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

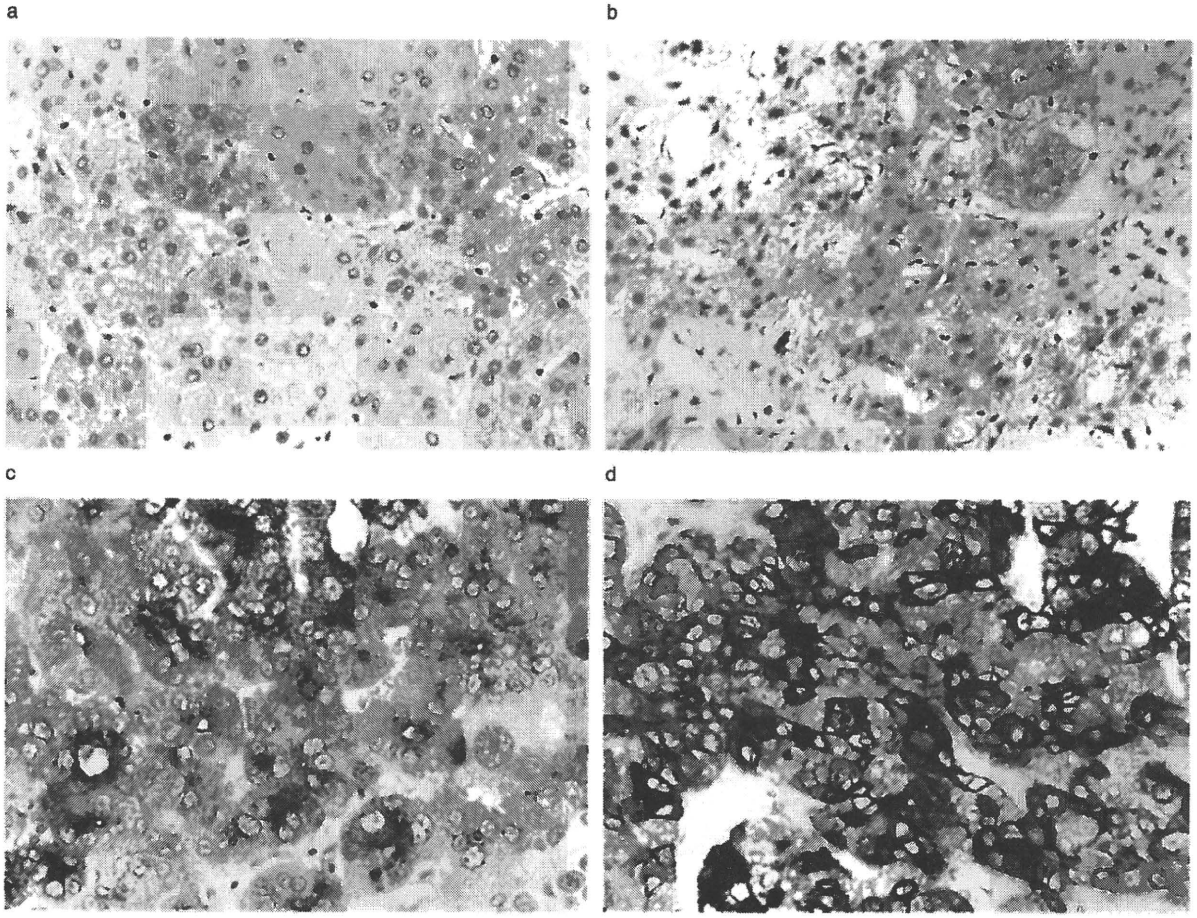

Figure 1 The degree of immunohistochemical staining for glypican-3. (a) No staining, (b) light staining, (c) moderate staining, (d) heavy staining.

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.

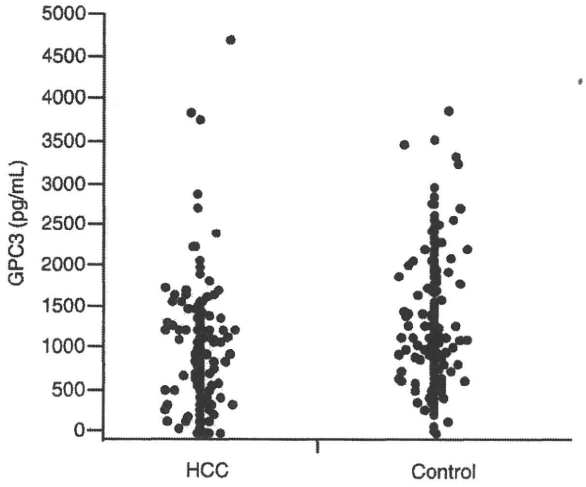

Figure 2 Serum glypican-3 (GPC3) level in patients with hepatocellular carcinoma (HCC) and in patients with chronic liver disease (CLD, control). Serum GPC3 level was higher in patients with CLD (1161.6 pg/mL) than those with HCC (924.8 pg/mL; $P < 0.0001$).

