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.

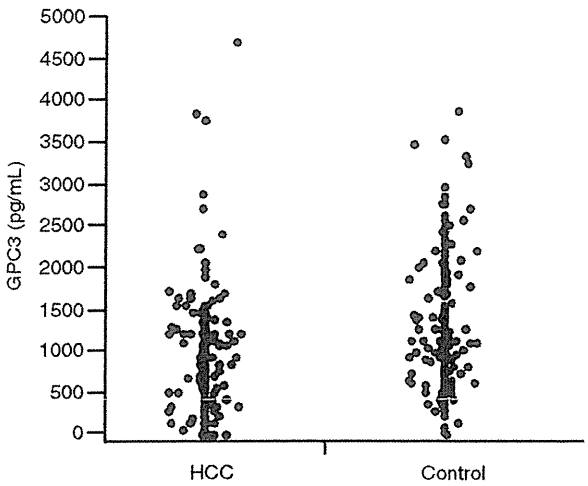


Figure 2 Serum glypican-3 (GPC3) level in patients with hepatocellular carcinoma (HCC) and in patients with chronic liver disease (CLD, control). Serum GPC3 level was higher in patients with CLD (1161.6 pg/mL) than those with HCC (924.8 pg/mL; $P < 0.0001$).

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

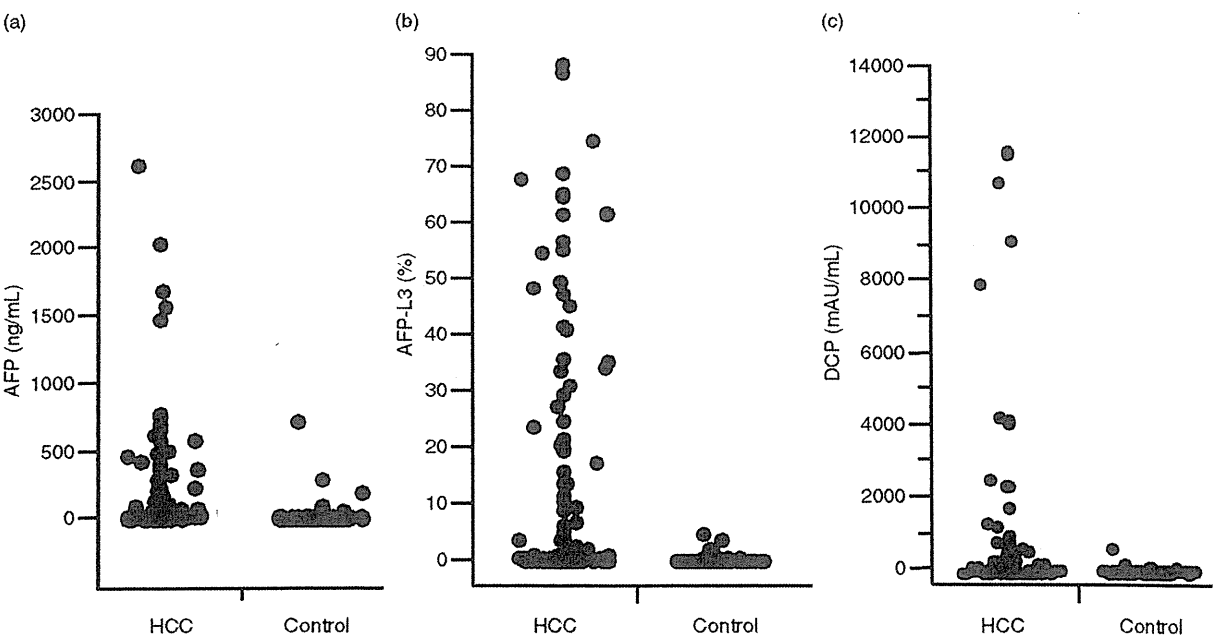


Figure 3 Serum alpha-fetoprotein (AFP), Lens culinaris agglutinin-reactive fraction of AFP (AFP-L3), and des-gamma carboxy prothrombin (DCP) levels in patients with hepatocellular carcinoma (HCC) and in patients with chronic liver disease (CLD, control). Serum AFP, AFP-L3, and DCP levels were significantly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

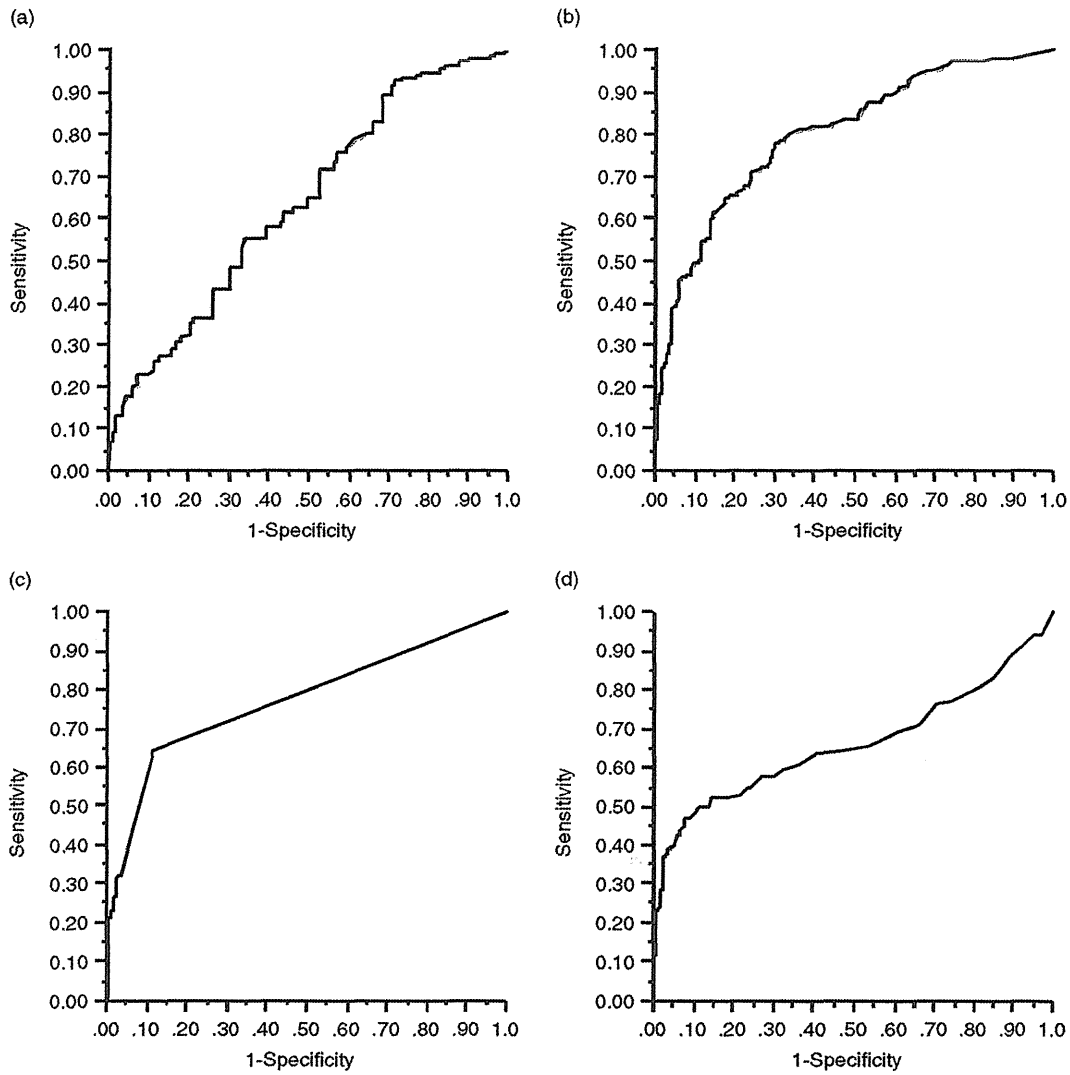


Figure 4 Area under the receiver-operating curve (AUROC) of (a) serum glypican-3 (GPC3), (b) alpha-fetoprotein (AFP), (c) Lens culinaris agglutinin-reactive fraction of AFP (AFP-L3), and (d) des-gamma carboxy prothrombin (DCP) for the diagnosis of hepatocellular carcinoma. AUROC was 0.64 for GPC3, 0.80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

