorical variables. An actuarial analysis of the cumulative incidence of HCC was performed using the Kaplan–Meier method, and differences were tested by a log-rank test. The Cox proportional hazard model and forward selection method were used to estimate the relative risk of HCC development associated with age (i.e., ≤40 years or >40 years), sex (i.e., male or female), HBeAg (i.e., positive or negative), HBV-DNA level (i.e., <5.0 or ≥5.0 log copies/ml), average ALT integration value (i.e., ≤20 or >20 IU/L), the change pattern of ALT (persistently normal ALT group or intermittently normal ALT group), average AST integration value (i.e., ≤40 or >40 IU/L), platelet count (i.e., <15.0 or ≥15.0 × 10^4/mm^3), average gamma-GTP integration value (i.e., ≤56 or >56 IU/L), total bilirubin (i.e., ≤1.2 or >1.2 mg/dl), average ALP integration value (i.e., ≤338 or >338 IU/L), cholinesterase (i.e., <431 or ≥431 IU/L), albumin (i.e., <3.5 or ≥3.5 g/dl), total cholesterol (i.e., <130 or ≥130 mg/dl), and average AFP integration value (i.e., ≤10 or >10 ng/ml). The lower and upper limits of the reference values at our institution were used as cut-off values for AST, platelet count, gamma-GTP, total bilirubin, ALP, cholinesterase, albumin, and total cholesterol. Statistical significance was defined as $P < 0.05$.

The study protocol was approved by the Ethics Committee at Ogaki Municipal Hospital and performed in compliance with the Helsinki Declaration.

RESULTS

Patient Characteristics

The median follow-up period was 8.6 years (range, 3.0–14.0 years). HCC developed in 17 of 381 patients

(4.5%) during the follow-up period. The 5- and 10-year cumulative incidence of HCC was 0.8% and 6.5%, respectively. Profiles and data from the 381 patients with normal ALT values are summarized in Table I.

Factors Associated With the Incidence of HCC

Factors associated with the incidence of HCC, as determined by univariate analysis, are listed in Table II. Male sex, high HBV-DNA levels, intermittently normal ALT, high AST levels, low platelet counts, low cholinesterase levels, low albumin levels, low total cholesterol levels, high AFP levels, and presence of cirrhosis were significantly associated with HCC development. The cumulative incidence of HCC was significantly higher in patients with platelet counts $<15.0 \times 10^4/mm^3$ (n = 70) than in patients with platelet counts $\geq 15.0 \times 10^4/mm^3$ (n = 311, $P < 0.001$, Fig. 4). The cumulative incidence of HCC was significantly higher in patients with HBV-DNA levels ≥5.0 log copies/ml (n = 90) than in patients with HBV-DNA levels <5.0 log copies/ml (n = 291, $P < 0.001$, Fig. 5).

Factors associated with incidence of HCC, as determined by the Cox proportional hazard model and the forward selection method, are listed in Table III. Male sex, high HBV-DNA levels, low platelet counts, and low total cholesterol levels were significantly associated with the development of HCC.

Baseline of patients with normal ALT according to HBV-DNA level and platelet counts.

HBV carriers with normal ALT levels were divided into four groups (A: HBV-DNA levels <5.0 log copies/ml and platelet counts $\geq 15.0 \times 10^4/mm^3$ [n = 257]; B: HBV-DNA levels <5.0 log copies/ml and platelet counts $<15.0 \times 10^4/mm^3$ [n = 45]; C: HBV-DNA levels ≥5.0 log copies/ml and platelet counts $\geq 15.0 \times 10^4/mm^3$ [n = 54]; and D: HBV-DNA levels ≥5.0 log copies/ml and platelet counts $<15.0 \times 10^4/mm^3$ [n = 25]). Positive rates of HBeAg were highest in Group C, total cholesterol levels were lowest in Group D, and ALT level, frequency of intermittently normal ALT, AFP levels, and presence

TABLE I. Patient Characteristics

Age (years)	49 (12–84)
Sex (F/M)	201/180
BMI (kg/m^2)	22.4 (17–36)
HBV genotype (A/B/C/D)	8/24/149/2
HBeAg (positive/negative)	59/322
HBV-DNA (log copies/ml)	3.7 (2.6–9.6)
ALT (IU/L)	22.6 (8.7–39.9)
Persistently normal ALT (+/−)[a]	182/199
AST (IU/L)	23.4 (13.3–74.3)
Platelet ($\times 10^4/mm^3$)	19.3 (3.3–39.5)
Gamma-GTP (IU/L)	19.5 (7.4–441.0)
Total bilirubin (mg/dl)	0.6 (0.3–4.7)
ALP (IU/L)	214.8 (82.4–621.3)
Cholinesterase (IU/L)	314.0 (99.6–483.9)
Albumin (g/dl)	4.2 (2.4–4.9)
Total cholesterol (mg/dl)	186.5 (102.0–332.1)
AFP (ng/ml)	2.4 (0.8–303.6)
Cirrhosis (−/+)[b]	341/40
Hepatocarcinogenesis (+/−)	17/364

F, female; M, male; BMI, body mass index; HBV, hepatitis B virus; HBeAg, hepatitis B e antigen; ALT, alanine aminotransferase: AST, aspartate aminotransferase; GTP, glutamyl transpeptidase; ALP, alkaline phosphatase; AFP, alpha-fetoprotein.
Values are expressed as median (range).
[a]Persistently normal ALT values includes patients with ≤40 IU/L.
[b]Cirrhosis diagnosed by ultrasound findings.