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

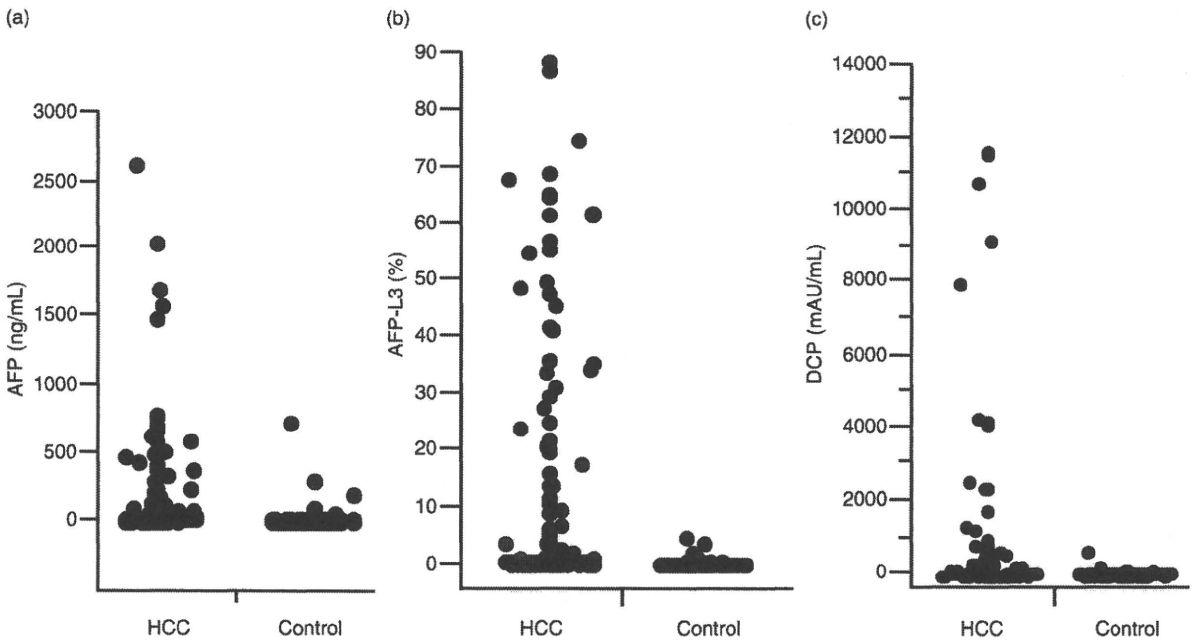

Figure 3 Serum alpha-fetoprotein (AFP), Lens culinaris agglutinin-reactive fraction of AFP (AFP-L3), and des-gamma carboxy prothrombin (DCP) levels in patients with hepatocellular carcinoma (HCC) and in patients with chronic liver disease (CLD, control). Serum AFP, AFP-L3, and DCP levels were significantly 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 $P < 0.0001$).

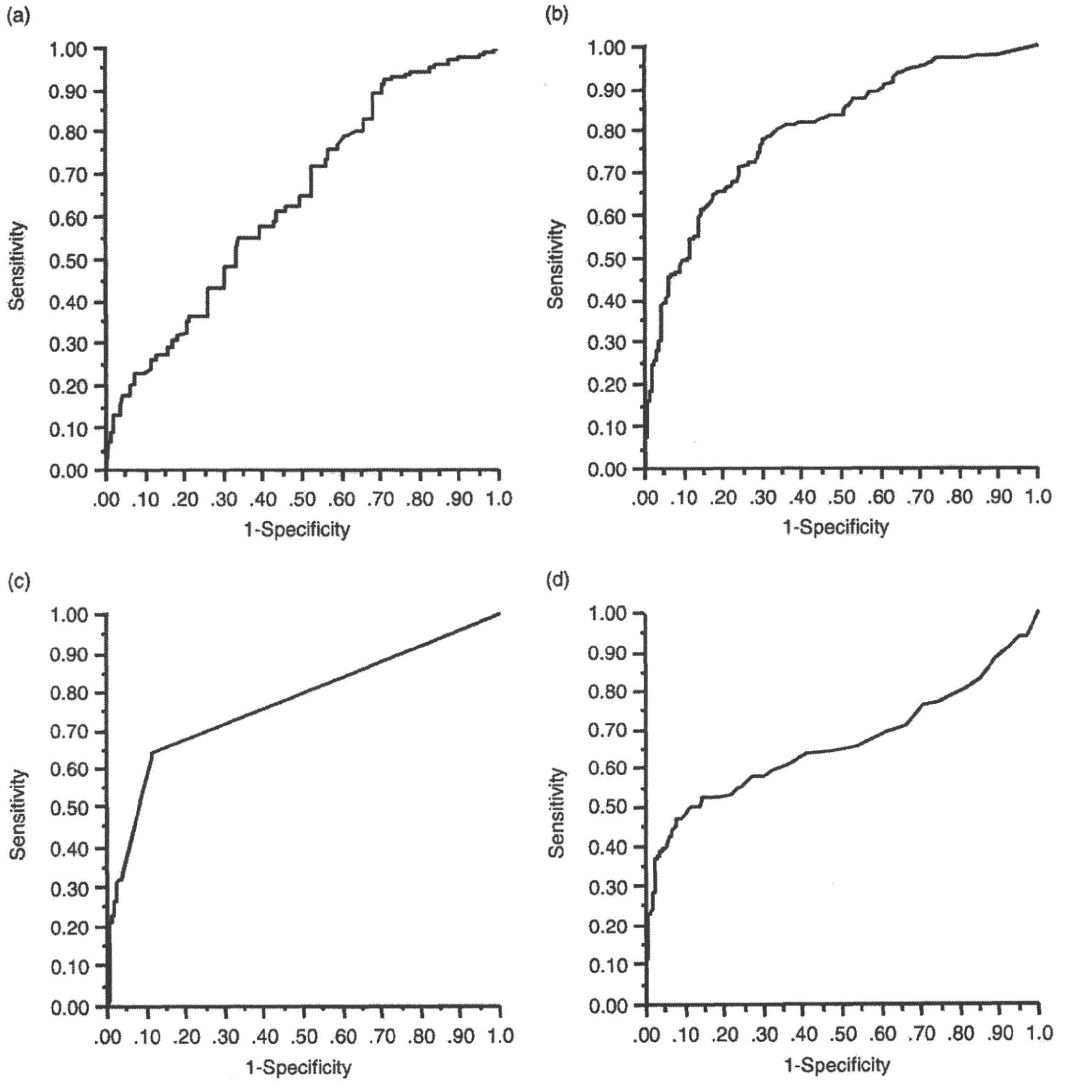

Figure 4 Area under the receiver-operating curve (AUROC) of (a) serum glypican-3 (GPC3), (b) alpha-fetoprotein (AFP), (c) Lens culinaris agglutinin-reactive fraction of AFP (AFP-L3), and (d) des-gamma carboxy prothrombin (DCP) for the diagnosis of hepatocellular carcinoma. AUROC was 0.64 for GPC3, 0.80 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]