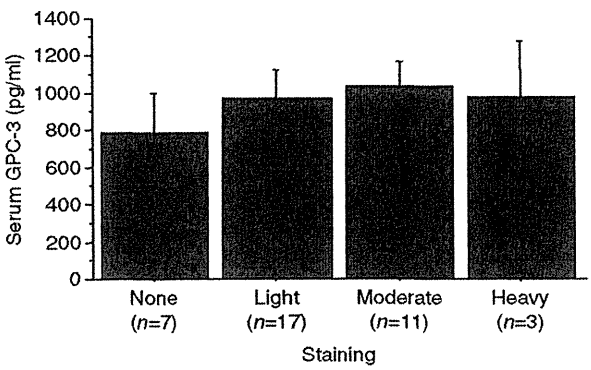


Figure 5 Serum glypican-3 (GPC3) level in 38 patients with hepatocellular carcinoma (HCC) who underwent hepatectomy according to the immunohistochemical staining of GPC3 on the resected HCC specimens. No association was found between serum GPC3 level and immunohistochemical staining of GPC3 on HCC tissues.

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.

REFERENCES

1 Parkin D, Bray F, Ferlay J, Pisani P. Global Cancer Statistics, 2002. *CA Cancer J Clin* 2002; **55**: 74–108.
2 Umemura T, Kiyosawa K. Epidemiology of hepatocellular carcinoma in Japan. *Hepatol Res* 2007; **37**: S95–100.
3 Zaman SN, Melia WM, Johnson RD, Portmann BC, Johnson PJ, Williams R. Risk factors in development of hepatocellular carcinoma in cirrhosis: prospective study of 613 patients. *Lancet* 1985; **1**: 1357–60.
4 Poynard T, Aubert A, Lazizi Y *et al.* Independent risk factors for hepatocellular carcinoma in French drinkers. *Hepatology* 1991; **13**: 896–901.
5 Colombo M, de Franchis R, Ninno ED *et al.* Hepatocellular carcinoma in Italian patients with cirrhosis. *N Engl J Med* 1991; **325**: 675–80.
6 Tsukuma H, Hiyama T, Tanaka S *et al.* The factors for hepatocellular carcinoma among patients with chronic liver disease. *N Engl J Med* 1993; **328**: 1797–801.
7 Chevret S, Trinchet JC, Mathieu D, Rached AA, Beaugrand M, Chastang C. A new prognostic classification for predicting survival in patients with hepatocellular carcinoma. *J Hepatol* 1999; **31**: 133–41.
8 Abelev GI. Production of embryonal serum alpha-globulin by hepatomas: review of experimental and clinical data. *Cancer Res* 1968; **28**: 1344–50.
9 O'Connor GI, Tatarinov YS, Abelev GI, Uriel J. A collaborative study for the evaluation of a serologic test for primary liver cancer. *Cancer* 1970; **25**: 1091–8.
10 Di Bisceglie AM, Hoofnagle JH. Elevations in serum alpha-fetoprotein levels in patients with chronic hepatitis B. *Cancer* 1989; **64**: 2117–20.
11 Di Bisceglie AM, Sterling RK, Chung RT *et al.* Serum alpha-fetoprotein levels in patients with advanced hepatitis C: results from the HALT-C Trial. *J Hepatol* 2005; **43**: 434–41.

12 Taketa K, Sekiya C, Namiki M *et al.* Lectin-reactive profiles of alpha-fetoprotein characterizing hepatocellular carcinoma and related conditions. *Gastroenterology* 1990; **99**: 508–18.
13 Taketa K, Endo Y, Sekiya C *et al.* A collaborative study for the evaluation of lectin-reactive a-fetoproteins in early detection of hepatocellular carcinoma. *Cancer Res* 1993; **53**: 5419–23.
14 Oka H, Saito A, Ito K *et al.* Multicenter prospective analysis of newly diagnosed hepatocellular carcinoma with respect to the percentage of Lens culinaris agglutinin-reactive a-fetoprotein. *J Gastroenterol Hepatol* 2001; **16**: 1378–83.
15 Liebman HA. Isolation and characterization of a hepatoma-associated abnormal (des-gamma carboxy) prothrombin. *Cancer Res* 1989; **49**: 6493–7.
16 Okuda H, Obata H, Nakanishi T, Furukawa R, Hashimoto E. Production of abnormal prothrombin (des-gamma-carboxy prothrombin) by hepatocellular carcinoma. A clinical and experimental study. *J Hepatol* 1987; **4**: 357–63.
17 Yano Y, Yamashita F, Kuwaki K *et al.* Clinical features of hepatitis C virus-related hepatocellular carcinoma and their association with alpha-fetoprotein and protein induced by vitamin K absence or antagonist-II. *Liver Int* 2006; **26**: 789–95.
18 Toyoda H, Kumada T, Kiriyama S *et al.* Prognostic significance of simultaneous measurement of three tumor markers in patients with hepatocellular carcinoma. *Clin Gastroenterol Hepatol* 2006; **4**: 111–17.
19 Filmus J, Church J, Buick R. Isolation of a cDNA corresponding to a developmentally regulated transcript in rat intestine. *Mol Cell Biol* 1988; **8**: 4243–9.
20 Filmus J, Selleck S. Glypicans: proteoglycans with a suprise. *J Clin Invest* 2001; **108**: 497–501.
21 Bernfield M, Gotte M, Park P *et al.* Function of cell surface heparan sulfate proteoglycans. *Annu Rev Biochem* 1999; **68**: 729–77.
22 Libbrecht L, Severi T, Cassiman D *et al.* Glypican-3 expression distinguishes small hepatocellular carcinomas from cirrhosis, dysplastic nodules, and focal nodular hyperplasia-like nodules. *Am J Surg Pathol* 2006; **30**: 1405–11.
23 Tommaso LD, Franchi G, Park Y *et al.* Diagnostic value of HSP70, glypican 3, and glutamine synthetase in hepatocellular nodules in cirrhosis. *Hepatology* 2007; **45**: 725–34.
24 Wang H, Anatelli F, Zhai Q, Adley B, Chuang S, Yang X. Glypican-3 as a useful diagnostic marker that distinguishes hepatocellular carcinoma from benign hepatocellular mass lesions. *Arch Pathol Lab Med* 2008; **132**: 1723–8.
25 Capurro M, Wanless I, Sherman M *et al.* Glypican-3: A novel serum and histochemical marker for hepatocellular carcinoma. *Gastroenterology* 2003; **125**: 89–97.
26 Nakatsura T, Yoshitake Y, Senju S *et al.* Glypican-3, overexpressed specifically in human hepatocellular carcinoma, is a novel tumor marker. *Biochem Biophys Res Commun* 2003; **306**: 16–25.
27 Hippo Y, Watanabe K, Watanabe A *et al.* Identification of soluble NH_2-terminal fragment of glypican-3 as a serological marker for early-stage hepatocellular carcinoma. *Cancer Res* 2004; **64**: 2418–23.
28 Torzilli G, Minagawa M, Takayama T *et al.* Accurate preoperative evaluation of liver mass lesions without fine-needle biopsy. *Hepatology* 1999; **30**: 889–93.
29 Kudo M. Imaging diagnosis of hepatocellular carcinoma and premalignant/ borderline lesions. *Semin Liver Dis* 1999; **19**: 297–309.
30 Katoh H, Nakamura K, Tanaka T, Satomura S, Matsuura S. Automatic and simultaneous analysis of Lens culinaris agglutinin-reactive alpha-fetoprotein ratio and total alpha-fetoprotein concentration. *Anal Chem* 1998; **70**: 2110–14.
31 Yamagata Y, Katoh H, Nakamura K, Tanaka T, Satomura S, Matsuura S. Determination of alpha-fetoprotein concentration based on liquid-phase binding assay using anion exchange chromatography and sulfated peptide introduced antibody. *J Immunol Methods* 1998; **212**: 161–8.
32 Okuda H, Nakanishi T, Takatsu K *et al.* Measurement of serum levels of des-gamma-carboxy prothrombin in patients with hepatocellular carcinoma by a revised enzyme immunoassay kit with increased sensitivity. *Cancer* 1999; **85**: 812–18.
33 Liver cancer Study Group of Japan. *The General Rules for the Clinical and Pathological Study of Primary Liver Cancer.* English edn. Tokyo: Kanehara & Co, 2003.
34 Wang X, Degos F, Dubois S *et al.* Glypican-3 expression in hepatocellular tumors: diagnostic value for preneoplastic lesions and hepatocellular carcinomas. *Hum Pathol* 2006; **37**: 1435–41.
35 Shirakawa H, Suzuki H, Shimomura M *et al.* Glypican-3 expression is correlated with poor prognosis in hepatocellular carcinoma. *Cancer Sci* 2009; **100**: 1403–7.
36 Beale G, Chattopadhyay D, Gray J *et al.* AFP, PIVKAII, GP3, SCCA-1 and follisatin as surveillance biomarkers for hepatocellular cancer in non-alcoholic and alcoholic fatty liver disease. *BMC Cancer* 2008; **8**: 200.
37 Capurro M, Filmus J. Glypican-3 as a serum marker for hepatocellular carcinoma. *Cancer Res* 2005; **65**: 372–3.

