Measurements of sequences of amino acids 70 and 91 of core region, amino acid 139 of E1 region, and amino acid sequence 2209–2248 of NS5A region of hepatitis C virus

Amino acids 70 and 91 of the core region of HCV, amino acid 139 of the E1 region of HCV, and amino acid sequence of 2209–2248 of the NS5A region of HCV were analyzed by direct nucleotide sequencing of each region according to previous reports.[3,7,8] Hepatitis C virus was defined as wild type for the amino acid sequence of 2209–2248 of the NS5A region when the number of amino acid substitutions in comparison with HCV-J strain[24] was 0 or 1, and as non-wild type when the number of substitutions was greater than 1.

Statistical analyses

Quantitative values are shown as mean ± SD. Between-group differences were analyzed by the chi-square test. Differences in quantitative values between the two groups were analyzed by the Mann-Whitney *U*-test. Multivariate analysis was performed for baseline factors that affected the response to the combination therapy using a logistic regression model. The factors analyzed were age, sex, body weight, history of previous IFN therapy, serum alanine aminotransferase activity, serum aspartate aminotransferase activity, serum gamma-glutamyl transpeptidase, serum alkaline phosphatase, serum albumin, serum total bilirubin, white blood cell count, hemoglobin, platelet count, hepatitis activity grade (A0 and A1 *versus* A2 and A3), grade of liver fibrosis (F0 and F1 *versus* F2 and F3), pretreatment HCV RNA concentration, amino acids 70 and 91 of the HCV core protein, amino acid 139 of HCV E1 protein, and amino acid sequence 2209–2248 of the HCV NS5A protein. All statistical tests were two-tailed; $P < 0.05$ was accepted as statistically significant.

The study protocol was approved by the institutional review board and was in compliance with the Helsinki Declaration. Written informed consent was obtained from all patients prior to the study for use of the laboratory data.

Results

All 107 patients completed the entire treatment duration; no patient dropped-out of the study. Although reduction of PEG-IFN dose and ribavirin dose were experienced by 29 patients (27.1%) and 49 patients (45.8%), respectively, no patient discontinued either PEG-IFN or ribavirin. Eight patients (7.5%) had achieved RVR and 40 patients (37.4%) had achieved cEVR. HCV RNA was undetectable in 77 patients (72.0%) at the end of treatment (ETR). Outcomes following combination therapy were SVR in 39 patients (36.5%), relapse in 38 patients (35.5%), and NR in 30 patients (28.0%). Amino acid position 70 of the HCV protein core region was Arginine (Arg, reportedly associated with a good response to IFN) in 70 patients (65.4%) and Glutamine (Gln, reportedly associated with a poor response) in 29 patients (27.1%). Of the other eight patients, five had Histidine at that position, and in the other three HCV with both Arg and Gln at this position was detected. Amino acid position 91 of the HCV core region was Leucine (Leu, reportedly associated with a good response to IFN) in 76 patients (71.0%) and Methionine (Met, reportedly associated with a poor response) in 29 patients (27.1%). In the remaining two patients, HCV with both Leu and Met at this position was detected. Amino acid position 139 of the HCV E1 region was Threonine (Thr, reportedly associated with a good response to IFN) in 55 patients (51.4%) and Alanine at this position (Ala, reportedly associated with a poor response) in 40 patients (37.4%). From the remaining patients, the amino acid at position 139 was Serine in two patients, in four patients HCV with both Thr and Ala was detected; in HCV from four patients this position was deleted. In two patients we failed to amplify this region. At amino acid range 2209–2248 of the HCV NS5A region, HCV of 61 patients (57.0%) was wild type and that of 46 patients (43.0%) was non-wild type. There was no difference in baseline characteristics, including the rate of patients with reduction of PEG-IFN dose or ribavirin dose, between patients with Arg and Glu at amino acid position 70, between patients with Leu and Met at amino acid position 91, between patients with Thr and Ala at amino acid position 139, and between patients with non-wild type and wild type at amino acids range 2209–2248, respectively (data not shown).

Pretreatment HCV RNA concentration, and reduction in HCV RNA concentration at 24 h after the single administration of conventional interferon and after the start of combination therapy according to amino acid sequences

Pretreatment HCV RNA concentration, the reduction of HCV RNA concentration at 24 h after a single administration test of conventional IFN, and the reduction of HCV RNA concentration at 24 h after the start of combination therapy with PEG-IFN and ribavirin was compared between patients according to the amino acid sequence of the HCV (Table 2). Pretreatment HCV RNA concentration in patients with Arg at position 70 was significantly higher than in those with Gln at that position ($P = 0.0260$). Pretreatment HCV RNA concentration in patients with the wild type sequence at position 2209–2248 was significantly higher than that in patients with the non-wild type sequence ($P = 0.0002$). We found no difference in pretreatment HCV RNA concentration according to the identities of amino acids 91 or 139. The reduction of HCV RNA concentration in patients with Arg at position 70 was significantly more marked than in patients with Gln at that position ($P < 0.0001$). We found no difference in reduction of HCV RNA concentration after a single administration of conventional IFN according to the identities of amino acids 91 or 139, or the 2209–2248 sequence. Similarly, the reduction of HCV RNA concentration at 24 h after the start of combination therapy in patients with Arg at position 70 was significantly greater than that in patients with Gln at position 70 ($P = 0.0025$). We found no difference in reduction of HCV RNA concentration after a single administration of conventional IFN according to the identities of amino acids 91 or 139, or the sequence 2209–2248.