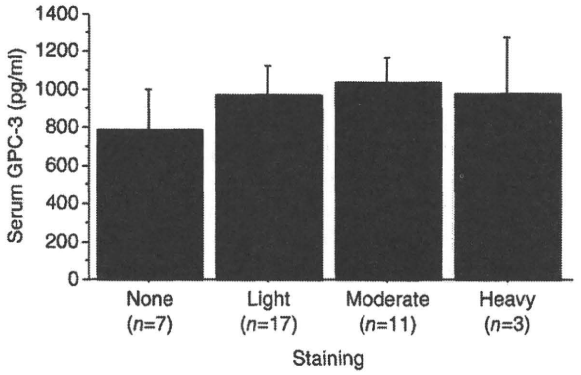

Figure 5 Serum glypican-3 (GPC3) level in 38 patients with hepatocellular carcinoma (HCC) who underwent hepatectomy according to the immunohistochemical staining of GPC3 on the resected HCC specimens. No association was found between serum GPC3 level and immunohistochemical staining of GPC3 on HCC tissues.

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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Original Article

Deregulation of miR-92a expression is implicated in hepatocellular carcinoma development

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MicroRNAs (miRNAs) belong to a class of the endogenously expressed non-coding small RNAs which primarily function as gene regulators. Growing evidence suggests that miRNAs have a significant role in tumor development and may constitute robust biomarkers for cancer diagnosis and prognosis. The *miR-17-92* cluster especially is markedly overexpressed in several cancers, and is associated with the cancer development and progression. In this study, we have demonstrated that miR-92a is highly expressed in hepatocellular carcinoma (HCC). In addition, the proliferation of HCC-derived cell lines was enhanced by miR-92a and inhibited by the anti-miR-92a antagomir. On the other hand, we have found that the relative amount of miR-92a in the plasmas from HCC patients is decreased compared with that from the healthy donors. Interestingly, the amount of miR-92a was elevated after surgical treatment. Thus, although the physiological significance of the decrease of miR-92a in plasma is still unknown, deregulation of miR-92 expression in cells and plasma should be implicated in the development of HCC.

Key words: hepatocellular carcinoma, microRNA, miR-638, miR-92a, plasma

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MicroRNAs (miRNAs) are small endogenous non-coding RNAs that regulate gene expression and have a critical role in many biological and pathological processes.[1] Recent studies have shown that deregulation of miRNA expression contributes to the multistep processes of carcinogenesis, and have shown promise as tissue-based markers for cancer classification and prognostication.[2,3] However, biological roles of only a small fraction of known miRNAs have been elucidated to date.

The miR-17-92 cluster at 13q31.3 is consists of six miRNAs: miR-17, miR-18a, miR-19a, miR-20a, miR-19b-1 and miR-92a-1, and plays an important role for development of lung cancer,[4] B-cell lymphomas,[5] chronic myeloid leukemia,[6] medulloblastomas,[7] colon cancer[8] and hepatocellular carcinoma (HCC).[9] In addition, mice deficient in the miR-17-92 cluster died shortly after birth with lung hypoplasia, and B-cell development was impaired in the mice.[10] It has been reported, however, that miR-92a increases cell proliferation by negative regulation of an isoform of the cell-cycle regulator p63.[11] Furthermore, miR-92a regulates angiogenesis.[12] Thus, it is clear that the miR-92a has some oncogenic characteristics. However, the specific biological role of miR-92a in the processes of human cancer development has remained unclear.

Here, we have revealed that miR-92a is implicated in human HCC development. Furthermore, we have demonstrated that miR-92a in human blood has the potential to be a noninvasive molecular marker for diagnosis of human HCC.

MATERIALS AND METHODS

In situ hybridization of miR-92a

Locked nucleic acid (LNA)-modified probes for miR-92a and negative control (miRCURY-LNA detection probe, Exiqon, Vedbaek, Denmark) were used. The probe sequences were as follows; *miR-92a*, 5′-ACAGGCCGGGACAAGTGCAATA-3′; and a scrambled oligonucleotides used for negative control, 5′-GTGTAACACGTCTATACGCCCA-3′. *In situ* hybridization was performed using the RiboMap *in situ* hybridization kit (Ventana Medical Systems, Tucson, AZ, USA) on the Ventana Discovery automated *in situ* hybridization instrument (Ventana Medical Systems). The *in situ* hybridization steps were performed as previously described.[13] Staining was evaluated by two investigators and graded as follows: negative (–), no or occasional (<5%) staining of tumor cells; positive (+), mild to strong (>5%) staining of tumor cells. Paraffin–embedded tissue samples of hepatocellular carcinoma (HCC) and adjacent non-tumorous liver cirrhosis (LC) were obtained from HCC patients at Ogaki Municipal Hospital (Ogaki, Japan). Details of the clinical data are provided in Table 1.