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

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

Takashi Kumada,* Hidenori Toyoda, Seiki Kiriyama, Yasuhiro Sone, Makoto Tanikawa, Yasuhiro Hisanaga, Akira Kanamori, Hiroyuki Atsumi, Makiko Takagi, Takahiro Arakawa, and Masashi Fujimori

Department of Gastroenterology, Ogaki Municipal Hospital, Ogaki, Gifu, Japan

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.

*Correspondence to: Takashi Kumada, 4-86, Minaminokawa-cho, Ogaki, Gifu 503-8052, Japan.
E-mail: hosp3@omh.ogaki.gifu.jp

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

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

MATERIALS AND METHODS

Patient Selection

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

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

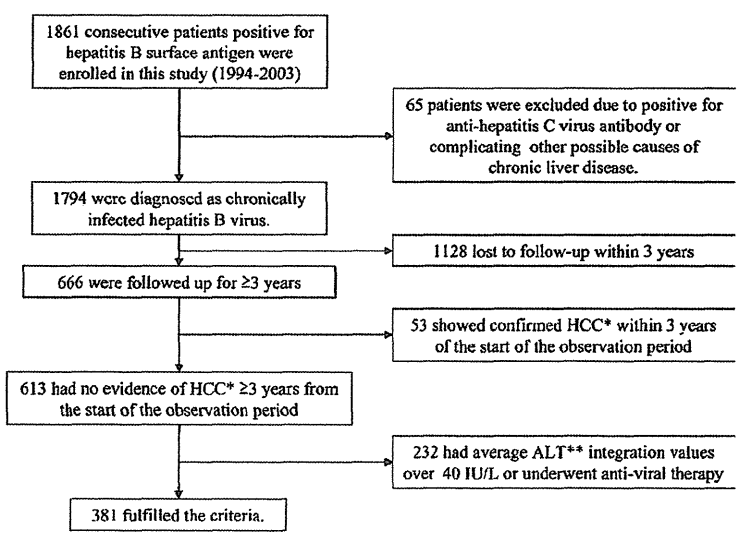


Fig. 1. Schematic flowchart of enrolled patients. *, hepatocellular carcinoma (HCC); **, alanine aminotransferase (ALT).

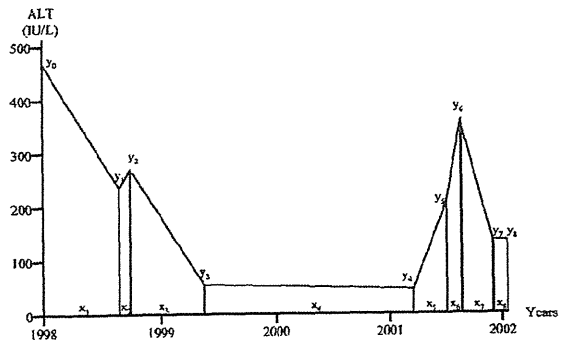


Fig. 2. Integration value of alanine aminotransferase (ALT). The integration value of ALT was calculated as follows: $(y_0+y_1)\times x_1/2+(y_1+y_2)\times x_2/2+(y_2+y_3)\times x_3/2+(y_3+y_4)\times x_4/2+(y_4+y_5)\times x_5/2+(y_5+y_6)\times x_6/2+(y_6+y_7)\times x_7/2+(y_7+y_8)\times x_8/2$. The integration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

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

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

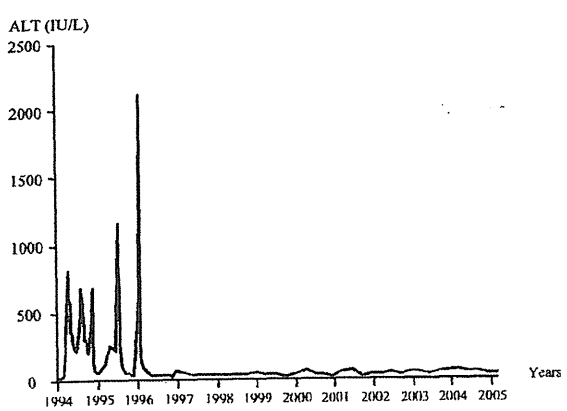


Fig. 3. Average integration value and arithmetic mean value of alanine aminotransferase (ALT) in a 26-year-old patient with hepatitis B virus (HBV). The patient was followed-up for 11.2 years. The number of ALT examinations was 96. The integration value of ALT was 955.2 IU/L × years. The average integration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

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

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

Statistical Analysis

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

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

RESULTS

Patient Characteristics

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

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

Factors Associated With the Incidence of HCC

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

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

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

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

TABLE I. Patient Characteristics

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

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

TABLE II. Factors Associated With Hepatocarcinogenesis (Univariate Analysis)

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

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

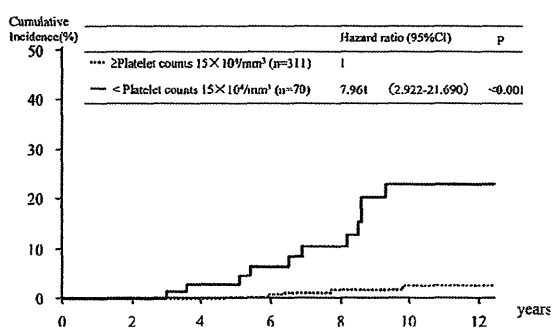


Fig. 4. Incidence of HCC according to platelet counts. The 5- and 10-year cumulative incidences of HCC was 0.4% and 2.6%, respectively, in patients with platelet counts $\geq 15.0 \times 10^4/mm^3$ (n = 311), and 2.9% and 22.9% in patients with platelet counts $<15.0 \times 10^4/mm^3$ (n = 70). The cumulative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