Response to combination therapy with peginterferon and ribavirin

The responses to combination therapy according to amino acid identity are summarized in Table 3. The rates of cEVR, ETR, and SVR were significantly higher in patients with Arg than in those with Gln at position 70 (all $P < 0.0001$), whereas we found no

Table 2 Association between amino acid substitutions and pretreatment HCV RNA concentration, reduction in HCV RNA concentration at 24 h after the single administration of conventional interferon (IFN) alpha-2b, and reduction in HCV RNA concentration at 24 h after the start of combination therapy with peginterferon (PEG-IFN) and ribavirin

	Core: amino acid70		Core: amino acid91	
	Arg ($n=70$)	Gln ($n=29$)	Leu ($n=76$)	Met ($n=29$)
Pretreatment HCV RNA concentration ($\times10^3$ IU/mL)	1943 ± 1294	1410 ± 895*	1755 ± 1151	1731 ± 1107
Reduction in HCV RNA concentration at 24 h after single administration ($\log_{10}$)	1.50 ± 0.45	0.95 ± 0.69**	1.41 ± 0.60	1.21 ± 0.49
Reduction in HCV RNA concentration at 24 h after combination therapy ($\log_{10}$)	1.34 ± 0.52	0.97 ± 0.57***	1.25 ± 0.54	1.30 ± 0.60
	E1: amino acid139		NS5A: amino acids 2209–2248	
	Thr ($n=55$)	Ala ($n=40$)	Non-wild ($n=46$)	Wild ($n=61$)
Pretreatment HCV RNA concentration ($\times10^3$ IU/mL)	1809 ± 985	1759 ± 1374	1290 ± 861	2115 ± 1205****
Reduction in HCV RNA concentration at 24 h after single administration ($\log_{10}$)	1.34 ± 0.64	1.40 ± 0.53	1.40 ± 0.60	1.32 ± 0.57
Reduction in HCV RNA concentration at 24 h after combination therapy ($\log_{10}$)	1.21 ± 0.53	1.24 ± 0.61	1.29 ± 0.57	1.25 ± 0.55

Mean ± SD.

*$P=0.0260$; **$P<0.0001$; ***$P=0.0025$; ****$P=0.0002$.

Ala, Alanine; Arg, Arginine; Gln, Glutamine; Leu, Leucine; Met, Methionine; Thr, Threonine.

Table 3 Association between amino acid substitutions and responses to combination therapy with peginterferon and ribavirin

	Core: amino acid70		Core: amino acid91		E1: amino acid139		NS5A: amino acids 2209–2248	
	Arg ($n=70$)	Gln ($n=29$)	Leu ($n=76$)	Met ($n=29$)	Thr ($n=47$)	Ala ($n=34$)	non-wild ($n=46$)	wild ($n=61$)
RVR	6 (55.7)	0	6 (43.4)	2 (44.8)	1 (46.8)	4 (38.2)	7 (15.2)	1 (1.6)**
cEVR	39 (55.7)	1 (3.5)*	33 (43.4)	13 (44.8)	22 (46.8)	13 (38.2)	22 (47.8)	24 (39.3)
ETR	58 (82.9)	12 (41.4)*	58 (76.3)	18 (62.1)	36 (76.6)	23 (67.6)	35 (76.1)	42 (68.9)
SVR	33 (47.2)	1 (3.5)*	31 (40.8)	8 (27.6)	19 (40.4)	11 (32.3)	21 (45.7)	18 (29.5)
Relapse	25 (35.6)	11 (37.9)	27 (35.5)	10 (34.5)	17 (36.2)	12 (35.4)	14 (30.4)	24 (39.3)
NR	12 (17.2)	17 (58.6)*	18 (23.7)	11 (37.9)	11 (23.4)	11 (32.3)	11 (23.9)	19 (31.1)

*$P<0.0001$; **$P=0.0229$.

Ala, Alanine; Arg, Arginine; cEVR, complete early virologic response; ETR, end-of-treatment response; Gln, Glutamine; Leu, Leucine; Met, Methionine; NR, no response; SVR, sustained virologic response; Thr, Threonine.

difference in the rates of cEVR, ETR, and SVR according to the amino acids at position 91 or 139, or the sequence 2209–2248. In contrast, the NR rate was significantly lower in patients with Arg at position 70 than in those with Gln at that position ($P<0.0001$). We found no difference in the rates of cEVR, ETR, or SVR according to the identities of amino acids 91 or 139, or the sequence 2209–2248. The results were the same when we focused on 72 treatment-naïve patients (data not shown; see Table S1).