Plasma collection, RNA isolation and quantitative RT-PCR

Whole blood samples were collected from healthy donors and the patients with HCC at Ogaki Municipal Hospital. This study was approved by the institutional review board (IRB) of Tokyo Medical University, and all subjects provided written informed consent under the institutional review board. Details of clinical data are provided in Table 1. Diagnoses were confirmed using the post-operated tissues. Blood samples of the patients (Cases 1–10) were collected one day before the operation and then properly stored. One week after operation, blood samples of the patients were collected again. Whole blood was separated into plasma and cellular fractions by centrifugation at 1600 *g* for 15 min. Total RNA in the

Table 1 Summary of clinical details of hepatocellular carcinoma (HCC)used for in situ hybridaization and serum analysis

	Year	Sex	Virus type	Histologic type	Stage	Child-Pugh	miR-92a
Case 1	53	Male	HBV	Poorly	I	A	+
Case 2	59	Male	HBV	Moderate	II	A	+
Case 3	79	Male	NBNC	Moderate	III	A	+
Case 4	73	Male	HCV	Well	I	A	+
Case 5	76	Female	HCV	Moderate	IV-A	A	+
Case 6	59	Male	HCV	Moderate	II	A	+
Case 7	69	Female	HCV	Moderate	I	A	+
Case 8	71	Male	HCV	Moderate	I	A	+
Case 9	59	Female	HBV	Well	I	A	–
Case 10	69	Male	NBNC	Moderate	IV-A	A	–
Case 11	61	Female	HBV	Poorly	IV-A	B	+
Case 12	73	Male	NBNC	Moderate	II	A	+
Case 13	67	Male	NBNC	Moderate	IV-A	A	+
Case 14	61	Male	NBNC	Moderate	III	A	+
Case 15	45	Male	HBV	Moderate	I	A	+
Case 16	68	Female	HCV	Moderate	III	A	+
Case 17	70	Male	NBNC	Poorly	II	A	+
Case 18	59	Male	HCV	Moderate	III	A	+
Case 19	43	Male	HBV	Moderate	II	A	+
Case 20	69	Male	HCV	Moderate	II	A	–
Case 21	76	Male	HCV	Moderate	III	A	–
Case 22	53	Male	HCV	Moderate	II	A	–

HCV, hepatitis C virus; HBV, hepatitis B virus; NBNC, non-B non-C virus.

Table 2 Summary of clinical details of hepatocellular carcinoma (HCC) used for qPCR analysis

Code no.	Year	Sex	Virus type	Histologic type	Non-tumorous tissue	AFP	PIVKA-II
91	53	Male	HCV	Moderate	LC	5	0.06
160	59	Male	HCV	Moderate	LC	NI	NI
O89	68	Male	HCV	Moderate	LC	8	25
O90	70	Male	HCV	Moderate	LC	686	962
K89	51	Male	HCV	Moderate	LC	NI	NI

LC, liver cirrhosis; HCV, hepatitis C virus; NI, no information.