TABLE II. Factors Associated With Hepatocarcinogenesis (Univariate Analysis)

	Hazard ratio (95% CI)	*P*-value
Sex		
F	1	
M	8.282 (1.892–36.259)	0.005
HBV-DNA (log copies/ml)		
≤5.0	1	
>5.0	7.133 (2.699–18.852)	<0.001
Persistently normal ALT[a]		
Presence	1	
Absence	3.939 (1.126–13.776)	0.032
AST (IU/L)		
≤40	1	
>40	4.046 (1.157–14.140)	0.029
Platelets ($\times 10^4/m^3$)		
≥15	1	
<15	7.961 (2.922–21.690)	<0.001
Cholinesterase (IU/L)		
≥431	1	
<431	4.865 (1.368–17.298)	0.015
Albumin (g/dl)		
≥3.5	1	
<3.5	8.086 (2.567–25.474)	<0.001
Total cholesterol (mg/dl)		
≥130	1	
<130	9.704 (2.740–34.367)	<0.001
AFP (ng/ml)		
≤10	1	
>10	6.779 (1.445–31.809)	0.015
Cirrhosis[b]		
Absence	1	
Presence	18.033 (6.6055–19.233)	<0.001

W, female; M, male; HBV, hepatitis B virus; AST, aspartate aminotransferase; GTP, glutamyl transpeptidase; AFP, alpha-fetoprotein.
P-values and hazard ratio were calculated by Cox proportional hazard model.
[a]Persistently normal ALT values includes patients with ≤40 IU/L.
[b]Cirrhosis diagnosed by ultrasound.

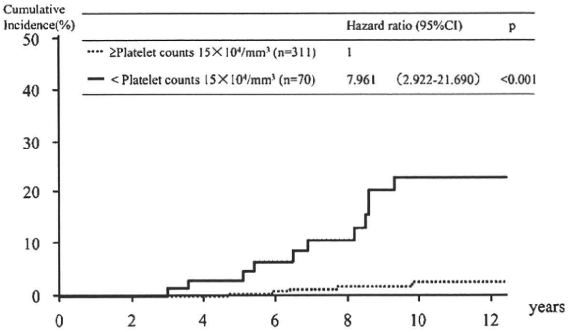

Fig. 4. Incidence of HCC according to platelet counts. The 5- and 10-year cumulative incidences of HCC was 0.4% and 2.6%, respectively, in patients with platelet counts $\geq 15.0 \times 10^4/mm^3$ (n = 311), and 2.9% and 22.9% in patients with platelet counts $<15.0 \times 10^4/mm^3$ (n = 70). The cumulative incidence of HCC was significantly higher in the latter group than in the former.

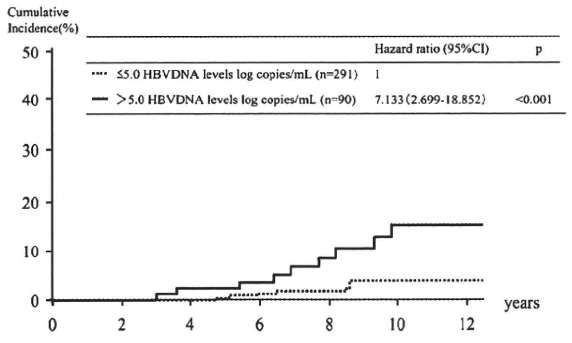

Fig. 5. Incidence of HCC according to serum HBV-DNA levels. The 5- and 10-year cumulative incidences of HCC was 0.4% and 3.7%, respectively, in patients with HBV-DNA levels <5.0 log copies/ml (n = 291) and 2.3% and 15.5%, respectively, in patients with HBV-DNA levels ≥5.0 log copies/ml (n = 90). The cumulative incidence of HCC was significantly higher in the latter group than in the former.

of cirrhosis were highest in Group D (Table IV). Group D showed the highest rate of incidence of HCC, followed by Groups B and C, as compared with Group A (Fig. 6).

DISCUSSION

The current studies revealed that the risk of developing HCC increases with decreasing platelet counts, decreasing total cholesterol levels, and increasing HBV-DNA levels in patients with average ALT integration values ≤40 IU/L.

ALT, AST, gamma-GTP, ALP, and AFP levels fluctuated within individual patients. Therefore, repeated measurements of these tests are important for accurate interpretation of the data. The arithmetic mean value is often used in the measurement of these tests; however, this value can be greatly affected by the period of time between measurements. Therefore, integral calculus was used to determine the value of these markers. Because this determination is strongly affected by the follow-up period, the average integration value was divided by the time of follow-up. The average integration value is more meaningful than the arithmetic mean value [Kumada et al., 2007].