Univariate and multivariate analyses for baseline factors affecting response to combination therapy with peginterferon and ribavirin

Univariate and multivariate analyses were conducted for baseline factors that could affect cEVR, ETR, or SVR. In univariate analysis, serum gamma-glutamyl transpeptidase level ($P=0.0025$), serum albumin level ($P=0.0008$), and the amino acid at position 70 of the HCV core region (Arg *versus* Gln, $P<0.0001$) were significantly associated with cEVR, and platelet count ($P=0.0707$) was associated with cEVR but not significantly. In multivariate analysis, the identity of amino acid 70 of the HCV core region ($P=0.0013$), serum albumin level ($P=0.0265$), and serum gamma-glutamyl transpeptidase level ($P=0.0308$) independently affected the rate of cEVR (Table 4). Serum gamma-glutamyl transpeptidase level ($P=0.0004$), serum albumin level ($P=0.0015$), white blood cell count ($P=0.0490$), and the identity of amino acid 70 ($P<0.0001$) were significantly associated with ETR, and serum alkaline phosphatase level ($P=0.0814$) was associated with ETR but not significantly by univariate analysis. By multivariate analysis, the identity of amino acid 70 ($P=0.0010$) and serum gamma-glutamyl transpeptidase level ($P=0.0055$) independently affected ETR (Table 5). In the analysis of factors affecting SVR, serum albumin level ($P=0.0069$), platelet count ($P=0.0238$), amino acid 70 of the HCV core region ($P<0.0001$) were significantly associated with SVR, and serum gamma-glutamyl transpeptidase level ($P=0.0832$) and the liver fibrosis grade (F0 and F1 *versus* F2 and F3, $P=0.0998$) were associated

Table 4 Multivariate analysis for factors affecting complete early virologic response

Factor		Parameter estimate	Standard error	X	Odds ratio (95% confidence interval)	*P* value
Gamma-glutamyl transpeptidase	by 1 IU	−0.0162	0.0075	4.66	0.0053 (0.0000–0.3807)	0.0308
Albumin	by 1.0 g/dL	2.3638	1.0650	4.93	181.33 (2.4858–25744)	0.0265
Platelet count	by 1.0 × 10^3/μL	0.0179	0.0554	0.10	1.5106 (0.1243–19.838)	0.7480
Core-70	1: Arginine				1	
	2: Glutamine	−1.7453	0.5429	10.33	0.0305 (0.0016–0.1719)	0.0013

Table 5 Multivariate analysis for factors affecting end-of-treatment response

Factor		Parameter estimate	Standard error	X	Odds ratio (95% confidence interval)	*P* value
Alkaline phosphatase	by 1 IU/L	−0.0028	0.0037	0.59	0.3034 (0.0137–6.6841)	0.4428
Gamma-glutamyl transpeptidase	by 1 IU	−0.0162	0.0058	7.72	0.0053 (0.0001–0.1738)	0.0055
Albumin	by 1.0 g/dL	1.2821	0.7700	2.77	16.787 (0.7440–626.72)	0.0959
White blood cell count	by 1/μL	0.0004	0.0002	3.09	12.603 (0.8685–269.45)	0.0787
Core-70	1: Arginine				1	
	2: Glutamine	−0.9882	0.3006	10.80	0.1386 (0.0400–0.4347)	0.0010

Table 6 Multivariate analysis for factors affecting sustained virologic response

Factor		Parameter estimate	Standard error	X	Odds ratio (95% confidence interval)	*P* value
Gamma-glutamyl transpeptidase	by 1 IU	−0.0015	0.0057	0.07	0.6201 (0.0121–20.764)	0.7962
Albumin	by 1.0 g/dL	1.4230	0.8788	2.62	22.888 (0.6793–1381.2)	0.1054
Platelet count	by 1.0 × 10^3/μL	0.0511	0.0556	0.84	3.2365 (0.2734–44.588)	0.3585
Fibrosis	1: F0/F1				1	
	2: F2/F3	0.0661	0.2976	0.05	1.1413 (0.3557–3.7626)	0.8242
Core-70	1: Arginine				1	
	2: Glutamine	−1.5097	0.5364	7.92	0.0488 (0.0026–0.2661)	0.0049

with SVR but not significantly by univariate analysis. By multivariate analysis, only amino acid 70 of the HCV core region independently affected SVR ($P = 0.0049$, Table 6).

Discussion

In the present study, we validated the association of several reported amino acid substitutions with IFN sensitivity and with the response to combination therapy with PEG-IFN and ribavirin. We found that total rate of SVR in the present study was considerably lower (36.5%) in comparison to previous reports on PEG-IFN and ribavirin combination therapy for patients with HCV genotype 1b. The reason for this was unknown. It could be partly because patients with a history of previous IFN therapy (retreatment cases) were included to the study population, partly because we focused on patients with higher pretreatment HCV RNA concentration, and partly because all patients completed the treatment at 48 weeks without elongation. In addition, more patients with HCV strain resistant to IFN might be included in the study patients, although we enrolled all patients who fulfilled the inclusion criteria.