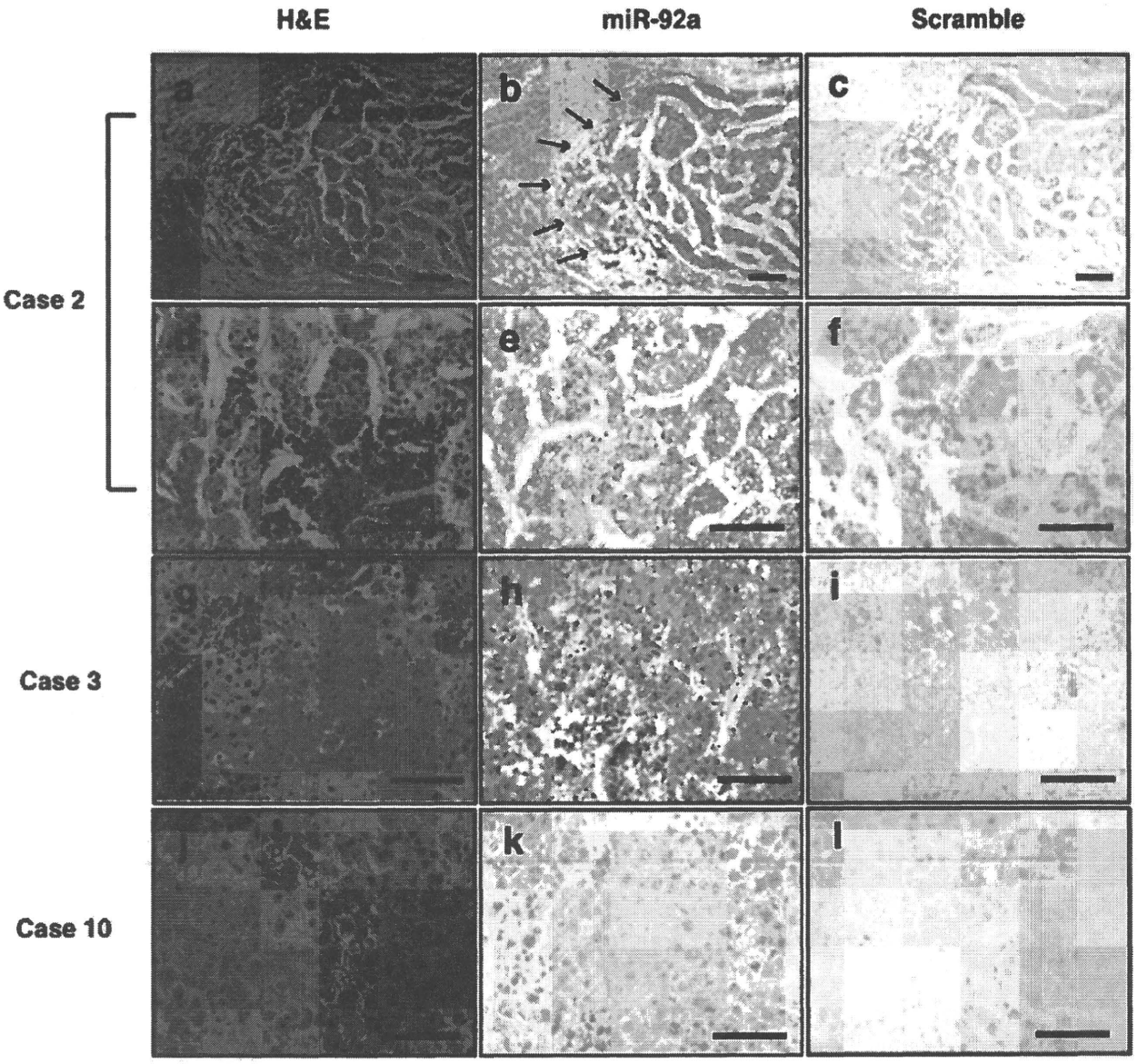

Figure 1 MiRNA expression in hepatocellular carcinoma (HCC). *In situ* hybridization was performed using Locked nucleic acid (LNA)-modified probes for miR-92a and negative control. Case 2 and Case 3 were positive cases for miR-92a. Case 10 was a negative case for miR-92a. (**a–c**) Low power field of boundary of HCC and non-tumor lesion. Arrowheads indicated a border. Only HCC regions were positive for miR-92a. (**d–l**) High power field of HCC. Blue signals represent positive for miR-92a. Bars indicate 100 μm.

plasma was isolated using Isogen-LS (NIPPON GENE, Tokyo, Japan) according to the manufacturer's instructions. The RNA sample was suspended in 20 μL of nuclease free water. In general, we obtained 400 ng of RNA from 1 mL of plasma. MiRNAs were quantified using TaqMan MiRNA Assays (Applied Biosystems, Life Technologies Corporation, Carlsbad, CA, USA) as previously described.[13]

For miR-92a quantification in tissue samples, five pairs of fresh HCC and non-tumorous LC samples were surgically resected from HCC patients (Table 2). All the patients or their guardians provided written informed consent, and the Ethics Committee of the Kyoto University Graduate School and Faculty of Medicine approved all aspects of this study. The amounts of miR-92a were normalized to RNU48 that is one of rRNAs (Applied Biosystems).

Cell culture and transfection

Hepatocellular carcinoma (HCC) cell lines HepG2, OR6 and SN1a were cultured in Dulbecco's modified Eagle's medium

(DMEM) (Sigma, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS). OR6 and SN1a are derived from the Huh7 HCC cell line and maintain hepatitis C virus (HCV) replicon.[14–16] The miR-92a oligonucleotide used in the transfection experiments is a synthetic double-strand 19 nucleotide RNA oligonucleotide (5′-UUGCACUUGUCCC GGCCUG-3′) purchased from B-Bridge International (Tokyo, Japan). The scrambled oligonucleotide represents a mix of two different frames of the miR-92 sequence (5′-UAUUGC ACUUGUCCCGGCCUGUCCCGGCC-3′ and 5′-AUUGCAC UUGUCCCGGCCUTT-3′). Locked nucleic acid (LNA) oligonucleotide miR-92 knockdown (antagomir) was obtained from Exiqon (Vedbaek, Denmark, http://www.exiqon.com). The oligonucleotides were individually transfected by Hiper-Fect (QIAGEN K. K., Tokyo, Japan) into the cells at a final concentration of 100 nM.

In vitro proliferation assays

The effects of miR-92a and the anti-miR-92a antagomir on the growth of HepG2, OR6 and SN1a were evaluated using the MTT cell growth assay kit (Cell Count Reagent SF, Nacalai tesque, Kyoto, Japan). The cells were transfected with miR-92a or the antagomir. The cell numbers were then assessed with MTT assay at 48 or 72 h after the transfection. The MTT assay was performed according to the manufacturer's recommendation. The reagents were added to each well and incubated at 37°C for 4 h. The MTT reduced by living cells into a formazan product was assayed with a multiwell scanning spectrophotometer at 450 nm.

RESULTS

Highly expression of miR-92a in HCC cells

We first examined whether or not miR-92a is expressed in hepatocellular carcinoma (HCC). We performed *in situ* hybridization using locked nucleic acid (LNA)-modified probes digoxigenin (DIG) labelled. We found that miR-92a was strongly expressed in cancer cells of 17 out of 22 HCC cases (Table 1 and Fig. 1). No significant differences were observed in age, sex, virus type, clinical stage and tumor differentiation of the clinical samples. In contrast, we did not detect miR-92a expression in non-cancerous hepatocytes around the HCCs.

Furthermore, we quantified miR-92a levels in HCC sections (n = 5) and their adjacent non-tumorous liver cirrhosis (LC) sections (n = 5) by *Taq*Man qRT-PCR (Table 2 and Fig. 2). The levels of miR-92a expression in HCC sections were higher than that in adjacent LC sections (Fig. 2).