TABLE III. Multivariate Analysis of Factors Associated With Development of Hepatocellular Carcinoma

Factor	Hazard ratio (95% CI)	*P*-value
Sex		
F	1	
M	6.011 (1.353–26.710)	0.018
HBV-DNA (log copies/ml)		
≤5.0	1	
>5.0	5.125 (1.880–13.973)	0.001
Platelets ($\times 10^4$/mm^3)		
≥15	1	
<15	4.803 (1.690–13.647)	0.003
Total cholesterol (mg/dl)		
≥130	1	
<130	5.983 (1.558–22.979)	0.009

F, female; M, male; HBV, hepatitis B virus.
P-values and hazard ratios were calculated using the Cox proportional hazard model.

In the present study, there was no difference between patients with average ALT integration values of 0–20 IU/L versus those with 21–40 IU/L. Thus, ALT levels are not good predictors of HCC development in patients with hepatitis B, as opposed to hepatitis C [Yuen et al., 2005; Sherman, 2005]. Furthermore, the change pattern of ALT was evaluated in the persistently normal ALT group and the intermittently normal ALT group. The results of the univariate analysis suggest that intermittently normal ALT levels, high AST levels, low cholinesterase levels, low albumin levels, and high AFP levels are associated significantly with HCC development; however, not all of these factors were significant in the multivariate analysis.

HBV-DNA levels at the start of the follow-up period correlated with the cumulative incidence of HCC. Chen et al. [2006] reported the adjusted hazard ratios for HCC development in HBeAg-seronegative subjects with normal ALT levels. Compared with participants in whom serum HBV-DNA levels were <300 copies/ml, the adjusted hazard ratio for developing HCC was 1.3 (95% confidence interval, 0.5–3.2; $P = 0.05$) for participants with serum HBV-DNA levels of 300–9,999 copies/ml; 2.7 (1.2–6.3; $P = 0.02$) for levels of 10,000–99,999 copies/ml; 7.2 (3.2–16.6; $P < 0.001$) for levels of 100,000–999,999 copies/ml; and 14.3 (6.2–32.8; $P < 0.001$) for levels of 1 million copies/ml and greater. It is emphasized that the cumulative incidence of HCC increases in patients with increased HBV-DNA levels, even if patients have normal ALT levels.

Lok and McMahon [2004] reported that HBV-DNA levels $>10^5$ copies/ml should be considered clinically significant. Their recommendation is supported by a meta-analysis of 26 trials of anti-HBV therapy which evaluated the association between viral load and hepatic inflammatory activity, as determined by hepatic histology and aminotransferase activity [Mommeja-Marin et al., 2003]. Thus, it is important for patients to maintain low HBV-DNA levels (i.e., $\leq 10^5$ copies/ml). These findings suggest that effective control of HBV replication, indicated by a decrease in serum HBV-DNA levels following antiviral therapy, may reduce the ultimate risk of developing HCC. Furthermore, it is believed that treatment with nucleosides or nucleotide analogues will decrease the cumulative incidence of HCC [Liaw et al., 2004; Piao et al., 2005].

The present study reveals that a low platelet count is a predictive factor for the development of HCC. Cirrhosis is an established risk factor for HCC in patients with HBV [Liaw et al., 1989; McMahon et al., 2001; Yu et al., 2002; Murata et al., 2005]. Ultrasonography produces detailed cross-sectional images of the liver and its surrounding structures. To distinguish cirrhosis patients from non-cirrhosis patients was attempted according to typical ultrasound findings [Caturelli et al., 2003; Iacobellis et al., 2005; Shen et al., 2006]. The presence of cirrhosis diagnosed by ultrasonography

TABLE IV. Patients Characteristics, According to HBVDNA Levels and Platelet Counts

HBV-DNA (log copies/ml) Platelets ($\times 10^4/m^3$)	Group A ≤ 5.0 $\geq 15 \times 10^4$ (n = 257)	Group B ≤ 5.0 $< 15 \times 10^4$ (n = 45)	Group C > 5.0 $\geq 15 \times 10^4$ (n = 54)	Group D > 5.0 $< 15 \times 10^4$ (n = 25)
Age (years)	49 (12–84)	51 (24–75)	47 (15–73)	52 (33–82)
Sex (F/M)	136/121	25/20	29/25	11/14
BMI (kg/m^2)	22.6 (14–36.3)	22.5 (16–28.2)	22.2 (16.7–32.4)	20.9 (16.9–36.4)
HBV genotype (A/B/C/D)	7/20/88/2	0/1/20/0	1/3/26/0	0/0/15/0
HBeAg (positive/negative)***	5/252	3/42	36/18	15/10
ALT (IU/L)***	19.7 (8.7–39.1)	25.3 (11.2–38.2)	29.8 (12.2–39.9)	32.1 (18.3–38.4)
Persistently normal ALT (+/−)[a],***	153/104	14/31	14/40	1/24
Total cholesterol (mg/dl)***	191.5 (114–332.1)	169.1 (102–259.2)	190.1 (147.1–254.4)	165.5 (112–234)
AFP (ng/ml)****	2.2 (0.8–119.8)	2.6 (0.8–20.8)	2.8 (0.8–45.5)	4.7 (1.1–303.6)
Cirrhosis (−/+)[b],***	253/4	27/18	50/4	11/14
Hepatucelluar carcinoma (+/−)***	2/255	5/40	4/50	6/19