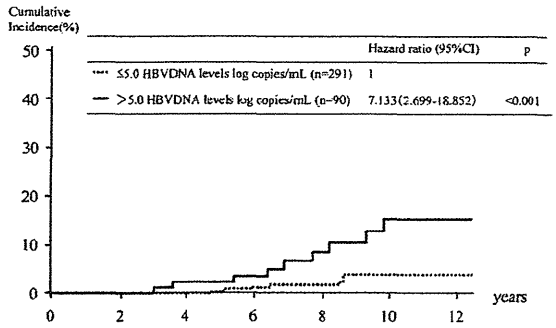


Fig. 5. Incidence of HCC according to serum HBV-DNA levels. The 5- and 10-year cumulative incidences of HCC was 0.4% and 3.7%, respectively, in patients with HBV-DNA levels <5.0 log copies/ml (n = 291) and 2.3% and 15.5%, respectively, in patients with HBV-DNA levels ≥5.0 log copies/ml (n = 90). The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

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

DISCUSSION

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

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

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

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

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

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

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

Lok and McMahon [2004] reported that HBV-DNA levels >10^5 copies/ml should be considered clinically significant. Their recommendation is supported by a meta-analysis of 26 trials of anti-HBV therapy which evaluated the association between viral load and hepatic inflammatory activity, as determined by hepatic histology and aminotransferase activity [Mommeja-Marin et al., 2003]. Thus, it is important for patients to maintain low HBV-DNA levels (i.e., ≤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.

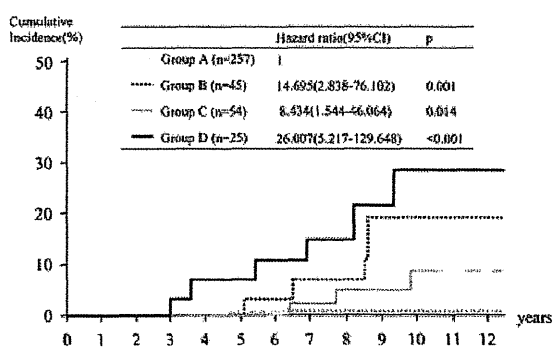


Fig. 6. The cumulative incidence of HCC according to HBV-DNA levels and platelet counts. HBV carriers with normal ALT levels were divided into four groups (A: HBV-DNA levels <5.0 log copies/ml and platelet counts ≥15.0 × 10^4/mm^3 [n = 257]; B: HBV-DNA levels <5.0 log copies/ml and platelet counts <15.0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

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

REFERENCES

Beasley RP. 1988. Hepatitis B virus. The major etiology of hepatocellular carcinoma. Cancer 61:1942–1956.

Beasley RP, Hwang LY, Lin CC, Chien CS. 1981. Hepatocellular carcinoma and hepatitis B virus. A prospective study of 22707 men in Taiwan. Lancet 2:1129–1133.

Caturelli E, Castellano L, Fusilli S, Palmentieri B, Niro GA, del Vecchio-Blanco C, Andriulli A, de Sio I. 2003. Coarse nodular US pattern in hepatic cirrhosis: Risk for hepatocellular carcinoma. Radiology 226:691–697.

Chan HL, Hui AY, Wong ML, Tse AM, Hung LC, Wong VW, Sung JJ. 2004. Genotype C hepatitis B virus infection is associated with an increased risk of hepatocellular carcinoma. Gut 53:1494–1498.

Chen CJ, Yang HI, Su J, Jen CL, You SL, Lu SN, Huang GT, Iloeje UH. 2006. REVEAL-HBV Study Group: Risk of hepatocellular carcinoma across a biological gradient of serum hepatitis B virus DNA level. JAMA 295:65–73.

D'Arienzo A, Manguso F, Scaglione G, Vicinanza G, Bennato R, Mazzacca G. 1998. Prognostic value of progressive decrease in serum cholesterol in predicting survival in Child-Pugh C viral cirrhosis. Scand J Gastroenterol 33:1213–1218.

EASL Jury. 2003. EASL International Consensus Conference on Hepatitis B. 13–14 September, 2002: Geneva, Switzerland. Consensus statement (short version). J Hepatol 38:533–540.

Iacobellis A, Fusilli S, Mangia A, Clemente R, Festa V, Giacobbe A, Facciorusso D, Niro G, Conoscitore P, Andriulli A. 2005. Ultrasonographic and biochemical parameters in the non-invasive evaluation of liver fibrosis in hepatitis C virus chronic hepatitis. Aliment Pharmacol Ther 22:769–774.

Ikeda K, Arase Y, Kobayashi M, Someya T, Hosaka T, Saitoh S, Sezaki H, Akuta N, Suzuki F, Suzuki Y, Kumada H. 2005. Hepatitis B virus-related hepatocellular carcinogenesis and its prevention. Intervirology 48:29–38.

Kato H, Orito E, Sugauchi F, Ueda R, Gish RG, Usuda S, Miyakawa Y, Mizokami M. 2001. Determination of hepatitis B virus genotype G by polymerase chain reaction with hemi-nested primers. J Virol Methods 98:153–159.

Kiyosawa K, Umemura T, Ichijo T, Matsumoto A, Yoshizawa K, Gad A, Tanaka E. 2004. Hepatocellular carcinoma: Recent trends in Japan. Gastroenterology 127:S17–S26.

Kudo M. 1999. Imaging diagnosis of hepatocellular carcinoma and premalignant/borderline lesions. Semin Liver Dis 19:297–309.

Kumada T, Toyoda H, Kiriyama S, Sone Y, Tanikawa M, Hisanaga Y, Kanamori A, Kondo J, Yamauchi T, Nakano S. 2007. Relation between incidence of hepatic carcinogenesis and integration value of alanine aminotransferase in patients with hepatitis C virus infection. Gut 56:738–739.

Liaw YF, Lin DY, Chen TJ, Chu CM. 1989. Natural course after the development of cirrhosis in patients with chronic type B hepatitis: A prospective study. Liver 9:235–241.

Liaw YF, Sung JJ, Chow WC, Farrell G, Lee CZ, Yuen H, Tanwandee T, Tao QM, Shue K, Keene ON, Dixon JS, Gray DF, Sabbat J. 2004. Cirrhosis Asian Lamivudine Multicentre Study Group. Lamivudine for patients with chronic hepatitis B and advanced liver disease. N Engl J Med 351:1521–1531.

Lok AS, McMahon BJ. 2004. Practice Guidelines Committee, American Association for the Study of Liver Diseases (AASLD): Chronic hepatitis B: Update of recommendations. Hepatology 39:857–861.

Lu SN, Wang JH, Liu SL, Hung CH, Chen CH, Tung HD, Chen TM, Huang WS, Lee CM, Chen CC, Changchien CS. 2006. Thrombocytopenia as a surrogate for cirrhosis and a marker for the identification of patients at high-risk for hepatocellular carcinoma. Cancer 107:2212–2222.

McMahon BJ, Holck P, Bulkow L, Snowball M. 2001. Serologic and clinical outcomes of 1536 Alaska Natives chronically infected with hepatitis B virus. Ann Intern Med 135:759–768.

Mommeja-Marin H, Mondou E, Blum MR, Rousseau F. 2003. Serum HBV DNA as a marker of efficacy during therapy for chronic HBV infection: Analysis and review of the literature. Hepatology 37:1309–1319.

Murata K, Sugimoto K, Shiraki K, Nakano T. 2005. Relative predictive factors for hepatocellular carcinoma after HBeAg seroconversion in HBV infection. World J Gastroenterol 11:6848–6852.

Orito E, Ichida T, Sakugawa H, Sata M, Horiike N, Hino K, Okita K, Okanoue T, Iino S, Tanaka E, Suzuki K, Watanabe H, Hige S, Mizokami M. 2001. Geographic distribution of hepatitis B virus (HBV) genotype in patients with chronic HBV infection in Japan. Hepatology 34:590–594.