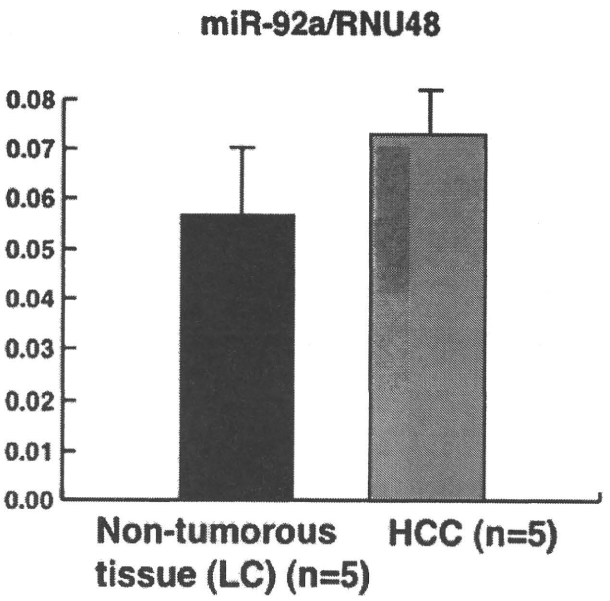

Figure 2 Quantification of miR-92a expression in hepatocellular carcinoma (HCC) tissue samples. The ratios of miR-92a to RNU48 in HCC tissues and their adjacent non-tumorous liver cirrhosis (LC) tissues were analyzed by *Taq*Man qRT-PCR. *Bars*, s.d.

Effects of miR-92a on a Hepatoma cell lines HepG2, OR6 and SN1a

Next, we investigated whether miR-92a affects cell proliferation of human HCC cell lines, HepG2, OR6 and SN1a. We transiently transfected either miR-92a or the anti-miR-92a antagomir into the cells. Antagomirs are single-stranded RNAs that are complementary to a specific miRNA and cause the depletion of the miRNA.[17] After the transfection, we found that all of the cells transfected with the anti-miR-92a antagomir showed lower proliferation rate than the cells transfected with a control RNA oligonucleotide (Fig. 3a). In contrast, the cells except for HepG2 showed increased proliferation rate when miR-92a was transfected (Fig. 3a). We also confirmed the amounts of miR-92a in the cells by quantitative real time PCR (Fig. 3b).

The ratio of miR-92a to miR-638 serves as a biomarker for HCC

Finally, we sought to determine whether the expression level of miR-92a in blood sera could discriminate HCC patients from healthy individuals. Previously, we have revealed that miR-92a is dramatically reduced in the plasmas of acute leukemia patients although in leukemic cells it is strongly expressed.[13] We analyzed the miR-92a levels in the plasma samples from normal individuals (n = 10) and HCC patients