F, female; M, male; BMI, body mass index; HBV, hepatitis B virus; HBeAg, hepatitis B e antigen; ALT, alanine aminotransferase; AFP, alpha-fetoprotein.
P-values were calculated using the Kruskal–Wallis test or the chi-square test. Values are expressed as median (range).
[a]Persistently normal ALT values includes patients with ≤40 IU/L.
[b]Cirrhosis diagnosed by ultrasound findings.
***$P < 0.0001$.
****$P < 0.0005$.

was strongly associated with the increased incidence of HCC by univariate analysis. Anatomical constraints and interobserver variability, however, remain limiting factors. In this study, histological confirmation was obtained in only 20 patients (6.3%). It is thought that this study had limitations because the liver histology was not obtained in many cases. Liver biopsy is still the "gold standard" for assessing liver fibrosis; however, it is not practical to undertake biopsies on all patients because of the potential complications which might arise from this procedure. Furthermore, results often differ depending on the pathologist, and results for liver fibrosis in liver biopsy specimens do not always reflect the grade of fibrosis in the entire liver. In contrast, the platelet count is a useful surrogate marker for the diagnosis of cirrhosis. Lu et al. [2006] reported that the best cutoff platelet count for a diagnosis of cirrhosis is $15.0 \times 10^4/mm^3$. The primary aim of this study was to identify serological markers associated with the development of HCC. Because of this, cirrhosis diagnosed by ultrasonography was excluded from the multivariate analysis. On the other hand, a low cholesterol level is associated with hepatocarcinogenesis, too. Hypocholesterolemia is found frequently in advanced liver disease because the liver is the most active site of cholesterol metabolism [D'Arienzo et al., 1998]. Four of 12 patients (33.3%) with <130 mg/dl serum total cholesterol developed HCC during follow-up period. It seemed that low platelet counts and hypocholesterolemia were confounding factors for identifying cirrhosis. Platelet counts were used as a parameter for cirrhosis in this study.

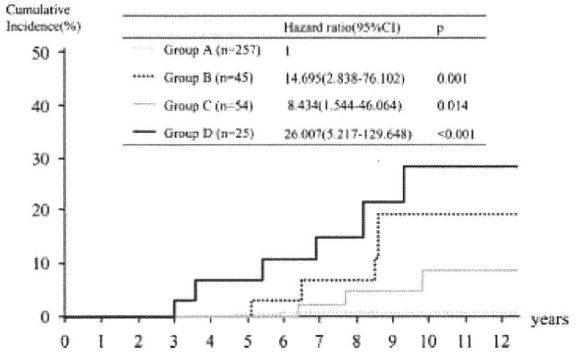

Fig. 6. The cumulative incidence of HCC according to HBV-DNA levels and platelet counts. HBV carriers with normal ALT levels were divided into four groups (A: HBV-DNA levels <5.0 log copies/ml and platelet counts $\geq 15.0 \times 10^4/mm^3$ [n = 257]; B: HBV-DNA levels <5.0 log copies/ml and platelet counts $< 15.0 \times 10^4/mm^3$ [n = 45]; C: HBV-DNA levels ≥5.0 log copies/ml and platelet counts $\geq 15.0 \times 10^4/mm^3$ [n = 54]; and D: HBV-DNA levels ≥5.0 log copies/ml and platelet counts $< 15.0 \times 10^4/mm^3$ [n = 25]). Group D had the highest incidence rate of HCC (26.007 [5.217–129.648], $P < 0.001$), followed by Group B (14.695 [2.838–76.102], $P = 0.001$) and Group C (8.434 [1.544–46.064], $P = 0.014$), as compared with Group A.

The HBV genotype is also predictive of the development of HCC [Chan et al., 2004; Yu et al., 2005]. In Japan, HBV genotype C is the predominant genotype [Orito et al., 2001]. Genotype C is associated with higher HBV-DNA levels and a greater risk of HCC than genotype B [Chan et al., 2004]. In the present study, 149 of 183 patients (81.4%) were infected with HBV genotype C. All eight patients with HCC in whom HBV genotype was determined were infected with genotype C. It was difficult to evaluate the relationship between HBV genotype and incidence of HCC in this study.

This study has some limitations such as the potential for selection bias due to a retrospective analysis of a cohort of patients. Therefore, an effort was made to minimize the influence of bias by using average integration values of various biochemical markers and a multivariate analysis.