Piao CY, Fujioka S, Iwasaki Y, Fujio K, Kaneyoshi T, Araki Y, Hashimoto K, Senoh T, Terada R, Nishida T, Kobashi H, Sakaguchi K, Shiratori Y. 2005. Lamivudine treatment in patients with HBV-related hepatocellular carcinoma–using an untreated, matched control cohort. Acta Med Okayama 59:217–224.

Prati D, Taioli E, Zanella A, Della Torre E, Butelli S, Del Vecchio E, Vianello L, Zanuso F, Mozzi F, Milani S, Conte D, Colombo M, Sirchia G. 2002. Updated definitions of healthy ranges for serum alanine aminotransferase levels. Ann Intern Med 137:1–10.

Rustgi VK. 1987. Epidemiology of hepatocellular carcinoma. Gastroenterol Clin North Am 16:545–551.

Shen L, Li JQ, Zeng MD, Lu LG, Fan ST, Bao H. 2006. Correlation between ultrasonographic and pathologic diagnosis of liver fibrosis due to chronic virus hepatitis. World J Gastroenterol 28:1292–1295.

Sherman M. 2005. Predicting survival in hepatitis B. Gut 54:1521–1523.

Szmuness W. 1978. Hepatocellular carcinoma and the hepatitis B virus: Evidence for a causal association. Prog Med Virol 24:40–69.

Torzilli G, Minagawa M, Takayama T, Inoue K, Hui AM, Kubota K, Ohtomo K, Makuuchi M. 1999. Accurate preoperative evaluation of liver mass lesions without fine-needle biopsy. Hepatology 30:889–893.

Wong CH, Chan SK, Chan HL, Tsui SK, Feitelson M. 2006. The molecular diagnosis of hepatitis B virus-associated hepatocellular carcinoma. Crit Rev Clin Lab Sci 43:69–101.

Yang HI, Lu SN, Liaw YF, You SL, Sun CA, Wang LY, Hsiao CK, Chen PJ, Chen DS, Chen CJ. 2002. Taiwan Community-Based Cancer Screening Project Group. Hepatitis B e antigen and the risk of hepatocellular carcinoma. N Engl J Med 347:168–174.

Yu MW, Chen CJ. 1994. Hepatitis B and C viruses in the development of hepatocellular carcinoma. Crit Rev Oncol Hematol 17:71–91.

Yu MW, Chang HC, Chen PJ, Liu CJ, Liaw YF, Lin SM, Lee SD, Lin SC, Lin CL, Chen CJ. 2002. Increased risk for hepatitis B-related liver cirrhosis in relatives of patients with hepatocellular carcinoma in northern Taiwan. Int J Epidemiol 31:1008–1015.

Yu MW, Yeh SH, Chen PJ, Liaw YF, Lin CL, Liu CJ, Shih WL, Kao JH, Chen DS, Chen CJ. 2005. Hepatitis B virus genotype and DNA level and hepatocellular carcinoma: A prospective study in men. J Natl Cancer Inst 97:265–272.

Yuen MF, Yuan HJ, Wong DK, Yuen JC, Wong WM, Chan AO, Wong BC, Lai KC, Lai CL. 2005. Prognostic determinants for chronic hepatitis B in Asians: Therapeutic implications. Gut 54:1610–1614.

肝腫瘤の超音波診断基準（案）

日本超音波医学会用語・診断基準委員会
委員長　貴田岡正史

「肝腫瘤の超音波診断基準（1988/11/30）の改訂」小委員会
委員長　熊田　卓[1]
副委員長　松田　康雄[2]
委員　飯島　尋子[3]　小川　眞広[4]　工藤　信樹[5]　小原　和史[6]　紺野　啓[7]　高倉　玲奈[8]
西田　睦[9]　南　康範[10]　森　秀明[11]　山田　昌彦[12]

1. 目　的

腹部超音波検査の中で肝腫瘤性病変の存在診断および質的診断の占める重要性は高い．1988年11月30日に「日本超音波医学会医用超音波診断基準に関する委員会」より公示された「肝腫瘤の超音波診断基準」は一般に広く受け入れられ活用され，大きな役割を果たしてきた．しかし，最近の超音波診断装置の進歩および疾患概念の変化により，以前作成された肝腫瘤の超音波診断基準では対応ができなくなった点が多く見られるようになった．今回，これらを解決すべく肝腫瘤の超音波診断基準の改訂を試みた．

2. 対　象

腹部超音波検査の適応となる全ての人が対象となる．特に，肝細胞癌の高危険群（B型慢性肝炎，C型慢性肝炎，非B非C型肝硬変）と超高危険群（B型肝硬変，C型肝硬変）ではそれぞれ，6ヵ月に一度と3-4ヵ月に1度の腹部超音波検査を行う必要がある[1)]．

3. 存在診断

存在診断は，確診，疑診，判定保留の3つの段階に分けて記載する．

1）確診
① 周囲肝組織との明らかなエコーレベルの相違もしくは明瞭な輪郭
② 2方向以上での描出

2）疑診
① 周囲肝組織との明らかなエコーレベルの相違もしくは明瞭な輪郭
② 1方向のみでの描出

3）判定保留
① 周囲肝組織とのわずかなエコーレベルの相違もしくは不明瞭な輪郭
② 1方向以上での描出

注1）存在診断はあくまで肝腫瘤性病変に限ったものではなく，確診の中には限局性の脂肪浸潤の程度の異なる部位も含まれる．
注2）疑診では正常解剖を理解し円靱帯などを，腫瘤性病変と間違わないように注意する．また，所見，随伴疾患から必要に応じてCT（computed tomography）あるいはMRI（magnetic resonance imaging）などの他の画像検査を行い確認する．
注3）判定保留の場合には必要に応じて他の画像診断（CTあるいはMRI）もしくは超音波検査による経過観察を行い確認する．
注4）血管の圧排，途絶，腫瘍の凹凸，肝表面のhump sign，辺縁のedge signは，腫瘍の存在診断の重要な間接所見であるが，本邦に多い肝硬変の存在を考えた時，付加所見として記載する．
注5）肝内胆管の限局性の拡張所見は肝内胆管癌（胆管細胞癌）を強く示唆する所見なので，要精査とする．
注6）エコーレベルに差はないが，周囲肝組織と異なるエコーパターンを示す部分の認められる場合は，判定保留とする．
注7）2方向以上で描出という場合の2方向は，肋弓下走査と肋間走査のように互いに直角に近い2方向が望ましいが不可能な場合はこの限りでない．

[1]大垣市民病院消化器科，[2]八尾徳州会総合病院肝臓外科，[3]兵庫医科大学超音波センター，[4]駿河台日本大学病院超音波室，[5]北海道大学工学部生体システム工学講座，[6]横須賀うわまち病院放射線科，[7]自治医科大学臨床検査医学講座，[8]大阪府立成人病センター検診部精密健康診断科，[9]北海道大学病院診療支援部，[10]近畿大学医学部消化器内科，[11]杏林大学医学部第3内科，[12]東京医科大学第4内科

Table 1 Bモード所見

主分類	細分類	形状	境界・輪郭	腫瘍辺縁	腫瘍内部	後方エコー	付加所見
肝細胞癌	結節型（2cm以下）	円形，類円形	やや不明瞭，整	辺縁低エコー帯（頻度少）	エコーレベルはさまざま（mosaic patternを認めることもある）	不変～時に増強	bright loop
	結節型（2cmを越える）	円形，類円形	明瞭，整	薄い辺縁低エコー帯（ハロー）	mosaic pattern, nodule in nodule,（大きさや分化度により異なる）	増強	外側エコーの増強
	塊状型	不整形	不明瞭		エコーレベルはさまざま		門脈や肝静脈の腫瘍栓を有する場合がある
肝内胆管癌（胆管細胞癌）		不整形	不明瞭		エコーレベルはさまざま 血管が腫瘍を貫く		末梢胆管の拡張を認める場合がある．また末梢胆管の拡張のみで腫瘤が描出されない例もある
転移性肝腫瘍		不整形で，小さなものは円形	明瞭，時に不明瞭，不整（あらい凹凸）	厚い辺縁低エコー帯（bull's eye pattern，target pattern）	高エコー，低エコー，中心部に無エコー域，石灰化		cluster sign，strong echo，全肝で多数の結節が見られることが多い
肝細胞腺腫		円形，類円形	明瞭，整		エコーレベルはさまざま 隔壁を認めない		腫瘍内出血は低エコー，脂肪変性は高エコー
肝血管腫		円形，類円形	明瞭，不整（細かい凹凸）	辺縁に高エコー帯を認めることもある（marginal strong echo）	高エコー型，辺縁高エコー型，混在型，低エコー型に分けられる		chameleon sign，wax and wane sign，disappearing sign
限局性結節性過形成（FNH）		不整形	不明瞭		低～高エコーさまざま．中心部高エコー		