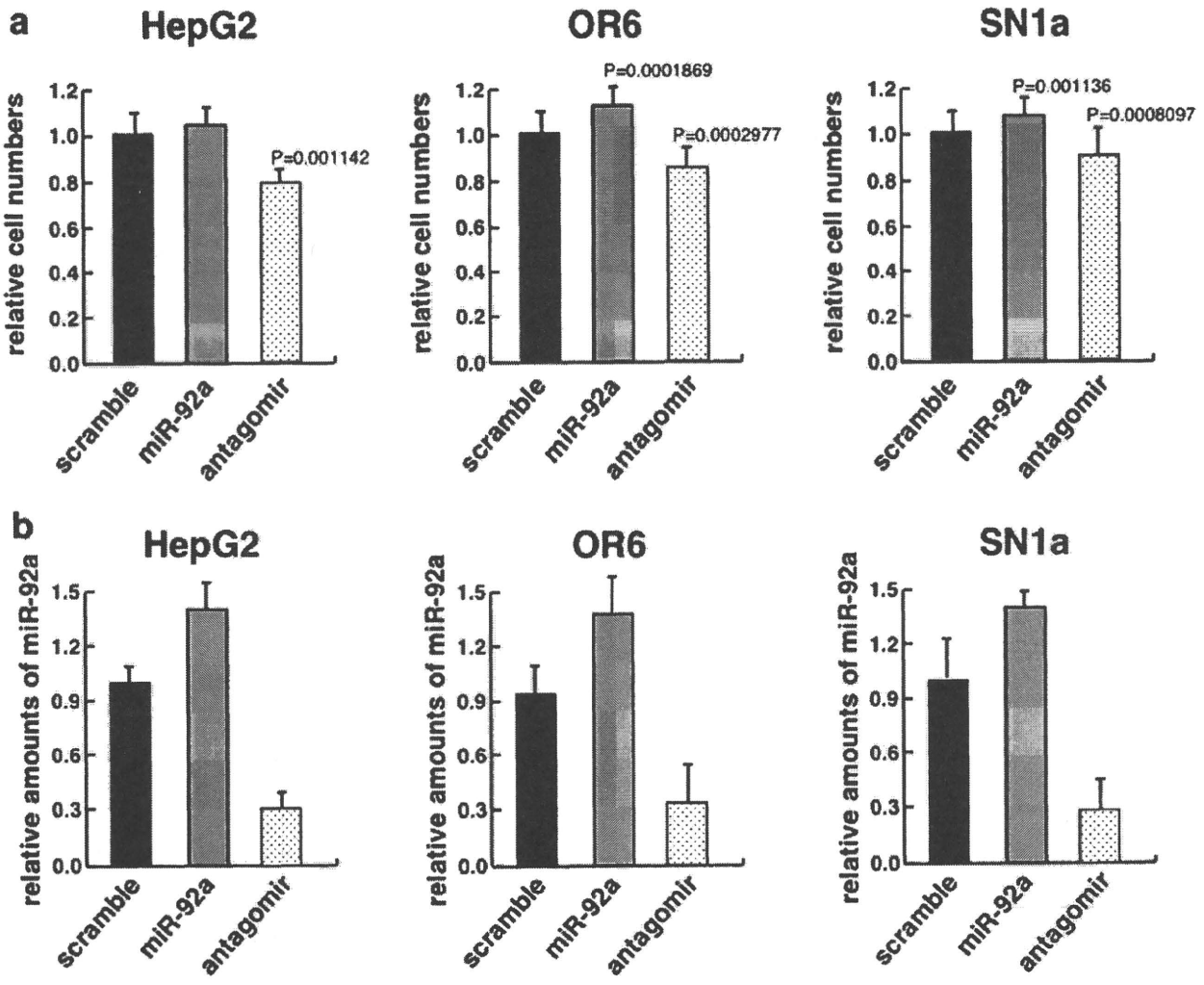

Figure 3 miR-92a modulates proliferation of HepG2, OR6 and SN1a cells. (**a**) Cell numbers of the HepG2, OR6 and SN1a cells transfected with synthetic miR-92a, anti-miR-92a antagomir, or scrambled control oligonucleotide were analyzed by MTT assays at 48 h for OR6 and SN1a and 72 h for HepG2 after transfection. *Bars*, s.d. (**b**) qRT-PCR analysis of miR-92a amounts in the cells transfected with miR-92a, anti-miR-92a antagomir or scrambled control at 48 h for OR6 and SN1a and 72 h for HepG2 after the transfection.

(n = 10) by *Taq*Man qRT-PCR. Because miR-638 is stably present in human plasmas,[13] we used miR-638 as the standard to improve the precision of the data. The ratio of miR-92a to miR-638 in the plasma samples from the HCC patients were decreased compared with that from the normal donors (Fig. 4a). Then, we further examined the ratio from the patients after surgical resection. Interestingly, the miR-92a/miR-638 levels were significantly higher than that in the plasmas from the patients before surgical resection (Fig. 4b).

DISCUSSION

In this study, we found that miR-92a was highly expressed in HCC (Figs 1,2). In addition, we demonstrated that the expression level of miR-92a affects the proliferation of hepatoma cell lines, HepG2, OR6 and SN1a (Fig. 3). These results suggest that miR-92a may play an important role in tumor progression of hepatocyte. We do not know why, but addition of miR-92a did not significantly increase the proliferation of HepG2 cells. It may be possible that HepG2 cells themselves already contain enough miR-92a to promote cancer cell proliferation. In addition, miR-92a is a part of the miR-17-92 cluster, which is actively involved in the development and progression of various cancers.[4–10] However, the molecular function of miR-92a is still unknown, and its mRNA targets have not been identified. Recently, it has been shown that one of the molecular mechanisms through which miR-92a increases cell proliferation is by negative regulation of an isoform of the cell-cycle regulator p63.[11] Thus, we examined

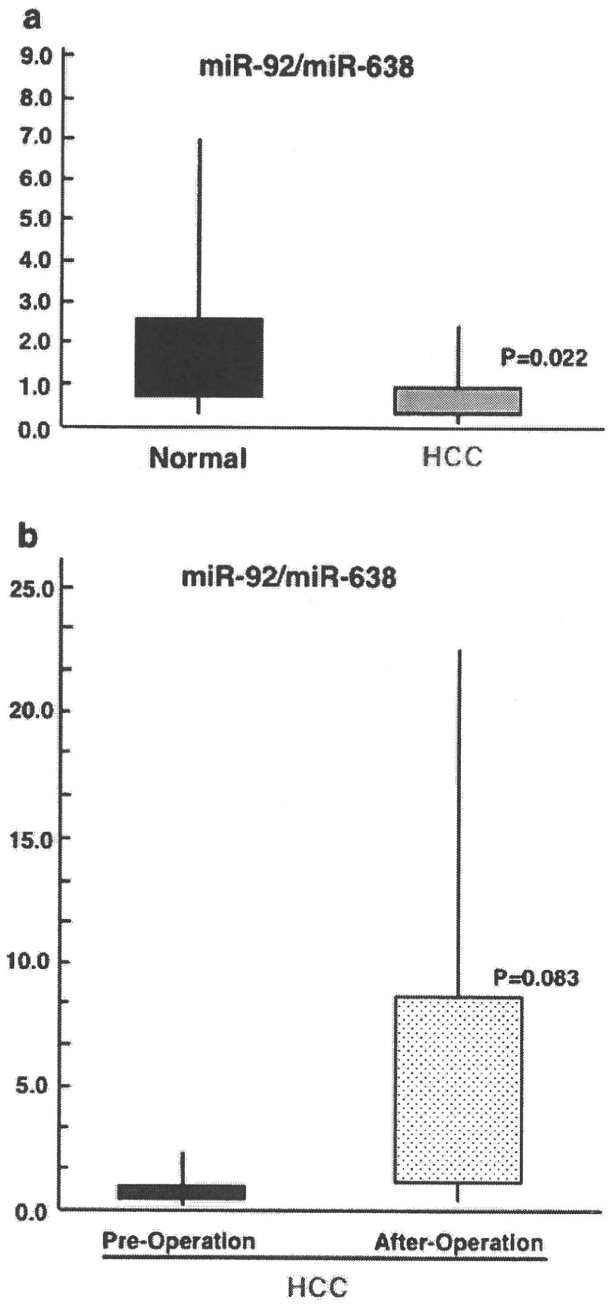

Figure 4 Comparison of miR-92a levels in the plasmas from normal individuals and hepatocellular carcinoma (HCC) patients. (**a**) The ratios of miR-92a to miR-638 in the plasmas from normal donors and HCC patients were analyzed by *Taq*Man qRT-PCR. Student's *t*-test was used to determine statistical significance. (**b**) The ratios of miR-92a to miR-638 in the plasmas from HCC patients before and after tumor resection were analyzed by *Taq*Man qRT-PCR.

the expression of p63 in HCC by immunohistochemistry. However, we could not find the positive nuclear staining both in HCC and normal hepatocyte (data not shown). On the other hand, the miRanda software found 300 different genes that have putative miR-92a binding sites conserved among *Homo sapiens*, *Mus musculus*, and *Rattus norvegicus* at the 3′-UTR regions of their transcripts. Therefore, at least in HCC, there may be novel miR-92a targets that are involved in cancer cell proliferation.