In conclusion, high HBV-DNA levels and low platelet counts are associated with an increased incidence of HCC in patients infected with hepatitis B who have normal ALT values. Therefore, maintenance of low HBV-DNA levels is important for the prevention for

HCC in patients with low platelet counts, even when the ALT values fall within the current normal range.

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Journal of Gastroenterology and Hepatology

JGHF

doi:10.1111/j.1440-1746.2009.06118.x

HEPATOLOGY

Prevalence and clinical characterization of patients with acute hepatitis B induced by lamivudine-resistant strains

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Key words
acute hepatitis, genotype, hepatitis B virus, lamivudine.

Accepted for publication 26 August 2009.

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Abstract

Background and Aims: Acute hepatitis caused by lamivudine (LMV)-resistant strains has not been reported, and the clinical impact of LMV-resistant strains on acute hepatitis is not known. The aim of this study was to investigate the molecular and clinical characteristics of patients with acute hepatitis B caused by LMV-resistant strains.

Methods: Forty-five patients with acute hepatitis B were studied. Hepatitis B virus (HBV) subgenotypes and LMV-resistance mutations were determined by direct sequencing of the preS and polymerase regions, respectively.

Results: HBV subgenotypes A2 ($n = 18$), B1 ($n = 1$), B2 ($n = 3$), B3 ($n = 2$), C1 ($n = 1$), C2 ($n = 19$) and C6 ($n = 1$) were detected in patients with acute hepatitis. LMV-resistance mutations were detected in two patients. LMV-resistance mutations (L180M, M204I) were detected in a patient with subgenotype C2 who had acute self-limited hepatitis. The other patient with LMV-resistance mutations (L180M, M204V) was infected with subgenotype A2 and had severe hepatitis.

Conclusion: LMV-resistant strains are rare, but they are starting to be found in patients with acute hepatitis B. Surveillance for detecting drug-resistant HBV strains would be important for clinical practice.

Introduction

Approximately 350 million people worldwide are infected with hepatitis B virus (HBV).[1] HBV infection causes a variety of clinical courses, such as self-limited acute hepatitis, fulminant hepatic failure, chronic hepatitis, and progression to cirrhosis and hepatocellular carcinoma.[2] Therefore, HBV infection is one of the most important global health problems. Most countries have performed universal vaccination to prevent HBV infection, but only high-risk groups, such as health-care workers and household contacts of HBV carriers, have received HBV vaccination in Japan.[3] Therefore, acute hepatitis is still a major problem in Japan. The frequencies of HBV strains that are rare in Japan have increased among Japanese patients with acute hepatitis B.[4–6] The distributions of the HBV strains in acute hepatitis are variable due to the changing social environment. Along the same lines, a study investigated acute hepatitis B induced by lamivudine (LMV)-resistant HBV strains, but acute hepatitis caused by an LMV-resistant strain has not been found, and the clinical impact of LMV-resistant strains on acute hepatitis is still unknown.[7] Surveillance of HBV strains associated with acute hepatitis B has been continued, and LMV-resistant strains have begun to be detected in patients with acute hepatitis B. Thus, the present study reports the clinical characteristics of patients in Japan with acute hepatitis B caused by LMV-resistant HBV strains.

Materials

Forty-five Japanese patients with acute hepatitis B who were treated at Nagoya University Hospital, Ogaki Municipal Hospital, Tosei Hospital, Yokkaichi Hospital, and Fujita Health University Hospital were enrolled in this study between January 2006 and September 2008. The patients were 37 men and eight women, with a mean age of 38.6 ± 12.9 years (range, 18–84 years). There were no patients who had received HBV vaccine. Acute hepatitis B was diagnosed as follows. Each patient had high titers of hepatitis B surface antigen (HBsAg) and immunoglobulin (Ig)M class antibody against HBV core antigen, elevated serum levels of alanine aminotransferase and absence of antibodies against other causative viruses, such as hepatitis A virus, hepatitis C virus, Epstein–Barr virus and cytomegalovirus. It was necessary to discriminate

between initial HBV infection and acute onset or reactivation of chronic HBV infection. Thus, serum HBsAg levels noted in previous medical records, blood donation screening, labor and delivery screening, or employment health screening, were obtained or were followed until negative of HBsAg and/or positive of hepatitis B surface antibody (HBsAb). No patients were using chemotherapeutic and immune modulating agents involved in HBV reactivation. Informed consent was obtained from all patients, and the study was carried out in accordance with the 1975 Helsinki Declaration. Serum was stored at −80°C for virological examinations.