We found a significant difference in pretreatment HCV RNA concentration according to the amino acid sequence 2209–2248 of the HCV NS5A protein. The original report[3] of an association between the amino acid sequence 2209–2248 of HCV NS5A and the response to IFN monotherapy showed a higher pretreatment HCV RNA concentration in patients with the wild type sequence of the HCV NS5A protein. Our results are in agreement with this result. We found a significant difference in the reduction in HCV RNA concentration at 24 h after a single administration of conventional IFN-alpha, according to the amino acid at position 70 of the HCV core protein. The reduction of HCV RNA concentration after a single administration of conventional IFN was significantly less marked in patients with Gln at position 70. The reduction after a single administration of conventional IFN was considered to reflect the resistance to IFN, and this result suggests a difference in sensitivity to IFN between HCV with Arg at position 70 and that with Gln at position 70. In previous studies, Jessner *et al.*[25,26] and Boulestin *et al.*[27] reported the importance of the decrease in serum HCV RNA concentration at 24 h after a single administration of conventional IFN in distinguishing HCV strains that are resistant to IFN-based antiviral therapy against HCV. We suggest that HCV with Glutamine at position 70 might be resistant to IFN-based therapy.

In the association between amino acid identities and the response to the combination therapy, the identity of the amino acid at position 70 was solely and strongly associated with cEVR, ETR, and SVR. In addition to the effect of the substitution of amino acid 70 of the HCV core region, Akuta *et al.*[8,28,29] reported the effect of the substitution of amino acid 91 of the HCV core region and Donlin *et al.*[7] suggested the effect of the substitution of amino acid 139 of the HCV E1 region on the response to the combination therapy with PEG-IFN and ribavirin in patients infected with HCV genotype 1b. More recently, Mori *et al.*[30] reported the effect of amino acid sequence 2209–2248 of the HCV NS5A region, in addition to the effect of amino acid 70 on the response to combination therapy in Japanese patients with HCV genotype 1b. In contrast to these reports, we did not find an association between these substitutions and the response to the combination therapy. The reason for this discrepancy is unknown. It might be partly because we focused on patients with high pretreatment HCV RNA concentration.

The predictive value for SVR of Arg at position 70 was not high (47.2%). In contrast, the predictive value for the lack of SVR of Gln at position 70 was extremely high (96.5%). The influence of amino acid identity at position 70 was therefore closely related to the resistance to IFN of HCV with Gln at position 70. Recently, Okanoue *et al.* reported the close association of amino acid identity at position 70 not with SVR but with the lack of SVR.[31] Our results supported their findings and, in addition, our results showed the direct resistance to IFN of HCV with Gln at position 70.

There are several limitations of the present study. The patients studied were all Japanese. The effect of amino acid 70 on combination therapy with PEG-IFN and ribavirin should therefore be verified in other ethnicities. Also, all patients studied had high pretreatment HCV RNA concentration ($>100 \times 10^5$ IU/mL). If HCV genotype 1b with amino acid 70 of Gln is resistant to IFN, the lower rates of cEVR, ETR, or SVR will be maintained in patients with HCV genotype 1b but with lower pretreatment HCV RNA concentration. This should also be clarified in the future. In addition, there are no reported mechanisms which could explain the effect of this amino acid substitution on the effectiveness of the combination therapy against HCV. This area should be studied in the future.

In conclusion, the results of the present study show that the substitution of amino acid 70 of the HCV core region was strongly associated with resistance to IFN and was independently associated with the response to the combination therapy with PEG-IFN and ribavirin in Japanese patients with HCV genotype 1b, presumably due to this resistance to IFN of HCV with Gln at position 70. The determination of the identity of amino acid 70 of the HCV core region can be useful for prediction of the resistance to combination therapy in this patient subpopulation, and can be useful for the decision of the indication of this therapy. Further studies in larger patient populations including multiple ethnicities will be needed to validate the significance of these amino acid substitutions and others which have been reported.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Table S1. Association between amino acid substitutions and responses to combination therapy with peginterferon and ribavirin in treatment-naïve patients.