4. 存在部位診断

1）小さな腫瘍ではCouinaud[2]の区域で記述する．また，2区域にまたがるような腫瘍の場合，多くの部分を占める区域を先に記載しその残りの区域を記載する（例：S_7－S_8にかけて腫瘤が存在しS_7が主体の場合には$S_{7,8}$としS_8が主体の場合には$S_{8,7}$とする）．

2）大きな腫瘍ではHealey[3]の区域で記述する．

3）Healeyの区域間に存在する腫瘍では，肝静脈や門脈との立体的位置関係につき記述する．

4）Couinaudの上下区域の診断に迷う場合は，Healeyの区域門脈枝の何番目の枝によって支配されているかを記述する．門脈枝の分岐が複雑な場合は図示する．

5. 質的診断

腹部超音波検査には肝腫瘤診断の役割として存在診断に加えて質的診断がある．

質的診断には①Bモード所見，②ドプラ所見，③造影所見の3種類あり，それぞれの役割は異なる．

鑑別診断に必要な代表的な所見をそれぞれについて，主に肝細胞癌，肝内胆管癌（胆管細胞癌），転移性肝腫瘍，肝細胞腺腫，肝血管腫，限局性結節性過形成（FNH）の6疾患について以下に記載する．

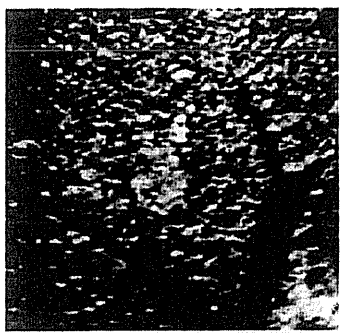
5.1 Bモード所見

超音波検査所見の基本となる．**Table 1**に示すごとく，形状，境界・輪郭，腫瘍辺縁，腫瘍内部，後方エコー，付加所見から鑑別診断を行う．肝細胞癌においては結節型，塊状型の肝細胞癌が対象である[4]．

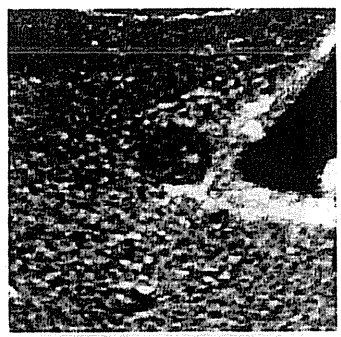
注1）いずれも典型的な所見を示した．転移性肝腫瘍（癌）は上皮性，非上皮性を区別していな