Assay methodology

Hepatitis B virus DNA was isolated from peripheral blood with a QIAamp DNA Mini Kit (QIAGEN, Hilden, Germany). Nested polymerase chain reaction (PCR) analysis and direct sequencing of the preS, polymerase and precore/core regions were performed as reported previously.[7] In brief, each 50-uL PCR reaction contained 100 nM each primer, 1 ng template DNA, 5 uL GeneAmp 10× PCR buffer, 2 uL deoxyribonucleotide triphosphate and 1.25 U AmpliTaq Gold (Applied Biosystems, Foster City, CA, USA). Primers were: preS region sense 5′-TCACCTATTCT TGGGAACAAGA-3′ and antisense 5′-GGCACTAGTAAACTG AGCCA-3′; polymerase region, sense 5′-CCTGCTGGTGGCT CCAGTTC-3′ and antisense 5′-GGTTGAGTCAGCAAACAC ACTTG-3′; and precore/core region, sense 5′-ATGTCGACAA CCGACCTTGA-3′ and antisense 5′-GTATGGTGAGGTGAAC AATG-3′. Amplification conditions consisted of 5 min at 94°C followed by 40 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 1 min in a thermal cycler (GeneAmp PCR System 9700; Applied Biosystems). The second PCR was done in the same reaction buffer with the first-round PCR product as template and the following sets of primers: preS region, sense 5′-TCACCTATTCTT GGGAACAAGA-3′ and antisense 5′-AGAAGATGAGGCATAG CAGC-3′; and polymerase region, sense 5′-GGATGTGTCTGC GGCGTTT-3′ and antisense 5′-ACCCCATCTTTTTGTTTTG TTAGG-3′. PCR products were detected by electrophoresis on 2% agarose gels, stained with ethidium bromide and visualized under ultraviolet light. PCR products were then purified and sequenced with the second-round PCR primers with a dye terminator sequencing kit (BigDye Terminator ver. 1.1 Cycle Sequencing Kit; Applied Biosystems) and an ABI 310 DNA Sequencer (Applied Biosystems). The neighbor-joining method[8] was used for phylogenetic analysis of the preS region to identify HBV subgenotypes. The bootstrap test with 1000 replicates was performed to confirm the reliability of the phylogenetic tree.[9]

Results

The results of the phylogenetic analyses of HBV subgenotypes of the 41 patients are shown in Figure 1. The HBV subgenotypes A2 ($n = 18$), B1 ($n = 1$), B2 ($n = 3$), B3 ($n = 2$), C1 ($n = 1$), C2 ($n = 19$) and C6 ($n = 1$) were detected. The prevalence of subgenotype A2 was increased, as previously reported. LMV resistance-associated mutations were detected within the HBV polymerase region (positions 116–214) by direct sequencing. Alignment of the amino acid sequence of the HBV polymerase region with LMV resistance-associated mutations was analyzed, and LMV-associated mutations could be detected in two patients at acute hepatitis onset. LMV-resistance mutations (L180M, M204I) were detected in a patient with subgenotype C2. The other patient with subgenotype A2 had LMV-resistance mutations (L180M, M204V). There were no resistant HBV mutants for other nucleoside/nucleotide analogs such as V173L, L180M or M204V/I. The clinical and virological characteristics of patients with LMV-resistant HBV strains are summarized in Table 1.

Discussion

Hepatitis B virus reverse transcriptase is an error-prone enzyme without proofreading capacity, and it is easy for frequent mutations to occur during viral replication. As a result, there are many well-known mutations that are associated with the pathogenesis of HBV infection.[10] LMV-resistant strains that have mutations in the polymerase region are induced by long-term administration of LMV.[11,12] LMV had been used widely for treatment for chronic hepatitis B and was available from 2000 in Japan. LMV-resistant strains have emerged in patients with chronic hepatitis. However, the prevalence and clinical impact of LMV-resistant strains in patients with acute hepatitis B are unknown. Thus, surveillance of LMV-resistant strains associated with acute hepatitis B had been conducted, but LMV-resistant strains could not be detected in 2006.[7] The possibility of acute hepatitis B caused by LMV-resistant strains exists, and the surveillance has continued. Of 45 patients with acute hepatitis, two were found to have LMV-associated mutations. We previously hypothesized that LMV-resistant strains may not have enough power to cause acute hepatitis. However, the present study demonstrated that LMV-resistant strains would have infectivity and would be capable of causing acute hepatitis. Less opportunity for infection may explain why previous studies failed to find acute hepatitis caused by LMV-resistant strains.

The infectious source of the LMV-resistant strains could not be confirmed. The subgenotypes of the patients infected with LMV-resistant strains were subgenotype A1 and C2, respectively. The patient infected with subgenotype C2 plus LMV-resistant strain had a history of sex with a prostitute 1 month before admission. Subgenotype C2 was the predominant subgenotype found in Japanese patients with chronic hepatitis B.[7,13–15] The infectious source would be a chronic hepatitis patient who developed resistant HBV mutants during long-term LMV treatment. The route of infection for the other patient with subgenotype A2 was unknown. HBV subgenotype A2 has been rarely reported in Japanese patients with chronic hepatitis B. However, subgenotype A2 has been increasing and has become responsible for the majority of patients with acute hepatitis B.[4,7,16] This study also confirmed that HBV subgenotype A2 has become widespread among Japanese patients with acute hepatitis. However, the origin of subgenotype A2 with an LMV-resistant mutation is not clear. The possibility of it coming from a patient with chronic hepatitis B is low, because subgenotype A2 is rarely found in Japanese patients with chronic hepatitis B who receive long-term LMV treatment. The other possible infectious source is a patient co-infected with HIV. Nucleoside/nucleotide analogs (NA) such as LMV were effective for both HBV and HIV. NA were used not only for treatment of HBV but also for treatment of HIV, and LMV-resistant strains have been reported.[17] HBV genotype A and HIV co-infection have been found among male