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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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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

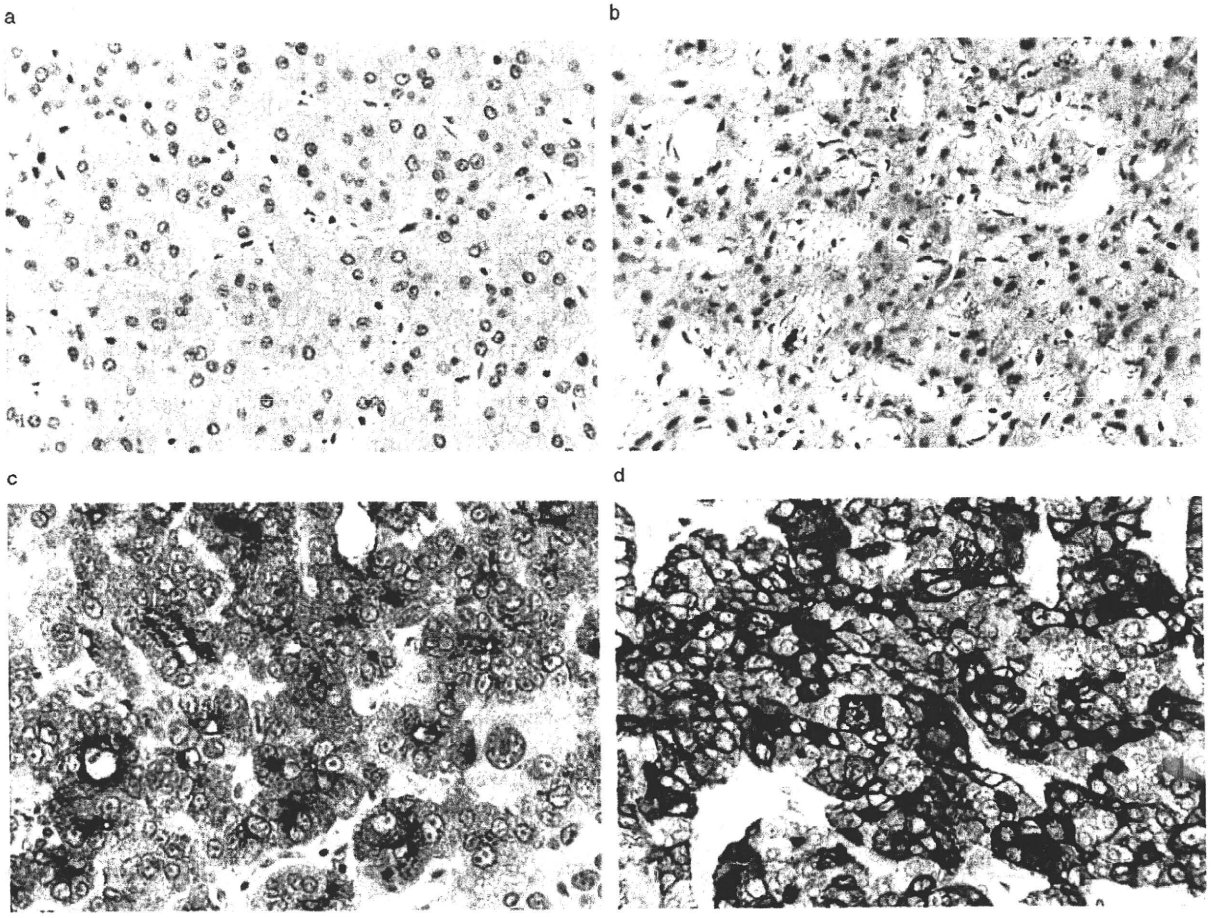

Figure 1 The degree of immunohistochemical staining for glypican-3. (a) No staining, (b) light staining, (c) moderate staining, (d) heavy staining.

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.

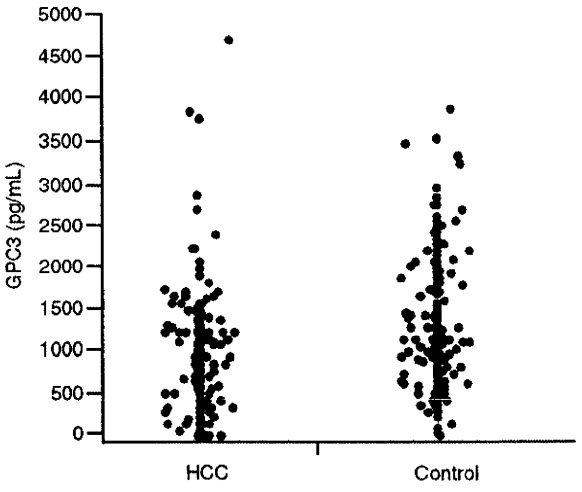

Figure 2 Serum glypican-3 (GPC3) level in patients with hepatocellular carcinoma (HCC) and in patients with chronic liver disease (CLD, control). Serum GPC3 level was higher in patients with CLD (1161.6 pg/mL) than those with HCC (924.8 pg/mL; $P < 0.0001$).