参考図

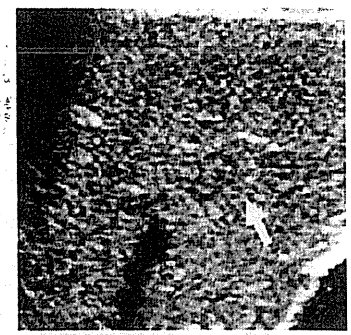
肝細胞癌



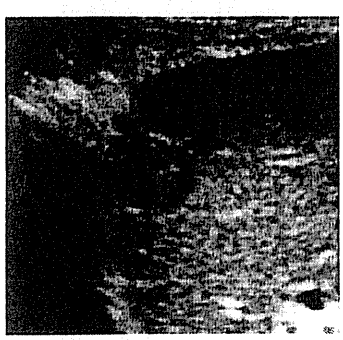
高エコー（結節型）



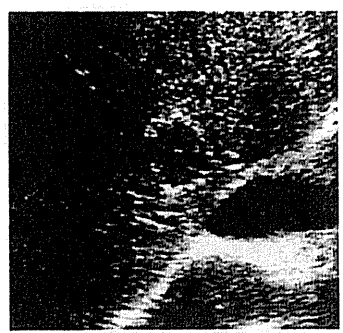
低エコー（結節型）



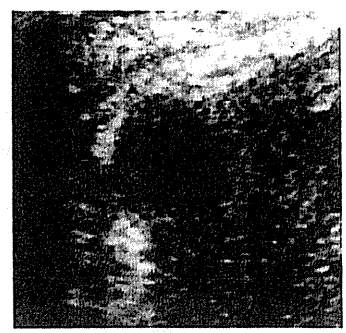
等エコー（結節型）



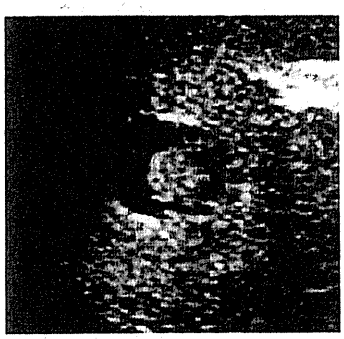
辺縁低エコー（結節型）



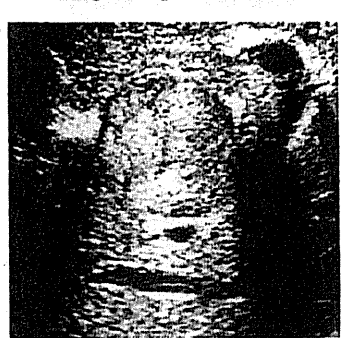
bright loop（結節型）



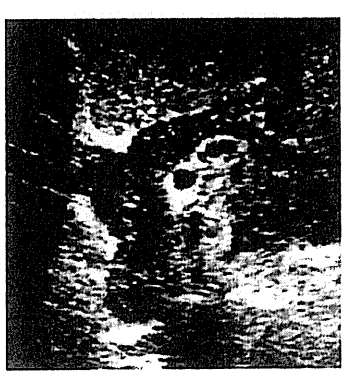
hump sign（結節型）



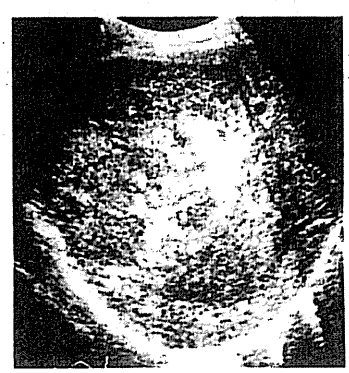
nodule in nodule



mosaic pattern



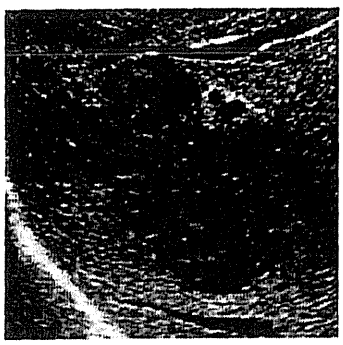
門脈腫瘍塞栓



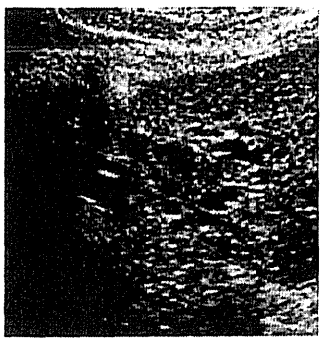
塊状型

参考図

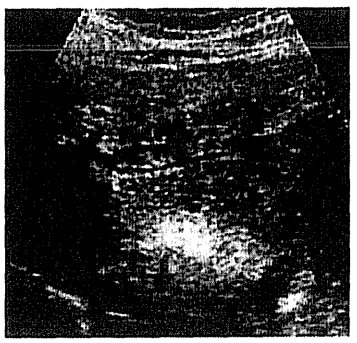
肝内胆管癌（胆管細胞癌）



境界不明瞭結節

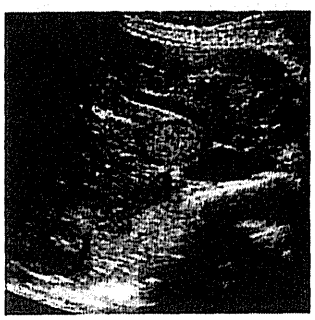


末梢胆管の拡張

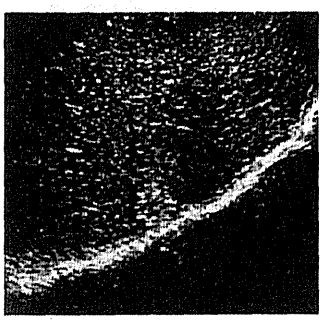


腫瘍を貫く血管

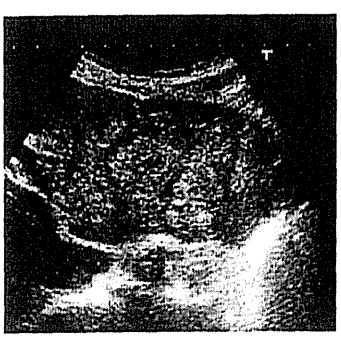
転移性肝腫瘍



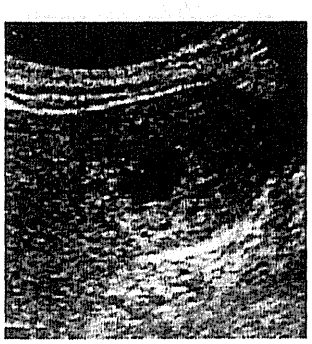
bull's eye pattern



高エコー



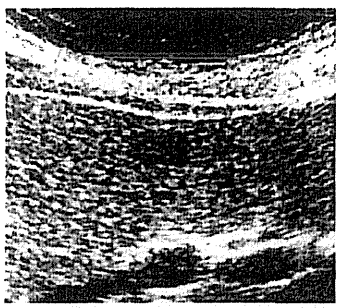
cluster sign



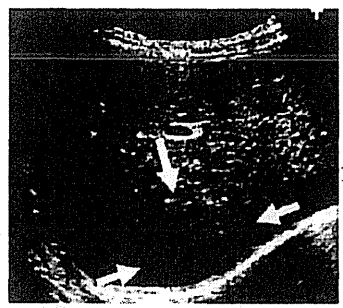
中心部に無エコー域

参考図

肝細胞腺腫

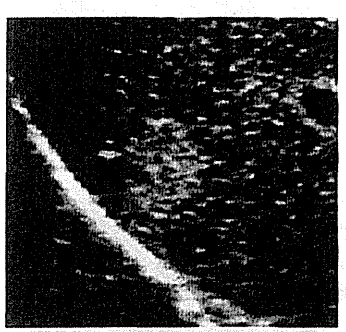


低エコー

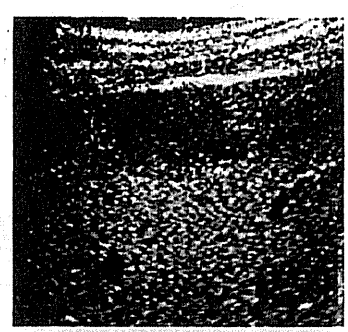


等エコー

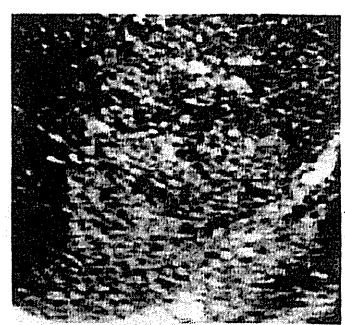
肝血管腫



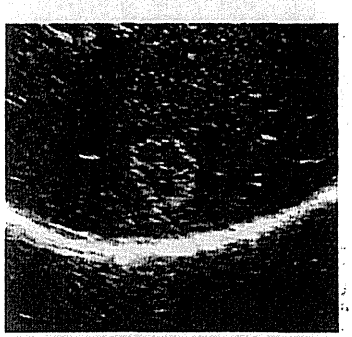
高エコー



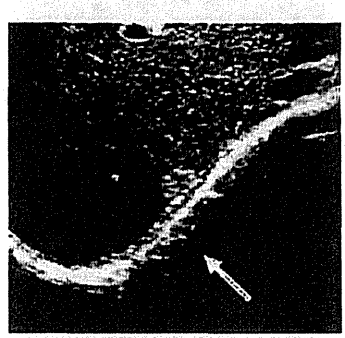
低エコー

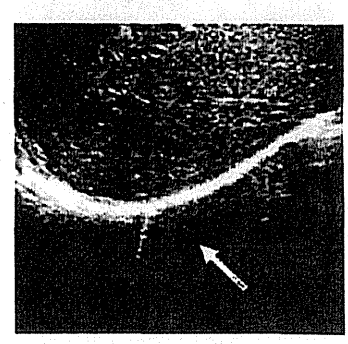


混合エコー



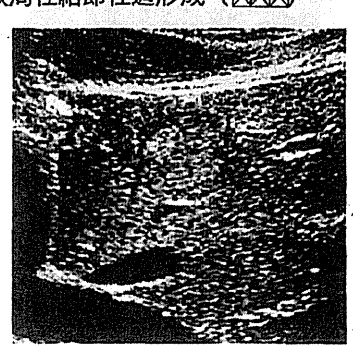
辺縁高エコー帯



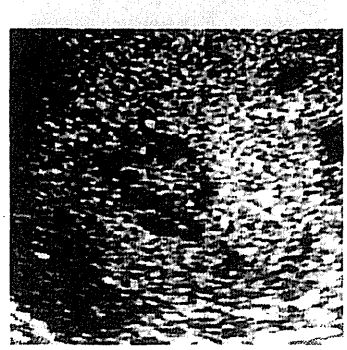


chameleon sign

限局性結節性過形成（XXX）



高エコー



中心高エコー

いため腫瘍としたが，主に胃癌や大腸癌などの消化器系の癌の典型像を示す．

注2) 腫瘍の大きさは質的診断において間接所見であるが，腫瘍の内部構造とは密接な関係があると考えられるので肝細胞癌の結節型においてのみサイズ別に代表する所見を記載した．

注3) 随伴所見や特徴的な形態変化は間接所見であるが，質的診断をするうえで重要な情報となりうるので付加所見として記載した．

注4) 肝細胞癌の肉眼分類として小結節境界不明瞭型，浸潤型，びまん型があるが，これらは腫瘤を形成せず，エコーレベルも肝実質との差が少なく存在が認識しにくいので診断基準からは除いた．しかし，びまん型や浸潤型は門脈や肝静脈の腫瘍栓を有する場合があり，この所見によって診断されることがある．小結節境界不明瞭型は組織学的には早期肝細胞癌に相当する．CTもしくはMRIなどの他の画像診断法の併用が必要となる．また，単純結節型，単純結節周囲増殖型，多結節癒合型は結節型として所見を記載した．