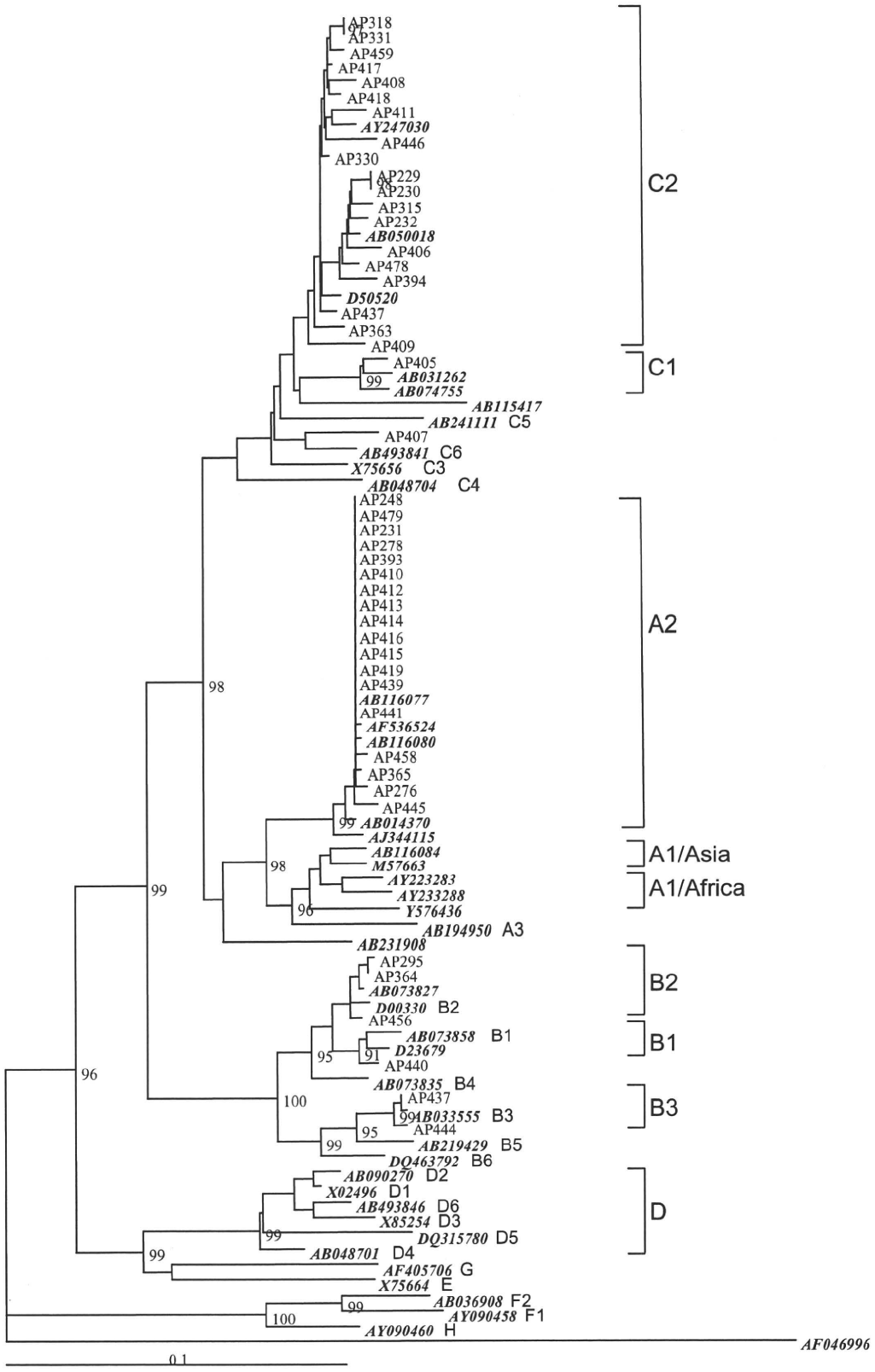

Figure 1 Results of phylogenetic analysis of 45 sequences from the preS region of hepatitis B virus (HBV) of acute hepatitis patients and 42 reference strains from a database and shown by accession number. Strains isolated from patients with acute hepatitis are indicated as AP. Phylogenetic analysis was performed by the neighbor-joining method with Woolly monkey HBV (AF046996) as out-group. Percentages of bootstrap values greater than 90% are shown on the nodes. The scale bar indicates genetic distance.