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

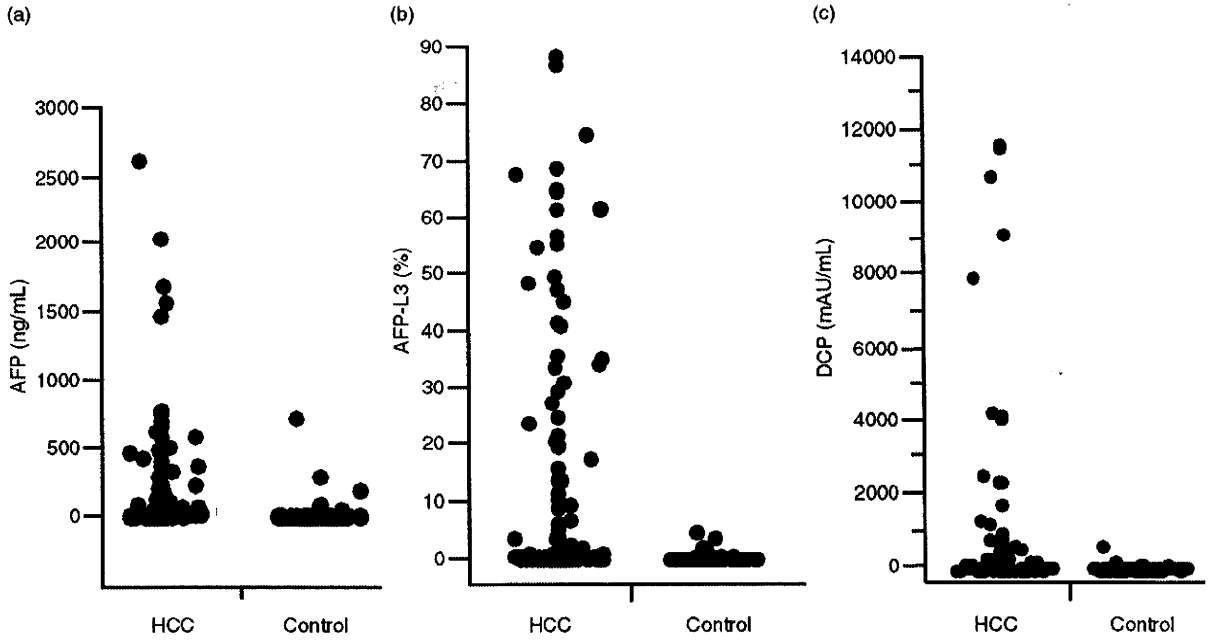

Figure 3 Serum alpha-fetoprotein (AFP), Lens culinaris agglutinin-reactive fraction of AFP (AFP-L3), and des-gamma carboxy prothrombin (DCP) levels in patients with hepatocellular carcinoma (HCC) and in patients with chronic liver disease (CLD, control). Serum AFP, AFP-L3, and DCP levels were significantly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