In this report, we have revealed that the value of miR-92a/miR-638 in plasma has potential as a very sensitive marker for HCC. We found that the ratio of miR-92a to miR-638 in the plasma samples from the HCC patients were decreased compared with that from the normal donors (Fig. 4a). We did not find any differences in the values of the ratios between hepatitis B virus (HBV) infection and hepatitis C virus (HCV) infection (data not shown). On the other hand, we recently observed decrease of miR-92a in plasma samples of acute leukemia.[13] These results suggest that the decrease of the miR-92a/miR-638 level in human plasma may serve as a valuable diagnostic marker for not only acute leukemia but also solid tumors such as HCC. Moreover, we observed increase of miR-92a/miR-638 levels in the plasmas from the HCC patients after tumor resection (Fig. 4b). Thus, the miR-92a/miR-638 levels in human plasmas may also be a potential noninvasive follow up marker of HCC. To confirm this notion, a large number of plasma samples should be examined. Nevertheless, the levels of miR-92a/miR-638 promise to be an effective biomarker for malignant tumors. The physiological significance of the decrease of miR-92a in plasma is still unknown.

In summary, we have shown that miR-92a may be involved in HCC development. In addition, we have demonstrated that the ratio of miR-92a/miR-638 in blood is expected to be useful for diagnosis of HCC patients. This study may also provide useful information for further investigations of functional association between miRNAs and HCC.

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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.

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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

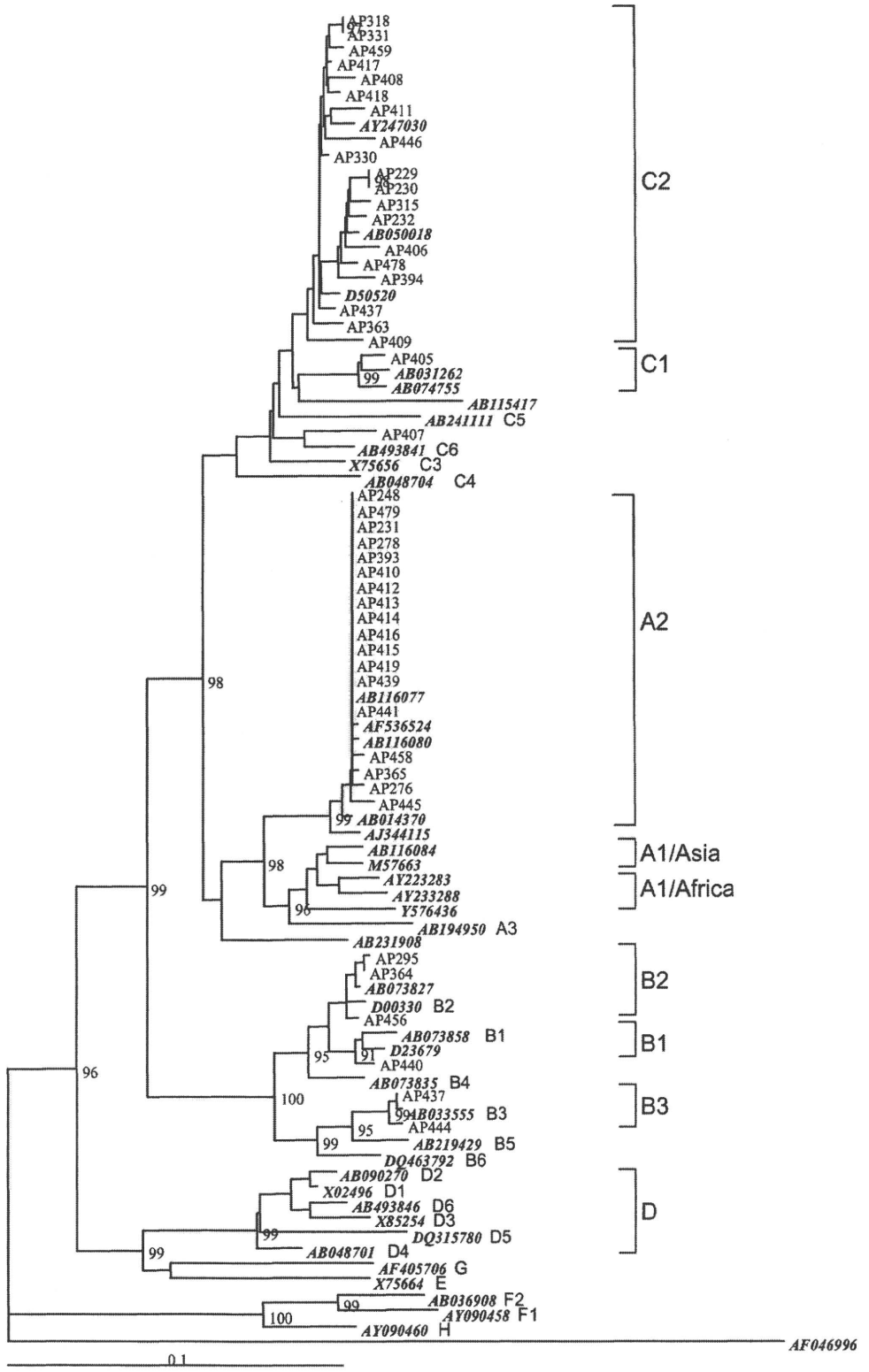

Figure 1 Results of phylogenetic analysis of 45 sequences from the preS region of hepatitis B virus (HBV) of acute hepatitis patients and 42 reference strains from a database and shown by accession number. Strains isolated from patients with acute hepatitis are indicated as AP. Phylogenetic analysis 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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