Table 1 Clinical characteristics

	Case 1	Case 2
Age (years)	32	32
Sex	Male	Male
ALT (IU/L)	4429	2820
AST (IU/L)	2709	1620
T Bil (mg/dL)	3.0	4.1
HBeAg	Positive	Positive
HBV (log copies/mL)	5.2	7.4
BCP1762/1764	T/A	A/G
PC1896	G	G
Route	STD	Unknown
Subgenotype	C2	A2

ALT, alanine aminotransferase; AST, aspartate aminotransferase; HBeAg, hepatitis B e-antigen; HBV, hepatitis B virus; STD, sexually transmitted disease; T Bil, total bilirubin.

patients who have sex with men in Japan.[18] Because the patient infected with subgenotype A2 that was LMV-resistant was not co-infected with HIV, this was also inconclusive. The other possibility was that the infectious source could have been a foreign patient with subgenotype A2 in whom an LMV-resistant strain emerged. This study has the following limitations: a small number of patients, patients without symptom were not recruited, the identification of the infectious source. Thus, further studies such as a nationwide survey including blood banks to investigate asymptomatic patients, the need to make conclusion of the prevalence of patients with acute hepatitis B induced by LMV-resistant strains in Japan.

The patient with LMV-resistant mutations with subgenotype C2 developed self-limited hepatitis, while the other patient with LMV-resistant mutations with subgenotype A1 developed severe acute hepatitis. Basal core promoter (BCP) and precore (PC) variants have been shown to be associated with the severity of the clinical course of acute hepatitis. In particular, mutations at BCP/PC of HBV subgenotype C2 and B1 can increase the risk of progression to fulminant hepatic failure. The clinical impacts of basal core promoter and precore variants in other genotypes are unclear.[7,16] In the present study, both patients with acute hepatitis caused by LMV-resistant strains had wild-type BCP/PC variants. The wild-type BCP/PC variants were linked with mild self-limited hepatitis in the patient with subgenotype C2. The clinical impact of LMV-resistant strains on acute hepatitis appears to be not serious for subgenotype C2. Meanwhile, the mutations in the BCP/PC regions were not associated with the severity of acute hepatitis in the patient with subgenotype A2. Therefore, LMV-resistant mutations in subgenotype A2 might be associated with the severity of the clinical course. However, the present sample size was too small to allow evaluation of the clinical course in acute hepatitis B with LMV-resistant strains and to determine whether LMV-resistant strains have different effects on each subgenotype. Further studies are needed to clarify the influence of LMV-resistant strains on the clinical course of acute hepatitis B.

Lamivudine has begun to be used to treat patients with acute hepatitis to prevent progression to fulminant hepatic failure or chronic hepatitis. Some reports have shown the safety and effectiveness of LMV for the treatment of acute hepatitis B.[19,20] However, one clinical study that has been published did not confirm its efficacy.[21] Thus, the administration of LMV in acute hepatitis B is controversial. The use of LMV for all acute hepatitis was not of benefit and was not recommended for use in all patients. However, selected patients who have a high risk for progression to fulminant hepatic failure and chronic infection may benefit from LMV to prevent disease progression. There is a small possibility that acute hepatitis B can be caused by LMV-resistant strains, but previous studies did not consider LMV-resistant strains before they started to use LMV. Caution must be exercised when determining whether LMV should be used to treat acute hepatitis B because of the possibility of the development of LMV-resistant strains. In the present study, the patient with LMV-resistant mutations who progressed to severe hepatitis was treated with LMV and steroid. Despite the limited efficacy of LMV in suppressing viral replication of LMV-resistant strains, this patient recovered from severe acute hepatitis. Patients with severe acute hepatitis have a high risk for progression to fatal liver failure. However, patients not treated with LMV may have a full recovery and not progress to fulminant liver failure, either because of the efficacy of other treatment, such as steroid, or because the patients' immune reaction could clear the HBV infection. It is difficult to judge the clinical role of LMV-resistant strains in acute hepatitis based on this case. The present study included insufficient information about the magnitude of screening for LMV-resistant strains in acute hepatitis.

Lamivudine is associated with a high incidence of resistance.[22] Thus, the first-line agent for HBV infection has been changed from LMV to adefovir or entecavir because of their powerful antiviral effect and the lower likelihood of drug resistance mutations emerging. The emergence of drug resistance during long-term adefovir or entecavir therapy in chronic hepatitis B was not frequent compared to that with LMV.[23,24] With adefovir or entecavir, the incidence of LMV-resistant strains would be remarkably decreased, but the risk for other HBV drug-resistant strains still remains. Clinical use of anti-HBV agents such as adefovir, entecavir, telbivudine, clevudine and tenofovir has started, and multiple anti-HBV drug-resistant strains could occur in patients undergoing long-term treatment in the near future. Therefore, maintaining surveillance to detect drug-resistant strains of HBV may have a small impact, but it is important for clinical practice.

In conclusion, LMV-resistant mutations were previously rare but now appear to be prevalent among patients in Japan with acute hepatitis B. LMV-resistant strains must be considered in patients with acute hepatitis B.

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