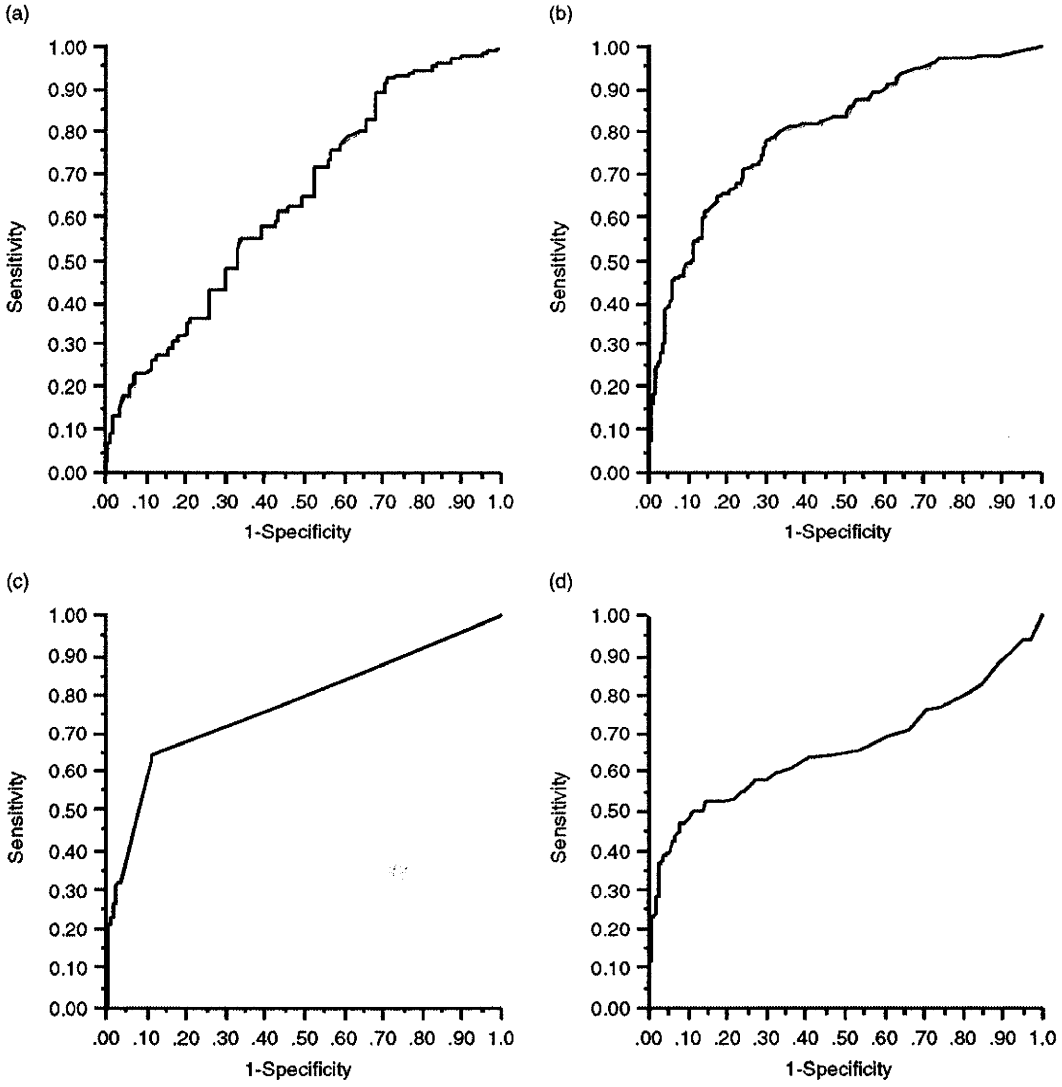

Figure 4 Area under the receiver-operating curve (AUROC) of (a) serum glypican-3 (GPC3), (b) alpha-fetoprotein (AFP), (c) Lens culinaris agglutinin-reactive fraction of AFP (AFP-L3), and (d) des-gamma carboxy prothrombin (DCP) for the diagnosis of hepatocellular carcinoma. AUROC was 0.64 for GPC3, 0.80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

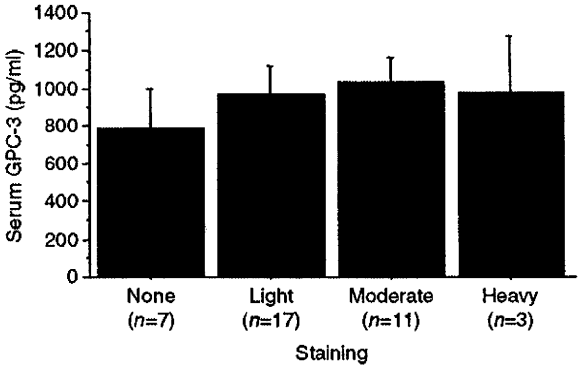

Figure 5 Serum glypican-3 (GPC3) level in 38 patients with hepatocellular carcinoma (HCC) who underwent hepatectomy according to the immunohistochemical staining of GPC3 on the resected HCC specimens. No association was found between serum GPC3 level and immunohistochemical staining of GPC3 on HCC tissues.

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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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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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

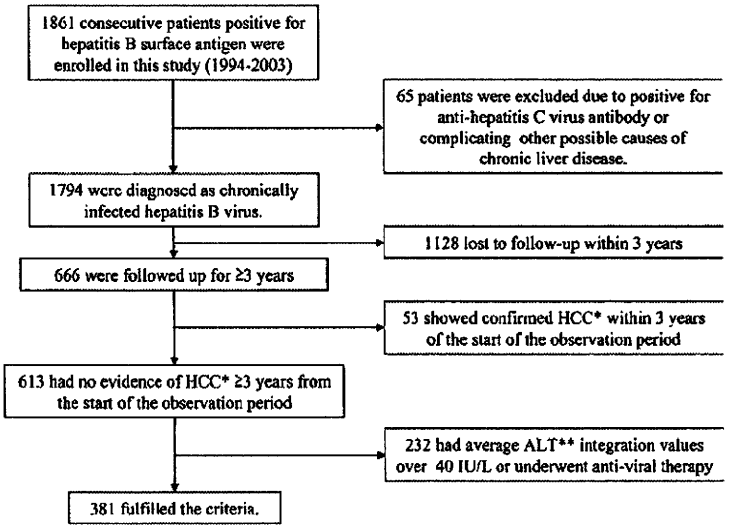

Fig. 1. Schematic flowchart of enrolled patients. *, hepatocellular carcinoma (HCC); **, alanine aminotransferase (ALT).

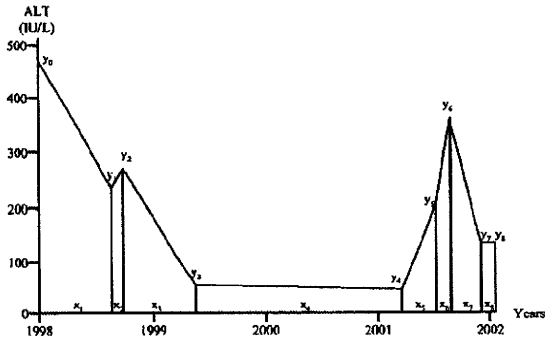

Fig. 2. Integration value of alanine aminotransferase (ALT). The integration value of ALT was calculated as follows: $(y_0+y_1)\times x_1/2+(y_1+y_2)\times x_2/2+(y_2+y_3)\times x_3/2+(y_3+y_4)\times x_4/2+(y_4+y_5)\times x_5/2+(y_5+y_6)\times x_6/2+(y_6+y_7)\times x_7/2+(y_7+y_8)\times x_8/2$. The integration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