注5) 肝辺縁に存在する肝細胞癌では腫瘤の一部が肝表面より突出する所見（hump sign）が認められることがある．

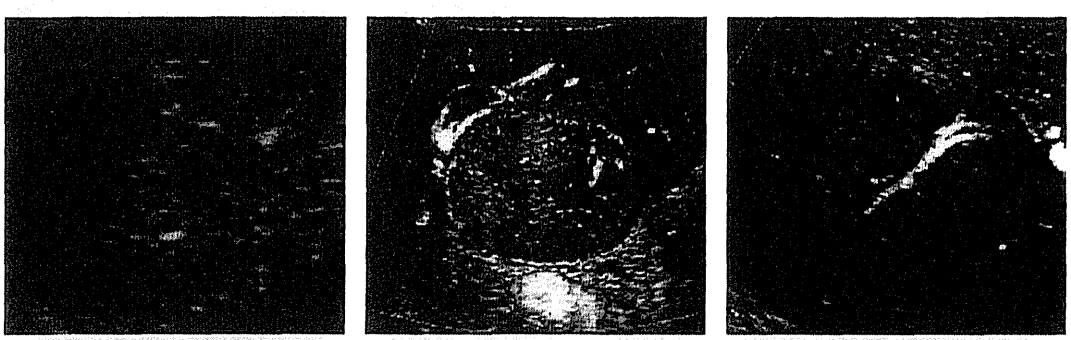
注6) 異型結節は基本的には肝細胞癌結節型（2 cm以下）の所見に類似し鑑別は困難である．

注7) 肝内胆管癌（胆管細胞癌）には腫瘤形成型，胆管浸潤型，肝内胆管発育型があるが，ここで記載した所見は腫瘤形成型の所見である．

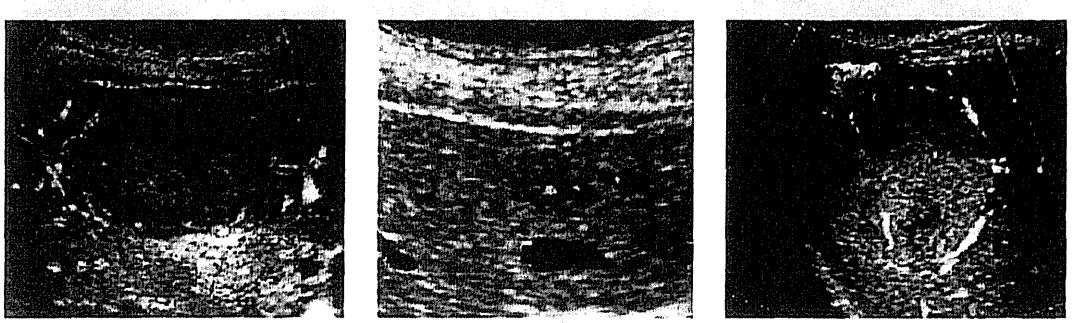
5.2 ドプラ所見

ドプラ所見は，**Table 2**に示すように腫瘤内の血流の多寡，血管の走行，血流性状（拍動波，定常波），付加所見などと[5,6]，Bモード所見と合わせて鑑別診断を行う．血流の方向についても評価することが望ましい．

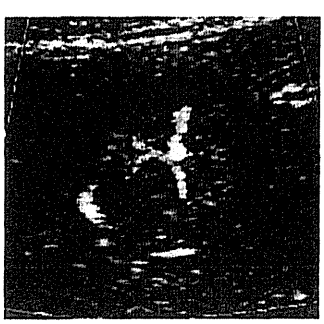
参考図



肝細胞癌（2 cm以下）　肝細胞癌（バスケットパターン）　肝内胆管癌



転移性肝腫瘍　肝細胞腺腫　肝血管腫



FNH

Table 2 ドプラ所見

主分類	細分類	血流の多寡	血管の走行	血流性状	付加所見
肝細胞癌	結節型（2 cm以下）	少ない	時に腫瘍内部および周辺に線状もしくは点状	定常性 時に拍動性	血流信号が認められないことが多い
	結節型（2 cmを越える）	多い	バスケットパターン（周辺から中心に向かう）	拍動性 時に定常性	A-P shuntや腫瘍塞栓を認めることもある
	塊状型	多い	不整な血管，バスケットパターン	拍動性	門脈内に拍動流を認める場合腫瘍塞栓やA-P shuntをの存在を疑う．
肝内胆管癌（胆管細胞癌）		少ない	腫瘍周辺に圧排 腫瘍内に既存血管の残存	拍動性 定常性	腫瘍周辺の一部のみ血流信号を認めることが多いが，内部でも見られる場合がある．
転移性肝腫瘍		少ない	腫瘍周辺に 圧排 腫瘍内に既存血管の残存	拍動性 定常性	腫瘍周辺部に血流信号を認めることが多いが，中心部はあまり認めない．原発巣によっては血流が多いことがある．
肝細胞腺腫		多い	腫瘍境界から取り囲むように内部に細い血管が流入	拍動性 時に定常性	
肝血管腫		少ない	腫瘍辺縁部に点状	定常性 時に拍動性	A-P shuntを認めることもある．血流が豊富な場合がある．
限局性結節性過形成（FNH）		多い	腫瘍中心部から流入し辺縁に広がる spoke-wheel pattern	拍動性	

注1）いずれも典型的な所見を示した．転移性肝腫瘍（癌）は上皮性，非上皮性を区別していないため腫瘍としたが，主に胃癌や大腸癌などの消化器系の癌の典型像を示す．

注2）肝細胞癌は腫瘍の大きさやパターンにより特有の血流パターンを示すためBモード所見の細分類を用いた．血流の方向を加味して解釈するのが望ましい．一部の肝細胞癌結節型（2 cm以下）は流入する定常性血流のみを認めることが多く，基本的には異型結節との鑑別は困難である．

注3）肝内胆管癌（胆管細胞癌）には腫瘤形成型，胆管浸潤型，肝内胆管発育型があるが，ここで記載した所見は腫瘤形成型のドプラ所見である．

5.3 造影所見（時相，イメージの定義）

肝臓は肝動脈（25～30%）と門脈（70～75%）の2重の血行支配であり，超音波造影剤を静脈から投与すると3つのオーバーラップする時相（phase，造影超音波検査における造影剤投与後の経時的撮像タイミング）が観察される．時相に関しては以下の如く定義する．

血管相（vascular phase，造影超音波検査において造影剤が血管内に存在している時相）と後血管相（post vascular phase，血管内の造影剤濃度が十分に低下し，造影剤による血管の造影効果が失われた時相）に分類し，血管相はさらに，動脈優位相（arterial [predominant] phase，臓器実質および腫瘍が動脈由来の造影剤により造影される時相）と門脈優位相（portal [predominant] phase，肝内門脈枝が造影された後肝実質が造影される時相）に分ける．動脈優位相では腫瘤内の血管構築像，腫瘤の灌流像が得られる．門脈優位相では腫瘤の造影剤のwashoutと肝実質相の染まりの輝度を比較する．動脈優位相で得られる画像を血管イメージ（vascular image）および灌流イメージ（perfusion image），後血管相で得られる画像を後血管イメージ（post vascular image）と呼ぶ．各疾患の造影所見を**Table 3**に示す．

注1）血管相は質的診断を，後血管相は存在診断を主目的として使用される．

注2）後血管イメージは，「クッパーイメージ（Kupffer image）」とも呼ばれるが，この点に関しては異論もあり今後の検討が必要である[7-10]．

注3）1つの目安であるが，動脈（優位）相は造影剤静脈内投与後約30秒まで，門脈（優位）相はそれ以後から約120秒まで，後血管相は約10分以降とされる．ただし，肝機能もしくは腫瘤の状態により個人差のあることには留意する[11]．

注4）いずれも典型的な所見を示した．転移性肝腫瘍は上皮性，非上皮性を区別していないため腫瘍としたが，主に胃癌や大腸癌などの消化器系の癌典型像を示す．

注5）後血管相の撮像時に血管相では気付かれなかった新たな病変が発見された場合は再度造影剤を注入してその結節の血管相を評価することが可能であ