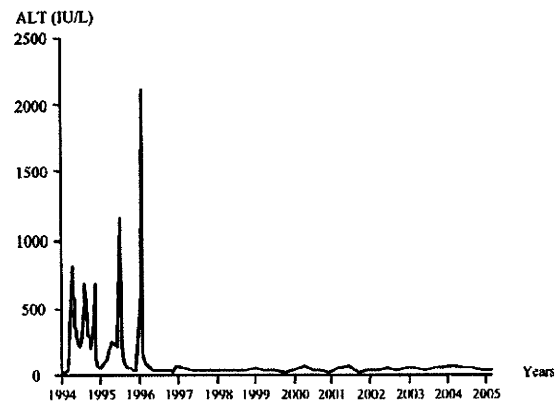

Fig. 3. Average integration value and arithmetic mean value of alanine aminotransferase (ALT) in a 26-year-old patient with hepatitis B virus (HBV). The patient was followed-up for 11.2 years. The number of ALT examinations was 96. The integration value of ALT was 955.2 IU/L × years. The average integration 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.

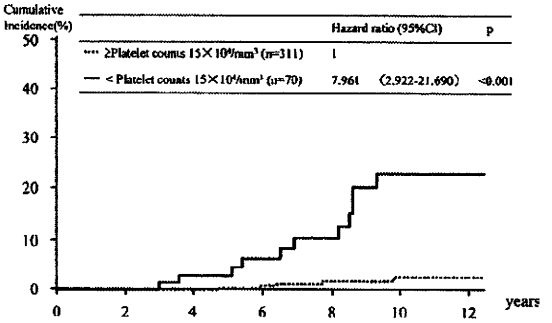

Fig. 4. Incidence of HCC according to platelet counts. The 5- and 10-year cumulative incidences of HCC was 0.4% and 2.6%, respectively, in patients with platelet counts $\geq 15.0 \times 10^4/mm^3$ (n = 311), and 2.9% and 22.9% in patients with platelet counts $<15.0 \times 10^4/mm^3$ (n = 70). The cumulative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

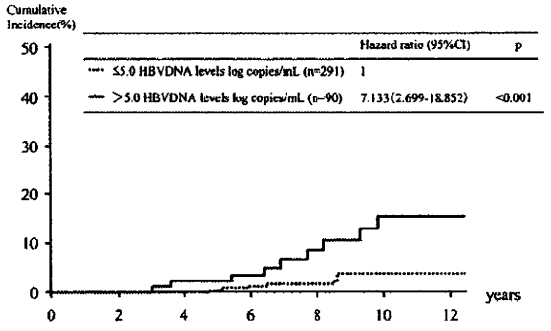

Fig. 5. Incidence of HCC according to serum HBV-DNA levels. The 5- and 10-year cumulative incidences of HCC was 0.4% and 3.7%, respectively, in patients with HBV-DNA levels <5.0 log copies/ml (n = 291) and 2.3% and 15.5%, respectively, in patients with HBV-DNA levels ≥5.0 log copies/ml (n = 90). The 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 ($\times 10^4$/mm^3)	Group A ≤5.0 ≥15 × 10^4 (n = 257)	Group B ≤5.0 <15 × 10^4 (n = 45)	Group C >5.0 ≥15 × 10^4 (n = 54)	Group D >5.0 <15 × 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 15.0×10^4/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.

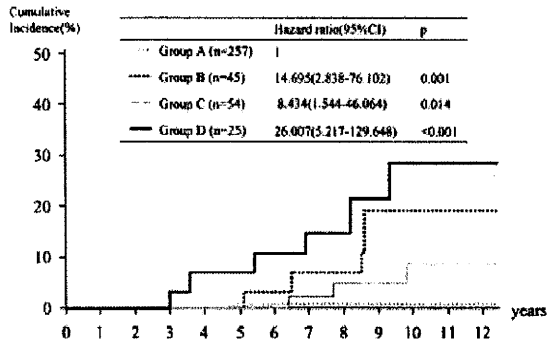

Fig. 6. The cumulative incidence of HCC according to HBV-DNA levels and platelet counts. HBV carriers with normal ALT levels were divided into four groups (A: HBV-DNA levels <5.0 log copies/ml and platelet counts ≥15.0 × 10^4/mm^3 [n = 257]; B: HBV-DNA levels <5.0 log copies/ml and platelet counts <15.0 × 10^4/mm^3 [n = 45]; C: HBV-DNA levels ≥5.0 log copies/ml and platelet counts ≥15.0 × 10^4/mm^3 [n = 54]; and D: HBV-DNA levels ≥5.0 log copies/ml and platelet counts <15.0 × 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